Edited by Stefan Dübel

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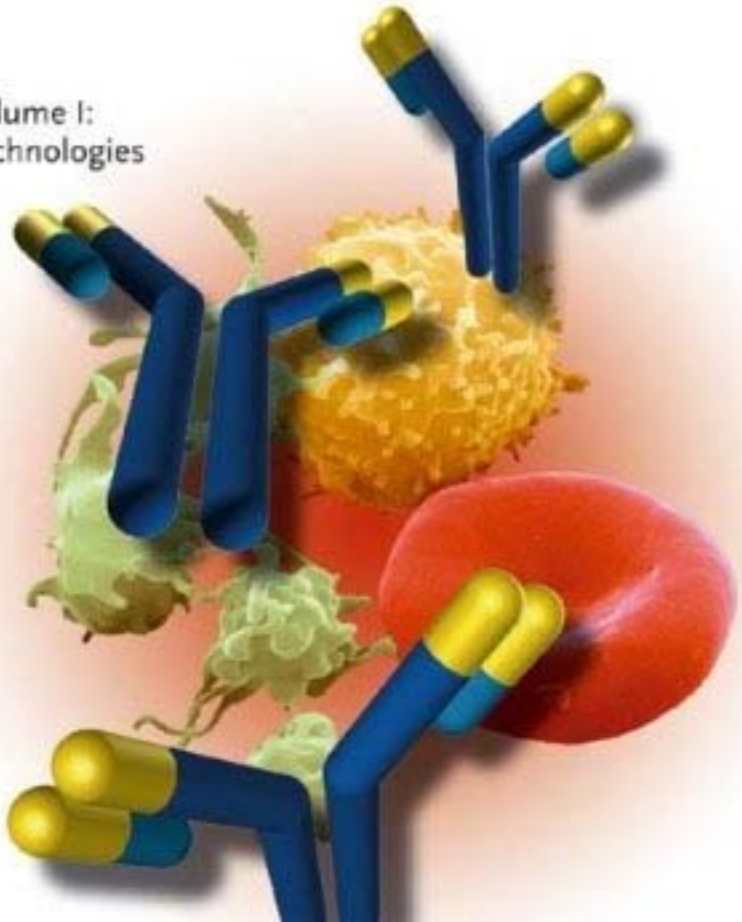
Handbook of Therapeutic Antibodies

Volume I:
Technologies



Handbook of
Therapeutic Antibodies

Edited by
Stefan Dübel

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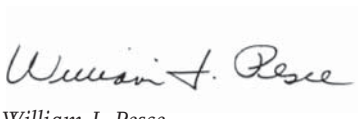

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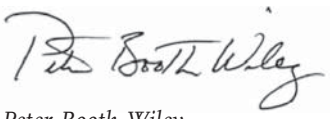

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Handbook of Therapeutic Antibodies

Volume I

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Dedication

To Inge, Hans, Ulrike and Tasso Dübel – the four best things in my life

Stefan Dübel

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Handbook of Therapeutic Antibodies. Edited by Stefan Dübel

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A Greeting by the Editor

Today, therapeutic antibodies are essential assets for physicians fighting cancer, inflammation, and infections. These new therapeutic tools are a result of an immense explosion of research sparked by novel methods in gene technology which became available between 1985 and 1995.

This handbook endeavors to present the fascinating story of the tremendous achievements that have been made in strengthening humanity's weapons arsenal against widespread diseases. This story not only includes the scientific and clinical basics, but covers the entire chain of therapeutic antibody production – from downstream processing to Food and Drug Administration approval, galenics – and even critical intellectual property issues.

A significant part is devoted to emerging developments of all aspects of this process, including an article showing that antibodies may only be the first generation of clinically used targeting molecules, making the IgG obsolete in future developments, and novel ideas for alternative therapeutic paradigms.

Finally, approved antibody therapeutics are presented in detail in separate chapters, allowing the clinicians to quickly gain a comprehensive understanding of individual therapeutics.

In such a fast-developing area, it is difficult to keep pace with the rapidly growing information. For example, a PubMed search with "Herceptin" yields more than 1500 citations. Consequently, we have tried to extract the essentials from this vast resource, offering a comprehensive basis of knowledge on all relevant aspects of antibody therapeutics for the researcher, the company expert, and the bedside clinician.

At this point, I express my deep gratitude to all the colleagues who wrote for these books. Without their enthusiasm this project would never have materialized. I would also like to thank Dr Pauly from the publisher's office, who paved the way for this three-volume endeavor, and the biologist Ulrike Dübel – my wife. Both played essential roles in keeping the project on track throughout the organizational labyrinth of its production. The hard work and continuous suggestions of all of these colleagues were crucial in allowing the idea of a comprehensive handbook on therapeutic antibodies to finally become a reality.

Braunschweig, December 2006 Stefan Dübel

Handbook of Therapeutic Antibodies. Edited by Stefan Dübel

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Foreword

The most characterized class of proteins are the antibodies. After more than a century of intense analysis, antibodies continue to amaze and inspire. This *Handbook of Therapeutic Antibodies* is not just an assembly of articles but rather a state-of-the-art comprehensive compendium, which will appeal to all those interested in antibodies, whether from academia, industry, or the clinic. It is an unrivaled resource which shows how mature the antibody field has become and how precisely the antibody molecule can be manipulated and utilized.

From humble beginnings when the classic monoclonal antibody paper by Kohler and Milstein ended with the line, "such cultures could be valuable for medical and industrial use" to the current Handbook you hold in your hand, the field is still in its relative infancy. As information obtained from clinical studies becomes better understood then further applications will become more streamlined and predictable. This Handbook will go a long way to achieving that goal. With the application of reproducible recombinant DNA methods the antibody molecule has become as plastic and varied as provided by nature. This then takes the focus away from the antibody, which can be easily manipulated, to what the antibody recognizes. Since any type, style, shape, affinity, and form of antibody can be generated, then what the antibody recognizes now becomes important.

All antibodies have one focus, namely, its antigen or more precisely, its epitope. In the realm of antibody applications antigen means "target." The generation of any sort of antibody and/or fragment is now a relatively simple procedure so the focus of this work has shifted to the target, and rightly so. Once a target has been identifed then any type of antibody can be generated to that molecule. Many of the currently US Food and Drug Administration (FDA) approved antibodies were obtained in this manner. If the target is unknown then the focus is on the specificity of the antibody and ultimately the antigen it recognizes.

As the field continues to mature the applications of antibodies will essentially mimic as much of the natural human immune response as possible. In this respect immunotherapy may become immunomanipulation, where the immune system is being manipulated by antibodies. With the success of antibody monotherapy the next phase of clinical applications is the use of antibodies with standard chemotherapy, and preliminary studies suggest the combination of

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these two modalities is showing a benefit to the patient. When enough antibodies become available then cocktails of antibodies will be formulated for medical use. Since the natural antibody response is an oligoclonal response then cocktails of antibodies can be created by use of various *in vitro* methods to duplicate this in a therapeutic setting. In essence, this will be oligotherapy with a few antibodies. After all, this is what nature does and duplicating this natural immune response may be effective immunotherapy.

And all of this brings us back full circle to where it all starts and ends, the antibody molecule. No matter what version, isotype, form, or combination used the antibody molecule must first be made and shown to be biologically active. Currently, many of the steps and procedures to generate antibodies can be obtained in kit form and therefore are highly reproducible, making the creation of antibodies a straightfoward process. Once the antibody molecule has been generated it must be produced in large scale for clinical and industrial applications. More often than not this means inserting the antibody genes into an expression system compatible with the end use of the antibody (or fragment). Since many of the steps in generating clinically useful antibodies are labor intensive and costly, care must be used to select antibodies with the specificity and activity of interest before they are mass produced. For commercial applications the FDA will be involved so their guidelines must be followed.

Stating the obvious, it would have been nice to have this Handbook series in the late 1970s when I entered the antibody field. It certainly would have made the work a lot easier! And here it is, about 30 years later, and the generation of antibodies has become "handbook easy." In this respect I am envious of those starting out in this field. The recipies are now readily available so the real challenge now is not in making antibodies but rather in the applications of antibodies. It is hoped that this Handbook will provide a bright beacon where others may easily follow and generate antibodies which will improve our health. The immune system works and works well; those using this Handbook will continue to amaze and inspire.

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December 2006

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Introduction

1
Therapeutic Antibodies – From Past to Future

Stefan Dübel

1.1
An Exciting Start – and a Long Trek

In the late nineteenth century, the German army doctor Emil von Behring (1854–1979), later first Nobel Laureate for Medicine, pioneered the therapeutic application of antibodies. He used blood serum for the treatment of tetanus and diphtheria ("Blutserumtherapie"). When his data were published in 1890 (Behring and Kitasato 1890), very little was known about the factors or mechanisms involved in immune defense. Despite this, his smart conclusion was that a human body needs some defense mechanism to fight foreign toxic substances and that these substances should be present in the blood – and therefore can be prepared from serum and used for therapy against the toxins or infections. It worked, and the success allowed him to found the first "biotech" company devoted to antibody-based therapy in 1904 – using his Nobel Prize money as "venture capital." The company is still active in the business today as part of ChironBehring.

In 1908, Paul Ehrlich, the father of hematology (Ehrlich, 1880) and the first consistent concept of immunology ("lateral chain theory," Fig. 1.1d, Ehrlich, 1908), got the second Nobel Prize related to antibody therapeutics for his groundbreaking work on serum, "particularly to the valency determination of sera preparations." Ehrlich laid the foundations of antibody generation by performing systematic research on immunization schedules and their efficiency, and he was the first to describe different immunoglobulin subclasses. He also coined the phrases "passive vaccination" and "active vaccination." His lateral chain theory ("Seitenketten," sometimes misleadingly translated to "side chain theory") postulated chemical receptors produced by blood cells that fitted intruding toxins (antigens). Through these chemical receptors, cells combine with antigens and the receptors are eventually released as circulating antitoxins (antibodies). Without any knowledge of molecular structure or biochemical binding mechanisms, Paul Ehrlich anticipated much of today's knowledge on immunoglobulin generation and antibody–antigen interaction, even class switching (Fig. 1.1d).

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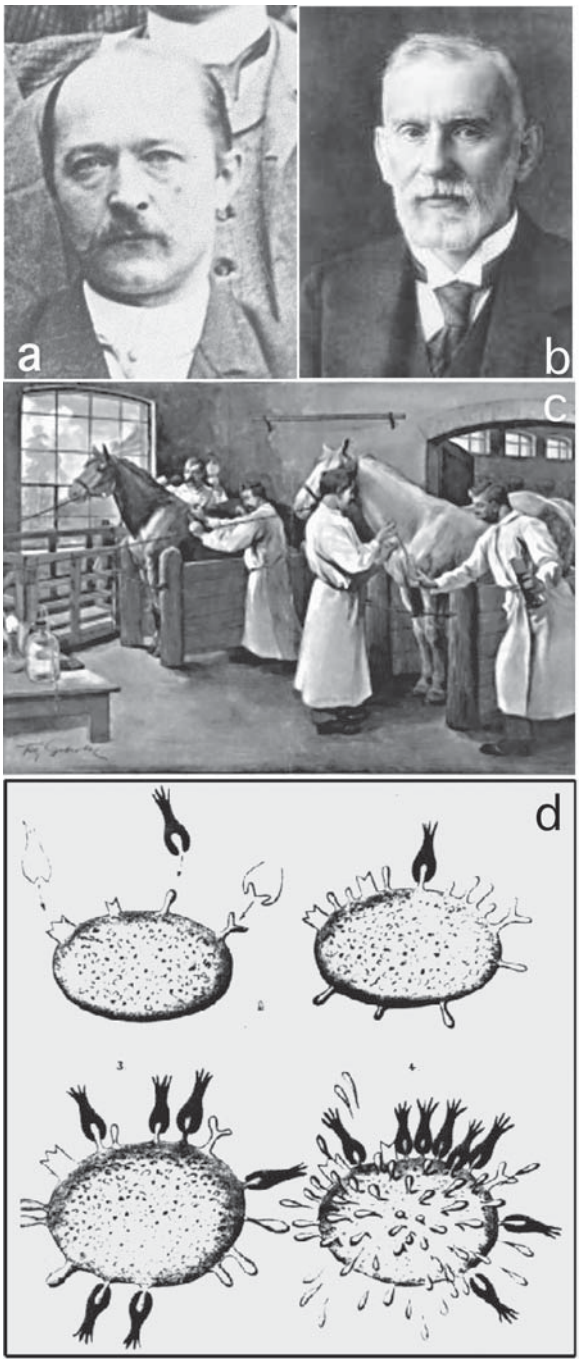

Fig. 1.1 We have come a long way since the first methods for the generation of antibody based therapeutics were established (c), pioneered by (a) Emil von Behring and (b) Paul Ehrlich in the last decade of the nineteenth century. (d) Drawing from Paul Ehrlich on the lateral chain theory (lateral chains = antibodies). He anticipated principles confirmed on a molecular basis many decades later, like the binding of antigens by different specific antibodies (the "lock and key" principle), the differentiation and maturation of B cells and the class switch, allowing the initially cell-bound antibodies to be released in large amounts. Photos: Deutsches Historisches Museum, Berlin.

Passive and active vaccines were developed in rapid succession at the beginning of the twentieth century, and were successful in saving many lives. Snake and insect bites could be treated specifically, and beneficial effects were even observed with human serum immunoglobulin G (IgG) preparations without prior specific immunization (e.g. protection against hepatitis A).

The enormous succes of all these blood products for the prevention and treatment of infections and intoxications, however, could not be expanded to other substantial disease areas, in particular cancer and autoimmunity. Here, the understanding of molecular processes in their etiology or at least the ability to identify molecules strongly correlated to their onset stimulated the desire to produce antibodies targeting these for therapeutic intervention. Unlike a snake bite, however, cancer and autoimmune diseases are chronic, and it was rapidly understood from animal models that antibodies have to be applied to the patient more than once. Immunologists knew very well at that time that repeated application of antigen during antiserum preparation is a good strategy to "booster" the immune response. When using animal serum antibodies to treat chronically ill humans, this must induce an immune response to the therapeutic agent. Further, a drug containing solely an IgG of defined specificity would be tremendously helpful to limit side effects and reach sufficient concentrations at the target site. These prerequisites could by no means be met by the well-established methods of serum antibody preparation.

Much has been learned about the antibody structure (Fig. 1.2) and its function since then (see Chapter 2, Vol I). Hopes were high when Cesar Milstein and Georg Köhler demonstrated that monoclonal antibodies could be produced in mouse cell culture (see Chapter 2, Vol I). However, the excitement of the late 1970s cooled rapidly when almost all first-generation monoclonal therapeutics failed during clinical evaluation. Only one of these products made it through to US Food and Drug Administration (FDA) approval in 1984 – Orthoclone (see Chapter 9, Vol III). However, this was a special case as the typical patient recieving this CD3 antibody was already immune suppressed by disease, a setup not commonly present in cancers or autoimmune diseases. Even more important, it was realized that simple binding to a target (inducing its neutralization) is in most cases not sufficient to make an antibody a good therapeutic agent to treat cancer or immune diseases. Effector functions, like complement activation or cellular responses triggered by Fc receptor binding, are obviously needed, but are not fully provided by the mouse monoclonals.

As a consequence, huge efforts were undertaken to exchange the antibody's effector domains (constant regions) for human ones – thereby also removing the most immunogenic parts from the mouse IgG. By using the then available methods of molecular cloning and recombinant expression, most mouse antibodies were humanized prior to clinical testing. Various methods (chimerization and CDR-exchange-based humanization being the most widely used) were employed (see Chapter 6, Vol I). When tinkering with the antibody sequence, most candidate antibodies were also affinity matured to typical nanomolar and subnanomolar affinities (see Chapter 7, Vol I). The technology for humanization

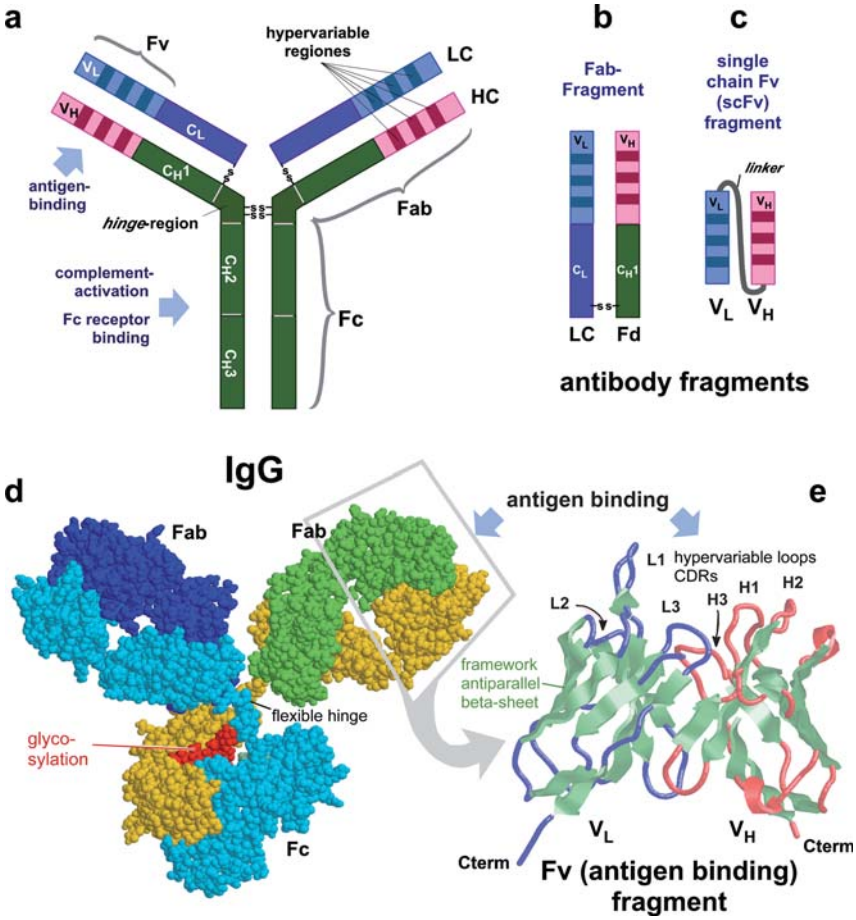

Fig. 1.2 Introduction to antibody structure. (a, d) IgG is a heterotetrameric protein assembled from two identical heavy and light chains (HC, LC), assembled by disulfide bonds. (b) Fab fragments contain the antigen-binding region, and can be generated by proteolysis or recombinant production. (c) In single-chain Fv fragments (scFv), the two antigen-contacting domains (variable regions of the heavy and light chains, V_H, V_L) are connected by an oligopeptide linker to form a single polypeptide. They can be produced in *E. coli* and are typically employed for the selection of human antibodies by phage display and other display systems. (d) Space fill model based on X-ray crystallographic data of an antibody. The typical Y shape is only one of the many conformations the Fab fragments of an antibody can assume relative to each other. T-shaped structures can be assumed, and the hinge region to the Fc part can also bend significantly relative to the Fab fragments. (e) Alpha carbon backbone of an Fv fragment, the antigen-binding fragment of an antibody located at the two tips of the Y- or T-shaped complex, emphasizing the typical antiparallel beta sheet framework structure which holds together the hypervariable loops (L1–3, H1–3) composing the antigen-binding surface (CDRs).

and affinity maturation became available from the mid-1980s. Taking into account that drug development, testing and approval needs about 10 years to reach the market, a growing number of therapeutic antibodies were approved starting from the mid-1990s. These "first generation" recombinant antibodies – cloned from rodent hybridoma cells and improved by gene engineering – dominate the current list of approved antibody therapeutics.

Early in the 1990s, two novel enabling technologies were developed that revolutionized the generation of therapeutic antibodies, as for the first time they provided a robust and reliable method to prepare specific antibodies of human origin. Phage display (see Chapter 3, Vol I) and transgenic mice (see Chapter 4, Vol I) allowed the production of antibodies that are genetically 100% identical to human immunoglobulins. Recently, even transgenic rabbits producing human antibodies have been generated (Buelow et al. 2006). Many other approaches for the *in vitro* selection of antibodies, such as yeast display or ribosomal display, followed and are still in various stages of maturation. These are all based on the selection of antibodies from a large antibody gene repertoire in a heterologous expression system (Fig. 1.3).

The experience with other recombinant human protein drugs (e.g. insulin) raised great hopes that these human antibodies are completely stealthy in respect of an anti-drug immune reaction. However, it was soon realized that immunoge-

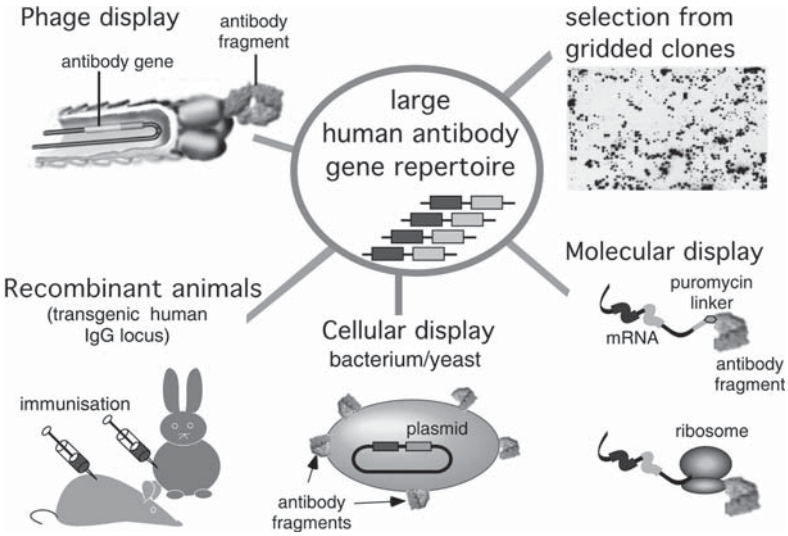

Fig. 1.3 Systems employed for the generation of human antibodies. They all include a heterologous expression of a repertoire of human immunoglobulin genes. Selection is achieved either *in vivo* by immunization (in case of the recombinant animals) or *in vitro* by binding to the antigen, allowing clonal selection of the gene host.

nicity was not switched off completely in most cases – Fc-glycosylation patterns resulting from recombinant production can be very diffferent from endogenous human IgG, and a variable human antibody region with its "lottery"-derived CDRs (complementarity determining regions) generated outside of the context of the human immune system can be quite immunogenic. However, these problems were in most cases minor compared with the effects observed previously with animal-produced immunoglobulins. Today, the first antibody with a completely "human" sequence origin reached the pharmacy shelves: Humira (see Chapter 1, Vol III). It was genetically assembled entirely *in vitro*, with an antigen-binding region selected from an *E. coli*-hosted gene repertoire by phage display (see Chapter 3, Vol I). Many more antibodies derived from human gene repertoires, selected by phage display or generated in transgenic mice carrying the human immunoglobulin locus, have entered clinical testing. The *in vitro* technologies, like phage display, offer an additional advantage when antibody generation in animals is difficult, for example due to the high homology (resulting in low immunogenicity) of the human antigen used for immunization to a mouse protein, or in case of highly toxic or deadly pathogenic antigens. A few animal or even human serum-derived antibody products are still available (Rohrbach et al. 2003) but recombinant human or humanized products vastly dominate the current clinical studies.

Approval time was also getting increasingly short for antibodies when compared with a typical small molecule drug, and success rates of clinical studies improved, mainly due to the more predictable pharmacokinetics and lower risk of toxicity and other side effects when using molecules almost identical to the IgG in our veins – of course with the usual unavoidable exceptions, usually due to effects caused by the antigen binding itself (e.g. seen with TGN1412).

It is a surprising side note of history that in contrast to so many other biomedical achievements, almost all of the enabling antibody technologies (polyclonal sera, monoclonal antibodies, production of functional antibodies in *E. coli* and phage display, humanization, among others) were pioneered in Europe, mainly in the UK and Germany.

1.2 The Gold Rush

A gold rush for new therapeutic agents started when it was realized that all enabling technologies are in place to develop and produce monospecific, nearly human antibodies that are only mildly immunogenic but provide high-affinity target binding and human effector functions, long serum half-life and other pharmacologic advances. Many promising new concepts for the treatment of a huge variety of different diseases were envisaged. In fact, there are only few theoretical restrictions for antibody treatments. First comes the necessity to find a molecular target (antigen) accessible from the bloodstream (i.e. typically a target

at the cell surface that is located solely or in a higher concentration on the cell compartment to be effected). Second, the antibody in most cases needs to activate some immune reaction at the binding site (e.g. to kill a tumor cell). Exceptions are antibodies that act by neutralization of an infectious agent or an overexpressed factor, which can be achieved by simple sterical inhibition of the binding of the agent to its natural receptor. Affinity is no practical limitation anymore, as with existing methods antibodies usually can be engineered to provide affinities better than those needed for a maximal therapeutic effect (see Chapter 7, Vol I). Specificity is always an issue, of course, as no antibody is a priori unsusceptible to a cross-reaction, but many strategies have been developed to tackle this problem. Most simply, large numbers of different human antibody clones can now easily be evaluated in parallel using high-throughput assays (see Chapter 4, Vol II).

Some commentators are pessimistic about the shortage of manufacturing capacity for antibodies, and their high cost of production in comparison with a small molecule drug. They have calculated that health systems could not afford all of these new, expensive drugs even if they were made available. However, a scale-up of capacity and novel alternative production systems (e.g. microbial, eukaryotic or plant-derived) may allow much cheaper production of antibodies for many applications, and may even allow the "expensive" antibodies to enter new, low-margin therapeutic markets (see Chapter 6, Vol II). An example already paving the way in this direction is a plant-produced anti-caries antibody which is in phase II clinical testing today.

1.3 Success and Disappointment

All these exciting opportunities offered by the new technologies have resulted in an explosion in the number of clinical studies and approvals (Fig. 1.4). After the slow start in the 1980s and no substantial increase between 1985 and 2000 (Reichert 2001), the number of clinical studies has changed dramatically. Today, more than 400 studies are ongoing, targeting a broad range of diseases, from various cancers to infection and autoimmunity (Tables 1.1 and 1.2). Cancer therapeutics clearly dominate the field, but infection/immunity applications are catching up considering the low number of already approved antibodies, with about a third of the ongoing studies now. The nature of the antigens is just as diverse, with the approved antibodies targeting both cell surface markers (e.g. CD11a, CD20, CD25, CD33, CD52, EGFr, Her2) and soluble molecules (e.g. TNF-α, RSV, VEGF), with three different molecular formats: IgG, Fab fragments, and radioconjugates. A range of new targets and conjugates will follow. This is perfectly illustrated by the antibody variants made to block the tumor necrosis factor (TNF)/TNFR interaction to downregulate overshooting inflammations (Fig. 1.5). Starting with a clinically failed mouse hybridoma antibody, the next steps were chimeric and then humanized antibodies. Finally, a completely human antibody

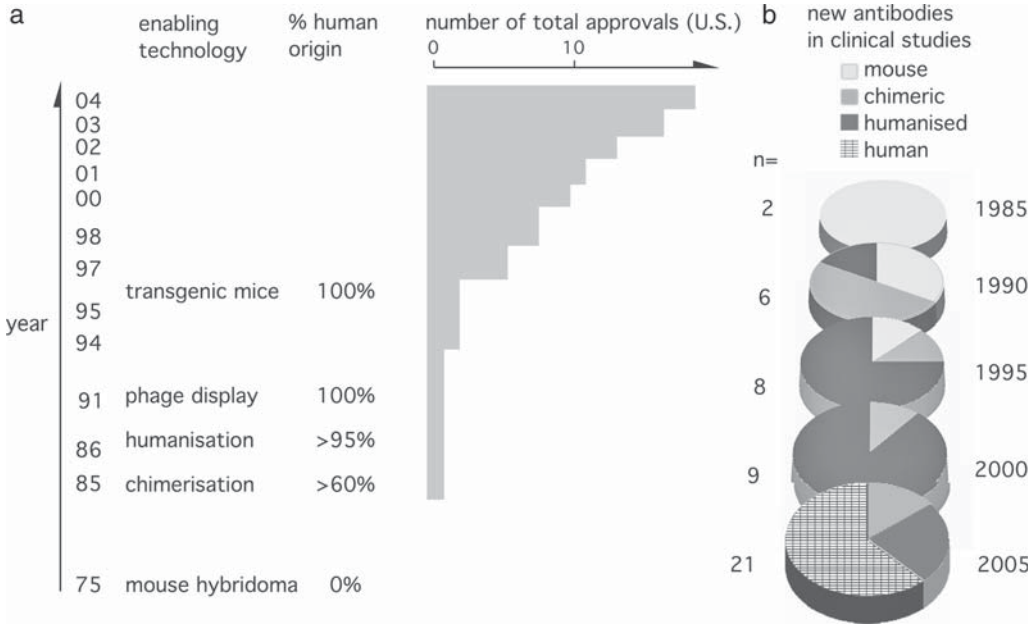

Fig. 1.4 Development history of therapeutic antibodies and the technologies for their generation. Data assembled from Reichert (2001), Reichert et al. (2005), Fabrizi (2005) and public domain sources of the companies.

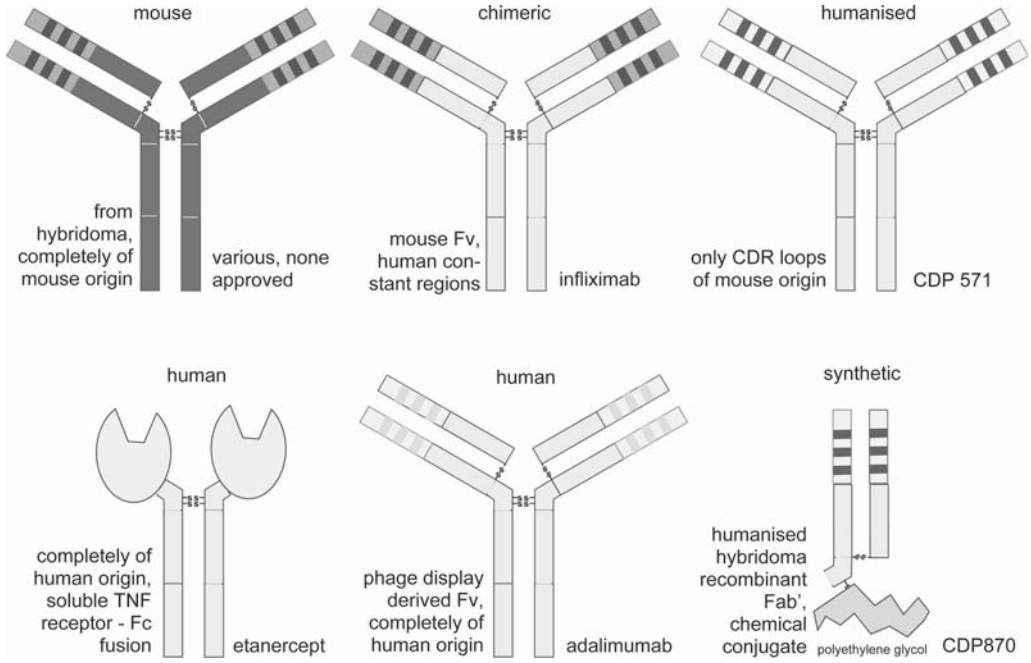

Fig. 1.5 The development of recombinant antibody variant TNF antagonists. Inspired by Rutgeerts et al. (2004).

Table 1.1 Clinical studies with therapeutic antibodies 2005.

Disease	*Clinical phase*			
	I	*II including I/II*	*III including II/III*	*Total*
Cancer				283
Systemic treatment	120	118	18	256
Ex-vivo purging				27
Infection and immunity	40	56	24	120
Total	160	174	42	403

Assembled from Bayes et al. (2005), Reichert et al. (2005), data presented on http://www.clinicaltrials.gov, http://www.centerwatch.com/, company homepages, other public domain sources and the Monoclonal Antibody Index.

and a human soluble receptor–Fc fusion were the next clinically tested and approved iterations of the idea. Now, a Fab fragment of human origin fused to PEG (polyethylene glycol) to improve its pharmacokinetics marks the sixth generation of TNF antagonist antibody constructs no longer resembling a molecule present in nature. Recombinant antibody technology may even reach its limits here, and a seventh-generation TNF antagonist could well be a novel synthetic binder of nonantibody structure (see Chapter 7, Vol II). A very good introduction to the clinical success story of recombinant antibodies is given by the review series by Reichert and colleagues (Reichert 2001, 2002; Reichert et al. 2005).

In February 2005, marketing of the therapeutic antibody natalizumab (marketed as Tysabri), a promising drug for the treatment of multiple sclerosis, was voluntarily suspended after only a few months of approval, based on reports of serious adverse events that have occurred in patients treated in combination with interferon beta-1a in clinical trials (Kleinschmidt-DeMasters and Tyler 2005). It seems, however, that this suspension will be released at time of print due to the reported positive effect of this drug. In December 2005, a voluntary market suspension due to serious and life-threatening cardiopulmonary events following the administration of NeutroSpec (technetium (^{99m}Tc) fanolesomab) was published. In March 2006, dramatic adverse effects not predicted from the animal studies were observed in clinical phase I for the anti-CD28 antibody TGN1412. These unfortunate stories of agents, some of which have even passed clinical studies for their approval, although far from final conclusion, as seen by the lift of the clinical hold for natalizumab after one year of evaluation, show that despite of all the theoretical advantages, there are still risks. These mainly originate from our still very incomplete understanding of the molecular and immunological processes, in particular in combination therapies. Here, great hopes are put on the

Table 1.2

*Drug name**	*FDA name ** (original name)*	*Antigen*	*Ab origin *****	*Trageted diseases*	*Used for*
Avastin	bevacizumab (rhuMAb-VEGF)	VEGF	humanised	colon, breast, kidney, lung cancer, myeloma, mesothelioma	combin. therapy
Bexxar	tositumomab (Bl)	CD20 (B cells)	murine Iodine 131I conjug.	lymphoma (NHL)	therapy
CEA-Scan	arcitumomab (IMMU-4)	CEA	Fab' fragment, 111In conjug.	colorectal, lung, breast cancer	imaging
Cotara	– (TNT-1)	histone H1, DNA	chimeric	lung, uterine cancer, glioma, sarcoma	therapy (China)
Erbitux	cetuximab (C225)	EGFR	chimeric	breast, head and neck, pancreatic, colorectal, lung cancer, melanoma	therapy
Herceptin	trastuzumab (rhuMAb-4D5-8)	p185/HER-2	humanised	breast, lung, ovarian, pancreatic cancer	therapy
HumaSpect-Tc	votumumab (88BV59H21-2V67-66)	CTAA 16.88	human BV-transformed cells 99mTc conjug.	Colorectal, breast, prostatic, lung, ovarian, pancreatic cancer	imaging
Humira	adalimumab (D2E7)	TNF-alpha	phage display, human library	rheumatoid arthritis, Crohn's disease	therapy
Indimacis-125	igovomab (OC125)	CA125	F(ab')2 fragment	Gastrointestinal, ovarian cancer	imaging
LeukoScan	sulesomab (MN-3)	NCA-90 (granulocytes)	Fab' fragment, 99Tcm conjug.	inflammation, infection, Crohn's disesase, Meckel's diverticulum, IBD	imaging
Leukosite	alemtuzumab (CAMPATH-1H)	CD52	humanised from rat	leukemia (CLL), lymphoma	therapy
Mylotarg	gemtuzumab ozogamicin (hP67.6)	CD33	humanised, conjugated to calicheamicin (cytostatic)	leukemia (AML)	therapy
Myoscint	imciromab pentetate RllDl0	myosin heavy chain	murine Fab, 111In conjug.	myocardial infarction	imaging
Neutrospec	fanolesomab (RB5)	CD15 granulocytes	murine IgM, 99Tcm conjug.	Inflammation, infection	imaging
OncoScint Oncorad	satumomab (B72.3n)	TAG-72 (CA72-4) / sialyl Tn	murine, 111In conjugate	breast, pancreatic, colorectal, ovarian, lung, endometrial cancer mesothelioma	imaging

Orthoclone	muromonab (OKT3)	CD3 (T cells)	murine	transplantation, rheumatoid arthritis	therapy
Panorex	edrecolomab (17-1A)	Ep-CAM	humanised	colon cancer	therapy (Germany)
ProstaScint	capromab (7Ell-C5.3)	PSA, PSMA	Murine, glycyl-tyrosyl-lysyl-DTPA konjugated	prostatic cancer	imaging
Raptiva	efalizumab (hu-1124)	CD2a	humanised	psoriasis, rheumatoid arthritis transplantation, autoimmunity	therapy
Remicade	infliximab (cA2)	TNF-alpha	chimeric	Crohn's disease, psoriasis Rheumatoid arthritis, asthma, Ankylosing spondylitis Wegener's granulomatosis,	therapy
ReoPro	abciximab (7E3)	gp lIb/IlIa Receptor complex (platelets)	chimeric Fab	Restenosis, heart ischemic complication	therapy
Rituxan	rituximab	CD20 (B cells)	chimeric	Non-hodgkin Lymphoma Rheumatoid arthritis,	therapy
Simulect	basiliximab (CHB-201)	CD25	chimeric	transplantation	therapy
Synagis	palivizumab (1129)	F protein of RS virus	humanised	RSV infection in infants	therapy
Tysabri	natalizumab (HP2/1)	Integrin a4-ß (T cells)	humanised	multiple sclerosis	therapy
Verluma	nofetumomab merpentan NR-LU-l0	ca. 40kDa glycoprotein	murine Fab, 99mTc conjug.	lung, colon, pancreatic, breast, ovarian, renal, gastric cancer	imaging
Xolair	omalizumab (rhuMAb-E25)	human IgE Fc	humanised	asthma, autoimmunity, peanut allergy	therapy
Zenapax	daclizumab (anti-Tac-H)	CD25	humanised	transplantation, asthma, autoimmunity, inflammation, multiple sclerosis	therapy
Zevalin	imbritumomab (C2B8)	CD20	chimeric	lymphoma (NHL), autoimmunity, transplantation, rheumatoid arthritis	therapy

Table 2 Approved Antibody Therapeutics. Please note that a listing does not indicate that the antibodies are still available, approved or in use at date of print! Quite a number of products, inclusing most of the radiolabelled mouse antibodies, have been withdrawn from the market or suspended. Some were approved by other bodies than the U.S. FDA (e.g. Panorex or Cotara).

* Trade names are Copyright of distributing companies.

** please see addendum for nomenclature.

*** chimeric / humanised indicates murine origin of V regions /CDRs, respectively, if not stated otherwise.

intense research going on worldwide into "-omics" and systems biology, which is intended to lead to a mathematical interaction model for all involved factors. Only then, and with quite a number of years to go, will there be a chance to better predict adverse effects of novel drugs and combination therapies on a truly rational basis.

1.4 The Gleaming Horizon

Despite all of the recent success stories of recombinant human-like IgGs, they do not mark the end of the development, but just the start. As we understand more and more of the complex molecular interactions between immune cells or in cancer tissues, and in expectation of a significant speed-up of knowledge gain from the "-omics" and systems biology approaches, we can endeavor to expand the design limits of an antibody drug. All approved drugs are based on IgG molecules close to the naive structure of the antibodies in our bloodstream (a few on the IgG–fragment Fab), sometimes conjugated to an effector or label. But this can only be the first step of engineering applied to this fascinating molecule. For example, by engineering the Fc glycosylation, dramatic improvements in efficiency can be obtained (Jefferis 2005).

Further, we should learn from nature by looking at the modular design it has used to create the highest diversity group of proteins from repeats of slightly changed domains with a single common basic structure (immunoglobulin fold). We can be inspired to utilize this modular approach for completely novel molecular designs. This has already successfully been attempted since the early 1990s, and led to a plethora of novel molecular designs. It allowed the creation of bispecific antibodies (see Chapter 2, Vol II), the adjustment of the size for optimal pharmacokinetics (Hu et al. 1996), and the addition of functions that nature does not provide with an IgG at all (see Chapters 1 to 3, Vol II). Unfortunately, clinical results with these new designs are sparse and frequently disappointing, although this may simply reflect the fact that the molecular design of today is still rather a result of trial and error than of an understanding of the underlying mechanisms, or is even dictated mainly by the developer's patent portfolio. Neverthless, this will change as well; an example is immunotoxins, which failed in many clinical studies for more than three decades, before novel concepts and acknowledgement of the vast body of knowledge collected thoughout this time brought optimization and new ideas (see Chapter 3, Vol II). Fascinating concepts are under evaluation in hundreds of labs. There are so many ideas and so many parameters affecting therapeutic efficiency to be learned about that the development of antibody therapeutics will not reach saturation for any foreseeable time. Furthermore, major technology patents which have blocked some developments in the past will expire in the foreseeable future (see Chapter 13). Gíven that only the first generation of antibody drugs just has been approved in significant numbers, given the availability of recombinant human antibodies and the number

of targets in the developer's pipelines plus the advent of completely novel therapeutic strategies using antibody fusion proteins, therapeutic antibodies look forward to a golden future.

Further Reading

Benny, K.C. (ed.) (2003) *Antibody Engineering: Methods and Protocols (Methods in Molecular Biology)*. Totowa, NJ: Humana Press (collection of relevant protocols).

Breitling, F., Dübel, S. (1999) *Recombinant Antibodies*. New York: John Wiley and Sons (introduction to basic principles and applications).

Brekke, O.H., Sandlie, I. (2003) Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nat Rev Drug Discov* 2: 52–62.

Carter, P.J. (2006) Potent antibody therapeutics by design. *Nat Rev Immunol* 6: 343–357.

Chowdhury, P.S., Wu, H. (2005) Tailor-made antibody therapeutics. *Methods* 36: 11–24.

Gavilondo, J.V., Larrick, J.W. (2000) Antibody engineering at the millennium. *Biotechniques* 29: 128–132.

Groner, B., Hartmann, C., Wels, W. (2004) Therapeutic antibodies. *Curr Mol Med* 4: 539–547.

Haurum, J., Bregenholt, S. (2005) Recombinant polyclonal antibodies: therapeutic antibody technologies come full circle. *IDrugs* 8: 404–409

Hudson, P., Souriau, C. (2003) Engineered antibodies. *Nature Med* 9: 129–134.

Huston, J.S., George, A.J. (2001) Engineered antibodies take center stage. *Hum Antibodies* 10: 127–142.

Kontermann, R., Dübel, S. (ed.) (2001) *Antibody Engineering*. Heidelberg, New York: Springer (collection of relevant protocols).

Monoclonal Antibodies and Therapies. Supplement of *Nat Biotechnol*, November 2004 (collection of reviews).

Monoclonal Antibody Index (MAI), Gallart Biotech SL, 2005 (collection of data on therapeutic antibodies, updated regularily).

Presta, L.G. (2002) Engineering antibodies for therapy. *Curr Pharm Biotechnol* 3: 237–256.

Subramanian, G. (ed.) (2004) *Antibodies: Volume 2: Novel Technologies and Therapeutic Use*. Heidelberg, New York: Springer.

Urch, C.E., George, A.J.T. (eds) (2000) *Diagnostic and Therapeutic Antibodies (Methods in Molecular Medicine)*. Totowa, NJ: Humana Press.

References

Bayes, M., Rabasseda, X., Prous, J.R. (2005) Gateways to clinical trials. *Methods Find Exp Clin Pharmacol* 27: 569–612.

Behring, E., Kitasato, S. (1890) Über das Zustandekommen der Diphterie-Immunität und der Tetanus-Immunität bei Thieren. *Dt Med Wschr* 16: 1113–1114.

Buelow, R., Puels, J., Hansen-Wester, I., Thorey, I., Schueler, N., Siewe, B., Niersbach, H., van Schooten, W., Platzer, J. (2006) Expression of a human antibody repertoire in transgenic rabbits. *Human Antibodies* 15: 19–23.

Ehrlich, P. (1880) Methodologische Beiträge zur Physiologie und Pathologie der verschiedenen Formen der Leukocyten. *Z Klin Med* 1: 553–560.

Ehrlich, P. (1908) Partial cell functions. Nobel Lecture, December 11: 1908.

Hu S.Z., Shively, L.E., Raubitschek, A., Sherman, M., Williams, L.E., Wong, J.Y.C., Shively J.E., Wu A.M. (1996) Minibody: a novel engineered anti-carcinoembryonic

antigen antibody fragment (single-chain Fv-C_H3) which exhibits rapid, high-level targeting of xenografts. *Cancer Res* 56: 3055–3061.

Jefferis, R. (2005) Glycosylation of recombinant antibody therapeutics. *Biotechnol Prog* 21: 11–16.

Kleinschmidt-DeMasters, B.K., Tyler, K.L. (2005) Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis. *N Engl J Med* 353: 369–374.

Rutgeerts, P.J., Targan, S.R, Hanauer, S.B., Sandborn, W.J. (2004) Challenges in Crohn's disease: the role for current and future TNF antagonists. www.medscape.com/ viewprogram/3261_pnt

Reichert, J.M. (2001) Monoclonal antibodies in the clinic. *Nat Biotechnol* 19: 819–822.

Reichert, J.M. (2002) Therapeutic monoclonal antibodies: trends in development and approval in the US. *Curr Opin Mol Ther* 4: 110–118.

Reichert, J.M., Rosenzweig, C.J., Faben, L. B., Dewitz, M.C. (2005) Monoclonal antibody successes in the clinic. *Nat Biotechnol* 23: 1073–1078.

Rohrbach, P., Broders, O., Toleikis, L., Dübel, S. (2003) Therapeutic antibodies and antibody fusion proteins. *Biotechnol/ Genet Eng Rev* 20: 129–155.

Part I
Selecting and Shaping the Antibody Molecule

2
Selection Strategies I: Monoclonal Antibodies

Gerhard Moldenhauer

2.1
Introduction

Since the late nineteenth century and the days of Paul Ehrlich, who considered antibodies as "magic bullets" (Ehrlich, 1900), immunologists have been attracted by the idea of destroying tumor cells with antibody molecules alone or conjugates made up of such molecules. Emil von Behring and Shibasaburo Kitasato were the first to demonstrate the efficacy of a heterologous polyclonal antiserum directed against the exotoxin produced by *Corynebacterium diphtheria* ("Diphtherie-Heilserum"), thereby saving the lives of many children (von Behring and Kitasato 1890). Although similar attempts were subsequently made to employ antisera for tumor treatment, the outcomes were less successful. With the advent of monoclonal antibody (mAb) technology 30 years ago, there was renewed enthusiasm for the development of a modern immunotherapy for cancer. This promise was fulfilled, however, only in some rare cases of non-Hodgkin's lymphomas (Levy and Miller 1990). The application of mouse mAbs for therapy has raised several problems. One major drawback was, for instance, that mouse antibodies are not usually able to activate human immunological effector functions as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). The formation of a human anti-mouse antibody (HAMA) response after repeated injection constitutes another reason for the low response rates observed (Khazaeli et al. 1994; DeNardo et al. 2003).

A breakthrough was achieved in 1997 with the approval of the chimeric (mouse/human) rituximab antibody for the treatment of relapsed/refractory low-grade non-Hodgkin's lymphomas by the US Food and Drug Administration (FDA) (McLaughlin et al. 1998). During the past decade molecular biology has provided the means to create chimeric, humanized or fully human antibodies for therapy. To date, eight antibody-based cancer therapeutics have been approved and are on the market. They comprise unmodified antibodies as well as conjugates with toxins or radionuclides. Thus, antibody engineering finally has led to a renaissance of antibody-guided tumor therapy. The new reagents can interact with

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human effector molecules and have been shown to synergize with or even substitute for conventional chemotherapeutic regimens (reviewed by Glennie and van de Winkel 2003; Brekke and Sandlie 2003; Ross et al. 2004; Stern and Herrmann 2005; Adams and Weiner 2005).

In this chapter I describe the principles of mAb creation by somatic cell hybridization, give an overview on frequently used screening procedures and modifications of the antibody molecule and finally outline the mechanisms by which antibodies exert their effector functions. For the sake of clarity, I have restricted my viewpoint mainly to the field of cancer research and oncology/hematology.

2.2 Historical Remarks

The immune system is capable of generating about 10^{11} to 10^{12} different antibody molecules. An individual B lymphocyte, however, synthesizes only the one distinct antibody it is genetically programmed for. How this enormous antibody repertoire is generated was a central question of immunology over the past 50 years. Starting from the natural clonal selection theory of antibody formation (Jerne 1955) it became obvious that two mechanisms affecting the immunoglobulin genes are of paramount importance: rearrangement of gene segments and somatic hypermutation. To study somatic mutations of antibody genes in more detail, an antibody-secreting cell line recognizing the same antigen was urgently needed. This was the aim of experiments performed by Georges Köhler and César Milstein, leading to the discovery of hybridoma technology for the production of mAbs (Köhler and Milstein 1975). One of the major methodological advances in biology and medicine, this work was honored by a Nobel Price in 1984, was achieved by answering an academic question (Milstein 1999). Recently, the immunologist Klaus Eichmann has published an interesting book on the scientific and historical background leading to the invention of hybridoma technology (Eichmann 2005).

Hybridoma production basically relies on the fusion of immunized lymphocytes from an experimental animal with immortal myeloma cells. The resulting cell hybrid contains the genetic material of both parents. From the tumor cell the hybrid acquires the capacity for indefinite growth while the B lymphocyte confers the ability to synthesize a specific antibody. After stabilization by repeated cell cloning, hybridomas produce fairly large quantities of identical mAb for years (Fig. 2.1). The early development of the method is documented in a couple of scientific anthologies (Melchers et al. 1978; Kennett et al. 1980; Hämmerling et al. 1981). Importantly, the first patient suffering from non-Hodgkin's lymphoma was treated in 1979 with a mouse mAb at the Dana Farber Cancer Center in Boston (Nadler et al. 1980). Taken together, mAbs have led to a revolution in basic sciences, medicine and industry during the past 30 years.

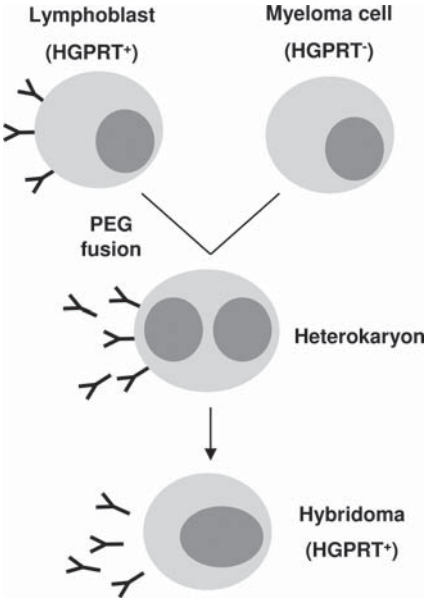

Fig. 2.1 Principle of hybridoma production by cell fusion. Both parents confer their key functions to the hybrid: production of an individual antibody (lymphoblast) and indefinite growth (myeloma cell). Introduction of a selectable marker (HGPRT) allows only hybridoma cells to proliferate.

2.3 Antibody Structure and Function

2.3.1 Membrane-bound and Secreted Forms of Antibodies

The basic structure of an IgG antibody has been elucidated as a symmetric monomer consisting of two identical heavy chains and two identical light chains, connected via disulfide bonds (Cohen and Porter 1964; Edelman et al. 1969). Five classes of immunoglobulins can be distinguished according to their distinct heavy chains: IgG, IgM, IgD, IgA, and IgE. Furthermore, each antibody contains one type of light chain, kappa or lambda. Both, heavy and light chains harbor a variable region of 110 amino acids at the N-terminus with three hypervariable segments called complementarity determining regions (CDRs). The hypervariable loops form the two antigen binding (or antigen-combining) sites of an IgG molecule and determine its specificity. In contrast, the constant part of the immunoglobulin (named the Fc portion) is responsible for secondary effects like activation of the complement system or binding to cellular Fc receptors (Fig. 2.2).

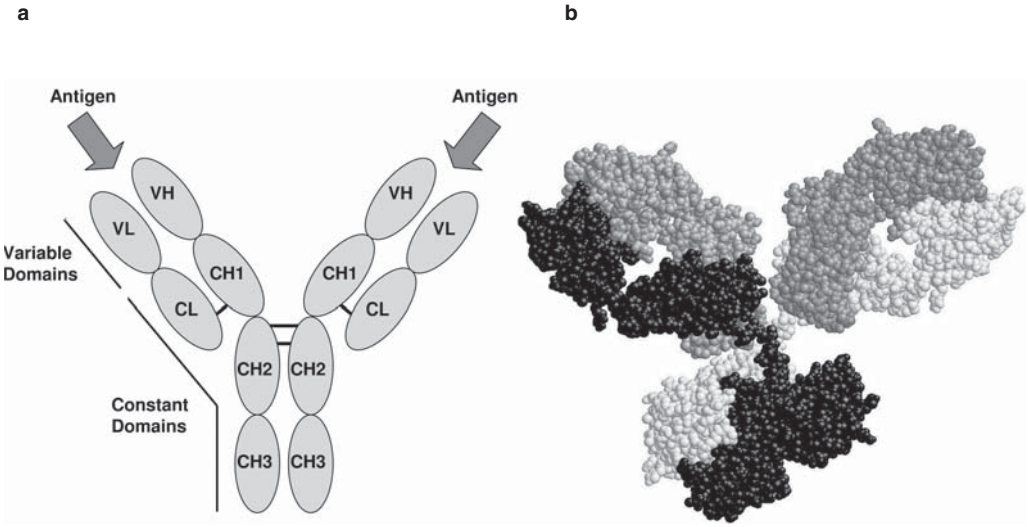

Fig. 2.2 Structure of an IgG molecule. The Y-shaped simplified representation in (a) shows the functional domains as well as the two antigen-binding sites. A more realistic space-fill model is depicted in (b) (courtesy of Dr C.W. von der Lieth, DKFZ Heidelberg).

Importantly, immunoglobulins display a dual function since they are exposed on the surface membrane of B lymphocyte as antigen receptors and are also secreted by plasma cells. They circulate in the blood and other body fluids and are able to bind, neutralize and eliminate foreign antigens, such as viruses, bacteria or toxins. Antibodies represent the effector molecules of the humoral immune system. Transmembrane and secreted forms of an antibody are generated by differential splicing of a primary transcript RNA. In the course of an ongoing immune response specific B lymphocytes undergo isotype switching, that is the transition of the IgM^+ and IgD^+ phenotype to IgG surface and secreted immunoglobulin. In addition, point mutations of the rearranged heavy and light chain variable genes occur in response to T lymphocyte signaling, giving rise to somatic hypermutation necessary to increase antibody affinity. These processes take place in the germinal centers of secondary lymphoid tissues such as lymph node and spleen. The enzyme activation-induced cytidine deaminase (AID) has been identified as a key player for both class switching and affinity maturation (Barreto et al. 2005). Detailed information on how an effective antibody response to antigen develops at a molecular level can be looked up in one of several excellent textbooks of immunobiology (Roitt et al. 2001; Abbas and Lichtman 2003; Janeway et al. 2005).

Immunoglobulin G (IgG, 150 kDa) is the most abundant immunoglobulin in serum, accounting for up to 80% of all secreted antibodies. There are four dif-

ferent IgG isotypes in humans (IgG1, IgG2, IgG3, and IgG4) and mouse (IgG1, IgG2a, IgG2b, and IgG3). Human IgG1 and IgG3 antibodies are potent activators of the complement system and also bind with high affinity to Fc receptors on phagocytic cells, resulting in ADCC. Immunoglobulin M (IgM, 900 kDa) accounts for approximately 10% of serum antibodies. It is expressed as monomer on B lymphocyte as antigen receptor, whereas the secreted form consists of a pentamer hold together by a J (joining) chain. Immunoglobulin D (IgD, 180 kDa) is only found in fairly low amounts in the serum but is, together with IgM, the major membrane-bound form expressed on mature B cells. Immunoglobulin A (IgA, 160 kDa) constitutes about 10–15% of serum antibodies. It represents the major antibody class, being secreted into tears, saliva, and mucus of the bronchial, genitourinary, and digestive tracts. While the prevalent form in the serum is a monomer, the secretory IgA usually consists of dimers covalently connected via J (joining) chains together with an additional polypeptide called secretory component. Immunoglobulin E (IgE, 180 kDa) is found in the serum only in trace amounts. IgE antibodies are responsible for immediate hypersensitivity and cause the symptoms of hay fever, asthma and anaphylactic shock. Mast cells and basophils bind IgE via Fc receptors and subsequent contact with an allergen will cause degranulation and release of histamine and other mediators.

2.3.2 Monoclonal Antibodies

The key feature of an mAb is its unique specificity. It recognizes only one particular antigenic determinant (called epitope) on a given molecule – that means it is monospecific. All antibodies secreted by an individual hybridoma represent identical immunoglobulin molecules that display identical binding strengths to its antigen (referred to as affinity) and have identical physicochemical properties (isotype, stability). This homogeneity will give rise to the same immunological effector functions. In principle, mAbs can be produced in unlimited quantities, the hybridoma cell itself survives after cryopreservation at least for decades.

When compared with polyclonal antisera (for instance from rabbit) the affinity of mAbs might sometimes be inferior. Because mAbs consist of homogeneous molecules of the same isotype they may not elicit certain biological responses. The majority of monoclonals are directed against conformational epitopes of an antigen and may lose reactivity when tested on denatured samples, for instances by Western blotting or by immunohistology on paraffin sections. In common with polyclonal reagents, mAbs may show unexpected cross-reactivity with antigens being expressed in unrelated tissues.

Being glycoproteins, antibodies are potent immunogens when injected into another species. There are three different types of antigenic determinants against which an immune response can be induced: anti-isotype, anti-allotype, and anti-idiotype. If a human being is injected several times with a mouse mAb, it is very likely a HAMA response will develop (Khazaeli et al. 1994; DeNardo et al. 2003).

2.4 Production of Monoclonal Antibodies

2.4.1 Immunization

The aim of an immunization is to elicit a strong immune response against a certain antigen.

For mAb production, most commonly mice and rats and less frequently hamsters and rabbits are immunized with antigen by distinct routes of administration. Antigen may consist of cellular components, purified proteins, peptides, carbohydrates, lipids, or nucleic acids and for each of these specific immunization protocols are available (Harlow and Lane 1988; Goding 1996; Coligan et al. 2004). The purity of the antigen used for immunization plays a major role in the outcome of antibody response. If rather impure preparations are used, problems may arise from the possible immunodominance of contaminants. This might occur when complete cells are employed as immunogen; on the other hand intact cells are highly immunogenic. Molecular biology allows the expression of fusion proteins in eukaryotic cells, a method that has largely improved the preparation of immunizing agents.

Especially for mounting an immune response against soluble antigens, the use of a strong adjuvant is highly recommended. Adjuvants are nonspecific stimulators of the immune system, the most famous representative being Freund's complete adjuvant (Freund 1956). This consists of mineral oil and inactivated *Mycobacterium tuberculosis* particles. When mixed with immunogen, a water-in-oil emulsion is prepared that allows the release of antigen over a long period of time. The mycobacteria give rise to an inflammatory response with the production of numerous cytokines. In general, for the first immunization it is appropriate to use a strong adjuvant (complete Freund's or *Bordetella pertussis*), whereas the second injection and following challenges can be given with incomplete Freund's (mineral oil only) or without any adjuvant. By repeated immunization the response of the animal is shifted against high-affinity antibodies of IgG isotype. Since previously activated lymphoblasts show preferential fusion with myeloma cells, the final booster immunization should be given 3 days prior to fusion to maximize the yield of hybrids.

If the amount of chosen protein antigen is short or if the antigen is not available at all, intrasplenic immunization with minute amounts of antigen (Spitz et al. 1984; Grohmann et al. 1991) or DNA immunization (Barry et al. 1994) might offer alternative approaches. During the past years a couple of useful antibodies were produced by immunizing with synthetic peptides coupled to immunogenic carriers like keyhole limpet hemacyanin (KLH) or bovine serum albumin (BSA). For this, only the amino acid sequence of the protein has to be known, which can be found in several databases. Coupling of peptides carrying an N- or C-terminal cysteine residue to the carrier is achieved by *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester or another heterobifunctional crosslinker (Green et al. 1982). One

drawback of the method is that such anti-peptide antibodies often exclusively react with the denatured but not with the native protein. *In vitro* immunization, invented to prime naive lymphocytes in cell culture, did not fulfill the expectations because in most instances solely an IgM response was induced (Borrebaeck 1983).

2.4.2 Myeloma Cell Lines

Multiple myeloma or plasmacytoma represents a malignancy of plasma cells in which large numbers of antibody-secreting cells residing in bone marrow are produced. They secrete monoclonal immunoglobulin, the specificity of which is usually not known, therefore they are regarded as "antibody without antigen." All available mouse myeloma lines for fusion are derived from the MOPC-21 tumor that has been induced in BALB/c mice by mineral oil injection into the peritoneal cavity (mineral oil-induced plasmacytoma) and was then adapted to growth in tissue culture. While the very first hybridomas were made with myeloma fusion partners that endogenously secreted complete antibody, later on loss variants were selected producing solely kappa light chains (e.g. P3-NS1-Ag4-1; Köhler et al. 1976) or no immunoglobulin. Such nonproducer lines are mostly used for cell fusion these days, prominent examples being X63-Ag8.653 (Kearney et al. 1979), Sp2/0-Ag-14 (Köhler and Milstein 1976) and F0 (Fazekas de St. Groth and Scheidegger 1980).

Similarly, for the production of mAbs against mouse antigens some rat myeloma lines have been established from the LOU/C strain. Frequently used are Y3-Ag1.2.3 (secreting kappa light chains; Galfrè et al. 1979) and line IR983F (nonproducer; Bazin et al. 1990). Since rats are not that much easier to handle than mice and since rat hybridomas are sometimes dependent on growth factors, making cell culture more complicated, interspecies hybrids have been constructed. For this, immune rat spleen cells were fused with a murine nonsecretor myeloma cell line. These rat/mouse hybrids turned out to be stable and secreted amounts of mAbs comparable to mouse/mouse hybridomas (Ledbetter and Herzenberg 1979). Mouse interspecies hybridomas have also been created with hamster and rabbit lymphoblasts to obtain respective mAbs (Sanchez-Madrid et al. 1983; Raybould and Takahashi 1988). A more advanced method for the production of mouse antibodies against mouse antigens is the use of knockout mice for immunization. Since they lack expression of the target antigen they are not tolerant and are able to mount a normal immune response.

2.4.3 Cell Fusion

In early experiments cell fusion was facilitated by means of agglutinating viruses like Sendai. The introduction of polyethylene glycol (PEG) as a fusing agent (Pontecorvo 1975) has simplified the procedure drastically and is used throughout

the field today. PEG renders the membrane of cells to be fused gluey, so that they stick together. Subsequently plasma membrane fusion occurs, giving rise to a cell with two (or more) nuclei called a heterokaryon. During cell division the nuclear membranes are degraded and the chromosomes are distributed into the daughter cell. These hybrid cells contain only one nucleus but the genetic material of both parents and are named synkaryons. The double set of chromosomes in hybrids causes genetic instability during further mitoses, leading to improper segregation or loss of chromosomes. If chromosomes coding for immunoglobulin heavy or light chain genes (in the mouse chromosomes 6, 12, and 16) are affected, antibody secretion of this hybrid will ultimately stop. To prevent overgrowth of early hybridoma culture by nonproducing variants, immediate cloning of cultures is mandatory. Following many cell divisions the hybrid line is stabilized in its chromosomal inventory. However, prior to mass production of hybridoma cells and in case the antibody titer of culture supernatant declines recloning should be performed (Fig. 2.3).

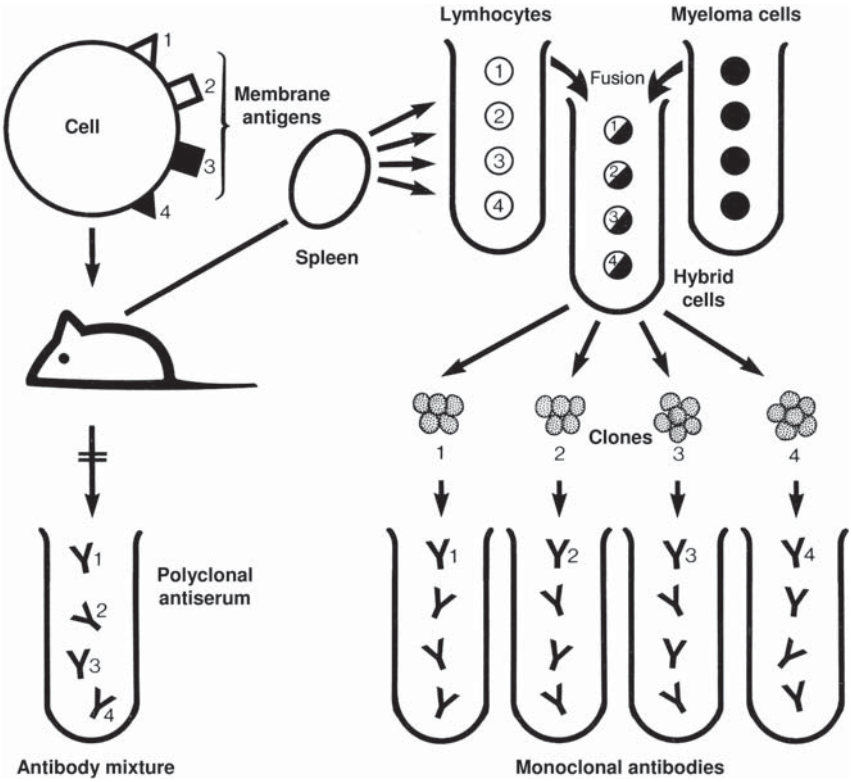

Fig. 2.3 Generation of monoclonal antibodies. Spleen cells from an immunized mouse are fused with myeloma cells to obtain hybridomas. After reduction to individual cell clones they secrete monoclonal antibodies of defined specificity.

Factors critically influencing the outcome of a fusion experiment are the choice of fetal calf serum (FCS) and the health status of the immunized mice. Depending on the content of the growth-promoting constituents, some FCS batches evolve better suited for hybridoma growth than others and it is worth testing for an optimal one. Bacterial and viral infections of rodents can cause severe immune suppression and will lead to low yields of hybridomas. Some protocols recommend the use of feeder cells as a source of growth factors as well as lysis of erythrocytes present in spleen cell preparations from immunized animal prior to fusion. In addition, repeated medium changes to remove potentially harmful substances derived from dying cells have been suggested. In my experience a simplified method works best, leaving the fused cells as untouched as possible. If a spleen from an immunized mouse (containing approximately 1–1.2 $\times$ 10^8 lymphocytes) is fused with an equal number of myeloma cells and the fusion mixture is distributed into 15–20 96-well microtiter plates (1440–1920 individual cultures), cell density is high enough to support hybridoma growth even without feeder cells and medium exchange (Fig. 2.4).

As an alternative to the PEG procedure described, electrically induced cell fusion has been developed. It is based on the delivery of high-voltage electrical field pulses to physically fuse lymphoblasts and myeloma cells (Ohnishi et al. 1987; Schmitt and Zimmermann 1989). More recently, a method was published

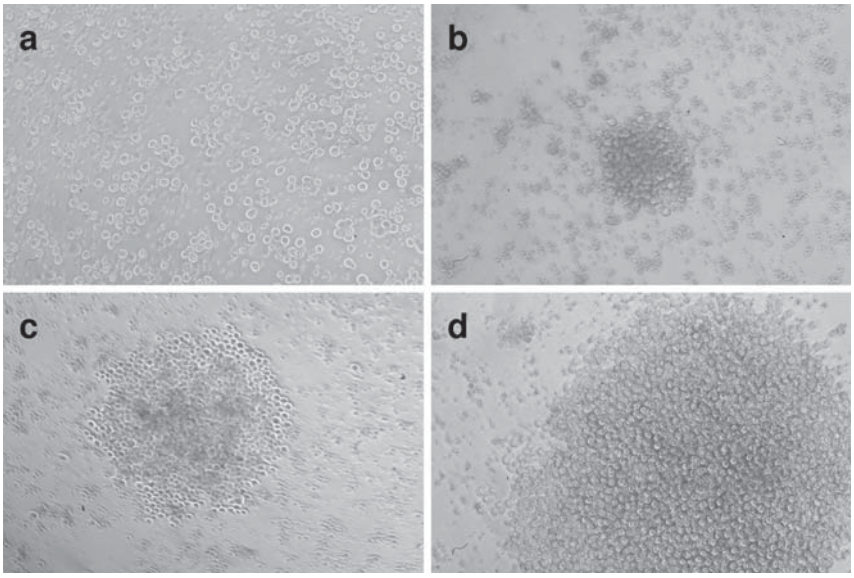

Fig. 2.4 Time course of hybridoma development visualized by phase-contrast light microscopy. Fusion mixture is plated on day 0 (a) and early hybridomas became visible after 4–6 days (b). At day 6–10 hybridomas grow vigorously (c) and are ready for screening at day 10–14 (d).

allowing the production of mAbs without hybridomas by using transgenic mice harboring a mutant temperature-sensitive simian virus-40 large tumor antigen (Pasqualini and Arap 2004).

2.4.4 Drug Selection of Hybridomas

During somatic cell hybridization only a small number of cells will actually fuse and only a minor proportion of them will develop into hybridomas (in the range of 1 in 10^5). Consequently, the culture is rapidly overgrown by myeloma cells or myeloma–myeloma fusions that also occur. This makes the introduction of a drug selection system indispensable. To accomplish this, enzyme-deficient myeloma lines are employed for cell fusion. In the system described by Littlefield (1964) the myeloma lacks the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). If the main biosynthetic pathway for purine and pyrimidine nucleotides required for both DNA and RNA synthesis is blocked by folic acid antagonists like aminopterin, cells can survive using an alternative "salvage pathway" that requires the enzymes HGPRT and thymidine kinase (Fig. 2.5). If the fusion mixture is cultured in medium containing hypoxanthine, aminopterin, and thymidine (HAT medium) only hybrids can actively grow. Normal spleen cells from the immunized mouse will die spontaneously *in vitro*. HGPRT-deficient myeloma cells undergo cell death in the presence of aminopterin since they cannot use the

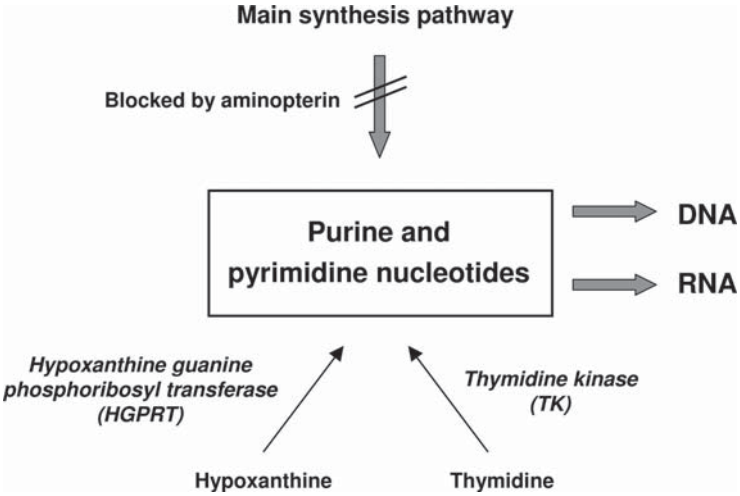

Fig. 2.5 Chemical selection of hybridomas. While the main pathway for synthesis of purine and pyrimidine nucleotides is blocked by the folic acid antagonist aminopterin, cells are forced to use the salvage pathway that requires the enzymes hypoxanthine guanine phosphoribosyl transferase (HGPRT) and thymidine kinase (TK).

salvage pathway. Hybridomas having acquired HGPRT from immune lymphoblast represent the only cell type that continues to proliferate.

HGPRT-negative myeloma mutants can be selected by culturing the cells in the presence of toxic purine analogs like 8-azaguanine or 6-thioguanine. Cells harboring the enzyme are killed after incorporation of the toxic nucleotide. Spontaneously arising mutants can be simply established because the enzyme is encoded on the X chromosome and only one gene locus has to be targeted.

2.4.5 Screening Hybridoma Cultures for Specific Antibody

Establishment of a reliable, sensitive, and fast screening assay for the detection of desired mAb is the most important prerequisite for successful hybridoma production. It is not recommended to start a fusion experiment before an appropriate screening assay has been set up. There are numerous different test types available for the initial screening that all are based on the measurement of antigen–antibody binding. Taking blood from the immunized animal for instance by puncture of the retro-orbital venous plexus will provide a serum sample that is very useful to establish a sophisticated assay system for screening.

During the decade following invention of hybridoma technology many laboratories have successfully applied solid-phase and cellular radioimmunoassay using ^{125}I-iodine-labeled second step antibodies or protein A (Bjork et al. 1972). This method as well as laborious rosette techniques employing sheep red blood cells as indicator for antibody binding are only of historical interest today. The most frequently performed tests for early hybridoma screening are discussed below. Of course much more tailor-made assays are now established to identify mAbs with special features, such as antibodies reacting with sugar or glycolipid epitopes, antibodies working in Western blotting or neutralizing antibodies against a bacterial toxin or a virus.

2.4.5.1 Enzyme-linked Immunosorbent Assay (ELISA)

Solid-phase ELISA (Engvall and Perlman 1971), where the antigen is immobilized on the well of a microtiter plate, represents a universal test system that can easily be customized and allows rapid analysis of many samples in parallel. Special ELISA plates (the surfaces of which are specifically treated) are coated first with (semi-) purified antigen or peptide that attaches to the plastic surface by adhesive forces. To prevent nonspecific binding plates are then blocked by incubation with a gelatin or bovine albumin solution. Plates can thus be stored in the cold for month. Hybridoma supernatants are allowed to react with immobilized antigen for a while. If the supernatant contains specific antibody it will be strongly retained by its corresponding antigen whereas all other contaminating proteins are subsequently washed away. Next, bound mAb is detected by an enzyme-labeled second step reagent, usually anti-mouse immunoglobulin linked to horseradish peroxidase or alkaline phosphatase, that attaches to the already formed

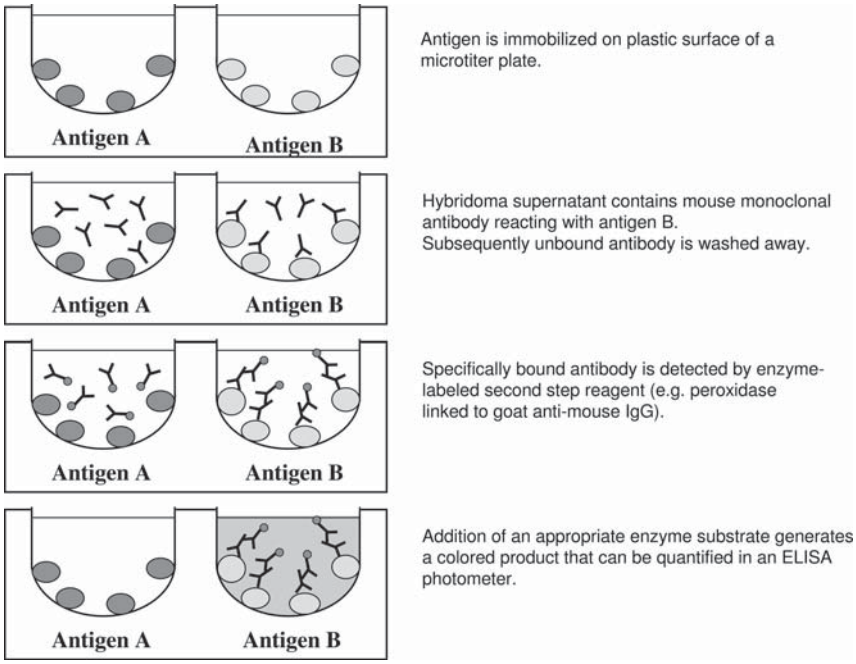

Fig. 2.6 Solid-phase ELISA for antibody screening.

antigen–antibody complex. Finally, the bound conjugate is visualized by a substrate reaction in which a colorless substrate is enzymatically converted into a dye. The reaction can be quantified by an ELISA photometer measuring the appropriate optical density in each well of the plate (Fig. 2.6).

ELISA is also the method of choice to determine the heavy and light chain subclasses of hybridoma antibodies. For this purpose, isotype-specific, enzyme-labeled secondary antibodies are supplied by commercial sources. Isotype-specific enzyme immunoassay was also engaged to select for isotype switch variants of hybridomas. The idea behind it implies that hybridoma clones at rather low frequency spontaneously perform class switching to the subsequent isotype encoded by the heavy chain immunoglobulin gene locus. These rare variants can be traced, enriched, and established by limiting dilution in combination with isotype-specific ELISA (Spira et al. 1984). Many variations of standard indirect ELISA (with immobilized antigen), such as sandwich and competitive ELISA, were suggested, allowing an increase in sensitivity that under normal conditions is not necessary. In addition, for the detection of antibodies directed against cell membrane-exposed antigens cellular ELISA protocols have been developed (Feit et al. 1983). These are especially suitable if a homogeneous cell population, as for instance a gene transfected cell line, is available for screening.

2.4.5.2 Flow Cytometry

The invention of the fluorescence-activated cell sorter (FACS) was a hallmark in the analysis of cell membrane antigens detected by polyclonal antibodies in the beginning and subsequently by mAbs (Bonner et al. 1972, Parks and Herzenberg 1984). First a cell suspension (for instance white blood cells) is incubated with hybridoma supernatant. Depending on the nature of the antigen recognized, a certain proportion of cells will bind the respective antibody to which a second layer, a fluorescently labeled anti-immunoglobulin (e.g. goat anti-mouse IgG coupled to fluorescein or phycoerythrin), is added. Single cells are then passed through a laser beam leading to excitation of the fluorochrome. Taking advantage of highly sophisticated optical and electronic devices, emitted fluorescence from stained cells is measured by a photomultiplier. Usually forward and side scatter signals providing information on the size and granularity of cellular subpopulations are also monitored. Dead cells can be discriminated in parallel by propidium iodide, a fluorescent dye that stains DNA and that is only taken up by injured cells.

In essence, fluorescence-activated cell analyzers and cell sorters provide extremely valuable tools for the rapid, reliable and quantitative screening of antibodies interacting with cell surface receptors. In addition, flow cytometry offers a broad spectrum of applications in immunology, cell biology, and other disciplines. Modern instruments equipped with argon and krypton lasers make the simultaneous use of multiple antibody–fluorochrome conjugates possible, thus allowing multi-parameter analysis of cellular subsets.

2.4.5.3 Immunohistology and Immunocytology

If morphological aspects play a major role in antibody screening immunohistology and immunocytology may provide appropriate methods. Several techniques have been advised to fix tissue or cell suspensions. In traditional immunohistology formalin-fixed and paraffin-embedded sections are mostly used. Unfortunately, many mAbs raised against protein antigens will not work with such material due to the destruction of native conformation by the fixation process. On the other hand, antibodies raised against protein-derived peptides are likely to react with paraffin sections because they often recognize linear epitopes of the antigen. Frozen sections fixed with acetone are well suited for initial antibody screening. Likewise, cells to be used for immunocytology should be fixed with acetone and subsequently air-dried. For this, adherent cells can be grown on coverslips, whereas cells growing in suspension are immobilized on a glass slide using a cytocentrifuge. The staining procedure is reminiscent of that described for ELISA. Briefly, it consists of a first incubation with the hybridoma-derived antibody followed by a secondary enzyme- or less frequently fluorochrome-labeled reagent. If the hybridoma secrets a mouse mAb, a secondary reagent like goat anti-mouse IgG conjugated to alkaline phosphatase or horseradish peroxidase may be applied. The reaction's sensitivity can be enhanced by employing preformed complexes consisting of enzyme and anti-enzyme antibodies (APAAP,

alkaline phosphatase anti-alkaline phosphatase and PAP, peroxidase anti-peroxidase). Another option for signal augmentation is the use of biotinylated secondary reagents that exhibit high-affinity binding to streptavidin linked to the enzyme. In contrast to ELISA, for immunohistology and immunocytology an insoluble substrate is needed that forms an insoluble colored precipitate at the site where the antibody has bound.

2.4.5.4 **Cytotoxicity Assays**

In case antibodies needs to be selected that do not only bind to a particular cell type but in addition should fix complement, a screening assay for cytotoxicity may be performed. This type of test, aimed at tracing CDC, is based on the measurement of cell membrane leakiness following complement attack. Target cells are plated in microtiter wells and mixed with hybridoma supernatants together with a source of complement proteins like rabbit or guinea-pig serum. If the antibody under investigation is able to activate the complement cascade, cell lysis will occur within minutes to hours. Killed target cells can be microscopically visualized by addition of dyes such as trypan blue or acridine orange. For quantitative evaluation either the chromium release test (that requires initial labeling of target cells with radioactive chromium-51) or flow cytometric staining with propidium iodide may be applied.

Similar assays have been established to detect antibodies capable of inducing ADCC. Here, target cells are lysed by Fc receptor-bearing effector cells (mainly natural killer (NK) cells and monocytes) that are attracted by and interact with the Fc portion of the antibody bound to the target cell (Sondel and Hanks 2001).

In very rare instances, antibody binding per se can cause target cell destruction. One prominent example is the crosslinking of certain death receptors such as CD95 exposed on the surface of lymphoid cells leading to apoptosis (Schulze-Bergkamen and Krammer 2004).

2.4.5.5 **Screening for Function**

A great variety of different assays have been set up to identify hybridoma antibodies with special features. To mention only few examples related to oncology here, those screening procedures relying on biological interference with tumor cell growth are of particular interest. A simple method to monitor cell proliferation is incorporation of ^{3}H-thymidine or bromodesoxyuridine into cellular DNA that can be quantified by beta-counting or ELISA, respectively. Antibodies directed against the growth factor interleukin 6 (IL-6) or its cellular receptor, IL-6R, can be recognized by growth inhibition of a sensitive multiple myeloma target cell. Similarly, epidermal growth factor receptor (EGFR) constitutes a growth-promoting receptor on colon cancer cells that can be used for screening. Recently, antibodies interfering with angiogenesis that bind to vascular endothelial growth factor (VEGF) or its receptors on endothelial cells have been successfully identified and brought to the clinics.

2.4.6
Cloning

Rapid cloning of hybridoma cultures is mandatory to select for stable antibody-secreting cell lines. There are mainly two reasons for single-cell cloning: first, as mentioned before, early hybridomas are sequestering chromosomes to stabilize their genetic inventory. Second, the culture of interest may contain two or even more individual antibody-producing hybrids, making the maintenance of the desired clone not an easy task. The method of choice for single-cell cloning is limiting dilution. In principle, hybridoma cells are distributed in 96-well plates so that one well will contain theoretically 0.5 or 1 cell. Based on the individual cloning efficiency of a particular hybridoma culture that actually is not known at time of cloning, few to many of the seeded cells will give rise to cell clones. Because single hybridoma cells are dependent on several poorly characterized growth factors and in addition require "cellular togetherness," feeder cells have to be added. Feeder cell cultures are usually prepared from BALB/c spleen cells, thymocytes, or peritoneal macrophages. After 10–14 days clones are reanalyzed for specificity. Ideally, every growing clone should secrete the mAb of interest. To be on the safe side, hybridoma cultures should be cloned at least twice. Interspecies hybrids like mouse/rat or mouse/human hybrids are often unstable and need repeated recloning to preserve antibody production. It is advisable to reclone any hybridoma before starting mass production.

2.4.7
Expansion and Freezing of Hybridoma Clones

After cloning and reanalysis cultures of interest are slowly expanded. Vigorous dilution can cause sudden death of the culture. At this time point hypoxanthine thymidine (HT) medium is gradually replaced by normal medium. To be on the safe side one should freeze a small cell aliquot as backup as soon as possible. If afterwards hybridoma cells divide rapidly, a couple of samples can be frozen and stored in liquid nitrogen for decades. Freezing medium usually consists of 20–90% fetal calf serum and 10% dimethyl sulfoxide.

One of the most adverse events during hybridoma culture is contamination with mycoplasma (Hay et al. 1989; Rottem and Barile 1993). Since mycoplasma infection can interfere with numerous cellular, biochemical, and molecular biological assays, early detection is essential. It is advisable to check all permanently growing cell lines in a laboratory on a regular basis by a sensitive method such as polymerase chain reaction (PCR) or ELISA (Uphoff et al. 1992). Some antibiotics can eliminate mycoplasma from cell culture but this needs a long lasting treatment procedure without guarantee of success and carries the risk of inducing resistant variants. Prevention of contamination by regular testing and clean cell culture working is of course the best way to solve the problem.

2.5 Purification and Modification of Monoclonal Antibodies

2.5.1 Mass Culture and Purification of Monoclonal Antibody

Once the hybridoma is established, large quantities of antibody can be produced employing modern cell culture devices for long-term propagation. At least two systems are on the market meeting the demands of laboratory-scale production because they can simply be installed in a normal CO_2 incubator and do not require complicated pumping and other sophisticated equipment. Both, the miniPERM modular minifermenter (Falkenberg et al. 1995) and the two-chamber cell culture device CELLine 1000 (Trebak et al. 1999) are easy to handle and allow culturing of hybridoma cells at high density (above 10^7 cells per mL). Harvest of the antibody-enriched product can be performed several times until productivity ceases. The antibody yield throughout is comparable to the formerly favored ascites production in mice that now is prohibited in most Western countries by animal protection laws.

Purification of mouse monoclonal IgG antibodies by affinity chromatography over protein A–Sepharose represents the method of choice (Seppälä et al. 1981). There are suitable protocols available for isolation of all different IgG isotypes. Mouse IgG antibodies are very robust molecules, easy to handle and of high efficiency in many biological assays. By contrast, only the rat IgG2c subtype will bind with sufficient strength to protein A, for the remaining IgG isotypes of rat protein G–Sepharose is the best-suited affinity matrix (Bjorck and Kronvall 1984). Monoclonals of IgM isotype, however, cause problems in purification, storage and handling. Therefore, one should carefully decide whether an IgM reagent is really useful for a certain application. If the epitope of interest is located on the carbohydrate or glycolipid portion of an antigen one has certainly to deal with IgM antibodies. IgM antibodies can be purified by a combination of gel filtration and ion exchange chromatography or alternatively by affinity chromatography on immobilized mannan-binding protein (Nevens et al. 1992).

2.5.2 Fragmentation of Monoclonal IgG Antibodies

There are certain applications where smaller versions of an antibody may perform better (e.g. in immunoscintigraphy). Furthermore, in some settings it is necessary to exploit monovalent binding of the antibody, thereby preventing crosslinking of the antigen. Another reason to use immunoglobulin fragments is to get rid of the antibody Fc portion that may bind in a nonspecific fashion to Fc receptors exposed on the surface of myelomonocytic cells.

Digestion of IgG (MW approx. 150 kDa) with the thiol protease papain results in two monovalent Fab fragments (MW approx. 50 kDa). The nonspecific protease pepsin cuts below the first disulfide bond in the hinge region, giving rise to a

$F(ab')_2$ fragment (MW approx. 100 kDa). Fragmentation of mouse mAbs is not an easy task and the protocol has to be adapted for each individual antibody. There is, however, a clear hierarchy with regard to the immunoglobulin subclasses (Parham 1983; Lamoyi and Nisonoff 1983). More recently a method has been reported that cleaves mouse IgG2b antibodies with lysyl endopeptidase to obtain $F(ab')_2$ fragments (Yamaguchi et al. 1995).

2.5.3
Labeling of Monoclonal Antibodies

For many applications, such as multicolor staining of cells, enzyme immunoassay, or affinity determination of antibody by Scatchard plot, directly labeled monoclonals are needed. Conjugation of purified antibody with either fluorescein isothiocyanate (FITC) and biotin or iodine-125 can be easily performed. FITC binds by a hydrolysis reaction to the free amino group of lysines in the immunoglobulin. Biotin connected by a spacer of variable length to succinimide ester is also covalently bound to the antibody via lysine. The biotin–streptavidin system is especially attractive because of its flexibility. In essence, streptavidin binds with such high affinity to biotin that this bond is rapidly formed and irreversible. In addition, one biotin can accommodate four streptavidins, leading to an amplification effect. There are many streptavidin conjugates commercially available containing fluorescent dyes, enzymes, or even particles for electron microscopy. If the antibody of choice contains many lysines in its combining site, conjugation may abolish binding activity. By contrast, sodium ^{125}I-iodide is coupled to immunoglobulin via tyrosine residues, usually by an oxidation reaction (for instance the chloramine-T method, the Iodogen method, or application of Iodobeads). Alternatively, radiolabeling of antibodies can be achieved via lysine by the Bolton–Hunter procedure (Bolton and Hunter 1973). Enzyme conjugation of antibodies is technically more demanding because the labeled products have to be separated from the unlabeled by biochemical means. Many conjugates of high quality are now commercially available and thus individual enzyme labeling of monoclonals is performed only in special cases.

2.6
Monoclonal Antibodies for Tumor Therapy

2.6.1
Leukocyte Differentiation Antigens

Shortly after the invention of mAb technology in laboratories all around the world a huge variety of reagents were raised against white blood cells and normal as well as malignant cells from numerous tissues. This led to a Babylonian confusion with regard to antibody names and designation of detected antigens. The problem was approached by the organization of the well-known Workshops and

Conferences on Human Leukocyte Differentiation Antigens (HLDA), the first of which took place 1982 in Paris, France, and the most recent 8th meeting was held 2004 in Adelaide, Australia (Bernard et al. 1984; Zola et al. 2005). Antibody samples submitted to the Workshop were grouped into panels and simultaneously analyzed by a couple of reference laboratories with expertise for particular methods such as flow cytometry, immunohistology, biochemistry, or molecular genetics. The results were subsequently compared and statistically evaluated. This allowed the identification of distinct "clusters of differentiation" that became the basis of CD antigen nomenclature. To date, 339 CD antigens have been defined and characterized in depth by approaches taking advantage of immunology, cell biology, biochemistry, and molecular biology.

Monoclonal antibodies have proven to be unique reagents for the analysis of surface antigens on lymphocytes that are expressed on certain stages of lymphocyte differentiation and maturation. Using a whole panel of such antibodies has facilitated the phenotyping of functional subpopulations of normal lymphocytes. Likewise, the malignant counterparts derived from the respective stages of differentiation can be classified. Figure 2.7 illustrates how individual B cell antigens show up and vanish during B cell development. The identification and diagnosis of distinct entities among malignant lymphomas is essentially based on immunohistological staining with a set of antibodies recognizing lymphocyte differentiation antigens (Harris et al. 2001). More recently, classical immunophenotyping

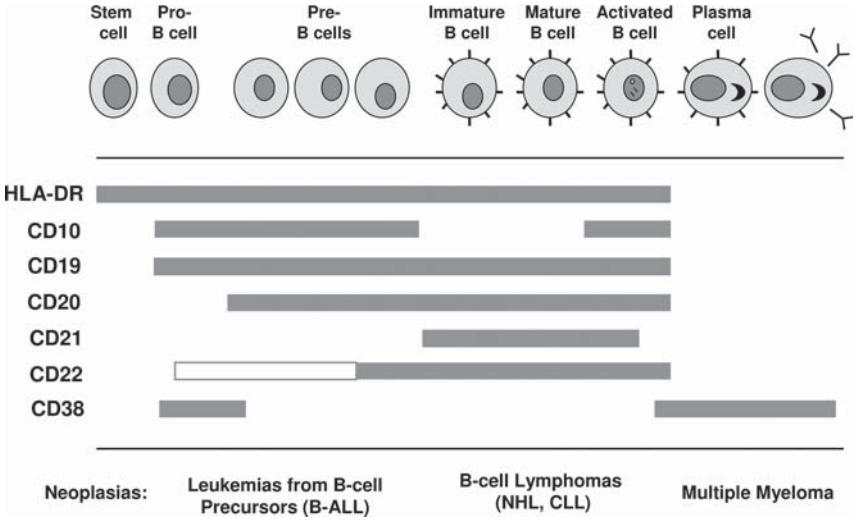

Fig. 2.7 Differentiation antigens of human B lymphocyte. Cluster of differentiation antigens (CD antigens) are expressed on certain maturation stages during B-cell ontogeny (upper panel). Monoclonal antibodies to CD antigens are also reactive with tumor-derived samples of corresponding counterparts (bottom panel), allowing for lymphoma phenotyping.

Table 2.1 Target antigens on lymphocytes for monoclonal antibody therapy.

Antigen	*Expression*	*Structure*	*Function*	*Antibody*	*Indication*
CD3	Mature T cells	Complex of five polypeptides	Signal transduction, TCR coreceptor	Muromonab	Transplant rejection
CD20	B cells	Pp35/37 kDa	Ion channel for Ca^{2+}	Rituximab	B-NHL
CD22	Mature B cells	Gp130/140 kDa heterodimer	Modulation of B cell activation, cell–cell adhesion	Epratuzumab	B-NHL
CD33	Monocytes, myeloid cells	Gp67	Cell–cell adhesion?	Gemtuzumab	AML
CD25	Activated B and T cells	Gp55 kDa	α-chain of interleukin 2 receptor	Daclizumab	Transplant rejection
CD52	Lymphocytes, monocytes	Gp21–28 kDa	Unknown	Alemtuzumab	B-CLL, T-lymphoma

Gp, glycoprotein; Pp, phosphoprotein (number given is the molecular weight under reducing conditions); TCR, T-cell receptor; NHL, Non-Hodgkin's lymphoma; AML, acute myelogenous leukemia; CLL, chronic lymphocytic leukemia.

of lymphomas was complemented by gene expression profiling technology (Staudt and Dave 2005).

Only a small proportion of the 339 CD antigens have yet evolved as valuable targets for antibody therapy not only of malignant lymphomas but also of certain autoimmune diseases and for the prevention of allograft rejection. In Table 2.1 the characteristics of six antigens that serve as targets for therapeutic antibodies already on the market are listed. These antigens differ with respect to their tissue distribution and show different traits concerning stability of surface expression and internalization. The CD22 antigen, for example, has a high internalization rate, making it an exquisite candidate for manufacturing an immunotoxin that has to reach the cytosol of a target cell to become effective (Messmann et al. 2000; Kreitman et al. 2001).

2.6.2 Epithelial Differentiation Antigens

The most frequent tumor type in humans, carcinoma, is derived from epithelial cells. Therefore, tremendous efforts have been made to identify tumor-associated or even tumor-specific membrane antigens on epithelial tumors by means of mAbs. With time it has emerged that all antigens initially regarded as tumor-specific were actually differentiation antigens and are also expressed on certain normal cells. Today it is clear that tumor-specific antigens recognized by antibodies most likely do not exist. The same experience emerged from studies focusing on other tumor types such as melanoma and brain tumors. We have

learned, however, that differentiation antigens, although not tumor-specific, are valuable targets for antibody-based tumor therapy.

Members of the epidermal growth factor receptor (EGFR) family, in particular, such as HER2/neu and EGFR, hold great promise as therapeutic targets since they are overexpressed in a variety of solid tumors (Hynes and Lane 2005). The induction of antitumor responses using the antibodies trastuzumab and cetuximab are discussed in Volume III in Chapters 14 and 4. Further good candidates for antibody therapy of solid tumors are the epithelial cell adhesion molecule, Ep-CAM, and the carcinoembryonic antigen, CEA. Ep-CAM represents a very stable marker even in highly de-differentiated adenocarcinomas and its overexpression is associated with poor prognosis in breast cancer (Gastl et al. 2000) whereas CEA appears to be a suitable target for radioimmunotherapy of colorectal and medullary thyroid cancer (Mayer et al. 2000; Sharkey et al. 2005).

2.6.3 Mechanisms of Action of Monoclonal Antibodies

The antitumor effects of antibodies can be induced by direct and indirect mechanisms (Table 2.2). In some instances antibody binding per se will lead to cell death. For instance, if a surface receptor is crosslinked that transmits an apoptosis signal, programmed cell suicide is started. Likewise binding to growth receptors or their ligands might abrogate vital signals required for cell proliferation. Antibodies against EGFR family members are prominent examples of this mode of action, as already mentioned. Recently, reagents interfering with angiogenesis have become increasingly attractive (Ferrara et al. 2003). Antibodies specific for VEGF or its receptors can prevent tumor vessel formation and thus deprive the tumor of nutrients. A rather special case is represented by anti-idiotype antibodies in B cell lymphoma. By mechanisms that are poorly understood, these antibodies are able to facilitate long lasting growth control of tumor cells (Davis et al. 1998).

The classical effector functions of antibody are CDC and ADCC. Depending on the isotype of the therapeutic antibody, complement component C1q is activated and triggers a cascade of enzymatic reactions resulting in recruitment of phagocytes and formation of a membrane-attack complex that finally leads to the

Table 2.2 Therapeutic effects of monoclonal antibodies.

Direct effects	Induction of apoptosis Inhibition of proliferation Blockade of growth factors or growth factor receptors Interference with angiogenesis
Indirect effects	Complement-dependent cytotoxicity (CDC) Antibody-dependent cellular cytotoxicity (ADCC) Vehicle for toxins, radionuclides and cytostatic drugs Anti-idiotype antibody formation Effector cell targeting using bispecific antibodies

lysis of tumor cells (Gelderman et al. 2004). In case of ADCC tumor cell-bound antibodies interact via their Fc portion with Fc receptors expressed at high density on NK cells, neutrophils, and monocytes (Ravetch and Bolland 2001). Upon activation, these effector cells release cytotoxic granules from the cytosol delivering a kiss of death to the tumor target. Unfortunately, many antibodies elicit neither direct nor indirect effects, this holds especially true for murine antibodies. However, these reagents can be successfully used as carriers for toxins, radionuclides, or chemotherapeutic substances. There has been much debate on the issue whether anti-idiotypic networks, forming an internal image of tumor antigens, really contribute to tumor regression. Finally, bispecific antibodies are synthetic molecules that carry two different antigen binding sites. By virtue of their dual specificity they can trigger effector cells via a membrane receptor and at the same time link them to a tumor cell. This interaction leads to the subsequent destruction of the tumor cell.

2.6.4 Human Monoclonal Antibodies

Great efforts have been made to take human myeloma cells in culture suitable for cell fusion in order to raise human mAbs. These attempts were largely hampered by the fact that most of the laboriously established lines later turned out to be Epstein–Barr virus (EBV) transformed lymphoblastoid B cell cultures. Although some human cell lines capable of producing human hybridomas have been described, for instance SK-007 (Olsson and Kaplan 1980), GM1500 (Croce et al. 1980) LICR-LON-Hmy2 (Edwards et al. 1982) and Karpas 707 (Karpas et al. 1982), the overall experience remains disappointing. In addition, for ethical reasons it is not possible to immunize a human volunteer with an experimental antigen. As already mentioned, *in vitro* immunization was not able to solve the problem due to predominant IgM responses.

In an alternative attempt, antigen-specific B lymphocytes were isolated from the peripheral blood of human donors and immortalized by EBV to establish permanent cell lines (Steinitz et al. 1977). Unfortunately, the production rate of the lines was low and decreased with time. It further turned out that the EBV-transformed lines were extremely difficult to clone. To circumvent those problems, the EBV hybridoma technique was developed, which combined EBV-induced immortalization of human antibody-secreting cells with fusion of a variant of the human myeloma line GM1500 to obtain human–human hybrids (Kozbor and Roder 1981). This method, however, is complex and often leads to instable hybridomas that require repeated recloning. Lacking a human non-secretor myeloma cell line with high fusion frequency the production of human mAbs by the hybridoma technique was no longer pursued for many years. Recently, the EBV method has been improved to immortalize memory B cells from a patient with severe acute respiratory syndrome (SARS) coronavirus infection. Neutralizing mAbs of high affinity against the virus, conferring protection in a mouse model, were successfully isolated (Traggiai et al. 2004).

At present, there are at least three alternative core technologies available allowing for the creation of human mAbs. The variable regions or only the CDRs from mouse heavy and light chains can be grafted onto a human IgG scaffold giving rise to chimeric or humanized antibodies, respectively (Carter 2001). Screening of large recombinant antibody libraries is exploited to build human antibodies with high specificity and affinity (Hoogenboom 2005). Transgenic mice carrying human immunoglobulin genes will respond to immunization with the production of entirely human antibodies. After fusion with mouse myeloma cells, these human antibodies are secreted by resulting hybridomas (Lonberg 2005). In addition, recombinant antibodies containing minimal binding fragments can be reconstructed to multivalent high-affinity reagents (Holliger and Hudson 2005).

2.7 Outlook

Monoclonal antibodies secreted by hybidoma cells have led to a revolution in biology, medicine, and many applied sciences due to their excellent specificity. After a first wave of innovation based on mouse monoclonals, molecular biology has provided tools for reshaping the antibody molecule to obtain chimeric, humanized, and fully human antibodies as well as recombinant antibody fragments. Therapeutic antibodies have evolved as effective pharmaceutical compounds not only for the treatment of malignant tumors but also of autoimmune diseases and infections. Currently we are encountering a third wave of scientific advancement by subtle antibody engineering, making it possible to tune the molecule in a way that it can meet special therapeutic demands (Weiner and Carter 2005). In the end there is no doubt that antibody-based therapeutics will play an outstanding role in several fields of modern medicine.

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References

Abbas, A.K., Lichtman, A.H. (2003) *Cellular and Molecular Immunology*, 5th edn. Philadelphia: Saunders.

Adams, G.P., Weiner, L.M. (2005) Monoclonal antibody therapy of cancer. *Nat Biotechnol* 23: 1147–1157.

Barreto, V.M., Ramiro, A.R., Nussenzweig, M.C. (2005) Activation-induced deaminase: controversies and open questions. *Trends Immunol* 26: 90–96.

Barry, M.A., Barry, M.E., Johnston, S.A. (1994) Production of monoclonal

antibodies by genetic immunization. *BioTechniques* 16: 616–618.

Bazin, H., Pear, W.S., Klein, G., SÜmegi, J. (1990) Rat immunocytomas (IR) In: *Rat Hybridomas and Rat Monoclonal Antibodies* (Bazin, H., ed.), pp. 53–68. Boca Raton: CRC Press.

Bernard, A., Boumsell, L., Dausset, J., Milstein, C., Schlossman S.F. (1984) *Leucocyte Typing*. Berlin, Heidelberg: Springer-Verlag.

Bolton, A.E., Hunter W.M. (1973) The labeling of proteins to high specific radioactivities by conjugation to a 125I-containing acylating agent. *Biochem J* 133: 529–539.

Bjorck, L., Kronvall, G. (1984) Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. *J Immunol* 133: 969–974.

Bjork, I., Petersson, B.A., Sjoquist, J. (1972) Some physicochemical properties of protein A from Staphylococcus aureus. *Eur J Biochem* 29: 579–584.

Bonner, W.A., Hulett, H.R., Sweet, R.G., Herzenberg, L.A. (1972) Fluorescence activated cell sorting. *Rev Sci Instrum* 43: 404–409.

Borrebaeck, C.A. (1983) In vitro immunization of mouse spleen cells and the production of monoclonal antibodies. *Acta Chem Scand B* 37: 647–648.

Brekke, O.H., Sandlie, I. (2003) Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nat Rev Drug Disc* 2: 52–62.

Carter, P. (2001) Improving the efficacy of antibody-based cancer therapies. *Nat Rev Cancer* 1: 118–129.

Cohen, S., Porter, R.R. (1964) Structure and biological activity of immunoglobulins. *Adv Immunol* 3: 287–349.

Coligan, J.E. Bierer, B.E., Margulies, D.H., Shevach, E.M., Strober, W. (2004) *Current Protocols in Immunology*. New York: John Wiley and Sons.

Croce, C.M., Linnenbach, A., Hall, W., Steplewski, Z., Koprowski, H. (1980) Production of human hybridomas secreting antibodies to measles virus. *Nature* 288: 488–489.

Davis, T.A., Maloney, G.G., Cerwinski, D.K., Liles, T.M., Levy, R. (1998) Anti-idiotype antibodies can induce long-term complete remissions in non-Hodgkin's lymphoma without eradicating the malignant clone. *Blood* 15: 1184–1190.

DeNardo, G.L., Bradt, B.M., Mirick, G.R., DeNardo, S.J. (2003) Human antiglobulin responses to forein antibodies: therapeutic benefit? *Cancer Immunol Immunother* 52: 309–316.

Edelman, G.M., Cunningham, B.A., Gall, W.E., Gottlieb, P.D., Rutishauser, U., Waxdal, M.J. (1969) The covalent structure of an entire γG immunoglobulin molecule. *Proc Natl Acad Sci USA* 63: 78–85.

Edwards, P.A.W., Smith, C.M., Neville, A.M., O'Hare, M. (1982) A human-human hybridoma system based on a fast-growing mutant of the ARH-77 plasma cell leukemia-derived line. *Eur J Immunol* 12: 641–648.

Ehrlich, P. (1900) On immunity with special reference to cell life. *Proc R Soc Lond* 66: 424–448.

Eichmann, K. (2005) *Köhler's Invention*. Basel-Boston-Berlin: Birkhäuser-Verlag.

Engvall, E., Perlman, P. (1971) Enzyme-linked immunosorbent assay (ELISA): Quantitative assay of immunoglobulin G. *Immunochemistry* 8: 871–879.

Falkenberg, F.W., Weichert, H., Krane, M., Bartels, I., Palme, M., Nagels, H.-O., Fiebig, H. (1995) In vitro production of monoclonal antibodies in high concentration in a new and easy to handle modular minifermenter. *J Immunol Methods* 179: 13–29.

Fazekas de St. Groth S., Scheidegger, D. (1980) Production of monoclonal antibodies: strategy and tactics. *J Immunol Methods* 47: 129–144.

Feit, C., Bartal, A.H., Tauber, G., Dymbort, G., Hirshaut, Y. (1983) An enzyme-linked immunosorbent assay (ELISA) for the detection of monoclonal antibodies recognizing antigens expressed on viable cells. *J Immunol Methods* 58: 301–308.

Ferrara, N., Gerber, H.-P., LeCouter, J. (2003) The biology of VEGF and its receptors. *Nat Med* 9: 669–676.

Freund, J. (1956) The mode of action of immunologic adjuvants. *Adv Tuberc Res* 7: 130–148.

Galfrè,G., Milstein, C., Wright, B. (1979) Rat x rat hybrid myelomas and a monoclonal

anti-Fd portion of mouse IgG. *Nature* 277: 131–133.
Gastl, G., Spizzo, G., Obrist, P., Dünser, M., Mikuz, G. (2000) Ep-CAM overexpression in breast cancer as a predictor of survival. *Lancet* 356: 1981–1982.
Gelderman, K.A., Tomlinson, S., Ross, G.D., Gorter, A. (2004) Complement function in mAb-mediated cancer immunotherapy. *Trends Immunol* 25: 158–164.
Glennie, M.J., van de Winkel, J.G.J. (2003) Renaissance of cancer therapeutic antibodies. *Drug Disc Today* 8: 503–510.
Goding, J.W. (1996) *Monoclonal Antibodies: Principles and Practice*. 3rd edn. London, San Diego: Academic Press.
Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T.M., Sutcliffe, J. G., Lerner, R.A. (1982) Immunogenic structure of the influenza virus hemagglutinin. *Cell* 28: 477–487.
Grohmann, U., Romani, L. Binaglia, L., Fioretti, M.C., Puccetti, P. (1991) Intrasplenic immunization for the induction of humoral and cell-mediated immunity to nitrocellulose-bound antigen. *J Immunol Methods* 137: 9–15.
Hämmerling, G.J., Hämmerling, U., Kearney, J.F. (Eds) (1981) *Monoclonal Antibodies and T-cell Hybridomas*. Amsterdam: Elsevier/North-Holland Biomedical Press.
Harlow, E., Lane, D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
Harris, N.L., Stein, H., Coupland, S.E., Hummel, M., Favera, R.D., Pasqualucci, L., Chan, W.C. (2001) New approaches to lymphoma diagnosis. *Hematology (Am Soc Hematol Educ Program)*, 194–220.
Hay, R.J., Macy, M.L., Chen, T.R. (1989) Mycoplasma infection of cultured cells. *Nature* 339: 487–488.
Holliger, P., Hudson, P. (2005) Engineered antibody fragments and the rise of single domains. *Nat Biotechnol* 23: 1126–1136.
Hoogenboom, H. (2005) Selecting and screening recombinant antibody libraries. *Nat Biotechnol* 23: 1105–1116.
Hynes, N.E., Lane, H.A. (2005) ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* 5: 341–354.
Janeway, C.A., Travers, P., Walport, M., Shlomchik, M.J. (2005) *Immunobiology. The Immune System in Health and Disease*, 6th edn. London: Garland Science.
Jerne, N.K. (1955) The natural selection theory of antibody formation. *Proc Natl Acad Sci USA* 41: 849–857.
Karpas, A., Fischer, P., Swirsky, D. (1982) Human plasmacytoma with an unusual karyotype growing in vitro and producing light-chain immunoglobulin. *Lancet* I: 931–933.
Kearney, J.F., Radbruch, A., Liesengang, B., Rajewsky, K. (1979) A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J Immunol* 123: 1548–1550.
Kennett, R.H., McKearn, T.J., Bechtol, K.B. (Eds) (1980) *Monoclonal Antibodies. Hybridomas: A New Dimension in Biological Analyses*. New York: Plenum Press.
Khazaeli, M.B., Conry, R.M., LoBuglio, A.F. (1994) Human immune response to monoclonal antibodies. *J Immunother* 15: 42–52.
Köhler, G., Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.
Köhler, G., Milstein, C. (1976) Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. *Eur J Immunol* 6: 511–519.
Köhler, G., Howe, S.C., Milstein. C. (1976) Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines. *Eur J Immunol* 6: 292–295.
Kozbor, D., Roder, J.C. (1981) Requirements for the establishment of high-titered human monoclonal antibodies using the Epstein-Barr virus technique. *J Immunol* 127: 1275–1280.
Kreitman, R.J., Wilson, W.H., Bergeron, K., Raggio, M., Stetler-Stevenson, M., FitzGerald, D.J., Pastan, I. (2001) Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia. *N Engl J Med* 345: 241–247.
Lamoyi, E., Nisonoff, A. (1983) Preparation of $F(ab')_2$ fragments from mouse IgG of

various subclasses. *J Immunol Methods* 56: 235–243.
Ledbetter, J.A., Herzenberg, L.A. (1979) Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol Rev* 47: 63–90.
Levy, R., Miller, R.A. (1990) Therapy of lymphoma directed at idiotypes. *J Natl Cancer Inst Monogr* 10: 61–68.
Littlefield, J.W. (1964) Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. *Science* 145: 709–710.
Lonberg, N. (2005) Human antibodies from transgenic animals. *Nat Biotechnol* 23: 1117–1125.
Mayer, A., Tsiompamou, E., O'Malley, D., Boxer, G.M., Bhatia, J., Flynn, A.A., Cester, K.A., Davidson, B.R., Lewis, A.A., Winslet, M.C., Dhillon, A.P., Hilson, A.J., Begent, R.H. (2000) Radioimmunoguided surgery in colorectal cancer using a genetically engineered anti-CEA single-chain Fv antibody. *Clin Cancer Res* 6: 1711–1719.
McLaughlin, P., Grillo-Lopez, A.J., Link, B. K., Levy, R., Czuczman, M.S., Williams, M.E., Heyman, M.R., Bence-Bruckler, I., White, C.A., Cabanillas, F., Jain, V., Ho, A.D., Lister, J., Wey, K., Shen, D., Dallaire, B. (1998) Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 16: 2825–2833.
Melchers, F., Potter, M., Warner, N.L. (Eds) (1978) Lymphocyte hybridomas. *Current Topics in Microbiology and Immunology*, Vol. 81. Berlin, Heidelberg, New York: Springer-Verlag.
Messmann, R.A., Vitetta, E.S., Headlee, D., Senderowicz, A.M., Figg, W.D., Schindler, J., Michiel, D.F., Creekmore, S., Steinberg, S.M., Kohler, D., Jaffe, E.S., Stetler-Stevenson, M., Chen, H., Ghetie, V., Sausville E.A. (2000) A phase I study of combination therapy with immunotoxins IgG-HD37-deglycosylated ricin A chain (dgA) and IgG-RFB4-dgA (Combotox) in patients with refractory CD19(+), CD22(+) B cell lymphoma. Clin. *Cancer Res* 6: 1302–1313.
Milstein, C. (1999) The hybridoma revolution: an offshoot of basic research. *BioAssays* 21: 966–973.
Nadler, L.M., Stashenko, P., Hardy, R., Kaplan, W.D., Button, L.N., Kufe, D.W., Antman, K.H., Schlossman, S.F. (1980) Serotherapy of a patient with a monoclonal antibody directed against a human lymphoma-associated antigen. *Cancer Res* 40: 3147–3154.
Nevens, J.R., Mallia, A.K., Wendt, M.W., Smith, P.K. (1992) Affinity chromatographic purification of immunoglobulin M antibodies utilizing immobilized mannan binding protein. *J Chromatogr* 597: 247–256.
Ohnishi, K., Chiba, J., Goto, Y., Tokunaga, T. (1987) Improvement in the basic technology of electrofusion for generation of antibody-producing hybridomas. *J Immunol Methods* 26: 181–189.
Olsson, L., Kaplan, H.S. (1980) Human-human hybridomas producing monoclonal antibodies of predefined antigenic specificity. *Proc Natl Acad Sci USA* 77: 5429–5434.
Parham, P. (1983) On the fragmentation of monoclonal IgG1: IgG2a and IgG2b from BALB/c mice. *J Immunol* 131: 2895–2902.
Parks, D.R., Herzenberg, L.A. (1984) Fluorescence-activated cell sorting: theory, experimental optinization, and application in lymphoid cell biology. *Methods Enzymol* 108: 197–241.
Pasqualini, R., Arap, W. (2004) Hybridoma-free generation of monoclonal antibodies. *Proc Natl Acad Sci USA* 101: 257–259.
Pontecorvo, G. (1975) Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. *Somatic Cell Genet* 1: 397–400.
Ravetch, J.V., Bolland, S. (2001) IgG Fc receptors. *Annu Rev Immunol* 19: 275–290.
Raybould, T.J.G., Takahashi, M. (1988) Production of stable rabbit-mouse hybridomas that secrete rabbit MAb of defined specificity. *Science* 240: 1788–1790.
Roitt, I., Brostoff, J., Male, D. (2001) *Immunology*, 6th edn. St Louis: Mosby.
Ross, J.S., Schenkein, D.P., Pietrusko, R., Rolfe, M., Linette, G.P., Stec, J., Stagliano, N.E., Ginsburg, G.S., Symmans, W.F., Pusztai, L., Hortobagyi, G.N. (2004) Targeted therapies for cancer 2004. *Am J Clin Pathol* 122: 598–609.

Rottem, S., Barile, M.F. (1993) Beware of mycoplasmas. *Trends Biotechnol* 11: 143–151.

Sanchez-Madrid, F., Szklut, P., Springer T.A. (1983) Stable hamster-mouse hybridomas producing IgG and IgM hamster monoclonal antibodies of defined specificity. *J Immunol* 130: 309–317.

Schmitt, J.J., Zimmermann, U. (1989) Enhanced hybridoma production by electrofusion in strongly hypo-osmolar solutions. *Biochim Biophys Acta* 983: 42–50.

Schulze-Bergkamen, H., Krammer, P.H. (2004) Apoptosis in cancer – implications for therapy. *Semin Oncol* 31: 90–119.

Seppälä, I., Sarvas, H., Péterfly, F., Mäkela, O. (1981) The four sub-classes of IgG can be isolated from mouse serum by using protein A-Sepharose. *Scand J Immunol* 14: 335–342.

Sharkey, R.M., Hajjar, G., Yeldell, D., Brenner, A., Burton, J., Rubin, A., Goldenberg, D.M. (2005) A phase I trial combining high-dose 90Y-labeled humanized anti-CEA monoclonal antibody with doxorubicin and peripheral blood stem cell rescue in advanced medullary thyroid cancer. *J Nucl Med* 46: 620–633.

Sondel, P.M., Hank, J.A. (2001) Antibody-directed, effector cell-mediated tumor destruction. *Hematol Oncol Clin North Am* 15: 703–721.

Spira, G., Bargellesi, A., Teillaud, J.L., Sharff, M.D. (1984) *J Immunol Methods* 74: 307–315.

Spitz, M., Spitz, L., Thorpe, R., Eugui, E. (1984) Intrasplenic primary immunization for the production of monoclonal antibodies. *J Immunol Methods* 11: 39–43.

Staudt, L.M., Dave, S. (2005) The biology of human lymphoid malignancies revealed by gene expression profiling. *Adv Immunol* 87: 163–208.

Steinitz, M., Klein, G., Koskimies, S., Mäkela, O. (1977) EB virus-induced B lymphocyte cell lines producing specific antibody. *Nature* 269: 420–422.

Stern, M., Herrmann, R. (2005) overview of monoclonal antibodies in cancer therapy: present and promise. *Crit Rev Oncol/ Hematol* 54: 11–29.

Traggiai, E., Becker, S., Subbarao, K., Kolesnikova, L., Uematsu, Y., Gismondo, M.R., Murphy, B.R., Pappuoli, R., Lanzavecchia, A. (2004) An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat Med* 10: 871–875.

Trebak, M., Chong, J.M., Herly, D., Speicher, D.W. (1999) Efficient laboratory-scale production of monoclonal antibodies using membrane-based high-density cell culture technology. *J Immunol Methods* 230: 59–70.

Uphoff, C.C., Brauer, S., Grunicke, D., Gignac, S.M., MacLeod, R.A.F., Quentmeier, H., Steube, K., Tummier, M., Voges, M., Wagner, B., Drexler, H.G. (1992) Sensitivity and specificity of five different mycoplasma detection assays. *Leukemia* 6: 335–241.

von Behring, E., Kitasato, S. (1890) Über das Zustandekommen der Diphtherie-Immunität und der Tatanus-Immunität bei Thieren. *Dtsch Med Wochenschr* 16: 1113–1114.

Weiner, L.M., Carter, P. (2005) Tunable antibodies. *Nat Biotechnol* 23: 556–557.

Yamaguchi, Y., Kim, H.H., Kato, K., Masuda, K., Shimida, I., Arata, Y. (1995) Proteolytic fragmentation with high specificity of mouse immunoglobulin G. *J Immunol Methods* 181: 259–267.

Zola, H., Swart, B., Nicholson, I., Aasted, B., Bensussan, A., Boumsell, L., Buckley, C., Clark, G., Drbal, K., Engel, P., Hart, D., Horejsí, V., Isacke, C., Macardle, P., Malavasi, F., Mason, D., Olive, D., Saalmueller, A., Schlossman, S.F., Schwartz-Albiez, R., Simmons, P., Tedder, T.M., Uguccioni, M., Warren, H. (2005) CD molecules 2005: human cell differentiation molecules. *Blood* 106: 3123–3126.

3
Selection Strategies II: Antibody Phage Display

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3.1
Introduction

The production of polyclonal antibodies by immunisation of animals is a method established for more than a century. The first antibody serum was directed against Diphterie and produced in horses (von Behring and Kitasato, 1890). Hybridoma technology was the next milestone, allowing the production of monoclonal antibodies by fusion of an immortal myeloma cell with an antibody producing spleen cell (Köhler and Milstein 1975). However, hybridoma technology has some limitations, the possible instability of the aneuploid cell lines, most of all its inability to produce human antibodies and to provide antibodies against toxic or highly conserved antigens (Winter and Milstein 1991).

To overcome the limitations of hybridoma technology, antibodies or antibody fragments can be generated by recombinant means (Fig. 1). The most common used antibody fragments are the Fragment antigen binding (Fab) and the single chain Fragment variable (scFv). The Fab fragment consists of the fd fragment of the heavy chain and the light chain linked by a disulphide bond. The variable region of the the heavy chain (V_H) and the variable region of the light chain (V_L) are connected by a short peptide linker in the scFv. A major breakthrough in the field of antibody engineering was the generation of antibody fragments as recombinant proteins in the periplasmatic space of *E. coli* (Better et al. 1988, Huston et al. 1988, Skerra and Plückthun 1988). To circumvent the instability of hybridoma cell lines, the genes encoding V_H and V_L of a monoclonal antibody can be cloned into an *E. coli* expression vector in order to produce antibody fragments in the periplasmatic space of *E. coli* which preserve the binding specificity of the parental hybridoma antibody (Toleikis et al. 2004).

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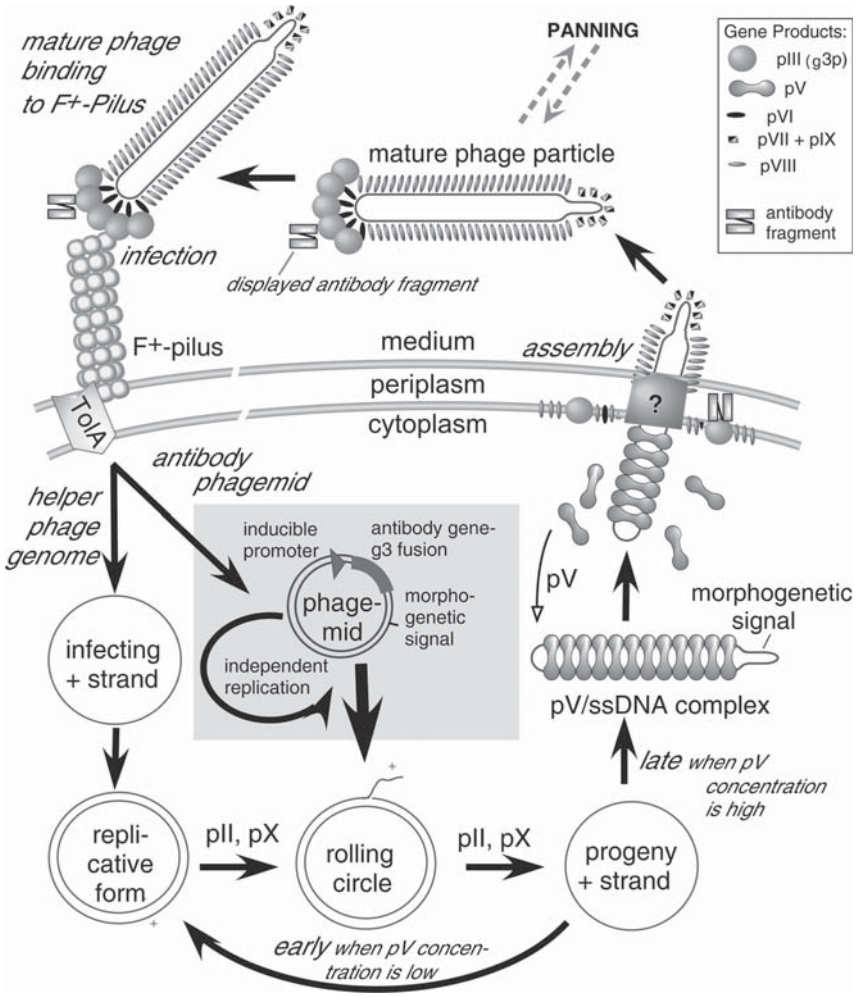

Fig. 3.1 Filamentous phage life cycle and antibody phage display. The plasmid encoding a fusion of an antibody fragment with pIII (as shown in the grey box) typically replicates and produces the fusion protein independently) from the phage genome. It carries the morphogenetic signal of the phage (which make it a "phagemid"). This allows it to be packaged into phage particles by the assembly machinery, typically up to 20 fold more effective than the phage genomes when replication deficient M13KO7 or its derivatives (like Hyperphage) are used. This limits the contamination of the panned antibody phage with "meaningless" helper phage genomes to less than 5%.

The production of mouse derived monoclonal antibody fragments in *E. coli* did not remove the major barrier for the broad application of antibodies in therapy as repeated administration of mouse derived antibodies causes a human anti-mouse antibody (HAMA) response (Courtenay-Luck et al. 1986). This problem can be overcome by two approaches: By humanisation of mouse antibodies (Studnicka

et al. 1994) or by employing repertoires of human antibody genes. The second approach was achieved in two ways. First, human antibody gene repertoires were inserted into the genomes of IgG-knockout-mice, allowing to generate hybridoma cell lines which produce human immunoglobulins (Jakobovits 1995, Lonberg und Huszar 1995, Fishwild et al. 1996). However, this method still requires immunisation and has limitations in respect of toxic and conserved antigens.

These restrictions do not apply for the more rational second approach: the complete *in vitro* generation of specific antibodies from human antibody gene repertoires. There, despite of the constant suggestion of novel methods like bacterial surface display (Fuchs et al. 1991, for review see Jostock and Dübel 2005), ribosomal display (Hanes and Plückthun 1997), puromycin display (Roberts and Szostak 1997) or yeast surface display (Boder and Wittrup et al. 1997), phage display has become the most widely used selection method (Table 3.1, chapter "An exciting start- and a long trek" Fig. 1.3), which is based on the groundbreaking work of Smith (1985). The genotype and phenotype of a polypeptide were linked by fusing short gene fragments to the minor coat protein III gene of the filamentous bacteriophage M13. This resulted in the expression of this fusion

Table 3.1 Comparison of recombinant antibody selection systems (modified from Hust et al. 2005).

Selection system	*Advantages*	*Disadvantages*
Transgenic mice	Somatic hypermutation	Immunisation requiered, not freely avaible
Cellular display		
Bacteria	N- and C-terminal and sandwich fusion	Not matured, requires individual sorting
Yeast	Display of larger proteins, N- and C-terminal and sandwich fusion	Requires individual sorting
Intracellular display		
Yeast two hybrid	Screening library versus library possible	Cytoplasm not optimal for antibody folding
Molecular display		
Puromycin/ribosomal	Largest achievable library size *in vitro*	Finicky method
Phage display		
Filamentous		
Genomic	*In vitro*, robust, multivalent display	Prone to mutation, only C-terminal fusion
Phagemid	*In vitro*, robust	Only C-terminal fusion
T7	Well suited for peptide display	No display of antibody fragments
Arrays		
Gridded clones	Robust, simple	Small library sizes

protein on the surface of phage, allowing affinity purification of the gene of interest by the polypeptide binding. The first antibody gene repertoires in phage were generated and screened by using the lytic phage Lambda (Huse et al. 1989, Persson 1991) with limited success. Consequently, antibody fragments were presented on the surface of M13, fused to pIII (McCafferty 1990, Barbas et al. 1991, Breitling et al. 1991, Clackson et al. 1991, Hogenboom et al. 1991, Marks et al. 1991). By uncoupling antibody gene replication and expression from the phage life cycle by locating them on a separate plasmid (phagemid), genetic stability, propagation and screening of antibody libraries was greatly facilitated (Barbas et al. 1991, Breitling et al. 1991, Hoogenboom 1991, Marks et al. 1991). To date, "single-pot" (see below) antibody libraries with a theoretical diversity of up to 10^{11} independent clones were assembled (Sblattero and Bradbury 2000) to serve as a molecular repertoire for phage display selections.

3.2 The Phage Display System

Due to its robustness and straightforwardness, phage display has been the selection method most widely used in the past decade. Display systems employing insertion of antibody genes into the phage genome have been developed for phage T7 (Danner and Balesco 2001), phage Lambda (Huse et al. 1989, Mullinax et al. 1990, Kang et al. 1991a) and the Ff class (genus *inovirus*) of the filamentous phages f1, fd and M13 (McCafferty 1990). Being well established for peptide display, the phage T7 is not well suited for antibody phage display because it is assembled in the reducing enviroment of the cytoplasm, thus leaving most antibodies unfolded (Danner and Balesco 2001). In contrast, the oxidizing milieu of the bacterial periplasm allows antibody fragments to be folded and assembled properly (Skerra and Plückthun 1988). The Ff class non-lytic bacteriophages are assembled in this cell compartment and allow the production of phages without killing the host cell (Fig. 1). This is a major advantage compared to the lytic phages Lambda (Huse et al. 1989). In addition, filamentous phages allow the production of soluble proteins by introducing an amber stop codon between the antibody gene and gene III when using phagemid vectors. In an *E. coli* supE suppressor strain, the fusion proteins will be produced, whereas soluble antibodies are made in a non suppressor strain (Marks et al. 1992a, Griffiths et al. 1994), but expression in suppressor strains is also possible (Kirsch et al. 2005). Therefore, the members of the Ff class are the phages of choice for antibody phage display.

To achieve surface display, five of the M13 coat proteins (Fig. 1) have been used in fusion to foreign proteins, protein fragments or peptides. In the commonly used system the antibody fragment is coupled to the N-terminus or second domain of the minor coatprotein pIII (Barbas et al. 1991, Breitling et al. 1991, Hoogenboom et al. 1991). The function of the 3–5 copies of pIII, in particular their N-terminal domain, is to provide interaction of the phage with the F pili

expressed on the surface of *E. coli* (Crisman and Smith 1984). The major coat protein pVIII has been considered as an alternative fusion partner, with only very few success reports in the past decade (Kang et al. 1991b). pVIII fusions are obviously more useful for the display of short peptides (Cwirla et al. 1990, Felici et al. 1991). Fusions to pVI have also been tried, but not yet with antibody fragments (Jespers et al. 1995). pVII and pIX were used in combination, by fusing the V_L domain to pIX and the V_H domain to pVII, allowing the presentation of a Fv fragment on the phage surface. Thus this format offers the potential for heterodimeric display (Gao et al. 1999). However, the fusion with pIII remains the most widely used system for antibody phage display and is still the only system of practical relevance.

Two different systems have been developed for the expression of the antibody:: pIII fusion proteins. First, the fusion gene can be inserted directly into the phage genome substituting the wildtype (wt) pIII (McCafferty et al. 1990). Second, the fusion gene encoding the antibody fusion protein can be provided on a separate plasmid with an autonomous replication signal, a promoter, a resistance marker and a phage morphogenetic signal, allowing this "phagemid" to be packaged into assembled phage particles. A helperphage, usually M13KO7, is necessary for the production of the antibody phage to complement the phage genes not encoded on the plasmid. Due to its mutated origin, the M13KO7 helperphage genome is not efficiently packaged during antibody phage assembly when compared to the phagemid (Vieira and Messing 1987).

In the system using direct insertion into the phage genome, every pIII protein on a phage is fused to an antibody fragment. This is of particular advantage in the first round of panning, where the desired binder is diluted in millions to billions of phages with unwanted specificity. The oligovalency of these phages improves the chances of a specific binder to be enriched due to the improvement of apparent affinity by the avidity effect. This advantage, however, has to be weighted to a number of disadvantages. The transformation efficiency of phagemids is two to three orders of magnitude better than the efficiency of phage vectors, thus facilitating the generation of large libraries. Second, the additional protein domains fused to pIII may reduce the function of pIII during reinfection. In a phagemid system, the vast majority of the pIII assembled into phage are wt proteins, thus providing normal pilus interaction. This may explain why only two "single-pot" antibody libraries (Griffiths et al. 1994, O'Connel et al. 2002) were made using a phage vector. In contrast, in phagemid systems both replication and expression of foreign fusion proteins are independent from the phage genome. There is no selection pressure, as propagation of the phagemid occurs in the absence of helperphage. The fusion proteins can be produced in adjustable quantities and allowing to use the amber/suppressor system for switching to soluble expression of antibody fragments without a pIII domain. Finally, despite usually not derived from highest copy plasmids, the dsDNA of phagemids is more easy to handle than phage DNA, facilitating cloning and analysis. Therefore, most pIII display systems use the phagemid approach. There is, however, a disadvantage which originates from the two independent sources for the pIII during phage

packaging. During assembly, the wt pIII of the phage are inserted into the phage particles with much higher rate than the pIII fusion protein. As a result, the vast majority of resulting phage particles carry no antibody fragments at all. The few antibody phages in these mixtures are mainly monovalent, with phage carrying two or more antibody fragments being extremely rare. This allows to select for antibodies with a high monovalent affinity, since avidity effects decreasing the dissociation rate from the panning antigen can be avoided. In the first panning round, however, when a few binders have to be fished out of a huge excess of unwanted antibody phages, the fact that only a few percent of the phages carry antibodies hampers the efficiency of the system (Barbas et al. 1991, Breitling et al. 1991, Hoogenboom et al. 1991, Garrard et al. 1991, Lowman et al. 1991, O'Connel et al. 2002). This problem can be overcome by using a newly developed helperphage "Hyperphage". Hyperphage does not have a functional pIII gene and therefore the phagemid encoded pIII antibody fusion is the sole source of pIII in phage assembly offering multivalent display for phagemid vectors. This method improves antibody phage display by two orders of magnitude and vastly improves panning efficiency (Rondot et al. 2001).

Multivalent display can be also achieved by the integration of two amber stop codons into the gIII gene of the helper phage genome, offering the production of a functional helper phage "Ex-phage" in an *E. coli* suppressor strain. In the associated phagemid pIGT3, the antibody::pIII fusion is made without an amber stop codon and the antibody phage is produced in an *E. coli* non suppressor strain (Baek et al. 2002). However, the necessary deletion of the amber stop codon in the phagemid makes it imperative to subclone the antibody gene or to use a protease to produce soluble antibodies in contrast to the Hyperphage system where the amber/suppressor system can be used for switching to soluble expression of antibody fragments without a pIII domain.

A niche application of phage display is the selective infective phage (SIP) technology. Here, antibody fragments are fused to the N-terminal domain of pIII by cloning into the phage genome, therefore every pIII carries an antibody and the deletion of the pIII N-terminal region made the phage non-infective. In turn, the antigen is fused to the C-terminal end of seperately produced soluble pIII N-terminal domain. The functional, F pili binding pIII is reconstituted when the antibody phage binds to the antigen, allowing only the correct antibody phage to infect *E. coli* and to be propagated (Spada and Plückthun 1997). However, due to the fast kinetics of pIII/pilin interactions and very low concentrations of the three reaction partners if not coexpressed in the same cell, this method is not applicable for the convenient panning of larger libraries.

3.3 Selection and Evaluation of Binders

The novel procedure for isolating antibody fragments by their binding activity *in vitro* was called "panning", referring to the gold washers tool (Parmley and Smith

1988). The antigen is immobilized to a solid surface, such as nitrocellulose (Hawlisch et al. 2001), magnetic beads (Moghaddam 2003), a column matrix (Breitling et al. 1991) or, most widely used, plastic surfaces as polystyrol tubes (Hust et al. 2002) or 96 well microtitre plates (Barbas et al. 1991). The antibody phages are incubated with the surface-bound antigen, followed by thorough washing to remove the vast excess of non-binding antibody phages. The bound antibody phages can subsequently be eluted and reamplified by infection of *E. coli*. This amplification allows detection of a single molecular interaction during panning, as a single antibody phage, by its resistance marker, can give rise to a bacterial colony after elution. This illustrates the tremendous sensitivity of the method. The selection cycle can be repeated by infection of the phagemid bearing *E. coli* colonies from the former panning round with a helperphage to produce new antibody phages, which can be used for further rounds of panning until a significant enrichment is achieved. The number of antigen specific antibody clones should increase with every panning round. Usually 2–6 panning rounds are necessary to select specifically binding antibody fragments (Fig. 3.2). For an

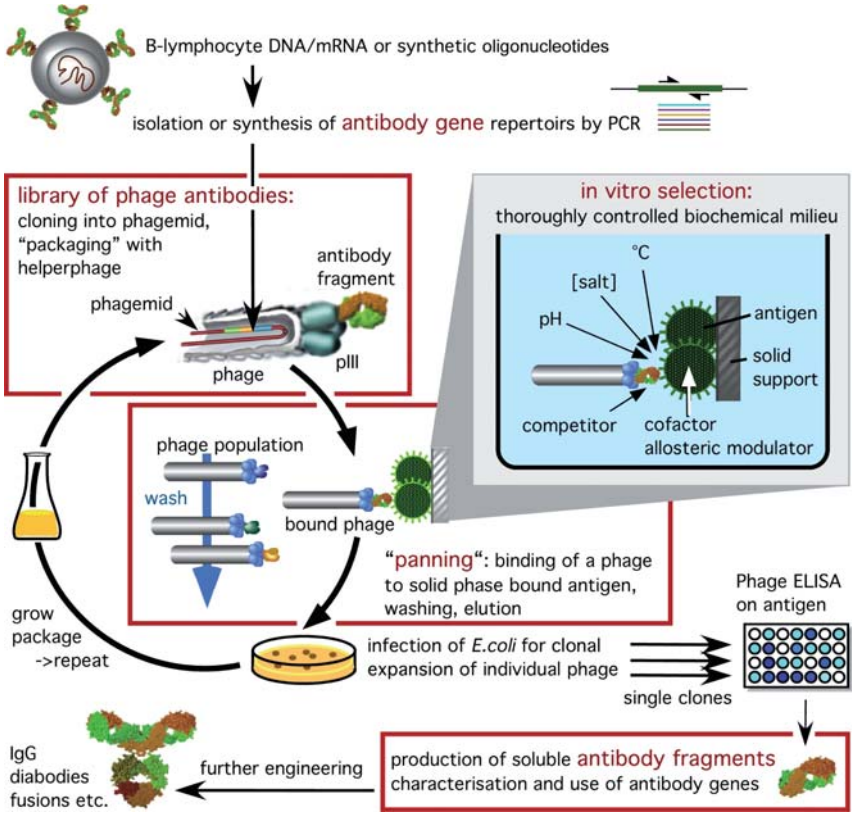

Fig. 3.2 Selection of antibodies from antibody libraries ("panning") by phage display.

overview of available stratagies and protocols, refer to McCafferty et al. (1996) and Kontermann and Dübel (2001). High throughput methods using microtitreplates and robotics can facilitate and enhance the panning procedure (for review see Konthur et al. 2005).

In most cases, the first step in the evaluation process of potential binders is an ELISA with polyclonal phage preparations from each panning round on coated target antigen and a control protein, e.g. BSA. The next step is the production of soluble monoclonal antibody fragments – from the panningrounds showing a significant enrichment of specific binders in polyclonal phage ELISA – in microtitreplates, followed by ELISA on coated antigen and in parallel on control protein. Soluble Fab fragments can be detected by their constant domains, wheras soluble scFvs can be detected by their engineered tags, e.g. his- or cmyc-tag. The clones producing specific antibody fragments will be further analysed by sequencing. Here, specific binders in duplicate can be rejected. A subcloning into *E. coli* expression vectors, like pOPE101 (Schmiedl et al. 2000) offers high scale production of antibody fragments for further analysis, e.g. analysis by flow cytometry (Schirrmann and Pecher 2005). Another important feature of an antibody is its affinity, which is analysed by surface plasmon resonance (BIAcore) (Lauer et al. 2005). After analysis of specificity and affinity the selected antibody fragment can be subcloned into other formats like IgG or scfv::Fc-fusion in order to achieve avidity and immunological effector functions (Jostock et al. 2004).

3.4 Phage Display Vectors

A large number of different phage display vectors have been constructed. Table 3.2 lists a selection of phage display vectors, without pretending to be complete. Some of them have not been used for the construction of a library up to now, but have been included since they offer ideas and alternatives, e.g. a system which allows to control the success of antibody gene cloning by green fluorescent protein (GFP) expression (Pascke et al. 2001).

A variety of different promoters have been employed for the expression of antibody fragments on the surface of phages. Widely used is the lac Z promoter (lacZ) derived from the lactose operon (Jacob and Monod 1961). The gIII promoter (gIII) from the bacteriophage M13 (Smith 1985), the tetracycline promoter (1x $tet^{o/p}$) (Zahn et al. 1999) and the phoA promotor of the *E. coli* alkaline phosphatase (Garrard et al. 1991) were also successfully used. It seems that very strong promoters, e.g. the synthetic promoter PAI/04/03 (Bujard et al. 1987), are rather a disadvantage (Dübel personnel communication). To our knowledge, a systematic comparison of the different promotors has not been done.

The targeting of the antibody fragments to the periplasmatic space of *E. coli* requires the use of signal peptides. The pelB leader of the pectate lyase gene of *Erwinia caratovora* (Lei et al. 1987) is commonly used. The gIII leader (Smith 1985), the phoA leader of the *E. coli* alkaline phosphatase and the ompA leader

Table 3.2 Antibody Phage display vectors in alphabetical order (modified from Hust and Dübel 2005).[1] Cre/lox recombination[2] λ recombination[3] Construction of the HuCAL library is described, but the pMorph vectorsystem is unpublished.

Phage display vector	*Promoter*	*Secretion*	*Antibody format used by reference*	*C-domains in vector*	*Sites heavy chain*	*Sites light chain*	*Tags*	*gIII*	*Expression of soluble Ab*	*Reference*
pAALFab	1x lac Z, 2x RBS	2x pelB	Fab	no	*Eco*RI – *Bst*PI	*Spe*I – *Xho*I	–	truncated	subcloning	Iba et al. (1997)
pAALFv	1x lac Z, 2x RBS	2x pelB	Fv	no	*Eco*RI – *Bst*PI	*Spe*I – *Xho*I	–	truncated	subcloning	Iba et al. (1997)
pAALSC	1x lac Z, 1x RBS	1x pelB	scFv	no	*Eco*RI – *Bst*PI	*Spe*I – *Xho*I	–	truncated	subcloning	Iba et al. (1997)
pAK100	1x lac Z, 1x RBS	1x pelB	scFv	no	*Sfi*I (tet resistance will be removed)		FLAG, myc	truncated	amber, supE strain	Krebber et al. (1997)
pAPIII$_6$ scFv	1x phoA, 1x RBS	1x OmpA	scFv	no	*Hind*III – *Sal*I		FLAG, His	truncated	*Sal*I – KI digest, deletion of gIII	Haidaris et al. (2001)
pCANTAB3his	1x lac Z, 1x RBS	1x g3p	scFv	no	*Nco*I/*Sfi*I – *Not*I		His, myc	full	amber, supE strain	McCafferty et al. (1994)
pCANTAB5his/ pCANTAB 6	1x lac Z, 1x RBS	1x cat	scFv	no	*Nco*I/*Sfi*I – *Not*I		His, myc	full	amber, supE strain	
pCANTAB 5 E	1x lac Z, 1x RBS	1x g3p	scFv	no	*Sfi*I – *Not*I		E tag	full	amber, supE strain	www.amershambiosciences.com
pCES1	1x lac Z, 2x RBS	1x gIII (L) 1x pelB (H)	Fab	yes	*Sfi*I – *Pst*I/*Bst*EII (VH)	*Apa*L1 – *Asc*I (L chain), *Apa*L1 – *Xho*I (VL)	His, myc	full	amber, supE strain	Haardt et al. (1999)
pComb3	2x lac Z, 2x RBS	2x pelB	Fab	no	*Xho*I – *Spe*I	*Sac*I – *Xba*I	–	truncated	*Nhe*I – *Spe*I digest, deletion of gIII	Barbas et al. (1991)
pComb3H	1x lac Z, 2x RBS	ompA (LC) pelB (HC)	Fab, scFv	yes	*Xho*I – *Spe*I	*Sac*I – *Xba*I	–	truncated	*Nhe*I – *Spe*I digest, deletion of gIII	Barbas et al. (2001)
pComb3X	1x lac Z, 2x RBS	ompA (LC) pelB (HC)	Fab, scFv	yes	*Xho*I – *Spe*I	*Sac*I – *Xba*I	His, HA	truncated	amber, supE strain	
pCW93/H, pCW99/L[1]	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Nhe*I	*Sac*I – *Bgl*II	myc	truncated	amber, supE strain	Tsurushita et al. (1996)
pDAN5[1]	1x lac Z, 1x RBS	undiscribed leader	scFv	no	*Xho*I – *Nhe*I	*Bss*HII – *Sal*I	SV5, his	full	amber, supE strain	Sblattero and Bradbury (2000)

Table 3.2 *Continued*

Phage display vector	*Promoter*	*Secretion*	*Antibody format used by reference*	*C-domains in vector*	*Sites heavy chain*	*Sites light chain*	*Tags*	*gIII*	*Expression of soluble Ab*	*Reference*
pDH188	2x phoA, 2x RBS	2x stII	Fab	no	n.d.	n.d.	–	truncated	subcloning	Garrard et al. (1991)
pDN322	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Not*I		FLAG, His	full	amber, supE strain	Pini et al. (1998)
pDNEK	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Not*I		FLAG, His	full	amber, supE strain	Viti et al. (2000)
pEXmide3	1x lac Z, 2x RBS	2x pelB	Fab	yes	*Sfi*I/*Nco*I – *Kpn*I/*Apa*I	*Eag*I/*Not*I – *Nhe*I/*Spe*I	–	full	amber, supE strain	Söderlind et al. (1993)
pEXmide4	1x lac Z, 1x RBS	1x pelB	scFv	CH1	*Nco*I – *Sal*I		–	full	amber, supE strain	Kobayashi et al. (1997)
pEXmide5	1x lac Z, 1x RBS	1x pelB	scFv	?	*Nco*I – *Sal*I		–	full	amber, supE strain	Jirholt et al. (1998)
pFAB4	2x lac Z, 2x RBS	2x pelB	Fab	no	*Sfi*II – *Not*I		–	truncated	amber, supE strain	Ørum et al. (1993)
pFAB4H	1x lac Z, 2x RBS	2x pelB	Fab	CH1	*Sfi*II – *Not*I		–	truncated	subcloning	Dziegel et al. (1995)
pFAB5c	2x lac Z, 2x RBS	2x pelB	Fab	no	*Sfi*I – *Not*I		–	truncated	amber, supE strain	Örum et al. (1993)
pFAB5c-His	1x lac Z, 2x RBS	2x pelB	scFv	no	*Sfi*I – *Not*I		his	truncated	amber, supE strain	Söderlind et al. (2000)
pFAB60	1x lac Z, 1x RBS	2x pelB	Fab	CH1	*Sfi*I – *Spe*I (VH) *Sfi*I – *Not*I (Fd)	*Nhe*I – *Asc*I (L chain)	his	truncated	*Eag*I digest, deletion of gIII	Johansen et al. (1995)
pFAB73H	1x lac Z, 1x RBS	2x pelB	Fab	CH1	*Nhe*I – *Apa*I (VH)	*Sfi*I – *Asc*I (L chain)	his	truncated	*Eag*I digest, deletion of gIII	Engberg et al. (1996)
pGP-F100	1x $tet^{o/p}$, 1x RBS	1x pelB	scFv	no	*Sfi*I (GFPuv will be removed)		myc	truncated	TEV protease site	Pascke et al. (2001)
pGZ1	1x $tet^{o/p}$, 1x RBS	1x pelB	scFv	no	*Sfi*I – *Not*I		myc	full	amber, supE strain	Zahn et al. (1999)
pHAL14	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Hind*III	*Mlu*I – *Not*I	Yol1/34, his, myc	full	amber, supE strain	Hust et al. (unpublished)

pHEN1	1x lac Z, 1x RBS	1x pelB	scFv, Fab, Fd, LC	no	*Sfi*I – *Not*I		myc	full	amber, supE strain	Hoogenboom et al. (1991)
pHEN1-Vλ3	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Xho*I	Vλ3 anti-BSA Ab chain	myc	full	amber, supE strain	Hoogenboom and Winter (1992)
pHEN2	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Xho*I	*ApaL*I – *Not*I	his, myc	full	amber, supE strain	http://www.mrc-cpe.cam.ac.uk
pHENIX	1x lac Z, 1x RBS	1x pelB	scFv	no	*Sfi*I/*Nco*I – *Sal*I/*Xho*I	*ApaL*1 – *Not*I	myc	full	amber, supE strain	Finnern et al. (1997)
pHG-1m/ A27Jκ1	1x lac Z, 1x RBS	1x pelB	scFv	no	*ApaL*I – *Sfi*I	A27Jκ1 (VL)	his, myc	full	amber, supE strain	Rojas et al. 2002
phh3mu-γ1	2x lac Z, 2x RBS	2x pelB	Fab bidirectional	yes	*Xho*I – *Eco*RI	*Sac*I – *Hind*III	–	truncated	subcloning	Den et al. (1999)
pIG10	1x lac Z, 1x RBS	1x OmpA	scFv	no	*Eco*RV – *Eco*RI		myc	full	amber, supE strain	Ge et al. (1995)
pIGT2 (vector)	1x lac Z, 1x RBS	1x g3p	scFv	no	*Sfi*I – *Not*I		myc	full	amber, supE strain	Baek et al. (2002)
pIGT3 (vector)	1x lac Z, 1x RBS	1x g3p	scFv	no	*Sfi*I – *Sfi*I		myc	full	subcloning	
pIT2	1x lac Z, 1x RBS	1x pelB	scFv	no	SfiI/*Nco*I – *Xho*I	*SalL*I – *Not*I	His, myc	full	amber, supE strain	Goletz et al. (2002)
pLG18	1x phoA, 2x RBS	2x stII	Fab	yes	*BssH*II – *Nco*I (CDR2-3)	*BstE*II – *Asp*718 (CDR1-3)	–	truncated	subcloning	Garrard and Henner (1993)
pM834, pM827[2]	2x lac Z, 2x RBS	2x pelB	Fab	no	*Xho*I – *Spe*I	*Sac*I – *Xba*I	–	full	amber, supE strain	Geoffroy et al. (1994)
pMorph series[3]	1x lac Z ?	1x phoA	scFv	no	*Xba*I – *Eco*RI		FLAG ?	?	subcloning	Knappik et al. (2000)
pScUAGΔcp3	1x lac Z, 1x RBS	1x pelB	scFv with Cκ	Cκ	*Xho*I – *Nhe*I	*Sst*I – *Bgl*II	–	truncated	amber, supE strain	Akamatsu et al. (1993)
pSEX	1x PA1/04/03, 1x RBS	1x pelB	scFv	no	–		Yol1/34	full	subcloning	Breitling et al. (1991)
pSEX20	1x PA1/04/03, 1x RBS	1x pelB	scFv	no	–		Yol1/34	full	subcloning	Dübel et al. (1993)
pSEX81	1x lac Z, 1x RBS	1x pelB	scFv	no	*Nco*I – *Hind*III	*Mlu*I – *Not*I	Yol1/34	full	subcloning	Welschof et al. (1997)

of *E. coli* outer membrane protein OmpA have also been used, being common to many protein expression vectors (Skerra et al. 1993, Skerra and Schmidt 1999). Further examples are the heat-stable enterotoxin II (stII) signal sequence (Garrard et al. 1991) and the bacterial chloramphenicol acetyltransferase (cat) leader (McCafferty et al. 1994).

Due to the inability of *E. coli* to assemble complete IgG, with one exception (Simmons et al. 2002), smaller antibody fragments are used for phage display. In particular, Fabs and scFvs have been shown to be the antibody formats of choice. As aforementioned, in Fabs, the fd fragment and light chain are connected by a disulphide bond. In scFvs, the V_H and V_L are connected by a 15–25 amino acid linker (Bird et al. 1988, Bird and Walker 1991, Huston et al. 1988). Soluble scFvs tend to form dimers, in particular when the peptide linker is reduced to three to twelve amino acid residues. Diabodies or tetrabodies are produced if the linker between V_H and V_L is reduced to a few amino acids (Kortt et al. 1997, Arndt et al. 1998, Le Gall et al. 1999). The dimerization agravates the determination of the affinity, due to the possible avidity effect of the antibody complex (Marks et al. 1992b). Furthermore, some scFvs show a reduced affinity up to one order of magnitude compared to the corresponding Fabs (Bird and Walker 1991). ScFvs with a higher affinity than the corresponding Fabs were rarely found (Iliades et al. 1998). Small antibody fragments like Fv and scFv can easily be produced in *E. coli*. The yield of functional Fvs expressed in *E. coli* is higher than the yield of the corresponding Fabs, due to a lower folding rate of the Fabs (Plückthun 1990, Plückthun 1991). In one example, the stability in long-term storage was much higher for Fabs than for scFvs. After 6 month the functionality of scFvs stored at 4°C was reduced by 50 %, Fabs, however, showed no significant loss of functionality after one year (Kramer et al. 2002). The overall yield of Fvs expressed in *E. coli* vary from 0.5 to 10 mg/l culture compared to 2 to 5 mg/l culture for Fabs (Ward 1993), but very high yields of 1–2 mg/l of soluble and functional F(ab')2 have been reported (Carter et al. 1992). Therefore, the choice of the antibody format, scFv or Fab, depends on the desired application.

For the expression of Fabs in *E. coli* two polypeptide chains have to be assembled. In the monocistronic systems, e.g. pComb3, the antibody genes are under control of two promoters and each has its own leader peptide (Barbas et al. 1991), whereas in plasmids like pCES1 with a bicistronic Fab operon, both chains are under control of a single promoter, leading to a mRNA with two ribosomal binding sites (De Haard et al. 1999). The bicistronic system is more sufficient for the expression of Fabs (Kirsch et al. 2005).

Two variants of the antibody::pIII-fusion have been made. Either full size pIII or truncated version of pIII were used. The truncated version was made by deleting the pIII N-terminal domain. This domain mediated the interaction with the F pili of *E. coli*. Infection is provided by wt pIII, as only a small percentage of phage in phagemid-based systems are carrying an antibody. These truncated vectors are therefore not compatible with the use of Hyperphage or Ex-phage, as the fullsize pIII is necessary for infection (Rondot et al. 2001, Baek et al. 2002). Some phagemids, e.g. pSEX81 (Welschof et al. 1997) allow the elution of antibody

phages during panning by protease digestion instead of pH shift. This is possible due to a protease cleavage site between pIII and the antibody fragment. Therefore complete recovery of specifically antigen bound antibody phages is possible, even in case of very strong antigen binding.

Most of the described phagemids have an amber stop codon between the antibody gene and gIII. This allows the production of soluble antibody fragments after transformation of the phagemid to a non suppressor bacterial strain like HB2151 (Griffiths et al. 1994). For phagemids like pComb, it is necessary to delete the gIII by digestion and religate the vector before tranformation into *E. coli* (Barbas et al. 1991). In the case of phagmids like pSEX81, the selected antibody genes have to be subcloned into a separate *E. coli* expression vector like the pOPE series (Breitling et al. 1991, Dübel et al. 1993, Schmiedl et al. 2000).

3.5 Phage Display Libraries

Various types of phage display libraries have been constructed. Immune libraries are generated by amplification of V genes isolated from IgG secreting plasma cells of immunised donors (Clackson et al. 1991). From immune libraries antibody fragments with monovalent dissociations constants in the nM range can be isolated. Immune libraries are typically created and used in medical research to select an antibody fragment against one particular antigen, e.g. an infectious pathogen, and therefore would not be the source of choice for the selection of a large number of different specificities. Naive, semi-synthetic and synthetic libraries have been subsumed as "single-pot" libraries, as they are designed to isolate antibody fragments binding to every possible antigen. A correlation is seen between the size of the repertoire and the affinities of the isolated antibodies. Antibody fragments with a μM affinity have been isolated from a "single-pot" library consisting of approximately 10^7 clones, whereas antibody fragments with nM affinities were obtained from a library consisting of 10^9 independent clones (Hoogenboom 1997). It is evident that the chance to isolate an antibody with a high affinity for a particular antigen increases almost linearly to the size of the library. According to the source of antibody genes, "single pot" libraries (Table 3.3) can be naive libraries, semisynthetic libraries or fully synthetic libraries. Naive libraries are constructed from rearranged V genes from B cells (IgM) of non-immunized donors. An example for this library type is the naive human Fab library constructed by de Haardt et al. (1999), yielding antibodies with affinities up to 2.7×10^{-9} M. Semi-synthetic libraries are derived from unrearranged V genes from pre B cells (germline cells) or from one antibody framework with genetically randomized complementary determining region (CDR) 3 regions, as described by Pini et al. (1998). The antibody fragments obtained from this library show affinities between 10^{-8} M and 10^{-9} M, with one scFv having a dissociation constant of 5×10^{-11} M. A combination of naive and synthetic repertoire was used by Hoet et al. (2005). They combined light chains from autoimmune patients

Table 3.3 Human "single-pot" phage display libraries (modified from Hust and Dübel 2004). [1]Sheets et al. 1998, [2]Persson et al. 1991, [3]Griffiths et al. 1994.

Library vector	*Library type*	*Antibody type*	*Library cloning stratagy*	*Library size*	*reference*
DY3F63	synthetic and naive repertoire	Fab	ONCL, 4 step cloning with integration of naive CDRH3 in synthetic HC	3.5×10^{10}	Hoet et al. 2005
fdDOG-2lox, pUC19-2lox	semi-synthetic	Fab	PCR with random CDR3 Primers, Cre-lox	6.5×10^{10}	Griffiths et al. 1994
fdTet	naive	scFv	recloning of a naive library[1]	5×10^{8}	O'Connel et al. 2002
pAALFab	semi-synthetic (anti-hen egg white lysozyme Ab framework)	Fab	PCR with random CDR Primers, assembly PCR	2×10^{8}	Iba et al. 1997
pAP-III$_6$ scFv	naive	scFv	assembly PCR	n.d.	Haidaris et al. 2001
pCANTAB 6	naive	scFv	assembly PCR	1.4×10^{10}	Vaughan et al. 1996
pCES1	naive	Fab	3 step cloning (L chain, VH)	3.7×10^{10}	Haardt et al. 1999
pComb3	semi-synthetic (anti-tetanus Ab framework[2])	Fab	PCR with random CDR H3 Primers	5×10^{7}	Barbas et al. 1992
pComb3	semi-synthetic (anti-tetanus Ab framework[2])	Fab	PCR with random CDR H3 Primers	$>10^{8}$	Barbas et al. 1993
pDAN5	naive	scFv	Cre-lox	3×10^{11}	Sblattero and Bradbury 2000
pDN322	semi-synthetic (VH DP47 and VL DPK22 V-genes)	scFv	random CDR3 Primer, assembly PCR	3×10^{8}	Pini et al. 1998
pDN322	semi-synthetic (anti-AMCV CP ab framework)	scFv	random CDR3 Primer, assembly PCR	3.75×10^{7}	Desiderio et al. 2001

pDNEK (ETH2 library)	semi-synthetic (VH DP47, Vλ DPL16 and Vκ DPK 22 V-genes)	scFv	random CDR3 Primer, assembly PCR	5×10^8	Viti et al. 2000
pEXmide5	semi-synthetic (germline VH-DP47 and VL-DPL3 framework)	scFv	assembly PCR, CDR shuffling	9×10^6	Söderlind et al. 1993
pFAB5c-His (n-CoDeR library)	semi-synthetic (germline VH-DP47 and VL-DPL3 framework)	scFv	assembly PCR, CDR shuffling	2×10^9	Söderlind et al. 2000
pHAL14 (HAL4)	naive, kappa	scFv	2 step cloning	2.2×10^9	Hust et al. unpublished
pHAL14 (HAL7)	naive, lambda	scFv	2 step cloning	2.8×10^9	Hust et al. unpublished
pHEN1	naive	scFv	assembly PCR	10^7–10^8	Marks et al. 1991
pHEN1	naive	scFv	assembly PCR	$2 \times 10^5 / 2 \times 10^6$	Marks et al. 1992a
pHEN1	naive	scFv	assembly PCR	6.7×10^9	Sheets et al. 1998
pHEN1-Vλ3	semi-synthetic (Vλ3 anti-BSA Ab light chain)	scFv	PCR with random CDR H3 Primers	10^7	Hoogenboom and Winter 1992
pHEN1-Vλ3	semi-synthetic (Vλ3 anti-BSA Ab light chain)	scFV	PCR with random CDR H3 Primers	$>10^8$	Nissim et al. 1994
pHEN1-Vκ3	semi-synthetic (VH)/naive (VL)	scFv	3 step cloning, PCR with random CDR H3 Primers	3.6×10^8	de Kruif et al. 1995
pHEN2 (Griffin 1. library)	semi-synthetic	scFv	recloning of the lox library in scFv format[3]	1.2×10^9	www.mrc-cpe.cam.ac.uk
pIT2 (Tom I/J library)	semi-synthetic (3x VH and 4x Vκ V-genes)	scFv	PCR with random CDR2 and CDR3 Primers	1.47×10^8/ 1.37×10^8	Goletz et al. 2002
pLG18	semi-synthetic (anti-HER2 Ab framework)	Fab	PCR with random CDR Primers, 2 step cloning	2–3×10^8	Garrard and Henner 1993

Table 3.3 *Continued*

Library vector	*Library type*	*Antibody type*	*library cloning stratagy*	*library size*	*reference*
pMorph series (HuCAL library)	synthetic	scFv	2 step cloning, CDR3 replacement	2×10^9	Knappik et al. 2000
pMorph series (HuCAL GOLD library)	synthetic	Fab	2 step cloning, all CDR replacement	1.6×10^{10}	www.morphosys.com
pMID21	synthetic and naïve repertoire	Fab	ONCL, 4 step cloning with integration of naïve CDRH3 in synthetic HC	1×10^{10}	Hoet et al. 2005
pScUAGDcp3	semi-synthetic	scFv connected to Ck	3 step cloning with random CDR3 Primers	1.7×10^7	Akamatsu et al. 1993
pSEX81	naive	scFv (with N-terminus of CH1 and CL)	2 step cloning	4×10^7	Dörsam et al. 1997
pSEX81	naive	scFv (with N-terminus of CH1 and CL)	2 step cloning	4×10^9	Little et al. 1999
pSEX81	naive	scFv (with N-terminus of CH1 and CL)	4 step cloning	1.6×10^7/ 1.8×10^7/ 4×10^7	Schmiedel et al. 2000
pSEX81	naive	scFv (with N-terminus of CH1 and CL)	2 step cloning	6.4×10^9	Løset et al. 2005

with a fd fragment containing synthetic CDR1 and CDR2 in the human V_H3-23 framework and naive, origined from autoimmune patients, CDR3 regions. The fully synthetic libraries have a human framework with randomly integrated CDR cassettes (Hayashi et al. 1994). Antibody fragments selected from fully synthetic libraries exhibit affinities between 10^{-6} M and 10^{-11} M (Knappik et al. 2000). All library types – immune, naive, synthetic and their intermediates – are useful sources for the selection of antibodies for diagnostic and therapeutic purposes.

3.6 Generation of Phage Display Libraries

Various methods have been employed to clone the genetic diversity of antibody repertoires. After the isolation of mRNA from the desired cell type and the preparation of cDNA, the construction of immune libraries is usually done by a two step cloning or assembly PCR (see below). Naive libraries are constructed by two or three cloning steps. In the two step cloning strategy, the amplified repertoire of light chain genes is cloned into the phage display vector first, as the heavy chain contributes more to diversity, due to its highly variable CDRH3. In the second step the heavy chain gene repertoire is cloned into the phagemids containing the light chain gene repertoire (Johansen et al. 1995, Welschof et al. 1997, Little et al. 1999). In the three step cloning strategy, separate heavy and light chain libraries are engineered. The V_H gene repertoire has then to be excised and cloned into the phage display vector containing the repertoire of V_L genes (De Haardt et al. 1999). Another common method used for the cloning of naive (McCafferty et al. 1994, Vaughan et al. 1996), immune (Clackson et al. 1991) or hybridoma (Krebber et al. 1997) scFv phage display libraries is the assembly PCR. The V_H and V_L genes are amplified seperately and connected by a subsequent PCR, before the scFv encoding gene fragments are cloned into the vector. The assembly PCR is usually combined with a randomization of the CDR3 regions, leading to semi-synthetic libraries. To achieve this, oligonucleotide primers encoding various CDR3 and J gene segments were used for the amplication of the V gene segments of human germlines (Akamatsu et al. 1993). The CDRH3 is a major source of sequence variety (Shirai et al. 1999). Hoogenboom and Winter (1992) and Nissim et al. (1994) used degenerated CDRH3 oligonucleotide primers to produce a semi-synthetic heavy chain repertoire derived from human V gene germline segments and combined this repertoire with an anti-BSA light chain. In some cases a framework of a well known antibody was used as scaffold for the integration of randomly created CDRH3 and CDRL3 (Barbas et al. 1992, Desiderio et al. 2001). Jirholt et al. (1998) and Söderlind et al. (2000) amplified all CDR regions derived from B cells before shuffling them into one antibody framework in an assembly PCR reaction. An example for an entirely synthetic library, Knappik et al. (2000) utilized seven different V_H and V_L germline master frameworks combined with six synthetically created CDR cassettes. The construction of large naive and semi-synthetic libraries (Hoet et al. 2005, Løset et al. 2005,

Little et al. 1999, Sheets et al. 1998, Vaughan et al. 1996) requires significant effort to tunnel the genetic diversity through the bottleneck of *E. coli* transformation, e.g. 600 transformations were necessary for the generation of a 3.5×10^{10} phage library (Hoet et al. 2005).

To move the diversity potentiating step of random V_H/V_L combination behind the bottleneck of transformation, the Cre-lox or lamda phage recombination system has been employed (Waterhouse et al. 1993, Griffiths et al. 1994, Geoffrey et al. 1994). However, libraries with more than 10^{10} independent clones have now been accomplished by conventional transformation, rendering most of these complicated methods unnecessary in particular as they may result in decreased genetic stability. A remarkable exception is the use of a genomically integrated CRE recombinase gene (Sblattero and Bradbury 2000) which is expected to solve the instability issue and allows the generation of libraries with complexities above the limit achievable by conventional cloning.

In summary, antibodies with nanomolar affinities can be selected from either type of library, naive or synthetic. If the assembly by cloning or PCR and preservation of molecular complexity is carefully controlled at every step of its construction, libraries of more than 10^{10} independent clones can be generated.

Antibody phage display is delivering and will deliver high affinity human antibodies and antibody fragments for research as well as for diagnostic and therapeutic applications in the future.

References

Akamatsu, Y., Cole, M.S., Tso, J.Y., Tsurushita, N. (1993) Construction of a human Ig combinatorial library from genomic V segments and synthetic CDR3 fragments. *J Immunol* 151: 4651–4659.

Arndt, K.M., Müller, K.M., Plückthun, A. (1998) Factors influencing the dimer to monomer transition of an antibody single-chain Fv fragment. *Biochemistry* 37: 12918–12926.

Baek, H., Suk, K.H., Kim, Y.H., Cha, S. (2002) An improved helper phage system for efficient isolation of specific antibody molecules in phage display. *Nucleic Acids Res* 30: e18.

Barbas III, C.F., Kang, A.S., Lerner, R.A., Benkovic, S.J. (1991) Assembly of combinatorial antibody libraries on phages surfaces: the gene III site. *Proc Natl Acad Sci* USA 88: 7987–7982.

Barbas III, C.F., Bain, J.D., Hoekstra, M., Lerner, R.A. (1992) Semisynthetic combinatorial antibody libraries: a chemical solution to the diversity problems. *Proc Natl Acad Sci* USA 89: 4457–4461.

Barbas III, C.F., Amberg, W., Simoncsitis, A., Jones, T.M., Lerner, R.A. (1993) Selection of human anti-hapten antibodies from semisynthetic libraries. *Gene* 137: 57–62.

Barbas III, C.F., Burton, D.R., Scott, J.K., Silverman, G.J. (2001) Phage display: A laboratory manual. *Cold Spring Harbor Laboratory Press.*

Better, M., Chang, C.P., Robinson, R.R., Horwitz, A.H. (1988) Escherichia coli secretion of an active chimeric antibody fragment. *Science* 240: 1041–1043.

Bird, R.E., Hardman, K.D., Jacobsen, J.W., Johnson, S., Kaufman, B.M, Lee, S.M., Lee, T., Pope, S.H., Riordan, G.S., Whitlow, M. (1988) Single-chain antigen-binding proteins. *Science* 242: 423–426.

Bird, R.E., Walker, B.W. (1991) Single chain variable regions. *Trends Biotech* 9: 132–137.

Boder, E.T., Wittrip, K.D. (1997)Yeast surface display for screening combinatorial polypeptide libraries. *Nat Biotech* 15: 553–558.

Breitling, F., Dübel, S., Seehaus, T., Kleewinghaus, I., Little, M. (1991) A surface expression vector for antibody screening. *Gene* 104: 1047–1153.

Bujard, H., Gentz, R., Lanzer, M., Stueber, D., Mueller, M., Ibrahimi, I., Haeuptle, M. T., Dobberstein, B. (1987) A T5 promoter-based transcription-translation system for the analysis of proteins in vitro and in vivo. *Methods Enzymol* 155: 416–433.

Carter, P., Kelley, R.F., Rodrigues, M.L., Snedecor, B., Covarrubias, M., Velligan, M. D., Wong W.L., Rowland, A.M., Kotts, C.E., Carver, M.E., et al. (1992) High level Escherichia coli expression and production of a bivalent humanized antibody fragment. *Biotechnology* 10: 163–167.

Clackson, T., Hoogenboom, H.R., Griffiths, A.D., Winter, G. (1991) Making antibody fragments using phage display libraries. *Nature* 352: 624–628.

Crissman, J.W., Smith, G.P. (1984) Gene 3 protein of filamentous phages: evidences for a carboxyl-terminal domain with a role in morphogenesis. *Virology* 132: 445–455.

Courtenay-Luck, N.S., Epenetos, A.A., Moore, R., Larche, M., Pectasides, D., Dhokia, B., Ritter, M.A. (1986) Development of primary and secondary immune responses to mouse monoclonal antibodies used in the diagnosis and therapy of malignant neoplasms. *Cancer Res.* 46: 6489–6493.

Cwirla, S.E., Peters, E.A., Barrett, R.W., Dower, W.J. (1990) Peptides on phage: a vast library of peptides for identifying ligands. *Proc Natl Acad Sci USA* 87: 6378–6382.

Danner, S., Belasco, J.G. (2001) T7 phage display: A novel genetic selection system for cloning RNA-binding protein from cDNA libraries. *Proc Natl Acad Sci* USA 98: 12954–12959.

De Kruif, J. Boel, E., Logtenberg, T. (1995) Selection and application of human single chain Fv antibody fragments from a semi-synthetic phage antibody display library with designed CDR3 regions. *J Mol Biol* 248: 97–105.

De Haardt, H.J., van Neer, N., Reurst, A., Hufton, S.E., Roovers, R.C., Henderikx, P., de Bruine, A.P., Arends, J.-W., Hoogenboom, H.R. (1999) A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies. *J Biol Chem* 274: 18218–18230.

Den, W., Sompuram, S.R., Sarantopoulos, S., Sharon, J. (1999) A bidirectional phage display vector for the selection and mass transfer of polyclonal antibody libraries. *J Immunol Meth* 222: 45–57.

Desiderio, A., Franconi, R., Lopez, M., Villani, A.E., Viti, F., Chiaraluce, R., Consalvi, V., Neri, D., Benvenuto, E. (2001) A semi-synthetic repertoire of intrinsically stable antibody fragments derived from a single-framework scaffold. *J Mol Biol* 310: 603–615.

Dörsam, H., Rohrbach, P, Kürschner, T. Kipriyanov, S., Renner, S., Braunnagel, M., Welschof, M., Little, M. (1997) Antibodies to steroids from a small human naive IgM library. *FEBS Letters* 414: 7–13.

Dübel, S., Breitling, F., Fuchs, P., Braunagel, M., Klewinghaus, I., Little, M. (1993) A family of vectors for surface display and production of antibodies. *Gene* 128: 97–101.

Dziegiel, M., Nielsen, L.K., Andersen, P.S., Blancher, A., Dickmeiss, E., Engberg, J. (1995) Phage display used for gene cloning of human recombinant antibody against the erythrocyte surface antigen, rhesus D. *J Immunol Meth* 182: 7–19.

Engberg, J. Andersen, P.S., Nielsen, L.K., Dziegiel, M., Johansen, L.K., Albrechtsen, B. (1996) Phage-display libraries of murine and human Fab fragments. *Mol Biotechnol* 6: 287–310.

Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R., Cesareni, G. (1991) Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *J Mol Biol* 222: 301–310.

Finnern, R., Pedrollo, E., Fisch, I., Wieslander, J., Marks, J.D., Lockwood, C. M., Ouwehand, W.H. (1997) Human autoimmune anti-proteinase 3 scFv from a phage display library. *Clin Exp Immunol* 107: 269–281.

Fishwild, D.M., O'Donnel, S.L., Bengoechea, T., Hudson, D.V., Harding, F., Bernhar, S. L., Jones, D., Kay, R.M., Higgins, K.M., Schramm, S.R., Lonberg, N. (1996) High-avidity human IgG kappa monoclonal antibodies from a novel strain of minilocus transgenic mice. *Nat Biotech* 14: 845–851.

Fuchs, P., Breitling, F., Dübel, S., Seehaus, T., Little, M. (1991) Targeting recombinant antibodies to the surface of E. coli: Fusion to a peptidoglycan associated lipoprotein. *Bio/Technology* 9: 1369–1372.

Gao, C., Mao, S., Lo, C.H., Wirsching, P., Lerner, R.A., Janda, K.D. (1999) Making artificial antibodies: a format for phage display of combinatorial heterodimeric arrays. *Proc Natl Acad Sci* USA 96: 6025–6030.

Garrard, L.J., Yang, M., O'Connel, M.P., Kelley, R., Henner, D.J. (1991) Fab assembly and enrichment in a monovalent phage display system. *Bio/Technology* 9: 1373–1377.

Garrard, L.J., Henner, D.J. (1993) Selection of an anti-IGF-1 Fab from a Fab phage library created by mutagenesis of multiple CDR loops. *Gene* 128: 103–109.

Ge, L., Knappik, A., Pack, P., Freund, C., Plückthun, A. (1995) Expressing antibodies in *Escherichia coli*, in: Antibody Engineering (Borrebaeck, C.A.K., ed.), Oxford University Press.

Geoffroy, F., Sodoyer, R., Aujame, L. (1994) A new phage display system to construct multicombinatorial libraries of very large antibody repertoires. *Gene* 151: 109–113.

Goletz, A., Cristensen, P.A., Kristensen, P., Blohm, D., Tomlinson, I., Winter, G., Karsten, U. (2002) Selection of large diversities of antiidiotypic antibody fragments by phage display. *J Mol Biol* 315: 1087–1097.

Griffiths, A.D., Williams, S.C., Hartley, O., Tomlinson, I.M., Waterhouse, P., Crosby, W., Kontermann, R.E., Jones, P.T., Low, N.M., Allison, T.J., Prospero, T.D., Hoogenboom, H.R., Nissim, A., Cox, J.P.L., Harrison, J.L., Zaccolo, M., Gherardi, E., Winter, G. (1994) Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J* 13: 3245–3260.

Haidaris, C.G., Malone, J., Sherrill, L.A., Bliss, J.M., Gaspari, A.A., Insel, R.A., Sullivan, M.A. (2001) Recombinant human antibody single chain variable fragments reactive with *Candida albicans* surface antigens. *J Immunol Meth* 257: 185–202.

Hanes, J., Plückthun, A. (1997) In vitro selection and evolution of functional proteins by using ribosome display. *Proc Natl Acad Sci* USA 94: 4937–4942.

Hawlisch, H., Müller, M., Frank, R., Bautsch, W., Klos, A., Köhl, J (2001) Sitespecific anti-C3a receptor single-chain antibodies selected by differential panning on cellulose sheets. *Analytical Biochemistry* 293: 142–145.

Hayashi, N., Welschoff, M., Zewe, M., Braunagel, M., Dübel, S., Breitling, F., Little, M. (1994) Simultaneous mutagenesis of antibody CDR regions by overlap extension and PCR. *Biotechniques* 17: 310–316.

Hoet, R.M., Cohen, E.H., Kent, R.B., Rookey, K., Schoonbroodt, S., Hogan, S., Rem, L., Frans, N., Daukandt, M., Pieters, H., van Hegelsom, R., Neer, N.C., Nastri, H.G., Rondon, I.J., Leeds, J.A., Hufton, S. E., Huang, L., Kashin, I., Devlin, M., Kuang, G., Steukers, M., Viswanathan, M., Nixon, A.E., Sexton, D.J., Hoogenboom, H. R., Ladner, R.C. (2005) Generation of high-affinity human antibodies by combining donor-derived and synthetic complementarity-determining-region diversity. *Nat Biotechnol* 23: 344–348.

Hoogenboom, H.R., Griffiths, A.D., Johnson, K.S., Chiswell, D.J., Hudson, P., Winter, G. (1991) Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucl Acids Res* 19: 4133–4137.

Hoogenboom, H.R., Winter, G. (1992) By-passing immunisation: Human antibodies from synthetic repertoires of germline V_H gene segments rearranged *in vitro*. *J Mol Biol* 227: 381–388.

Hoogenboom, H.R. (1997) Designing and optimizing library selection strategies for generating high-affinity antibodies. *Trends Biotech* 15: 62–70.

Huse, W.D., Sastry, L., Iverson, S.A., Kang, A.S., Alting-Mees, M., Burton, D.R.,

Benkovic, S.J., Lerner, R. (1989) Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. *Science* 246: 1275–1281.

Hust, M., Maiss, E., Jacobsen, H.-J., Reinard, T. (2002) The production of a genus specific recombinant antibody (scFv) using a recombinant Potyvirus protease. *J Virol Meth* 106: 225–233.

Hust, M., Dübel, S. (2004) Mating antibody phage display with proteomics. *Trends in Biotechnology* 22: 8–14.

Hust, M., Dübel, S. (2005) Phage display vectors for the in vitro generation of human antibody fragments in: Immunochemical Protocols, 3rd ed., Ed: Burns, R., *Meth Mol Biol* 295: 71–95.

Hust, M., Toleikis, L., Dübel, S. (2005) Antibody Phage Display. *Mod Asp Immunobiol* 15: 47–49.

Huston, J.S., Levinson, D., Mudgett, H.M., Tai, M.S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoloreri, R.E., Haber, E., Crea, R., Oppermann, H. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digosin single-chain Fv analogue produced in *Escherichia coli Proc Natl Acad Sci* USA 85: 5879–5883.

Iba, Y., Ito, W., Kurosawa, Y. (1997) Expression vectors for the introduction of higly diverged sequences into the six complementarity-determining regions of an antibody. *Gene* 194: 35–46.

Iliades, P., Dougan, D.A., Oddie, G.W., Metzger, D.W., Hudson, P.J., Kortt, A.A. (1998) Single-chain Fv of anti-idiotype 11–1G10 antibody interacts with antibody NC41 single-chain Fv with a higher affinity than the affinity for the interaction of the parent Fab fragments. *J Protein Chem* 17: 245–254.

Jacob, F., Monod, J. (1961) Genetic regulatory mechanism in the synthesis of proteins. *J Mol Biol* 3: 318–356.

Jakobovits, A. (1995) Production of fully human antibodies by transgenic mice. *Curr Opin Biotechnol* 6: 561–6.

Jespers, L.S., Messens, J.H., de Keyser, A., Eeckhout, D., van den Brande, I., Gansemans, Y.G., Lauwerey, M.J., Vlasuk, G.P., Stanssens, P.E. (1995) Surface expression and ligand based selection of cDNAs fused to filamentous phage gene VI. *Bio/Technology* 13: 378–381.

Jirholt, P., Ohlin, M., Borrebaeck, C.A.K., Söderlind, E. (1998) Exploiting sequences space: shuffling *in vivo* formed complementarity determining regions into a master framework. *Gene* 215: 471–476.

Johansen, L.K., Albrechtsen, B., Andersen, H.W., Engberg, J. (1995) pFab60: a new, efficient vector for expression of antibody Fab fragments displayed on phage. *Prot Engineering* 8: 1063–1067.

Jostock, T., Vanhove, M., Brepoels, E., Van Gool, R., Daukandt, M., Wehnert, A., Van Hegelsom, R., Dransfield, D., Sexton, D., Devlin, M., Ley, A., Hoogenboom, H., Mullberg, J. (2004) Rapid generation of functional human IgG antibodies derived from Fab-on-phage display libraries. *J Immunol Methods* 289: 65–80.

Jostock, T., Dübel, S. (2005) Screening of Molecular Repertoires by Microbial Surface Display. *Comb Chem High Throughput Screen* 8: 127–133.

Kang, A.S., Jones, T.M., Burton, D.R. (1991a) Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries. *Proc Natl Acad Sci* USA 88: 11120–11123.

Kang, A.S., Barbas, C.F., Janda, K.D., Bencovic, S.J., Lerner, R.A. (1991b) Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. *Proc Natl Acad Sci* USA 88: 4363–4366.

Kirsch, M., Zaman, M., Meier, D., Dübel, S., Hust, M. (2005) Parameters affecting the display of antibodies on phage. *J Immunol Meth* 301: 173–185.

Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wölle, J., Plückthun, A., Virnekäs, B. (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus framework and CDRs randomized with trinucleotides. *J Mol Biol* 296: 57–86.

Köhler, G., Milstein, C. (1975) Continous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.

Kobayashi, N. Söderlind, E., Borrebaeck, C. A.K. (1997) Analysis of assembly of synthetic antibody fragments: Expression of functional scFv with predifined specificity. *BioTechniques* 23: 500–503.

Kontermann, R., Dübel, S. (eds.) (2001) Antibody Engineering. Springer-Verlag, New York.

Konthur, Z., Hust, M., Dübel, S. (2005) Perspectives for systematic in vitro antibody generation. *Gene* 364: 19–29.

Kortt, A.A., Lah, M., Oddie, G.W., Gruen, L. C., Burns, J.E., Pearce, L.A., Atwell, J.L., McCoy, A.J., Howlett, G.J., Metzger, D.W., Webster, R.G., Hudson, P.J. (1997) Single chain Fv fragments of anti-neurominidase antibody NC10 containing five and ten residue linkers form dimers and with zero residue linker a trimer. *Prot Eng* 10: 423–428.

Kramer, K., Fiedler, M., Skerra, A., Hock, B. (2002) A generic strategy for subcloning antibody variable regions from the scFv phage display vector pCANTAB 5 E into pASK85 permits the economical production of Fab fragments and leads to improved recombinant immunoglobulin stability. *Biosensors & Bioelectronics* 17: 305–313.

Krebber, A., Bornhauser, S., Burmester, J., Honegger, A., Willuda, J., Bosshard, H.R., Plückthun, A. (1997) Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. *J Immunol Meth* 201: 35–55.

Lauer, B., Ottleben, I., Jacobsen, H.J., Reinard, T. (2005) Production of a single-chain variable fragment antibody against fumonisin B1. *J Agric Food Chem* 53: 899–904.

Le Gall, F., Kipriyanov, S.M., Moldenhauer, G., Little, M. (1999) Di-, tri- and tetrameric single chain Fv antibody fragments against human CD19: effect of valency on cell binding. *FEBS Letters* 453: 164–168.

Lei, S.-P., Lin, H.-C, Wang, S.-S., Callaway, J., Wilcox, G. (1987) Characterization of the *Erwinia caratovora pelB* gene and its product pectate lyase. *J Bacteriol* 169: 4379–4383.

Little, M., Welschof, M., Braunagel, M., Hermes, I., Christ, C., Keller, A., Rohrbach, P., Kürschner, T., Schmidt, S., Kleist, C., Terness, P. (1999) Generation of a large complex antibody library from multiple donors. *J Immunol Meth* 231: 3–9.

Lonberg, N., Huszar, D. (1995) Human antibodies from transgenic mice. *Int Rev Immunol* 13: 65–93.

Løset, G.Å., Løbersli, I., Kavlie, A., Stacy, J. E., Borgen, T., Kausmally, L., Hvattum, E., Simonsen, B., Hovda M.B., Brekke, O.H. (2005) Construction, evaluation and refinement of a large human antibody phage library based on the IgD and IgM variable gene repertoire. *J Immunol Meth* 299: 47–62.

Lowman, H.B., Bass, S.H., Simpson, N., Wells, J.A. (1991) Selecting High-Affinity binding proteins by monovalent phage display. *Biochemistry* 30: 10832–10838.

Marks, J.D., Hoogenboom, H.R., Bonnert, T. P., McCafferty, J., Griffiths, A.D., Winter, G. (1991) By-passing immunization: human antibodies from V-gene libraries diplayed on phage. *J Mol Biol* 222: 581–597.

Marks, J.D., Griffiths, A.D., Malmqvist, M., Clackson, T.P., Bye, J.M., Winter, G. (1992a) By-passing immunization: building high affinity human antibodies by chain shuffling. *Bio/Technology* 10: 779–783.

Marks, J.D., Hoogenboom, H.R., Griffiths, A.D., Winter, G. (1992b) Molecular evolution of proteins on filamentous phage. *J Biol Chem* 267: 16007–16010.

McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J. (1990) Phage antibodies: filamentous phage displaying antibody variable domain. *Nature* 348: 552–554.

McCafferty, J., Fitzgerald, K.J., Earnshaw, J., Chiswell, D.J., Link, J., Smith, R., Kenten, J. (1994) Selection and rapid purification of murine antibody fragments that bind a transition-state analog by phage-display. *Appl Biochem Biotech* 47: 157–173.

McCafferty, J., Hoogenboom, H.R., Chiswell, D.J. (eds.) (1996) Antibody Engineering. IRL Press, Oxford.

Moghaddam, A., Borgen, T., Stacy, J., Kausmally, L., Simonsen, B., Marvik, O.J., Brekke, O.H., Braunagel, M. (2003) Identification of scFv antibody fragments that specifically recognise the heroin

metabolite 6-monoacetylmorphine but not morphine. *J Immunol Meth* 280: 139–155.
Mullinax, R.L., Gross, E.A., Amberg, J.R., Hay, B.N., Hogreffe, H.H., Kubitz, M.M., Greener, A., Alting-Mees, M., Ardourel, D., Short, J.M., Sorge, J.A., Shopes, B. (1990) Identification of human antibody fragment clones specific for tetanus toxoid in a bacteriophage λ immunoexpression library. *Proc Natl Acad Sci* USA 87: 8095–8099.
Nissim, A., Hoogenboom, H.R., Tomlinson, I.M., Flynn, G., Midgley, C., Lane, D., Winter, G. (1994) Antibody fragments from a "single pot" phage display library as immunochemical reagents. *EMBO J* 13: 692–698.
O'Connel, D., Becerril, B., Roy-Burman, A., Daws, M., Marks, J.D. (2002) Phage versus phagemid libraries for generation of human monoclonal antibodies. *J Mol Biol* 321: 49–56.
Ørum, H., Andersen, P.S., Øster, A., Johansen, L.K., Riise, E., Bjørnevad, M., Svendsen, I., Engberg, J. (1993) Efficient method for constructing comprehensive murine Fab antibody libraries displayed on phage. *Nucl Acids Res* 21: 4491–4498.
Parmley, S.F., Smith, G.P. (1988) Antibody selectable filamentous fd phage vectors: affinity purification of target genes. *Gene* 73: 305–318.
Paschke, M., Zahn, G., Warsinke, A., Höhne, W. (2001) New series of vectors for phage display and prokaryotic expression of proteins. *BioTechniques* 30: 720–726.
Persson, M.A.A., Caothien, R.H., Burton, D. R. (1991) Generation of diverse high-affinity human monoclonal antibodies by repertoire cloning. *Proc Natl Acad Sci* USA 88: 2432–2436.
Pini, A, Viti, F., Santucci, A., Carnemolla, B., Zardi, L., Neri, P., Neri, D. (1998) Design and use of a phage display library. *J Biol Chem* 273: 21769–21776.
Plückthun, A. (1990) Antibodies from *Escherichia Coli Nature* 347: 497–498.
Plückthun, A. (1991) Antibody Engineering: Advances from the use of *Escherichia coli* expression systems. *Bio/Technology* 9: 545–551.
Roberts, R.W., Szostak, J.W. (1997) RNA-peptide fusions for the in vitro selection of peptides and proteins. *Proc Natl Acad Sci* USA 94: 12297–12302.
Rojas, Gertrudis, Almagro, J.C., Acevedo, B., Gavilondo, J.V. (2002) Phage antibody fragments library combining a single human light chain variable region with immune mouse heavy chain variable regions. *J Biotech* 94: 287–298.
Rondot, S., Koch, J., Breitling, F., Dübel, S. (2001) A helperphage to improve single chain antibody presentation in phage display. *Nat Biotech* 19: 75–78.
Sblattero, D., Bradbury, A. (2000) Exploiting recombination in single bacteria to make large phage antibody libraries. *Nat Biotech* 18: 75–80.
Schirrmann, T., Pecher, G. (2002) Human natural killer cell line modified with a chimeric immunoglobulin T-cell receptor gene leads to tumor growth inhibition *in vivo*. *Cancer Gene Ther* 4: 390–398.
Schmiedl, A., Breitling, F., Dübel, S. (2000) Expression of a bispecific dsFv-dsFv' antibody fragment in Escherichia coli. *Protein Engineering* 13: 725–734.
Sheets, M.D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R., Hemingsen, G., Wong, C., Gerhart, J.C., Marks, J.D. (1998) Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. *Proc Natl Acad Sci* USA 95: 6157–6162.
Shirai, H., Kidera, A., Nakamura, H. (1999) H3-rules: identification of CDR3-H3 structures in antibodies. *FEBS Letters* 455: 188–197.
Simmons, L.C., Reilly, D., Klimowski, L., Raju, T.S., Meng, G., Sims, P., Hong, K., Shields, R.L., Damico, L.A., Rancatore, P., Yansura, D.G. (2002) Expression of full-length immunoglobulins in Escherichia coli: rapid and efficient production of aglycosylated antibodies. *J Immunol Meth* 263: 133–47.
Skerra, A., Plückthun, A. (1988) Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli Science* 240: 1038–1041.
Skerra, A., Pfitzinger, I., Plückthun, A. (1993) The functional expression of antibody Fv fragments in *Escherichia coli*:

improved vectors and a generally applicable purification technique. *Bio/Technology* 9: 273–278.

Skerra, A., Schmidt, T.G.M. (1999) Applications of a peptide ligand for streptavidin: the Strep-tag. *Biomol Engineer* 16: 79–86.

Smith, G.P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228: 1315–1317.

Söderlind, E., Lagerkvist, A.C.S., Dueňas, M., Malmborg, A.-C., Ayala, M., Danielsson, L., Borrebaeck, C.A.K. (1993) Chaperonin assisted phage display of antibody fragments of filamentous bacteriophages. *Bio/Technology* 11: 503–507.

Söderlind, E., Strandberg, L., Jirholt, P., Kobayashi, N., Alexeiva, V., Aberg, A.-M., Nilsson, A., Jansson, B., Ohlin, M., Wingren, C., Danielsson, L., Carlsson, R., Borrebaeck, C.A.K. (2000) Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. *Nat Biotech* 18: 852–856.

Spada, S., Plückthun, A. (1997) Selectivity infective phage (SIP) technology: A novel method for *in vivo* selection of interacting protein-ligand pairs. *Nature Medicine* 3: 694–696.

Studnicka, G.M., Soares, S., Better, M., Williams, R.E., Nadell, R., Horwitz, A.H. (1994) Human-engineered monoclonal antibodies retain full specific binding activity by preserving non-CDR complementarity-modulating residues. *Protein Eng* 6: 805–14.

Toleikis, L., Broders, O., Dübel, S. (2004) Cloning single-chain antibody fragments (scFv) from hybridoma cells. *Meth Mol Med* 94: 447–458.

Tsurushita, N., Fu, H., Warren, C. (1996) Phage display vectors for *in vivo* recombination of immunoglobulin heavy and light chain genes to make large combinatorial libraries. *Gene* 172: 59–63.

Vaughan, T.J., Williams, A.J., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, J.C., McCafferty, J., Hodits, R.A., Wilton, J., Johnson, K.S. (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat Biotech* 14: 309–314.

Vieira, J., Messing, J. (1987) Production of single-stranded plasmid DNA. *Methods Enzymol* 153: 3–11.

Viti, F., Nilsson, V., Demartis, S., Huber, A., Neri, D. (2000) Design and use of phage display libraries for the selection of antibodies and enzymes. *Meth Enzymol* 326: 480–497.

Von Behring, E., Kitasato, S. (1890) Über das Zustandekommen der Diphtherie-Immunität und der Tetanus-Immunität bei Thieren. *Deutsche Medizinische Wochenzeitschrift* 16: 1113–1114.

Ward, E.S. (1993) Antibody engineering using *Escherichia coli* as host. *Adv Pharmacol* 24: 1–20.

Waterhouse, P., Griffiths, A.D., Johnson, K.S., Winter, G. (1993) Combinatorial infection and *in vivo* recombination: a stratagie for making large phage antibody repertoires. *Nucl Acids Res* 21: 2265–2266.

Welschof, M., Terness, P., Kipriyanov, S., Stanescu, D., Breitling, F., Dörsam, H., Dübel, S., Little, M., Opelz, G. (1997) The antigen binding domain of a human IgG-anti-F(ab')2 autoantibody. *Proc Natl Acad Sci* USA 94: 1902–1907.

Winter, G., Milstein, C. (1991) Man-made antibodies. *Nature* 349: 293–299.

Zahn, G., Skerra, A., Höhne, W. (1999) Investigation of a tetracycline-regulated phage display system. *Protein Eng* 12: 1031–1034.

4
Selection Strategies III: Transgenic Mice

Marianne Brüggemann, Jennifer A. Smith, Michael J. Osborn, and Xiangang Zou

4.1
Introduction

Antibodies of diverse specificity are produced in all mammals. Indeed, all jawed vertebrates contain the genes to allow combinatorial immune responses and can produce specific antibodies following immunization. The primary repertoire is generated after V (variable) D (diversity) J (joining) gene rearrangement of the Ig (immunoglobulin) heavy (H) chain locus and VJ joining of a κ or λ light (L) chain locus. This usually creates a limited diversity of low-affinity binders. In a second wave of diversification, initiated by repeated antigen encounter, low-affinity antibodies can be edited by undergoing somatic hypermutation. *In vivo* strategies using transgenic mice have been successful in obtaining high-affinity human antibodies while *in vitro* methods have allowed the selection of single-domain binders by library display technology (Neuberger and Brüggemann 1997).

Here we present a broad review of the methodologies used to express fully human antibody repertoires in mice. Transgenic animals carrying human Ig H and L chain loci on YACs or chromosome fragments have been derived from manipulated embryonic stem cells, oocytes, and fibroblasts (Fig. 4.1). This allows the expression of diverse repertoires, somatic mutation, and class switching in a background with silenced endogenous gene loci. In addition, alternative technologies are emerging that utilize the expression of specific single-chain antibodies and iterative affinity maturation of rearranged Ig genes transfected into cell lines.

4.2
Human Ig Genes and Loci

The human Ig loci (IgH, Igκ, and Igλ) have been cloned and all the genes have been sequenced (summarized in Lefranc and Lefranc 2001). The human IgH

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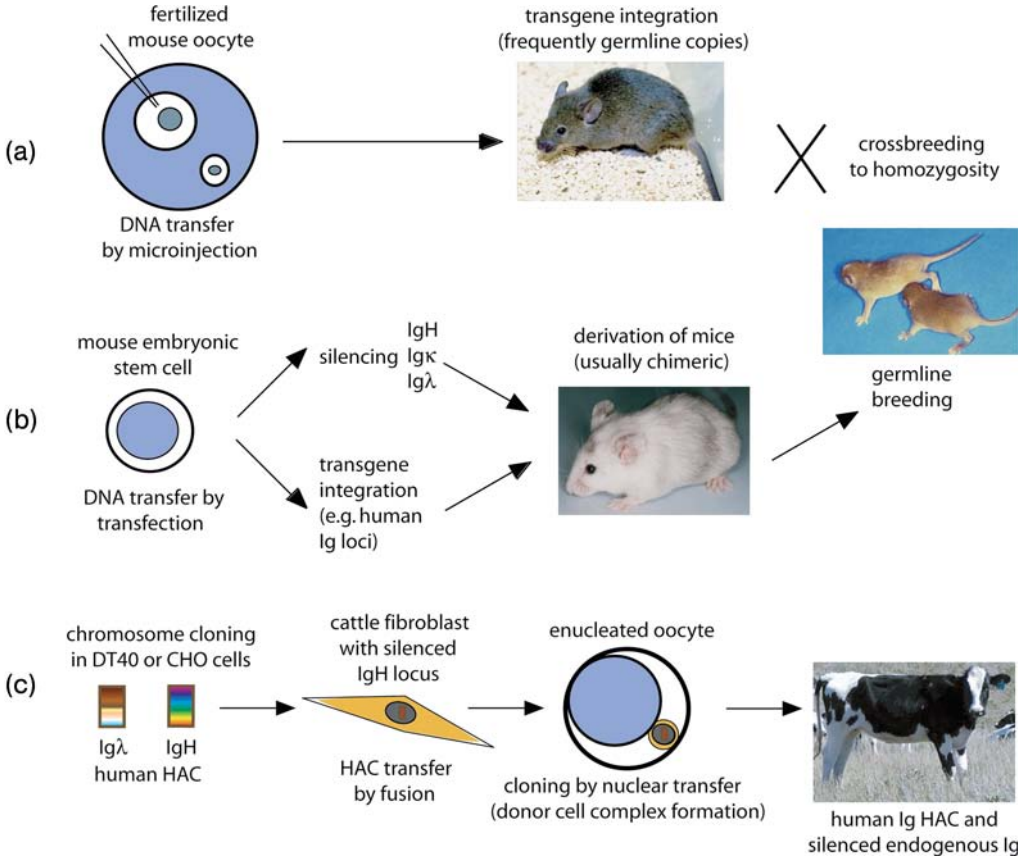

Fig. 4.1 Derivation of new mouse lines using (a) DNA microinjection into oocytes, (b) gene targeting in embryonic stem (ES) cells, and (c) nuclear transfer of manipulated fibroblasts. DNA microinjection into the male pronucleus of fertilized mouse oocytes is used for the production of transgenic animals. ES cells can be manipulated by transfection (e.g. DNA electroporation or protoplast fusion using YAC-containing yeast cells), which facilitates random integration and, using homology constructs, site-specific integration. From manipulated ES cells chimeric mice can be produced and further breeding may establish germline transmission and homozygous animals. A chromosome cloning system using DT40 or CHO host cells was used for the integration of human chromosome fragments or artificial chromosomes (HACs) containing the IgH and IgL loci. Human HACs were transferred into cattle fetal fibroblast cells by fusion. In addition gene targeting of the IgH locus has been achieved in fibroblasts. Animals expressing human Ig were produced from the manipulated fibroblasts by nuclear transfer. Extensive cross-breeding allowed the expression of, for example, human antibody H- and L-chains in a knockout background where equivalent endogenous genes are non-functional (Brüggemann et al. 1989; Taylor et al. 1994; Mendez et al. 1997; Nicholson et al. 1999; Kuroiwa et al. 2002, 2004).

locus is about 1.3 Mb in size with 38–46 functional Vs, 23 Ds, 6 Js, and 9 C region genes (Hofker et al. 1989; Cook et al. 1994; Corbett et al. 1997; Matsuda et al. 1998). The human κ L chain locus is accommodated on a ~1.8 Mb region with 17–19 functional Vs located at the proximal cluster, separated by about 800 kb from the distal V_κ cluster with 15–17 functional genes, followed by 5 Js and one C_κ (Roschenthaler et al. 2000; Zachau 2000). The human Igλ locus is just over 1 Mb in size with 29–33 functional Vs and 4–5 J-C genes (Frippiat et al. 1995; Kawasaki et al. 1995). The V_λ families form group clusters while members of the different V_H and V_κ gene families are interspersed. Members of the IgH as well as Igλ chain gene segments are assembled in the same transcriptional orientation, which permits conventional DNA rearrangement and deletion, whilst V_κ genes are organized in both transcriptional orientations, which allows deletional and inversional joining (Frippiat et al. 1995; Weichhold et al. 1990). In translocus mice (animals bearing an introduced [Ig] locus), rearrangement and expression of human V_κ genes assembled in either transcriptional orientation has been achieved (Xian et al. 1998). The layout of the human Ig loci and the established transloci with their gene content, using plasmids, YACs, and chromosome fragments, is illustrated in Fig. 4.2 and Table 4.1.

4.2.1 Minigene Constructs

Phenotypic modification of mice by gene transfer of microinjected DNA into fertilized oocytes was established in the early 1980s (Gordon and Ruddle 1983). The use of embryonic stem (ES) cells (Evans 1989) for gene targeting followed a few years later (Capecchi 1989) and more recent approaches allowed the removal or introduction of quite large gene loci in mice (Davies et al. 1993; Ren et al. 2004). The early experiments demonstrated that exogenous genes could be expressed but it had to be determined how efficiently they would interact with endogenous polypeptides. For example, could human Ig genes introduced into mice rearrange and interact with the cellular signaling machinery to permit B-cell receptor assembly and antigen-induced differentiation events?

The size limit for the different IgH and IgL minigene constructs (15–180 kb) restricted both the number of genes and the distances between gene segments or exons that could be included. For this reason human V, (D), J, and C gene segments in germline configuration were placed in artificially close proximity. The number of V_H and V_κ genes was between one and five and the number of D segments for the IgH chain was between 3 and 15 (Brüggemann et al. 1989, 1991; Taylor et al. 1992, 1994; Lonberg et al. 1994; Xian et al. 1998). In the initial IgH gene constructs a ~15 kb region including DQ52, J_H, Eμ, switch μ and C_μ was largely retained, while for an Igκ construct a V_κ was added to the J_κ–C_κ cluster (Fig. 4.2). The advantage of maintaining the region between J and C was that functional activity of the intronic enhancer, important for transcriptional activation after DNA rearrangement, could be preserved. Thus, with rather small constructs, DNA rearrangement and expression was obtained whilst the addition of

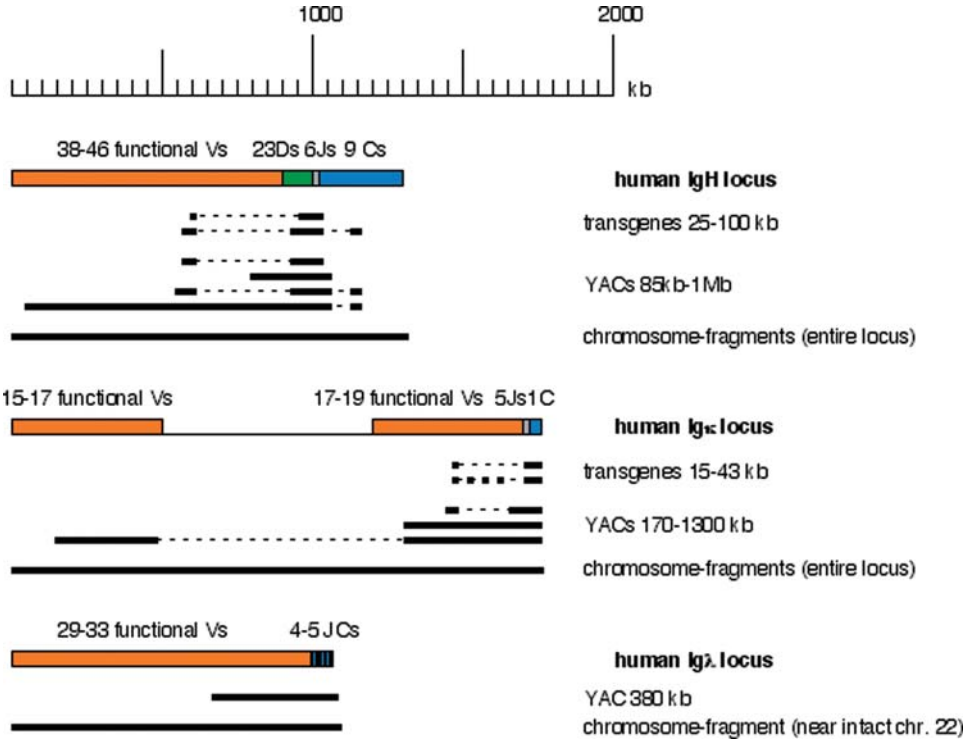

Fig. 4.2 Layout of the introduced human Ig loci expressed in mice. Linear constructs were obtained by gene assembly with size and gene content indicated. Human IgH, Igκ and Igλ loci in germline configuration are illustrated above (Ig loci summarized in Lefranc and Lefranc 2001; Ig transgenes summarized in Brüggemann 2004).

another C_H gene showed that switching from C_μ to $C_\gamma 1$ was possible (Taylor et al. 1992).

Transgenic animals, produced by microinjection, sometimes contain many (at times several hundred) identical copies of a human Ig construct resulting in higher levels of expression. Also, tandem integration events can occur when two or more constructs are injected together. For example, this has resulted in head to tail integration of two cosmids creating a ~100 kb IgH locus which rearranged and expressed a combination of segments from both cosmids (Brüggemann et al. 1991; Wagner et al. 1994a,b). A further increase in size was achieved by the use of the P1 cloning system and microinjection of three overlapping regions of ~80 kb each (Wagner et al. 1996). Following homologous recombination with each other, ~180 kb of the human IgH locus was reconstituted. This comprised five V_H genes, all D and J segments and C_μ and C_δ, containing the core region of the

Table 4.1 V and C gene content of mice carrying human IgH, Igκ and Igλ transloci.

	V genes	*C genes*	*Reference*
H chain			
HulgH	2	μ	Brüggemann et al. 1989; Wagner et al. 1994a,b
HulgH^{cos}	2	μ	Brüggemann et al. 1991; Wagner et al. 1994a,b
HC1	1	μ, γ1	Taylor et al. 1992
HC2	4	μ, γ1	Lonberg et al. 1994; Taylor et al. 1994
J1-3	2	μ, δ	Choi et al. 1993
HulgH^{p1-2}	5	μ, *d	Wagner et al. 1996
HulgH	5	μ, δ	Nicholson et al. 1999; Mundt et al. 2001
yH1	5	μ, δ	Green et al. 1994
yH2	~40	μ, δ, γ2 or γ1 or γ4	Mendez et al. 1997; Davis et al. 1999; Green 1999
hCF(SC20)	~40 (whole locus)	μ, δ, γs, ε, αs	Tomizuka et al. 2000; Tomizuka et al. 1997
κ L chain			
KC1	1	1 κ	Taylor et al. 1992
KCo4	4		Lonberg et al. 1994
HulgHκML	5		Xian et al. 1998
yK1	2		Green et al. 1994
HulgκYAC	2		Davies et al. 1993; Xian et al. 1998
HucoslgκYAC	~80		Xian et al. 1998; Zou et al. 1996
KCo5	~26		Fishwild et al. 1996
yK2	~25		Mendez et al. 1997
hCF(2-W23)	~50 (whole locus)		Tomizuka et al. 2000; Tomizuka et al. 1997
λ L chain			
lgl	15	7 Jλ (incl. 3 ψλs)	Popov et al. 1999
hCF[MH(ES)22-1]	~30 (whole locus)	4–5 Jλ	Tomizuka et al. 1997

locus required for DNA rearrangement and expression. For the Igκ locus, co-injection of two minigene constructs and homologous recombination between V genes achieved integration of a contiguous 43 kb translocus (Lonberg et al. 1994).

These early human Ig constructs, some with tightly assembled exons and control regions, established that human Ig gene segments in germline configuration could be rearranged and expressed in mouse lymphocytes. Additional mouse sequences, initially assumed to be beneficial to drive transgene expression, appeared to be unnecessary if equivalent human sequences, such as enhancer regions, were included. However, small constructs carrying few gene segments express relatively poorly and fully human antibody repertoires have not been obtained in mice that still rearrange and express their endogenous Ig genes.

4.2.2 Yeast Artificial Chromosomes (YACs)

In yeast, YACs can be used as cloning vehicles to accommodate large loci. In addition, YACs can be easily modified by site-specific recombination (Anand 1992; Davies et al. 1992). To create antibody repertoires comparable to the diversity obtained in humans, a large number of genes and perhaps the transfer of whole Ig loci may be necessary. For this reason much attention has been focused on using YACs to accommodate large genomic regions to which individual V genes of the different families, either on minigene constructs (for example, obtained by PCR) or in authentic configuration, could be added (Lonberg et al. 1994; Fishwild et al. 1996; Popov et al. 1996, 1999; Mendez et al. 1997). Defined genomic regions, ranging from a few hundred kilobases to well over 1 Mb, can be assembled from overlapping BACs (bacterial artificial chromosomes) or cosmids, or from direct cloning of human genomic DNA into YAC libraries. The cloned DNA can be modified by targeted retrofitting or sequence removal, and also can be easily extended by mating of yeast clones carrying overlapping YACs (Markie 1996). The core regions of the human IgH locus and the Igκ locus have been cloned separately on YACs of up to ~300 kb (reviewed in Brüggemann and Neuberger 1996). Impressive V gene additions by stepwise recombination have been made to these YACs (Zou et al. 1996; Mendez et al. 1997). This has resulted in a ~1 Mb human IgH YAC with ~66 V_H genes, Ds, J_Hs, C_μ, and C_δ, and a ~800 kb Igκ YAC with ~32 V_κ genes, J_κs, and C_κ all in authentic configuration (Mendez et al. 1997). The efficiency of the yeast host in homologous integration has also been exploited by extending a 300 kb Igκ YAC (Davies and Brüggemann 1993) to 1.3 Mb by multiple integration of a 50 kb cosmid with five V_κ genes (Zou et al. 1996). As no YAC containing the core region of the human Igλ locus (V_λ–JC_λs) was available, this had to be constructed from overlapping cosmids. Three cosmids with their 5′ and 3′ regions ligated to YAC vector arm sequences were co-transfected into yeast and YACs with correctly reconstituted Igλ core region were identified by Southern hybridization (Popov et al. 1996). Further extension was achieved by yeast mating, which allowed homologous recombination of the overlapping region and resulted in Igλ YACs with an authentic 380 kb region accommodating 15 functional V_λ genes (Popov et al. 1999). Therefore manipulation of YACs using stepwise recombination has been a valuable technique in creating human Ig loci with large or near authentic regions over 1 Mb in size.

Several strategies have been used successfully for YAC introduction into the mouse germline: DNA purification and microinjection into fertilized oocytes (Fishwild et al. 1996); co-lipofection of ES cells with a mixture of size-fractionated YAC DNA in agarose and a selectable marker gene (Choi et al. 1993); and fusion of yeast protoplasts with ES cells (Davies et al. 1993, 1996). Despite establishing very efficient methods for YAC purification and microinjection (Schedl et al. 1993) large DNA molecules are difficult to integrate as one complete copy of a human Ig translocus (Taylor et al. 1992). Improvements allowing complex and large (human Ig) loci in their intact form to be transferred into the mouse genome

came with the use and manipulation of ES cells (Hogan et al. 1994). Lipid-mediated DNA transfer (lipofection) of ES cells avoids the handling of naked DNA and if co-transfection is applied, removes the requirement to retrofit a selectable marker into the YAC or BAC (Choi et al. 1993). However, a recurring problem is that frequently only a portion of the introduced region is integrated into the host genome. To overcome this difficulty, protoplast fusion previously used for YAC integration into differentiated mammalian cells (Pachnis et al. 1990; Pavan et al. 1990) has been adapted for YAC transfer into ES cells (Davies et al. 1993, 1996; Zou et al. 1996). The preparation of YAC-containing yeast spheroplasts does not involve DNA handling or gel separation, but requires the YAC to be retrofitted with a selectable marker gene. Fusion of yeast protoplasts with ES cells is similar to the method employed for the generation of hybridomas, but usually only a few clones are obtained. Nevertheless, the approach achieved a reliable integration of complete single-copy Ig YACs into the mouse genome (Mendez et al. 1997; Nicholson et al. 1999).

The expression of multiple V_H and V_L transgenes in mice allowed the production of different combinations of V region pairs, generating a diverse antibody spectrum. Specific antibodies were obtained by hybridoma and/or PCR technologies which, upon re-expression, allowed bulk production of monoclonal human antibodies (reviewed in Maynard and Georgiou 2000). Initially, less emphasis was put on the addition of C_H region genes to permit class switching. The reason was that even small constructs, with a limited number of Vs, Ds, and Js, rearranged and expressed as μ H-chain which permitted surface IgM or B-cell receptor expression. This was followed by normal differentiation events to produce secreted Ig and diversification of V_H gene sequences by hypermutation (Wagner et al. 1996). Some larger IgH loci, identified from YAC libraries, included C_δ, downstream of C_μ. However, these rarely produced specific IgD antibodies (Choi et al. 1993; Green et al. 1994; Wagner et al. 1996; Brüggemann and Neuberger 1996). Further efforts were made to include one C_γ gene (Mendez et al. 1997; Davis et al. 1999; Green 1999) to allow isotype switching. Differentiation events associated with IgG expression allow a cell to enter the recirculating B-cell pool and become established as a long-lived memory B cell accumulating high levels of somatic mutation. Engineering mouse lines that express particular C_γ genes provides antibodies with tailor-made effector functions. For example, strong (IgG1 and IgG3) or weak (IgG2 and IgG4) binding to the Fc receptors (Fc_γR I, II, and III) could be useful to permit or avoid, respectively, interaction with macrophages or natural killer (NK) cells. Similarly, IgG1 and IgG3 antibodies have the ability to fix complement and can initiate extensive hemolytic activity which, in context, may or may not be a useful attribute. To produce defined isotype repertoires, such as IgG1 for the destruction of target cells and IgG2 or IgG4 to block or compete for binding without initiating cell lysis, the respective C_γ gene was placed immediately 3′ of C_δ (Mendez et al. 1997; Davis et al. 1999). Although switching from μ to γ was achieved, such close proximity of two C genes disregards the importance of many intervening regions exerting important control functions critical in securing high expression levels. In the human IgH locus the ~50 kb C_δ–C_γ3

interval is such a region, rich in transcription factor-binding motifs and with lymphocyte-specific enhancer activity (Mundt et al. 2001). It would be interesting to compare antibody titers and gene usage in mice carrying either closely assembled C genes or C genes in authentic configuration. As yet, no parallel immunizations with the same antigen have been carried out using the different human Ig mouse lines.

To secure good expression levels the IgH and IgL chain YACs contain at least one but usually two transcriptional enhancers. For the (human) IgH locus the activity of four enhancers (E) has been described: EDQ52, Eμ, Eδ–γ3, and E3′α in a multiple E site region (reviewed in Magor et al. 1999; Mundt et al. 2001; Arulampalam et al. 1997 and references therein). Human Eμ is present on all IgH transloci and as a second control region the rat or mouse enhancer downstream of the last C gene, E3′α (also termed HS1,2 to identify the precise location), has been added 3′ of C_γ (Taylor et al. 1994; Mendez et al. 1997). In the IgL loci there are transcriptional enhancers proximal to both C_κ and C_λ, making it unnecessary to add further enhancers to these YACs. Human, mouse, and rat enhancer sequences have all been used to drive human IgH and IgL chain transcription but there is little information about their requirement. For example, with the H chain the inclusion of additional enhancers in the presence of Eμ may be unnecessary because similar expression levels have been obtained in mice with and without a second enhancer (Brüggemann and Neuberger 1996).

4.2.3 Chromosome Fragments

Human chromosome fragments or human artificial chromosomes (HACs) can be maintained in the cell as distinct minichromosomes, which have the advantage that they provide whole loci when expressed in the mouse. In contrast, minigene constructs or YACs are integrated into a host chromosome and do not usually provide the full gene content. Individual chromosomes or their fragments, tagged with a selectable marker gene (neomycin, hygromycin, or puromycin) introduced by transfection, have been transferred by microcell-mediated fusion of human fibroblasts with somatic cell lines such as the mouse fibroblast line A9, the Chinese hamster ovary cell line CHO and the avian leukosis virus (ALV)-induced chicken tumor line DT40 (Fournier and Ruddle 1977; Koi et al. 1989; Shinohara et al. 2000). Hybrid libraries with different chromosomal regions, maintained under selection, include the human IgH, Igκ, and Igλ locus and adjacent loci identified by chromosomal marker analysis. Microcell-mediated fusion into ES cells allowed the derivation of chimeric mice (Tomizuka et al. 1997). Due to stability problems, maintenance of the complete transferred chromosome varies extensively, ranging from only a few per cent of the original sequence to nearly an intact transchromosomal region (Tomizuka et al. 1997).

Transchromosome stability and germline transmission were first obtained with a human chromosome 2-derived fragment containing the Igκ locus (Tomizuka

et al. 1997). Germline transmission was essential to achieve homozygous breeding into Ig knockout lines. This was accomplished for the IgH locus with a fragment of human chromosome 14 and for Igκ using a minichromosome (Tomizuka et al. 2000).

Further efforts have concentrated on site-directed chromosome truncation to overcome instability (somatic mosaicism) of HACs, which would prevent germline transmission (Kuroiwa et al. 1998, 2000; Kakeda et al. 2005). In addition, attempts have been made to perform recombination of two nonhomologous chromosomal fragments to increase the size of the HACs. Kuroiwa and coworkers achieved both these aims by modifying the unstable human Igλ locus from chromosome 22 by targeted insertion of a new telomeric region and *loxP* sequences either side of the locus. Recombination-proficient cells (DT40) containing this altered chromosome fragment were fused with cells carrying a stable HAC accommodating the human IgH locus and a *loxP*-modified RNR2 locus. After transfection with Cre, an HAC of ~10 Mb containing both the Igλ and IgH loci was produced (Kuroiwa et al. 2000). Integration of the HACs into ES cells established mouse lines expressing human IgH and IgL chain loci (Kuroiwa et al. 2000). Transchromosomic calves expressing Ig from human IgH and Igλ loci have been produced using a similarly generated HAC transfected into bovine fetal fibroblasts followed by transfer into bovine oocytes (Kuroiwa et al. 2002).

4.3 Transgenic Ig Strains

Experiments to derive human antibody repertoires in transgenic mice started almost 20 years ago (Brüggemann et al. 1989). From the early approaches it became clear that introduced human Ig genes in germline configuration could undergo rearrangement and be expressed in the mouse. Although human antibodies were clearly detectable in mouse serum their concentration was quite low compared with the level of endogenous Ig (summarized in Brüggemann and Neuberger 1996). The reason for this was that only a few per cent of transgenic mouse B cells were positive for human IgH, the majority of lymphocytes expressing mouse Ig. Despite this drawback human IgM titers of up to 100 $\mu g\,mL^{-1}$ have been achieved in some transgenic lines (Brüggemann et al. 1989; Brüggemann and Neuberger 1991). Comparing expression levels achieved using Ig (mini)loci suggested that larger regions may favor better expression. Furthermore, L chain loci may be more efficiently expressed than H chain loci, perhaps because of their more compact layout with the presence of enhancers in the natural configuration.

Significant improvements in the levels of human Ig expression in the mouse were achieved by the use of gene targeting technology and the derivation of knockout animals (Capecchi 1989). Silencing of the mouse Ig loci, first

accomplished by Rajewsky and coworkers for the H chain locus and later for the κ locus (Kitamura et al. 1991; Zou et al. 1993b), proved invaluable to secure human antibody expression without mouse H and κ L chain interference. The currently used mouse strains that express fully human antibody repertoires have been produced by the integration of human H and L chain (κ and/or λ) YACs and/or HACs, and crossing with animals in which the endogenous H and κ L chain loci have been silenced by gene targeting (Lonberg et al. 1994; Mendez et al. 1997; Nicholson et al. 1999; Tomizuka et al. 2000). Recently the mouse Igλ locus has been silenced (Zou et al. 2003) and suitable breeding could secure a mouse strain entirely free of any endogenous Ig production. Figure 4.2 summarizes the layout of the various human IgH, Igκ, and Igλ transloci. Websites illustrating the generation and use of these mice include http://www.babraham.ac.uk, http://www.abgenix.com, http://www.medarex.com, and http://www.tcmouse.com.

4.3.1 Stability of the Transloci

Miniloci and YACs have been transferred by both microinjection into oocytes, and ES cell technology. The former produces germline mice, but the latter generates chimeric animals, which require further breeding to establish heterozygous and homozygous mouse strains. Homozygosity for a combination of five features (human IgH, human Igκ, human Igλ, mouse IgH knockout, mouse Igκ knockout) has been readily obtained, indicating that these loci largely integrate in random fashion and not at preferred sites (Nicholson et al. 1999). No reports show the actual chromosomal integration sites of minigene constructs or YACs, while the maintenance of introduced HACs, as separate single units, has been well documented (Tomizuka et al. 2000).

Both YACs and HACs have advantages and disadvantages when used for the integration and expression of large human loci in the mouse. The advantage of transferring YACs is that integration into a mouse chromosome secures perfect stability and transmission, and in addition the gene content of the YAC is essentially known from sequence analysis. A disadvantage is that current YACs are hardly larger than 1 Mb and may not accommodate complete Ig loci. The generation of HACs offers the advantage of allowing the transfer of defined large regions. However, a drawback of using HACs is their somatic mosaicism, resulting in unpredictable transmission rates: HACs can be easily maintained in ES cells under selection but their germline transmission and maintenance in somatic cells appears to be significantly reduced (Shinohara et al. 2000). This means that every resulting mouse has to be analyzed for the level of HAC-positive B cells. Breeding analyses revealed a variable transmission efficiency that reached an impressive 38% for one particular HAC compared with the ideal 50% transmission rate of conventional genes in heterozygous configuration. One reason for HAC instability appears to be imprecise separation at mitosis due to poor centromere function (Shen et al. 1997).

4.3.2
Silenced Endogenous Loci

Establishing extensive fully human antibody repertoires has not been possible in the normal mouse background. The two major reasons are a generally low-level expression of human Ig and the presence of mixed molecules, such as human H chain associated with mouse L chain. Both problems have been overcome by breeding human IgH and IgL mice with animals in which the endogenous Ig genes had been silenced by gene targeting. In μMT mice (Kitamura et al. 1991) the μ transmembrane exons were disabled by the introduction of a selectable marker gene, which impeded B-cell development in the C57BL/6 background. The μMT knockout strain has been used to allow the production of authentic human Igs (Nicholson et al. 1999) although when breeding this particular knockout feature into other mouse backgrounds (e.g. BALB/c), endogenous IgG is still produced (Hasan et al. 2002; Orinska et al. 2002). The endogenous mouse IgH locus can interfere with human antibody production by trans-switching or trans-splicing events, allowing the expression of human–mouse chimeric heavy chains (Wagner et al. 1994b; Knight et al. 1995; Brüggemann and Taussig 1997). In the other extensively used IgH knockout strain, the J_H segments have been removed by gene targeting (Chen et al. 1993b; Jakobovits et al. 1993; Lonberg et al. 1994). The advantage of the J_H deficiency is that DNA rearrangement of the endogenous H chain locus is prohibited. An alternative approach, to prevent any undesired usage, has been the deletion of all constant region genes from the IgH locus (200 kb in total) (Ren et al. 2004).

Most attempts at silencing mouse L chain production have focused on the Igκ locus. These have resulted in knockout strains without J_κs and C_κ (Chen et al. 1993a) or a removed or disrupted Cκ (Zou et al. 1993b, 1995; Sanchez et al. 1994). Although κ L chain Ig is predominantly expressed in the mouse, silencing of the κ locus leads to a much-increased production of Igλ. Silencing of the λ L chain locus has been achieved by Cre-*loxP*-mediated removal of all J_λs and C_λs in a ~120 kb region (Zou et al. 2003). Complete ablation of IgL by breeding of κ and λ knockout mice resulted in a block in B cell development which can be overcome by expression of human L chain loci.

4.3.3
Immune Responses and Affinity of Human Ig

The introduced human IgH and IgL (κ and λ) loci are well expressed in a background where the endogenous IgH and Igκ loci have been silenced by targeted gene removal or disruption. Whilst human IgH and IgL chains can be expressed from minigene constructs containing one or a few of each of the V, (D), J, and C genes and one C proximal enhancer, better expression and repertoire formation is achieved from integration of larger Ig regions. Human antibody expression in the normal mouse background can reach up to 50 $\mu g\,mL^{-1}$ for Ig with human H chain and a somewhat higher level, 15–400 $\mu g\,mL^{-1}$ for Ig with human L chains.

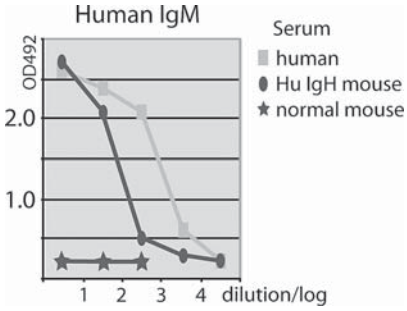

Fig. 4.3 Serum IgM in mice carrying a human H-chain locus in a background where the endogenous mouse IgH locus had been silenced by gene targeting. The titration was carried out in an ELISA assay with the level of human IgM produced in translocus mice >100 μg mL^{-1}.

However, as mouse Ig in the mg mL^{-1} range dominates expression, transgenic human Ig H and L chains are rarely associated with each other when forming antibodies. In the knockout background the level of human Ig reaches in many cases a few hundred μg mL^{-1} (Fig. 4.3), which suggests that expression levels are determined by the transferred Ig core region. Further improvements in Ig expression can be seen in YAC-based mouse strains where human antibody levels can exceed 1000 μg mL^{-1}, suggesting that larger Ig transloci with more gene segments are potentially better expressed. Disappointingly, Ig expression in the transchromosomal mice carrying complete H and L chain loci (Tomizuka et al. 1997 and 2000) is not increased further but is only about half the level found in IgH and Igλ YAC mice (Mendez et al. 1997; Popov et al. 1999). A plausible reason is that maintenance of the transferred HAC as a separate human chromosome in the mouse cells may interfere with recognition by the cellular machinery affecting chromatin structure and locus accessibility (Jenuwein and Allis, 2001). In all the transgenic Ig strains a large proportion of the "human" antibodies contain mouse λ L chain and thus are in chimeric configuration (Fishwild et al. 1996; Mendez et al. 1997; Nicholson et al. 1999; Magadán et al. 2002). Although the mouse Igλ locus has been silenced by gene targeting (Zou et al. 2003), human Ig mice with a background in which all three endogenous mouse Ig loci (IgH, Igκ and Igλ) have been rendered non-functional, and are thus unable to express any endogenous mouse Ig, have not yet been established by cross-breeding.

The high avidity but generally low affinity of IgM can be advantageous for some applications (Okada and Okada 1999), but other isotypes are also desirable. Early experiments adding a γ1 C gene to allow switching from IgM proved successful and established genomic recombination between the transgene μ and γ1 switch regions (Taylor et al. 1992, 1994). The human IgM and IgG1 concentration in serum improved significantly when the animals were crossed with endogenous H and κ L chain knockout mice. Also, the addition of more V_H and V_κ genes increased human antibody levels (Lonberg et al. 1994; Taylor et al. 1994). The

most notable advantages of an introduced human IgH locus on a 1 Mb YAC have been described by Mendez *et al.* (1997): good expression, extensive V_H gene diversity (including hypermutation) and switching to the desired isotype. However, although switching from C_μ to the added C_γ is achieved, the serum levels of human IgG are considerably lower than those of human IgM and do not represent the ratio or levels (1–2 mg mL^{-1} IgM and ~10 mg mL^{-1} IgG) found in human serum (Frazer and Capra 1999).

B-cell development in mice, carrying a human IgH, human Igκ, and human Igλ translocus in a background where the function of the endogenous IgH and Igκ loci have been disabled, is illustrated in Fig. 4.4. Flow cytometry analysis of these five-feature mice shows an up to two-thirds B cell recovery rate with, for example, the number of Ig$^+$ splenic lymphocytes being ~20% in these human Ig mice compared with ~30% found in normal mice kept under the same conditions (Nicholson et al. 1999; Brüggemann, 2004). With the availability of an introduced Igκ and Igλ locus Ig$^+$ lymphocytes predominantly express human λ L chain. This is linked to a more efficient B cell recovery and much reduced expression of endogenous λ L chain. Although human Igκ antibodies can be easily obtained from five-feature mice, it may be an advantage to use individual four-feature strains, which either express human IgH,λ or human IgH,κ antibodies, to select the type of response (Lonberg et al. 1994; Mendez et al. 1997; Nicholson et al. 1999; Magadán et al. 2002; Protopapadakis et al. 2005).

The therapeutic applications of antibodies necessitate that a diverse range of specificities can be easily obtained (e.g. high-affinity monoclonal antibodies after immunization) and that the immunogenicity of such antibodies is abolished or at least reduced (Waldmann and Cobbold 1993; Klingbeil and Hsu 1999). Expression of human H and L chain loci in an animal not expressing its own Ig genes and which can be immunized meets these demands. Immunizations of mice expressing human antibodies have been carried out in the same way as described for conventional mouse strains. However, evaluation of immune responses

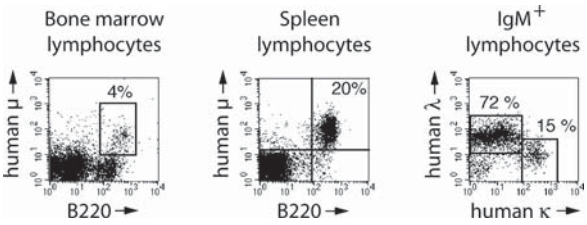

Fig. 4.4 Flow cytometry analysis of five-feature mice (carrying three human transloci, IgH, Igκ, and Igλ and two disabled endogenous loci mouse IgH and Igκ). The analysis shows a 1/3 to 2/3 B-cell recovery rate with, for example, the number of Ig$^+$ splenic lymphocytes being ~20% in the human Ig mice compared with ~30% found in normal mice kept under the same conditions (Nicholson et al. 1999; Brüggemann 2004).

showed that human antibody titers are reduced compared with those of normal nonmanipulated animals (Lonberg et al. 1994; Wagner et al. 1994a,b; Jakobovitz et al. 1995; Magadán et al. 2002). Despite low-serum titers, human Ig loci mice are capable of mounting antibody responses to a wide range of antigens and, similar to normal mice, increased levels of specific antibodies are visible 2–3 weeks after primary immunization and can be further increased by secondary immunization. Some antigen-specific antibodies were fully human (human H and L chain), while others were chimeric (consisting of human/mouse H/L chain combinations or a mixed H chain obtained by trans-switching).

The formation of mixed molecules or polypeptides led to disappointment particularly when some immunizations produced a substantial number of chimeric human antibodies with mouse λ L chain (Russell et al. 2000; Magadán et al. 2002). Nevertheless antigen-specific fully human antibodies were produced and the use of hybridoma technology (for background and methods see King 1998) established a large number of human monoclonal antibodies (mAbs) of good affinity (Table 4.2). Expression levels varied from a few $\mu g\,mL^{-1}$ in conventional tissue culture plates up to $400\,mg\,L^{-1}$ in serum-free, fed-spinner cultures (Ball et al. 1999; Nicholson et al. 1999; Davis et al. 1999; Green 1999). The mAbs exhibited a good usage of the different V, (D), J, and C genes. Hypermutation and the addition of N-sequences, to establish junctional diversity, allowed the creation of extensive repertoires. The length of the complementarity determining region (CDR) 3 regions established by nucleotide additions at the V to D and D to J joins (7–19 amino acids) is comparable to those identified in humans and considerably longer than those found in the mouse (Mendez et al. 1997; Nicholson et al. 1999).

Antibodies specific for the epidermal growth factor receptor (EGFR), overexpressed on many types of tumors, showed the preferential use of the closely related V_H and V_κ genes in combination with different D and J segments (Davis et al. 1999; Yang et al. 2001). The V genes were diversified by somatic mutation but most strikingly eight V_H genes coded for an aspartate residue in CDR1 at position 33. Analysis of the five-feature mice described by Nicholson et al. (1999)

Table 4.2 Antigen spectrum and affinity of fully human IgG monoclonal antibodies.

Antigen	*H chain*	*L chain*	*affinity*	*Reference*
Digoxin	γ1	κ	$2.5–22\,nmol\,L^{-1}$	Ball et al. 1999
Human CD4	γ1	κ	$11\,nmol\,L^{-1}–27\,pmol\,L^{-1}$	Lonberg et al. 1994; Fishwild et al. 1996
Human IL-8	γ2	κ	$0.2–0.9\,nmol\,L^{-1}$	Mendez et al. 1997; Green 1999
Human EGFR	γ2	κ	$0.8\,nmol\,L^{-1}–30\,pmol\,L^{-1}$	Mendez et al. 1997; Green 1999
Human TNFα	γ2	κ	$0.2–0.8\,nmol\,L^{-1}$	Mendez et at. 1997; Green 1999
CD4	γ[a]	κ	$32–77\,pmol\,L^{-1}$	Ishida et al. 2002
GCSF	γ[a]	κ	$0.2–0.3\,pmol\,L^{-1}$	Ishida et al. 2002

a isotype not defined.

revealed that the emergence of human IgH,κ or human IgH,λ Ig appears to be antigen-driven and that these mice do not produce significant levels of chimeric human antibodies with mouse λ L chain (Magadán et al. 2002; Brüggemann 2004). Immunization of transchromosomal mice resulted in human antibody responses including all Ig subclasses (Yoshida et al. 1999; Ishida et al. 2002). Problems with obtaining hybridomas, due to the instability of the Igκ locus-bearing chromosome fragment, were overcome by crossbreeding the H chain transchromosomal mice with mice carrying an Igκ YAC (Fishwild et al. 1996; Ishida et al. 2002).

Diverse human antibody repertoires with mAbs of desired specificity and high affinity in the picomolar range have been obtained from the translocus mouse strains. As an alternative to hybridoma production, immunization of translocus mice was combined with ribosome display technology to select high-affinity human V_H–V_κ fragments binding to progesterone (He et al. 1999). In this rapid approach antigen-specific V(D)J segments are selected which can be used for further manipulation, for example, the addition of a particular C_γ gene. But despite this success, immune responses of human translocus mice, their Ig levels and antibody diversity are not as refined as those of a normal animal. Extensive mutation in V_H and V_L have been described but it seems that the mouse strains with larger and indeed complete Ig loci (Ishida et al. 2002) may produce a more diverse repertoire with choice and selection optimized to produce superior antigen-binders.

Currently four promising human IgG,κ mAbs have reached phase III trials (Reichert et al. 2005; Lonberg 2005). Treatment with anti-EGF receptor antibodies is aimed at eradicating colorectal cancer, nonsmall cell lung cancer, and renal cell carcinoma; anti-CTLA-4 targets melanoma and possibly other cancers; anti-RANKL treatment is beneficial in osteoporosis; and anti-CD4 is used for the removal of lymphoma cells.

4.3.4
Ig Replacement

Attempts to retain the high levels of antibody expression and hypermutation of the mouse immune system for human Ig production concentrated on targeted substitution of mouse genes with human genes. This allowed site-specific integration of human C_κ and $C_\gamma1$ replacing mouse C_κ and $C_\gamma1$ or $C_\gamma2a$ (Zou et al. 1993a, 1994; Pluschke et al. 1998). In these two targeting constructs human C_κ and human γ1, adjacent to selectable marker genes, were flanked by the appropriate mouse homology sequences. This produced animals that rearranged and expressed chimeric Igκ and IgG1 antibodies with human C regions (Zou et al. 1993a; Pluschke et al. 1998). In another approach mouse $C_\gamma1$, excluding the transmembrane exons, was replaced by human $C_\gamma1$ (Zou et al. 1993a). Here the homology region and selectable marker gene were flanked by *loxP* sequences, which allowed their removal by Cre-mediated deletion. This permitted seamless insertion of a human γ1 C gene into the previous location of mouse γ1. The

resulting mouse produced chimeric human IgG1 in serum at levels similar to those of mouse IgG1 in normal animals. Immunizations induced a normal immune response and produced a diverse repertoire of chimeric human IgH and IgL chains (Zou et al. 1994). Replacement of mouse with human genes appears to be successful when small DNA regions, up to a few kilobases in size, are exchanged. Future improvements may permit targeted replacements using larger human regions, perhaps whole loci, appropriately regulated by integration adjacent to endogenous control regions.

4.4 Complementary Strategies

Major efforts have focused on the expression of modified antibodies with the aim of altering the specificity of particular V genes by hypermutation. *In vivo* selection regimes can be used to increase the affinity of cloned mAbs either re-expressed in the mouse or transfected into cell lines. In another complementary attempt H chain antibodies, normally only produced in camelids, have been expressed in the mouse. This approach aims at generating a new type of antibody without L chain. H-chain-only antibodies have a propensity to recognize grooved surfaces found on many viruses rather than flat or less contoured areas predominantly recognized by conventional antibodies (van der Linden 2000).

4.4.1 H-chain-only Ig

Conventional antibodies consist of multiple units of paired H and L chains (Padlan 1994), which are present in all jawed vertebrates studied to date (Litman et al. 1999). In addition to these conventional heteromeric antibodies, sera of camelids (suborder Tylopoda, which includes camels, dromedaries, and llamas) contain a major type of IgG composed solely of paired H chains (Hamers-Casterman et al. 1993). Homodimeric H chain antibodies in camelids, illustrated in Fig. 4.5, lack the first C domain (C_H1), which is spliced out during mRNA maturation, and use distinctive yet diverse V region genes termed V_HH (Muyldermans et al. 1994; Nguyen et al. 2000). The lack of C_H1 in H chain antibodies is most likely to be the crucial factor in allowing their release from cells in the absence of L chains (Haas and Wabl 1983; Munro and Pelham, 1987; Hendershot, 1990). Using structural analysis it has been concluded that the hydrophilic nature of particular amino acids in $V_HH–D–J_H$ prohibits association with L chain.

In conventional antibodies association between L and H chains is regarded as important in securing a diverse repertoire for antigen-binding. Nevertheless, antibodies composed solely of paired H chains are efficient antigen binders. In particular they recognize clefts on the antigen surface that are normally less immunogenic for conventional antibodies (Lauwereys et al. 1998). This may allow H chain antibodies to recognize viral structures much more readily, and perhaps

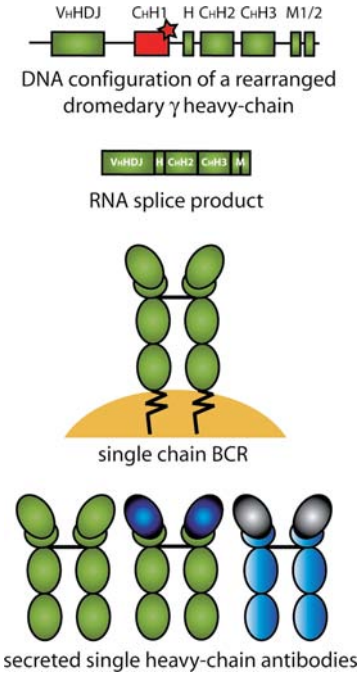

Fig. 4.5 Dromedary H-chain-only antibodies. The configuration of a rearranged dromedary γ H-chain gene is illustrated at the top. The C_HH1 exon, with the splice sequence mutation shown as a star, is indicated in red. The transcription product with removed C_HH1 is shown below. A repertoire of homodimeric H-chain antibodies with different V and C regions is illustrated at the bottom, with a hypothetical single-chain B-cell receptor (BCR) illustrated above.

exclusively, compared to large heterodimeric antibodies. In addition, H chain antibodies retain a dimeric configuration that is essential for antigen crosslinking, which can be followed by phagocytosis to remove an invader. H chain antibodies are absent in other mammals except in pathological situations, such as heavy chain disease, where they occur in mutated form (Alexander et al. 1982).

Recently it has been shown that antigen-specific dromedary H chain antibodies can be expressed in cultured cells and in mice (Nguyen et al. 2003; Zou et al. 2005). In the past, the aim of exploring how H-chain-only Ig could be expressed has been considered to present insurmountable obstacles and, indeed, without L chains, B-cell differentiation events are blocked (Zou et al. 2003). However, in transgenic mice carrying a dromedary H chain gene B cell development was found to be near normal and H chain antibodies were present on the cell surface and secreted in serum. This suggested that mice, just like camels, can remove the C_H1 exon by splicing. The finding that dromedary H chain antibodies can be readily produced is a crucial step towards the generation of a diverse human H-chain-only antibody repertoire in mice. Production of H chain antibodies by a mouse, which already has a human H chain locus integrated into the genome (Nicholson et al. 1999), would offer great advantages.

Already major advances towards expressing H-chain-only antibodies from a "camelized" human H chain locus have been made in the Drabek laboratory, Erasmus MC, Rotterdam, Netherlands (http://www2.eur.nl/fgg/ch1/cellbiology.

html). In the near future a spectrum of human antibodies with novel specificities may be available. For example, after immunization of the mice with human virus or viral proteins, not normally recognized by conventional antibodies, important therapeutic agents could be produced.

4.4.2 *In vivo* Mutation

H and L chain genes cloned from antibodies isolated by hybridoma or library display technology can be further modified by *in vivo* mutation and selection. Antibody expression in transgenic mice from rearranged Ig genes has been performed by random integration or by site-specific knockin strategies (Sohn et al. 1993; Li et al. 2005). In both approaches mature B cells expressed the transgene and underwent multiple cycles of hypermutation and/or receptor editing, which created extensive diversity (Bertrand et al. 1998; Jena et al. 2003). After immunization new specificities emerged from introduced V_HDJ_H segments (Li et al. 2005). The use of highly recombinogenic cells like the chicken DT40 line has allowed the selection of antigen-specific antibodies with nanomolar affinities generated by iterative affinity maturation in tissue culture (Cumbers et al. 2002; Harris et al. 2002). The mechanisms and key enzymes, such as activation-induced cytidine deaminase (AID), that operate on Ig diversification have been described in detail (Petersen-Mahrt 2005). Introduction of a *loxP*-flanked AID construct into $AID^{-/-}$ DT40 cells allowed induction of a reversible hypermutation regime (Kanayama et al. 2005). Similarly, AID expression in hybridoma cells induced a high rate of V gene mutation (Martin et al. 2002; Ronai et al. 2005). It appears that specificity and affinity of (human) antibodies can be proficiently improved by the above methods. In addition, cells expressing novel or high-affinity antigen binders can be easily identified by microarray technologies and flow cytometry.

4.5 Outlook

The success in expressing fully human antibody repertoires in mice and, more recently, in cattle is now extensively exploited by many different pharmaceutical and biotechnology companies. Immunized animals provide high-affinity antibodies and a diverse antibody repertoire, generating monoclonal human anti-human Ig from mice and highly specific polyclonal human Ig from large animals. The therapeutic human anti-human mAbs studied to date fall into three major categories: oncological, immunological, and anti-infective (Reichert et al. 2005). Emerging alternatives have focused on the production of single-chain Ig in the mouse. This has resulted in the expression of homodimeric H-chain-only antibodies retaining isotype effector functions. The small H chain Ig appears to recognize additional specificities not seen by conventional antibodies, which may

provide novel therapeutic agents in combating viral infections. Approaches to tailor the affinity of individual antibodies rely on diversification by the immune system of the mouse and on the selection of new specificities in hypermutating cell lines.

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The anti-EGF receptor antibody (page 83) has been approved by the U.S. Food and Drug Administration and is under the name of Vectibix for use in colon cancer patients. New results on the Generation of heavy-chain-only antibodies in mice[1] are described by Janssens et al., (2006) *Proc Natl Acad Sci USA* 103: 15130–15135.

References

Alexander, A., Steinmetz, M., Barritault, D., Frangione, B., Franklin, E.C., Hood, L., Buxbaum, J.N. (1982) γ Heavy chain disease in man: cDNA sequence supports partial gene deletion model. *Proc Natl Acad Sci USA* 79: 3260–3264.

Anand, R. (1992) Yeast artificial chromosomes (YACs) and the analysis of complex genomes. *Trends Biotechnol* 10: 35–40.

Arulampalam, V., Eckhardt, L., Pettersson, S. (1997) The enhancer shift: a model to explain the developmental control of IgH gene expression in B-lineage cells. *Immunol Today* 18: 549–554.

Ball, W.J. Jr., Kasturi, R., Dey, P., Tabet, M., O'Donnell, S., Hudson, D., Fishwild, D. (1999) Isolation and characterization of human monoclonal antibodies to digoxin. *J Immunol* 163: 2291–2298.

Bertrand, F.E., Golub, R., Wu, G.E. (1998) V_H gene replacement occurs in the spleen and bone marrow of non-autoimmune quasi-monoclonal mice. *Eur J Immunol* 28: 3362–3370.

Brüggemann, M. (2004) Human monoclonal antibodies from translocus mice. In: Honjo, T., Alt, F.W., Neuberger, M.S. (eds) *Molecular Biology of B-Cells*. Amsterdam: Elsevier, pp. 547–561.

Brüggemann, M., Caskey, H.M., Teale, C., Waldmann, H., Williams, G.T., Surani, M. A., Neuberger, M.S. (1989) A repertoire of monoclonal antibodies with human heavy-chains from transgenic mice. *Proc Natl Acad Sci USA* 86: 6709–6713.

Brüggemann, M., Neuberger, M.S. (1991) Generation of antibody repertoires in transgenic mice. *Methods: A Companion to Methods Enzymol* 2: 159–165.

Brüggemann, M., Neuberger, M.S. (1996) Strategies for expressing human antibody repertoires in transgenic mice. *Immunol Today* 17: 391–397.

Brüggemann, M., Spicer, C., Buluwela, L., Rosewell, I., Barton, S., Surani, M.A., Rabbitts, T.H. (1991) Human antibody production in transgenic mice: Expression from 100 kb of the human IgH locus. *Eur J Immunol* 21: 1323–1326.

Brüggemann, M., Taussig, M.J. (1997) Production of human antibody repertoires in transgenic mice. *Curr Opin Biotechnol* 8: 455–458.

Capecchi, M.R. (1989) The new mouse genetics: altering the genome by gene targeting. *Trends Genet* 5: 70–76.

Chen, J., Trounstine, M., Kurahara, C., Young, F., Kuo, C.-C., Xu, Y., Loring, J.F., Alt, F.W., Huszar, D. (1993a) B cell

development in mice that lack one or both immunoglobulin κ light chain genes. *EMBO J* 12: 821–830.

Chen, J., Trounstine, M., Alt, F.W., Young, F., Kurahara, C., Loring, J.F., Huszar, D. (1993b) Immunoglobulin gene rearrangement in B cell deficient mice generated by targeted deletion of the J_H locus. *Int Immunol* 5: 647–656.

Choi, T.K., Hollenbach, P.W., Pearson, B.E., Ueda, R.M., Weddell, G.N., Kurahara, C. G., Woodhouse, C.S., Kay, R.M., Loring, J.F. (1993) Transgenic mice containing a human heavy chain immunoglobulin gene fragment cloned in a yeast artificial chromosome. *Nat Genet* 4: 117–123.

Cook, G.P., Tomlinson, I.M., Walter, G., Riethman, H., Carter, N.P., Buluwela, L., Winter, G., Rabbitts, T.H. (1994) A map of the human immunoglobulin VH locus completed by analysis of the telomeric region of chromosome 14q. *Nat Genet* 7: 162–168.

Corbett, S.J., Tomlinson, I.M., Sonnhammer, E.L., Buck, D., Winter, G. (1997) Sequence of the human immunoglobulin diversity (D) segment locus: a systematic analysis provides no evidence for the use of DIR segments, inverted D segments, "minor" D segments or D-D recombination. *J Mol Biol* 270: 587–597.

Cumbers, S.J., Williams, G.T., Davies, S.L., Grenfell, R.L., Takeda, S., Batista, F.D., Sale, J.E., Neuberger, M.S. (2002) Generation and iterative affinity maturation of antibodies in vitro using hypermutating B-cell lines. *Nat Biotechnol* 20: 1129–1134.

Davies, N.P., Brüggemann, M. (1993) Extension of yeast artificial chromosomes by cosmid multimers. *Nucleic Acids Res* 21: 767–768.

Davies, N.P., Rosewell, I.R., Brüggemann, M. (1992) Targeted alterations in yeast artificial chromosomes for inter-species gene transfer. *Nucleic Acids Res* 20: 2693–2698.

Davies, N.P., Rosewell, I.R., Richardson, J. C., Cook, G.P., Neuberger, M.S., Brownstein, B.H., Norris, M.L., Brüggemann, M. (1993) Creation of mice expressing human antibody light chains by introduction of a yeast artificial chromosome containing the core region of the human immunoglobulin κ locus. *Bio/technology* 11: 911–914.

Davies, N.P., Popov, A.V., Zou, X., Brüggemann, M. (1996) Human antibody repertoires in transgenic mice: Manipulation and transfer of YACs. In: McCafferty, J., Hoogenboom, H.R., Chiswell, D.J. (eds) *Antibody Engineering: A Practical Approach*. Oxford: IRL Press, pp. 59–76.

Davis, C.G., Gallo, M.L., Corvalan, R.F. (1999) Transgenic mice as a source of fully human antibodies for the treatment of cancer. *Cancer Metastasis Rev* 18: 421–425.

Evans, M.J. (1989) Potential for genetic manipulation of Mammals. *Mol Biol Med* 6: 557–565.

Fishwild, D.M., O'Donnell, S.L., Bengoechea, T., Hudson, D.V., Harding, F., Bernhard, S.L., Jones, D., Kay, R.M., Higgins, K.M., Schramm, S.R., Lonberg, N. (1996) High-avidity human Igκ monoclonal antibodies from a novel strain of minilocus transgenic mice. *Nat Biotechnol* 14: 845–851.

Fournier, R.E., Ruddle, F.H. (1977) Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells. *Proc Natl Acad Sci USA* 74: 319–323.

Frazer, J.K., Capra, J.D. (1999) Immunoglobulins: structure and function. In: Paul, W.E. (ed.) *Fundamental Immunology*, 4th edn. Philadelphia: Lippincott-Raven, pp. 37–74.

Frippiat, J.P., Williams, S.C., Tomlinson, I. M., Cook, G.P., Cherif, D., Le Paslier, D., Collins, J.E., Dunham, I., Winter, G., Lefranc, M.-P. (1995) Organization of the human immunoglobulin lambda light-chain locus on chromosome 22q11.2. *Hum Mol Genet* 4: 983–91.

Gordon, J.W., Ruddle, F.H. (1983) Gene transfer into mouse embryos: production of transgenic mice by pronuclear injection. *Methods Enzymol* 101: 411–433.

Green, L.L., Hardy, M.C., Maynard-Currie, C.E., Tsuda, H., Louie, D.M., Mendez, M.J., Abderrahim, H., Noguchi, M., Smith, D.H., Zeng, Y., David, N.E., Sasai, H., Garza, D., Brenner, D.G., Hales,

J.F., McGuinness, R.P., Capon, D.J., Klapholz, S., Jakobovits, A. (1994) Antigen-specific human monoclonal antibodies from mice engineered with human Ig heavy and light chain YACs. *Nat Genet* 7: 13–21.

Green, L.L. (1999) Antibody engineering via genetic engineering of the mouse: XenoMouse strains are a vehicle for the facile generation of therapeutic human monoclonal antibodies. *J Immunol Methods* 231: 11–23.

Haas, I.G., Wabl, M. (1983) Immunoglobulin heavy chain binding protein. *Nature* 306: 387–389.

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E.B., Bendahman, N., Hamers, R. (1993) Naturally occurring antibodies devoid of light chains. *Nature* 363: 446–448.

Harris, R.S., Sale, J.E., Petersen-Mahrt, S.K., Neuberger, M.S. (2002) AID is essential for immunoglobulin V gene conversion in a cultured B cell line. *Curr Biol* 12: 435–438.

Hasan, M., Polic, B., Bralic, M., Jonjic, S., Rajewsky, K. (2002) Incomplete block of B cell development and immunoglobulin production in mice carrying the μMT mutation on the BALB/c background. *Eur J Immunol* 32: 3463–3471.

He, M., Menges, M., Groves, M.A., Corps, E., Liu, H., Brüggemann, M., Taussig, M.J. (1999) Selection of a human anti-progesterone antibody fragment from a transgenic mouse library by ARM ribosome display. *J Immunol Methods* 231: 105–117.

Hendershot, L.M. (1990) Immunoglobulin heavy chain and binding protein complexes are dissociated in vivo by light chain addition. *J Cell Biol* 111: 829–837.

Hofker, M.H., Walter, M.A., Cox, D.W. (1989) Complete physical map of the human immunoglobulin heavy chain constant region gene complex. *Proc Natl Acad Sci USA* 86: 5567–5571.

Hogan, B., Beddington, R., Costantini, F., Lacy, E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Ishida, I., Tomizuka, K., Yoshida, H., Tahara, T., Takahashi, N., Ohguma, A., Tanaka, S., Umehashi, M., Maeda, H., Nozaki, C., Halk, E., Lonberg, N. (2002) Production of human monoclonal and polyclonal antibodies in transchromo animals. *Cloning Stem Cells* 4: 91–102.

Jakobovits, A., Vergara, G.J, Kennedy, J.L., Hales, J.F., McGuinness, R.P., Casentini-Borocz, D.E., Brenner, D.G., Otten, G.R. (1993) Analysis of homozygous mutant chimeric mice: deletion of the immunoglobulin heavy-chain joining region blocks B-cell development and antibody production. *Proc Natl Acad Sci USA* 90: 2551–2555.

Jakobovits, A., Green, L.L., Hardy, M.C., Maynard-Currie, C.E., Tsuda, H., Louie, D.M., Mendez, M.J., Abderrahim, H., Noguchi, M., Smith, D.H., Zeng, Y., David, N.E., Sasai, H., Garza, D., Brenner, D.G., Hales, J.F., McGuinnes, R.P., Capaon, D.J., Klapholz, S. (1995) Production of antigen-specific human antibodies from mice engineered with human heavy and light chain YACs. *Ann N Y Acad Sci* 764: 525–535.

Jena, P.K., Smith, D.S., Zhang, X., Aviszus, K., Durdik, J.M., Wysocki, L.J. (2003) Somatic translocation and differential expression of Ig μ transgene copies implicate a role for the *IgH* locus in memory B cell development. *Mol Immunol* 39: 885–897.

Jenuwein, T., Allis, C.D. (2001) Translating the histone code. *Science* 293: 1074–1080.

Kakeda, M., Hiratsuka, M., Nagata, K., Kuroiwa, Y., Kakitani, M., Katoh, M., Oshimura., M., Tomizuka, K. (2005) Human artificial chromosome (HAC) vector provides long-term therapeutic transgene expression in normal human primary fibroblasts. *Gene Ther* 12: 852–856.

Kanayama, N., Todo, K., Reth, M., Ohmori, H. (2005) Reversible switching of immunoglobulin hypermutation machinery in a chicken B cell line. *Biochem Biophys Res Commun* 327: 70–75.

Kawasaki, K., Minoshima, S., Schooler, K., Kudoh, J., Asakawa, S., de Jong, P.J., Shimizu, N. (1995) The organization of the human immunoglobulin λ gene locus. *Genome Res* 5: 125–135.

King, D.J. (1998) *Applications and Engineering of Monoclonal Antibodies*. London: Taylor and Francis.

Kitamura, D., Roes, J., Kühn, R., Rajewsky, K. (1991) A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ chain gene. *Nature* 350: 423–426.

Klingbeil, C., Hsu, D.H. (1999) Pharmacology and safety assessment of humanized monoclonal antibodies for therapeutic use. *Toxicol Pathol* 27: 1–3.

Knight, K.L., Kingzette, M., Crane, M.A., Zhai, S.K. (1995) Transchromosomally derived Ig heavy chains. *J Immunol* 155: 684–691.

Koi, M., Shimizu, M., Morita, H., Yamada, H., Oshimura, M. (1989) Construction of mouse A9 clones containing a single human chromosome tagged with neomycin-resistance gene via microcell fusion. *Jpn J Cancer Res* 80: 413–418.

Kuroiwa, Y., Shinohara, T., Notsu, T., Tomizuka, K., Yoshida, H., Takeda, S., Oshimura, M., Ishida, I. (1998) Efficient modification of a human chromosome by telomere-directed truncation in high homologous recombination-proficient chicken DT40 cells. *Nucleic Acids Res* 26: 3447–3448.

Kuroiwa, Y., Tomizuka, K., Shinohara, T., Kazuki, Y., Yoshida, H., Ohguma, A., Yamamoto, T., Tanaka, S., Oshimura, M., Ishida, I. (2000) Manipulation of human minichromosomes to carry greater than megabase-sized chromosome inserts. *Nat Biotechnol* 18: 1086–1090.

Kuroiwa, Y., Kasinathan, P., Choi, Y.J., Naeem, R., Tomizuka, K., Sullivan, E.J., Knott, J.G., Duteau, A., Goldsby, R.A., Osborne, B.A., Ishida, I., Robl, J.M. (2002) Cloned transchromosomic calves producing human immunoglobulin. *Nat Biotechnol* 20: 889–894.

Kuroiwa, Y., Kasinathan, P., Matsushita, H., Sathiyaselan, J., Sullivan, E.J., Kakitani, M., Tomizuka, K., Ishida, I., Robl, J.M. (2004) Sequential targeting of the genes encoding immunoglobulin-μ and prion protein in cattle. *Nat Genet* 36: 775–780.

Lauwereys, M., Ghahroudi, M.A., Desmyter, A., Kinne, J., Holzer, W., De Genst, E., Wyns, L., Muyldermans, S. (1998) Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *EMBO J* 17: 3512–3520.

Lefranc, M.-P., Lefranc, G. (2001) *The Immunoglobulin Factsbook*. London: Academic Press, pp. 45–68.

Li, J., Geissal, E.D., Li, W., Stollar, D. (2005) Repertoire diversification in mice with an IgH-locus-targeted transgene for the rearranged VH domain of a physiologically selected anti-ssDNA antibody. *Mol Immunol* 42: 1475–1484.

Linden, van der R., Geus, de B., Stok, W., Bos, W., Wassenaar, van D., Verrips, T., Frenken, L. (2000) Induction of immune responses and molecular cloning of the heavy chain antibody repertoire of *Lama glama*. *J Immunol Methods* 240: 185–195.

Litman, G.W., Anderson, M.K., Rast, J.P. (1999) Evolution of antigen binding receptors. *Annu Rev Immunol* 17: 109–147.

Lonberg, N. (2005) Human antibodies from transgenic animals. *Nature Biotechnol* 23: 1117–1125.

Lonberg, N., Taylor, L.D., Harding, F.A., Troustine, M., Higgins, K.M., Schramm, S.R., Kuo, C.-C., Mashayekh, R., Wymore, K., McCabe, J.G., Munoz-O'Regan, D., O'Donnell, S.L., Lapachet, E.S.G., Bengoechea, T., Fishwild, D.M., Carmack, C.E., Kay, R.M., Huszar, D. (1994) Antigen-specific human antibodies from mice comprising four distinct genetic modifications. *Nature* 368: 856–859.

Magadán, S., Valladares, M., Suarez, E., Sanjuán, I., Molina, A., Ayling, C., Davies, S.L., Zou, X., Williams, G.T., Neuberger, M.S., Brüggemann, M., Gambón, F., Díaz-Espada, F., González-Fernández, A. (2002) Production of antigen-specific human monoclonal antibodies: comparison of mice carrying IgH/Iκ or IgH/Iκ/Iλ transloci. *BioTechniques* 33: 680–690.

Magor, B.G., Ross, D.A., Pilstrom, L., Warr, G.W. (1999) Transcriptional enhancers and the evolution of the IgH locus. *Immunol Today* 20: 13–17.

Markie, D. (1996) YAC protocols. In: *Methods in Molecular Biology*, Vol. 54. Totowa, NJ: Humana Press.

Martin, A., Bardwell, P.D., Woo, C.J., Fan, M., Shulman, M.J., Scharff, M.D. (2002) Activation-induced cytidine deaminase turns on somatic hypermutation in hybridomas. *Nature* 415: 802–806.

Matsuda, F., Ishii, K., Bourvagnet, P., Kuma, K., Hayashida, H., Miyata, T., Honjo, T. (1998) The complete nucleotide sequence of the human immunoglobulin heavy chain variable region locus. *J Exp Med* 188: 2151–2162.

Maynard, J., Georgiou, G. (2000) Antibody engineering. *Annu Rev Biomed Eng* 2: 339–376.

Mendez, M.J., Green, L.L, Corvalan, J.R., Jia, X.-C., Maynard-Currie, C.E., Yang, X.-D., Gallo, M.L., Louie, D.M., Lee, D.V., Erickson, K.L., Luna, J., Roy, C.M., Abderrahim, H., Kirschenbaum, F., Noguchi, M., Smith, D.H., Fukushima, A., Hales, J.F., Klapholz, S., Finer, M.H., Davis, C.G., Zsebo, K.M., Jakobovits, A. (1997) Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. *Nat Genet* 15: 146–156.

Mundt, C.A., Nicholson, I., Zou, X., Popov, A.V., Ayling C., Brüggemann, M. (2001) Novel control motif cluster in the IgH δ-γ3 interval exhibits B-cell specific enhancer function in early development. *J Immunol* 166: 3315–3323.

Muyldermans, S., Atarhouch, T., Saldanha, J., Barbosa, J.A., Hamers, R. (1994) Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. *Protein Eng* 7: 1129–1135.

Munro, S., Pelham, H.R. (1987) A C-terminal signal prevents secretion of luminal ER proteins. *Cell* 48: 899–907.

Neuberger, M., Brüggemann, M. (1997) Mice perform a human repertoire. *Nature* 386: 25–26.

Nguyen, V.K., Hamers, R., Wyns, L., Muyldermans, S. (2000) Camel heavy-chain antibodies: diverse germline V_HH and specific mechanisms enlarge the antigen-binding repertoire. *EMBO J* 19: 921–930.

Nguyen, V.K., Zou, X., Lauwereyes, M., Brys, L., Brüggemann, M., Muyldermans, S. (2003) Heavy-chain only antibodies derived from dromedary are secreted and displayed by mouse B-cells. *Immunology* 109: 93–101.

Nicholson, I.C., Zou, X., Popov, A.V., Cook, G.P., Corps, E.M., Humphries, S., Ayling, C., Xian, J., Taussig, M.J., Neuberger, M.S., Brüggemann, M. (1999) Antibody repertoires of four and five feature translocus mice carrying human immunuglobulin heavy chain and kappa and lambda light chain YACs. *J Immunol* 163: 6898–6906.

Okada, N., Okada, H. (1999) Human IgM antibody therapy for HIV-1 infection. *Microbiol Immunol* 43: 729–736.

Orinska, Z., Osiak, A., Lohler, J., Bulanova, E., Budagian, V., Horak, I., Bulfone-Paus, S. (2002) Novel B cell population producing functional IgG in the absence of membrane IgM expression. *Eur J Immunol* 32: 3472–3480.

Pachnis, V., Pevny, L., Rothstein, R., Costantini, F. (1990) Transfer of a yeast artificial chromosome carrying human DNA from *Saccharomyces cerevisiae* into mammalian cells. *Proc Natl Acad Sci USA* 87: 5109–5113.

Padlan, E.A. (1994) Anatomy of the antibody molecule. *Mol Immunol* 31: 169–217.

Pavan, W.J., Hieter, P., Reeves, R.H. (1990) Modification and transfer into an embryonal carcinoma cell line of a 360-kilobase human-derived yeast artificial chromosome. *Mol Cell Biol* 10: 4163–4169.

Petersen-Mahrt, S. (2005) DNA deamination in immunity. *Immunol Rev* 203: 80–97.

Pluschke, G., Joss, A., Marfurt, J., Daubenberger, C., Kashala, O., Zwickl, M., Stief, A., Sansig, G., Schläpfer, B., Linkert, S., van der Putten, H., Hardman, N., Schröder, M. (1998) Generation of chimeric monoclonal antibodies from mice that carry human immunoglobulin Cγ1 heavy or Cκ light chain gene segments. *J Immunol Methods* 215: 27–37.

Popov, A.V., Bützler, C., Frippiat, J.-P., Lefranc, M.-P., Brüggemann, M. (1996) Assembly and extension of yeast artificial chromosomes to build up a large locus. *Gene* 177: 195–205.

Popov, A.V., Zou, X., Xian, J., Nicholson, I. C., Brüggemann, M. (1999) A human immunoglobulin λ locus is similarly well expressed in mice and humans. *J Exp Med* 189: 1611–1620.

Protopapadakis, E., Kokla, A., Tzartos, S.J., Mamalaki, A. (2005) Isolation and characterization of human anti-acetylcholine receptor monoclonal antibodies from transgenic mice expressing

human immunoglobulin loci. *Eur J Immunol* 35: 1960–1968.

Reichert, J.M., Rosensweig, C.J., Faden, L. B., Dewitz, M.C. (2005) Monoclonal antibody success in the clinic. *Nature Biotechnol* 23: 1073–1078.

Ren, L., Zou, X., Smith, J.A., Brüggemann, M. (2004) Silencing of the immunoglobulin heavy chain locus by removal of all 8 constant region genes on a 200 kb region. *Genomics* 84: 686–695.

Ronai, D., Iglesias-Ussel, M.D., Fan, M., Shulman, M.J., Scharff, M.D. (2005) Complex regulation of somatic hypermutation by cis-acting sequences in the endogenous IgH gene in hybridoma cells. *Proc Natl Acad Sci USA* 102: 11829–11834.

Roschenthaler, F., Hameister, H., Zachau, H.G. (2000) The 5′ part of the mouse immunoglobulin kappa locus as a continuously cloned structure. *Eur J Immunol* 30: 3349–3354.

Russell, N.D., Corvalan, J.R., Gallo, M.L., Davis, C.G., Pirofski, L. (2000) Production of protective human antipneumococcal antibodies by transgenic mice with human immunoglobulin loci. *Infect Immun* 68: 1820–1826.

Sanchez, P., Drapier, A.M., Cohen-Tannoudji, M., Colucci, E., Babinet, C., Cazenave, P.A. (1994) Compartmentalization of lambda subtype expression in the B cell repertoire of mice with a disrupted or normal C kappa gene segment. *Int Immunol* 6: 711–719.

Schedl, A., Larin, Z., Montoliu, L., Thies, E., Kelsey, G., Lehrach, H., Schütz, G. (1993) A method for the generation of YAC transgenic mice by pronuclear microinjection. *Nucleic Acids Res* 21: 4783–4787.

Shen, M.H., Yang, J., Loupart, M.L., Smith, A., Brown, W. (1997) Human mini-chromosomes in mouse embryonal stem cells. *Hum Mol Genet* 6: 1375–1382.

Shinohara, T., Tomizuka, K., Takehara, S., Yamauchi, K., Katoh, M., Ohguma, A., Ishida, I., Oshimura, M. (2000) Stability of transferred human chromosome fragments in cultured cells and in mice. *Chromosome Res* 8: 713–725.

Sohn, J., Gerstein, R.M., Hsieh, C.L., Lemer, M., Selsing, E. (1993) Somatic hypermutation of an immunoglobulin μ heavy chain transgene. *J Exp Med* 177: 493–504.

Taylor, L.D., Carmack, C.E., Schramm, S.R., Mashayekh, R., Higgins, K.M., Kuo, C.-C., Woodhouse, C., Kay, R.M., Lonberg, N. (1992) A transgenic mouse that expresses a diversity of human sequence heavy and light chain immunoglobulins. *Nucleic Acids Res* 20: 6287–6295.

Taylor, L.D., Carmack, C.E., Huszar, D., Higgins, K.M., Mashayekh, R., Sequar, G., Schramm, S.R., Kuo, C.-C., O'Donnell, S.L., Kay, R.M., Woodhouse, C.S., Lonberg, N. (1994) Human immunoglobulin transgenes undergo rearrangement, somatic mutation and class switching in mice that lack endogenous IgM. *Int Immunol* 6: 579–591.

Tomizuka, K., Yoshida, H., Uejima, H., Kugoh, H., Sato, K., Ohguma, A., Hayasaka, M., Hanaoka, K., Oshimura, M., Ishida, I. (1997) Functional expression and germline transmission of a human chromosome fragment in chimaeric mice. *Nat Genet* 16: 133–143.

Tomizuka, K., Shinohara, T., Yoshida, H., Uejima, H., Ohguma, A., Tanaka S., Sato, K., Oshimura, M., Ishida, I. (2000) Double trans-chromosomic mice: Maintenance of two individual human chromosome fragments containing immunoglobulin heavy and kappa loci and expression of fully human antibodies. *Proc Natl Acad Sci USA* 97: 722–727.

Wagner, S.D., Williams, G.T., Larson, T., Neuberger, M.S., Kitamura, D., Rajewsky, K., Xian, J., Brüggemann, M. (1994a) Antibodies generated from human immunoglobulin miniloci in transgenic mice. *Nucleic Acids Res* 22: 1389–1393.

Wagner, S.D., Popov, A.V., Davies, S.L., Xian, J., Neuberger, M.S., Brüggemann, M. (1994b) The diversity of antigen-specific monoclonal antibodies from transgenic mice bearing human immunoglobulin gene miniloci. *Eur J Immunol* 24: 2672–2681.

Wagner, S.D., Gross, G., Cook, G.P., Davies, S.L., Neuberger, M.S. (1996) Antibody expression from the core region of the human IgH locus reconstructed in transgenic mice using bacteriophage P1 clones. *Genomics* 35: 405–414.

Waldmann, H., Cobbold, S. (1993) The use of monoclonal antibodies to achieve immunological tolerance. *Immunol Today* 14: 247–251.

Weichhold, G.M., Klobeck, H.G., Ohnheiser, R., Combriato, G., Zachau, H.G. (1990) Megabase inversions in the human genome as physiological events. *Nature* 347: 90–92.

Xian, J., Zou, X., Popov, A.V., Mundt, C.A., Miller, N., Williams, G.T., Davies, S.L., Neuberger, M.S., Brüggemann, M. (1998) Comparison of the performance of a plasmid-based human Igκ minilocus and YAC-based human Igκ transloci for the production of a human antibody repertoire in transgenic mice. *Transgenics* 2: 333–343.

Yang, X.D., Jia, X.C., Corvalan, J.R., Wang, P., Davis, C.G. (2001) Development of ABX-EGF, a fully human anti-EGF receptor monoclonal antibody, for cancer therapy. *Crit Rev Oncol Hematol* 38: 17–23.

Yoshida, H., Tomizuka, K., Uejima, H., Satoh, K., Ohguma, A., Oshimura, M., Ishida, I. (1999) Creation of hybridomas from mice expressing human antibody by introduction of a human chromosome. In: Kitagawa, Y., Matuda, T., Iijima, S. (eds) *Animal Cell Technology: Basic and Applied Aspects*, Vol. 10. Dordrecht: Kluwer Academic Publishers, pp. 69–73.

Zachau, H.G. (2000). The immunoglobulin kappa gene families of human and mouse: a cottage industry approach. *Biol Chem* 381: 951–954.

Zou, Y.R., Gu, H., Rajewsky, K. (1993a) Generation of a mouse strain that produces immunoglobulin kappa chains with human constant region. *Science* 262: 1271–1274.

Zou, Y.R., Takeda, S., Rajewsky, K. (1993b) Gene targeting in the Ig kappa locus: efficient generation of lambda chain-expressing B cells, independent of gene rearrangements in Ig kappa. *EMBO J* 12: 811–820.

Zou, Y.R., Müller, W., Gu, H., Rajewsky, K. (1994) Cre-loxP-mediated gene replacement: a mouse strain producing humanized antibodies. *Curr Biol* 4: 1099–1103.

Zou, X., Xian, J., Popov, A.V., Rosewell, I.R., Müller, M., Brüggemann, M. (1995) Subtle differences in antibody responses and hypermutation of λ light chains in mice with a disrupted κ constant region. *Eur J Immunol* 25: 2154–2162.

Zou, X., Xian, J., Davies, N.P., Popov, A.V., Brüggemann, M. (1996) Dominant expression of a 1.3 Mb human Igκ locus replacing mouse light chain production. *FASEB J* 10: 1227–1232.

Zou, X., Piper, T.A., Smith, J.A., Allen, N. D., Xian, J., Brüggemann, M. (2003) Block in development at the pre B-II to immature B-cell stage in mice without Igκ and Igλ light chain. *J Immunol* 170: 1354–1361.

Zou, X., Smith, J.A., Nguyen, V.K., Ren, L., Muyldermans, S., Brüggemann, M. (2005) Expression of a dromedary heavy-chain-only antibody and B-cell development in the mouse. *J Immunol* 175: 3769–3779.

5
Bioinformatics Tools for Antibody Engineering

Andrew C.R. Martin and James Allen

5.1
Introduction

From the bioinformatics perspective, antibodies present a number of unique challenges (for example, in sequence analysis and search tools) as well as a number of unique opportunities (standardized numbering schemes, high-quality structural modeling). This chapter will survey a number of these aspects and will attempt to guide the user towards tools and resources that will fulfill these requirements. It is assumed that the reader is familiar with the natural generation of antibody diversity. Concepts of antibody structure will be reviewed only briefly insofar as they influence the rest of the discussion.

5.1.1
Brief Review of Antibody Structure

The four-chain model of antibody structure consisting of two identical light chains and two identical heavy chains, was first proposed by Porter (1959). There are many reviews of antibody structure (Alzari et al. 1988; Padlan 1994; Searle et al. 1994, for example) and of the interactions between antibodies and antigens (Padlan 1977; Mariuzza et al. 1987; Davies et al. 1990; Wilson and Stanfield 1993, for example); the reader is referred to these reviews for more information. In their pioneering work, Wu and Kabat (1970) examined the sequences of the variable domain. They aligned the sequences and generated a "variability plot." While the method used to calculate variability has since been criticized (Valdar and Thornton 2001; Valdar 2002), the trends are remarkably clear and enabled them to identify "hypervariable" regions which they suggested form the actual antigen combining site. They proposed that in the three-dimensional structure, these regions adopt loop conformations supported on a relatively conserved framework. They termed these stretches of hypervariable sequence the "complementarity determining regions" (CDRs).

Electron microscopy revealed the "Y" shape (Valentine and Green 1967) of IgG, the best studied of the immunoglobulin classes. Each arm, or Fab fragment,

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consists of a complete light chain (V_L and C_L domains), and half of a heavy chain (V_H and C_H1 domains). The remainder of each heavy chain (C_H2 and C_H3 domains) pairs to form the stem of the "Y" known as the F_c fragment (IgM and IgE have an additional C_H4 domain). A dimer of V_H and V_L domains is known as the Fv fragment. The first X-ray crystal structure of a Fab fragment, solved by Poljak in 1973 confirmed that CDRs defined by Kabat and Wu corresponded approximately to structural loops which come together to form the antigen-binding site (Poljak et al. 1973).

In the 1980s, Chothia and Lesk showed that the CDRs were much less variable in structure than might have been expected (Chothia and Lesk 1987). They found that for a given length of CDR, if certain amino acids were present at key locations in the CDR and in the structurally adjacent framework, then the conformation of the CDR would be conserved. In general, the amino acids at other positions within the CDRs could be varied freely without any major change in the conformation of the CDR. They defined a set of "canonical conformations" into which the majority of CDRs would be expected to fall. The application of this observation to three-dimensional modeling is discussed later.

The rest of this chapter will be confined to discussion of the Fv fragment (i.e. the V_L and V_H domains) which encompass the variability of antibodies and their ability to interact with antigens.

5.1.2 Conventions Used in this Chapter

In this chapter, the following conventions will be used. The letters "L" and "H" will be used to refer to the light and heavy chains respectively and when referring to a particular residue number, the chain label will be prepended onto the residue number. For example, the 10th residue in the light chain will be referred to as L10. Any of the six CDRs will be referred to using the letters "CDR-" followed by the chain name and the CDR number. For example, the first CDR of the light chain will be referred to as CDR-L1. This serves to avoid confusion with the first residue of the light chain (residue L1). In addition, CDR*n* (e.g. CDR3) is used to refer to both the heavy and light chain CDRs. Similarly the framework regions (those regions outside the CDRs) are termed LFR1, LFR2, LFR3, and LFR4 in the light chain, and HFR1, HFR2, HFR3, and HFR4 in the heavy chain. FR*n* (e.g. FR3) is used to refer to a framework region in both heavy and light chains.

5.2 Numbering Schemes for Antibodies

One of the major advantages of working with antibodies from a bioinformatics viewpoint is the availability of a standardized numbering scheme. This provides a standard way of identifying specific locations within an antibody sequence. In addition, it may provide a standard link between sequence and structure. Unfor-

tunately in the case of antibodies, the idea of having a standard numbering scheme is now so popular that there are at least four different such schemes!

An extremely useful comparison of the different numbering schemes is presented by Honegger (see http://www.biochem.unizh.ch/antibody/).

5.2.1 The Kabat Numbering Scheme

The most commonly used scheme is that introduced by Wu and Kabat when they performed their analysis of sequence variability. This "Kabat numbering scheme," universely recognized by immunologists, was developed purely on the basis of sequence alignment. Insertions in the sequence relative to the standard numbering scheme are indicated using insertion letter codes. For example, residues inserted between residues L27 and L28 are indicated as L27A, L27B, etc. Deletions relative to the standard scheme are simply accommodated by skipping numbers.

Ideally, such schemes are designed in the light of both large amounts of sequence information and multiple structures. Insertion sites (i.e. residue L27A, etc.) are placed only in loop regions (or form β bulges) and have structural meaning such that topologically equivalent residues get the same label.

The numbering scheme for the light and heavy chains is shown in Table 5.1. The residues considered to be part of the six CDRs as defined by Wu and Kabat are CDR-L1: L24–L34; CDR-L2: L50–L56; CDR-L3: L89–L97; CDR-H1: H31–H35B; CDR-H2: H50–H65; CDR-H3: H95–H102.

While the Kabat numbering scheme is the most widely adopted, it was derived from the analysis of a rather limited set of sequence data and, as a result, has some problems.

The numbering adopts a very rigid specification such that the allowed insertions at each position are specified. For example, in CDR-H3, insertions occur between H100 and H101 and the Kabat specification allows insertion letters up to K: (i.e. H100, H100A . . . H100K, H101). This accommodates CDR-H3 loops with lengths up to 19 residues. More than 200 heavy chain sequences are now known in which CDR-H3 is longer than this, some being 30 residues in length and therefore needing insertion letters up to H100U. However, the Kabat standard does not allow insertion letters beyond H100K so there is no agreed way of numbering these very long loops. The Kabat data files place these additional insertions at varying positions. While CDR-H3 is the prime position at which such problems occur, similar situations can arise at other locations.

Even more importantly, when Chothia and Lesk performed their analysis of CDR conformation, they found that the insertion sites within CDR-L1 and CDR-H1 did not correspond to the sites of structural insertions. Therefore, when one looks at the three-dimensional structures, one finds that topologically equivalent residues in these loops are not assigned the same number, leading to the requirement for a structurally correct numbering scheme.

Table 5.1 Kabat numbering scheme.

Light chain										
L0	L1	L2	L3	L4	L5	L6	L7	L8	L9	
L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	
L20	L21	L22	L23	L24	L25	L26	L27			
L27A	L27B	L27C	L27D	L27E	L27F					
								L28	L29	
L30	L31	L32	L33	L34	L35	L36	L37	L38	L39	
L40	L41	L42	L43	L44	L45	L46	L47	L48	L49	
L50	L51	L52	L53	L54	L55	L56	L57	L58	L59	
L60	L61	L62	L63	L64	L65	L66	L67	L68	L69	
L70	L71	L72	L73	L74	L75	L76	L77	L78	L79	
L80	L81	L82	L83	L84	L85	L86	L87	L88	L89	
L90	L91	L92	L93	L94	L95					
					L95A	L95B	L95C	L95D	L95E	L95F
						L96	L97	L98	L99	
L100	L101	L102	L103	L104	L105	L106				
						L106A				
							L107	L108	L109	
Heavy chain										
H0	H1	H2	H3	H4	H5	H6	H7	H8	H9	
H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	
H20	H21	H22	H23	H24	H25	H26	H27	H28	H29	
H30	H31	H32	H33	H34	H35					
					H35A	H35B				
						H36	H37	H38	H39	
H40	H41	H42	H43	H44	H45	H46	H47	H48	H49	
H50	H51	H52								
		H52A	H52B	H52C						
			H53	H54	H55	H56	H57	H58	H59	
H60	H61	H62	H63	H64	H65	H66	H67	H68	H69	
H70	H71	H72	H73	H74	H75	H76	H77	H78	H79	
H80	H81	H82								
		H82A	H82B	H82C						
			H83	H84	H85	H86	H87	H88	H89	
H90	H91	H92	H93	H94	H95	H96	H97	H98	H99	
H100										
H100A	H100B	H100C	H100D	H100E	H100F	H100G	H100H	H100I	H100J	H100K
	H101	H102	H103	H104	H105	H106	H107	H108	H109	
H110	H111	H112	H113							

5.2.1.1 The Chothia Numbering Scheme

The problem of topological equivalents in CDR-L1 and CDR-H1 led Chothia and Lesk to introduce the Chothia numbering scheme. This is identical to the Kabat scheme with the exception of CDR-L1 and CDR-H1, where the insertions are placed at the structurally correct positions such that topologically equivalent residues do get the same label. The extreme variability of conformation in CDR-H3 and the lack of structures with very long CDR-H3 loops means that it has not

been possible to assign a definitive numbering scheme with topological equivalence for this CDR.

Unfortunately, Chothia and co-workers confused issues from 1989 (Chothia et al. 1989) when they erroneously changed their numbering scheme such that insertions in CDR-L1 were placed after residue L31 rather than the structurally correct L30. This was corrected in 1997 (Al-Lazikani et al. 1997).

The correct version of the Chothia numbering (as used before 1989 and since 1997) for the light and heavy chains is shown in Table 5.2.

Table 5.2 Chothia numbering scheme.

Light chain										
L0	L1	L2	L3	L4	L5	L6	L7	L8	L9	
L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	
L20	L21	L22	L23	L24	L25	L26	L27	L28	L29	
L30										
L30A	L30B	L30C	L30D	L30E	L30F					
	L31	L32	L33	L34	L35	L36	L37	L38	L39	
L40	L41	L42	L43	L44	L45	L46	L47	L48	L49	
L50	L51	L52	L53	L54	L55	L56	L57	L58	L59	
L60	L61	L62	L63	L64	L65	L66	L67	L68	L69	
L70	L71	L72	L73	L74	L75	L76	L77	L78	L79	
L80	L81	L82	L83	L84	L85	L86	L87	L88	L89	
L90	L91	L92	L93	L94	L95					
					L95A	L95B	L95C	L95D	L95E	L95F
						L96	L97	L98	L99	
L100	L101	L102	L103	L104	L105	L106				
						L106A				
							L107	L108	L109	
Heavy chain										
H0	H1	H2	H3	H4	H5	H6	H7	H8	H9	
H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	
H20	H21	H22	H23	H24	H25	H26	H27	H28	H29	
H30	H31									
	H31A	H31B								
		H32	H33	H34	H35	H36	H37	H38	H39	
H40	H41	H42	H43	H44	H45	H46	H47	H48	H49	
H50	H51	H52								
		H52A	H52B	H52C						
			H53	H54	H55	H56	H57	H58	H59	
H60	H61	H62	H63	H64	H65	H66	H67	H68	H69	
H70	H71	H72	H73	H74	H75	H76	H77	H78	H79	
H80	H81	H82								
		H82A	H82B	H82C						
			H83	H84	H85	H86	H87	H88	H89	
H90	H91	H92	H93	H94	H95	H96	H97	H98	H99	
H100										
H100A	H100B	H100C	H100D	H100E	H100F	H100G	H100H	H100I	H100J	H100K
	H101	H102	H103	H104	H105	H106	H107	H108	H109	
H110	H111	H112	H113							

5.2.2
The IMGT Numbering Scheme

An alternative numbering scheme has been introduced by Lefranc for use with the Immunogenetics (IMGT) database (Lefranc 1997). The advantage of this scheme, based on germline sequences, is that it unifies numbering across antibody lambda and kappa light chains, heavy chains and T-cell receptor alpha, beta, gamma, and delta chains.

However, because the scheme is based on germline sequences it does not span CDR3 or FR4. In addition, insertions and deletions increase in size unidirectionally.

5.2.3
Honegger and Plückthun Numbering Scheme

Yet another numbering scheme was introduced by Honegger and Plückthun in 2001 (Honegger and Plückthun 2001). This takes the same approach as the IMGT scheme, but addresses the problem in the IMGT scheme of being cut short in CDR3. In addition, insertions and deletions, rather than growing unidirectionally, are placed symmetrically around a key position. Furthermore, whereas length variations in CDR1 and CDR2 are accounted for by a single gap position in IMGT, this scheme has two locations at which gaps may be introduced.

In conclusion, while the latter numbering schemes, in particular that of Honneger and Plückthun, have distinct advantages over the earlier schemes, the Kabat scheme in particular is so well established amongst immunologists, it is hard for them to gain acceptance. The Chothia scheme is routinely used by other groups in structural analysis, but these other schemes are rarely used outside the groups who have developed them.

5.3
Definition of the CDRs and Related Regions

The complementarity determining regions (CDRs) were defined by Wu and Kabat from their variability plot. Tips for identifying the location of the CDRs through visual inspection are available on Andrew Martin's website. However, others have also provided definitions of regions related to the CDRs. It is important to note that, in the main, these are not attempts to redefine the CDRs, they are simply alternative subsets of residues, overlapping the CDRs, which are important in different ways. However, confusingly, some authors do use terms such as "Chothia CDRs" although the regions defined by Chothia have a different meaning.

Chothia defined the "structural loops" – those regions likely to vary in conformation between different antibody structures. With the exception of CDR-H1, all the structural loops are contained within the CDRs; in the case of CDR-H1 the structural loop and the CDR overlap one another. Thus the analysis of canonical

classes performed by Chothia is based on these structural loops rather than the full CDRs. To make things a little more confusing, the precise boundaries of the structural loops have varied somewhat between different papers from the Chothia group. These differences have been the result of finding changes in conformational variability as new structures have become available and have been added to the analysis. For example, the 1997 paper from Chothia's group (Al-Lazikani et al. 1997) changed from defining the CDR-H2 structural loop as residues H52–H56 to residues H50–H58.

Another region, known as the "AbM loops" was introduced by Martin et al. (1989). These regions are a compromise between the Kabat sequence-variability-defined CDRs and Chothia structural loops. This region is probably the most useful definition to use when trying to generate three-dimensional models of the conformations of the loops likely to interact with antigen.

An analysis of the contact residues from a set of antibody–antigen complexes by MacCallum et al. (1996) introduced the "contact region." Since these are the residues that are most likely to take part in interactions with the antigen, it is likely to be the most useful region for people wishing to perform mutagenesis to modify the affinity of an antibody.

IMGT has introduced another range of residues for the loops which form the combining site and confusingly they do term these CDRs.

These alternative regions are summarized in Table 5.3. Note that when using the Kabat numbering scheme, the C-terminal end of the Chothia structural loop changes residue number depending on the length of the loop.

Table 5.3 Residue ranges for different definitions of regions around the CDRs.

Loop	*Kabat CDR*	*AbM*	*Chothia*	*Contact*	*IMGT*
CDR-L1	L24–L34	L24–L34	L23–L34	L30–L36	L27–L32
CDR-L2	L50–L56	L50–L56	L50–L56	L46–L55	L50–L52
CDR-L3	L89–L97	L89–L97	L89–L97	L89–L96	L89–L97
CDR-H1	H31–H35B (Kabat numbering)	H26–H35B	H26–H32 . . . 34	H30–H35B	H26–H35B
CDR-H1	H31–H35 (Chothia numbering)	H26–H35	H26–H32	H30–H35	H26–H35
CDR-H2	H50–H65	H50–H58	H50–H58	H47–H58	H51–H57
CDR-H3	H95–H102	H95–H102	H95–H102	H93–H101	H93–H102

Note that for the Chothia definition, the C-terminal end of CDR-H1 varies in location under the Kabat numbering scheme depending on the length of the CDR (i.e. if neither H35A nor H35B is present then the loop ends at H32; if only H35A is present it ends at H33 and if both H35A and H35B are present then it ends at H34). It should also be noted that different papers by Chothia use slightly different definitions of the structural loops (for example earlier papers used H52–H56 for the CDR-H2 loop); the most recent definitions are shown.

5.4 Antibody Sequence Data

For those wishing to search and analyze antibody sequences, the standard databanks (Genbank/EMBL for DNA, Genpept/trEMBL/SwissProt for protein data) and search tools are generally not suitable. The sequence databanks make a deliberate effort to avoid including rearranged somatically mutated antibody sequences since the vast number of these can confuse significance statistics and more advanced profile-based search methods. Sequence search tools such as BLAST and FASTA are designed to identify homologs. Of course this is not an issue when comparing antibody sequences as all the sequences are homologous by definition.

Once the standard numbering scheme has been applied to antibody protein sequences, they are effectively multiply aligned. This allows much finer search criteria to be applied with the right tools. For example, one should be able to search a set of antibody sequences to find all examples with a 10-residue long CDR-L1 and a valine at position L30. Tools such as BLAST, FASTA, or SSEARCH are still valuable to search a database of antibody sequences to find the most similar sequence(s) or to rank sequences on the basis of sequence identity. However, the calculation of significance scores (*P*-values and *E*-values) is meaningless when the database contains only closely related homologs. If you wish to use one of these tools to calculate sequence identities with all antibody sequences in a database, you must set an extremely poor *E*-value cutoff (e.g. 10 000) to ensure that all sequences are compared.

5.4.1 Antibody Sequence Databanks

There are two major resources that collect antibody sequence data. Probably the best known, and certainly the oldest, resource is the Kabat database. This collection of data was started by Wu and Kabat in the 1970s when they started their work on analyzing sequence variability. It grew into the book *Sequences of Immunological Interest* (Kabat et al. 1991). The last edition of this appeared in 1991 when it was replaced by an internet-based resource.

The Kabat data have been available as a downloadable resource and as a web-based resource allowing interactive queries. The raw sequence data may be downloaded for local analysis from either the National Center for Biotechnology Information (NCBI) or European Bioinformatics Institute (EBI) FTP sites. The most up-to-date raw data is in the fixlen subdirectory (or in FASTA format in the fasta format subdirectory). The "fixlen" data format contains the sequences with the standard Kabat numbering scheme applied. Unfortunately, these freely available data have not been updated since April 2000 as the Kabat database has now become a paid-for resource. This can be accessed on the Kabat Database website (http://www.kabatdatabase.com/), but requires registration and payment for both commercial and academic use.

The second major resource is IMGT (Lefranc 2001). The data in IMGT are updated regularly and may be downloaded from the EBI FTP site. A huge advantage of IMGT is the adoption of an ontology to describe various features of the data (Giudicelli and Lefranc 1999). The ontology includes terms for species, loci, genes, chains, structure, localization, and specificity amongst numerous other terms. This makes it much easier to perform reliable analyses by allowing direct comparison of sequence characteristics. There are, however, some disadvantages compared with the Kabat data. First the data are only available as EMBL-style files, or as DNA FASTA files (at the time of writing, there are no FASTA files for protein translations). The EMBL-style files have translations that can be extracted, but no standard numbering scheme has been applied. Numbered sequences may be accessed via the web interface at the IMGT database website, but further confusion has been introduced by the IMGT numbering scheme described above. There is also some confusion about the copyright terms on the data. The user manual available from the EBI IMGT data manual FTP site states that the "manual and the database it accompanies may be copied and redistributed freely, without advance permission". On the other hand the IMGT database warranty web page states that "The IMGT software and data are provided as a service to the scientific community to be used only for research and educational purposes. [. . .] Any other use of IMGT material needs prior written permission of the IMGT coordinator and of the legal institutions."

5.4.2 Germline Sequence Databases

The Kabat and IMGT resources collect rearranged, somatically mutated, and expressed antibody sequences (though germline data may also be included). In contrast, Tomlinson's VBase (see the VBase website, http://vbase.mrc-cpe.cam.ac.uk/) is a comprehensive directory of all human germline variable region sequences (Tomlinson et al. 1992). The database was developed over a period of several years, but is now considered to be complete and is no longer updated. The sequences (both DNA and amino acid translation) can be viewed on the site and saved to disk through cut-and-paste. The site includes nucleotide alignments for all functional segments, scale maps of all human V gene loci, DNAPLOT alignment software allowing rearranged genes to be assigned to their closest germline counterparts and various compiled statistics (numbers of functional segments belonging to each V gene family, cuts by different restriction enzymes and polymerase chain reaction (PCR) primers for amplifying rearranged V genes).

Mouse germline data are collected by Almagro (Almagro et al. 1998) in the ABG database (http://www.ibt.unam.mx/vir/V_mice.html). This resource provides access to sequences for the mouse germline sequences together with alignments of murine V_H and V_κ sequences. Like VBase, pseudogene and fragment data are stored, but there is also information on the particular strain of mouse

from which the sequence data are obtained. Currently there are no data on murine V_λ sequences, which are relatively rare.

5.4.3 Web Resources for Sequence Analysis

5.4.3.1 Kabat Data

Access to the Kabat data is available from the Kabat Database website. The web interface allows searches of the annotations (ID, name, species, authors, etc.) by keyword, sequence searches using patterns (and allowing for mismatches), selection of specific sequence types, and positional correlations. The website also allows alignment of a light chain sequence against the data and provides an assortment of analyses such as variability, length distribution, and general statistics. However, as stated above, access to these tools requires registration and payment.

The publicly available Kabat protein sequence data (up to April 2000) may also be searched using KabatMan (Martin 1996). This is a specialized database for the analysis of Kabat antibody sequence data which may be queried using a language similar to the standard database query language SQL ("structured query language") using a full web interface, or via a simplified point and click interface both available through Andrew Martin's KabatMan web pages (http://www.bioinf.org.uk/abs/kabatman.html and http://www.bioinf.org.uk/abs/simkab.html). KabatMan is particularly suited to global analysis of the antibody data. It allows searches to specify individual amino acids or the contents of one of the six CDRs. For example, to identify all the antibodies which bind to DNA, but do not contain arginine in CDR-H3 one could use the query:

```
SELECT name, h3
WHERE antigen inc "DNA"
h3 <> " " and
h3 inc "R" not and
```

In this query, the "SELECT" clause specifies which data are to be returned (here, the name of the antibody and the sequence of CDR-H3). The "WHERE" clause specifies that the antigen should be DNA, then requires that the sequence of CDR-H3 should not be blank and that CDR-H3 should not include the letter "R" (i.e. arginine). Detailed examples are given in the KabatMan paper and in the online help.

KabatMan allows selection of antibody name, antigen, CDR sequences and lengths, framework region sequences, light chain class, species, citation, sequences of light and heavy chains, Chothia canonical conformational classes (see below), Kabat identifiers, human subgroups and earliest publication date. All of these properties can be restricted in the "WHERE" clause. While the Kabat data do not provide a link between paired light and heavy chains, KabatMan adds this information and the requirement for a "complete" antibody can be specified in the search.

Almagro (Ramirez-Benitez et al. 2001) has also provided a search interface (VIR-II) to access the public Kabat data (see http://www.ibt.unam.mx/vir/VIR/vir_index.html). This interface allows a subset of sequences to be extracted on the basis of type, gross or fine specificity, sequence completeness, and the presence of paired light and heavy chains. At the time of writing, however, this search facility is not available.

5.4.3.2 **IMGT Data**

The IMGT data may be accessed via a sequence retrieval service (SRS) interface at the EBI's IMGT website (http://www.ebi.ac.uk/imgt/). The main IMGT database website in France allows searching on the basis of accession number, keywords, name, date, length, species, functionality, specificity, group, subgroup, and reference. The interface to the data is hierarchical in nature, allowing one to home in on a particular sequence. However this is not suited to global analysis of the data. In future, IMGT data will be integrated into KabatMan to enable global analysis.

5.5 Antibody Structure Data

In contrast to the sequence data, structural data for antibodies are stored in the Protein Databank together with other protein structure data (Berman et al. 2000). Therefore, standard resources like the CATH database (http://cathwww.biochem.ucl.ac.uk/) contain information on antibody structures (Orengo et al. 1997). The antibody protein fold falls into the CATH class 2.60.40.10.

In addition, three specialist resources provide summaries of antibody structure data. The earliest of these, SACS (Allcorn and Martin, 2002), may be accessed at the SACS Database website (http://www.bioinf.org.uk/abs/sacs/). This resource is maintained in a fully automated manner with a brief manual check before data are made available. It is updated every 3–6 months. The resource provides names, light chain class, species, antigen, crystal structure details, fragment type and lengths, and sequences of the six CDRs. The data may be sorted on various criteria, including lengths of the CDRs, and the whole dataset may be downloaded. Almagro maintained another similar resource (see VIR Structures, http://www.ibt.unam.mx/vir/structure/structures.html), but at the time of writing this has not been updated since February 2001. Recently, IMGT has introduced a new summary, IMGT/3Dstructure-DB (Kaas et al. 2004) available at the IMGT3D website (http://imgt3d.igh.cnrs.fr/). This allows searches on the basis of species, group, subgroup, gene or allele, CDR length, sequence pattern within the CDRs, specific amino acids, accessible surface area, and backbone conformational details, as well as residue contact information.

5.6 Sequence Families

Ever since the first antibodies were studied, there have been attempts to group them into distinct sets. Initially this enabled estimates of the number of genes and their chromosomal locations, but it also allowed for comparisons between antibodies from different species, generally humans and mice. Groups of sequences are usually defined by analysis of sequence identity at the amino acid or nucleotide level, but have also been defined by antigen specificity and chromosomal location. The term "family" is usually applied to a set of sequences that are defined by protein sequence similarity or identity, and a "subgroup" generally refers to a set defined according to nucleotide sequence identity. In the IMGT-ONTOLOGY a subgroup is defined as a "set of genes which belong to the same group [V, D, J, or C], in a given species, and which share at least 75% identity at the nucleotide level" (Giudicelli and Lefranc 1999). This definition is not universally accepted, however, and "family" and "subgroup" are sometimes used interchangeably, or in a more general sense to denote grouping by any method. The number and definition of families/subgroups in the literature has varied, both because comprehensive data and sufficiently powerful computers have only recently become available, and because the grouping process is necessarily somewhat arbitrary. This arbitrariness is not a problem if the groupings are useful for some particular purpose.

5.6.1 Families and Subgroups

Inspection of amino acid sequences suggests that the variable region genes are arranged in subgroups, which evolved by gene duplication, and gene diversity is partly due to the expansion and contraction of the subgroups. DNA sequence analysis has confirmed the existence of subgroups for V_L and V_H genes and at least some of the hypervariability in CDR-H1 and CDR-H2 is present in the germline V_H genes, suggesting that an evolutionary process has caused substitution in the hypervariable regions.

Subdivision at the gene level can be made by cross-hybridization of different V genes with cDNA probes. There is not always a correspondence between groups defined on the basis of protein and nucleotide sequences, but the correlation is often good (Rechavi et al. 1983).

The V gene subgroups are multigene families that have been maintained throughout evolution; a subgroup preserves some characteristics in the noncoding segments that differ in other subgroups. It is difficult to relate subgroups from different species based on amino acid similarity; the germline structure, including the noncoding regions, provides a better understanding of the evolutionary relationship of V subgroups within, and subsequently between, species. Each cluster of V genes that constitutes a multigene family is likely to undergo

concerted evolution, with preservation of the sequence characteristics of the subgroup (Kroemer et al. 1991; Rechavi et al. 1983).

5.6.2 Human Family Chronology

6.6.2.1 Human Heavy Chain Variable Genes (V_H)

Kabat et al. (1991) classified V_H genes into three groups (I, II, and III) according to amino acid sequence identity; this grouping was confirmed by analysis at the nucleotide level (Kodaira et al. 1986). An additional family, homologous to a mouse family, was defined by Lee et al. (1987), and a further small family, with an unusual DNA sequence, was determined by Shen et al. (1987). Berman et al. (1988) defined six families; Kabat's subgroup II was divided into two families, one of which corresponds to Lee's family (named IV), and the fifth group (V) corresponds to Shen's family. The new sixth family (VI), which has 70% identity with family IV, was determined with Southern blot analysis and nucleotide identity.

A set of V_H sequences, previously classified as members of family I, differ at a clustered region and were proposed as a seventh family (VII) by Schroeder et al. (1990). This family can be considered as a subfamily of I, or as a family in transition to independence – either way, the classification has generally been accepted (Cook and Tomlinson 1995). Membership of a family is generally defined by >80% sequence identity at the nucleotide level, and this definition is supported by phylogenetic analysis (Honjo and Matsuda 1995).

Kabat et al. (1991) noted that the threshold of 80% nucleotide identity is somewhat arbitrary, and in the 1994 version of the Kabat database, the sequences are divided into families based on amino acid identity, where members of a family differ by 12 amino acids or less (Déret et al. 1995). This criterion creates 14 V_H families, but the classification into seven families described above is more generally used.

5.6.2.2 Human Light Chain Variable Genes (V_κ and V_λ)

In 1984, human V_κ sequences were classified into four families (I–IV) on the basis of amino acid sequence similarity (Pech et al. 1984), and Kabat et al. (1991) continued to use this grouping. Kroemer et al. (1991) found that analysis of nucleotide sequence identity largely paralleled this classification, but some sequences could not be assigned to a family using a threshold of 80% identity. Four sequences were grouped into three additional families, partly based on nucleotide identity and partly based on similarity to mouse V_κ families. A phylogenetic analysis of human V_κ genes showed four major clusters, and three groups with a single sequence (Sitnikova and Nei 1998), corresponding to the seven families defined by sequence similarity.

Based on >75% nucleotide sequence identity, human V_λ genes were placed into 10 subgroups (Frippiat et al. 1995), a result which agrees with later phylogenetic analysis (Williams et al. 1996).

In the 1994 version of the Kabat database, V_κ and V_λ sequences are grouped into six and nine families, respectively.

5.6.3 Mouse Family Chronology

5.6.3.1 Mouse Heavy Chain Variable Genes (V_H)

Amino acid sequence similarity defined seven families of mouse V_H sequences, each family having a different specificity. However, this grouping was not proposed as definitive, since the analyzed data did not constitute a representative sample (Dildrop 1984). These families corresponded with those determined by Southern blot analysis, and all nucleotide sequences available at that time had >80% sequence identity to members of one of these seven families (Brodeur and Riblet 1984). Two additional families were later defined (Dildrop et al. 1985).

Kabat divided the three subgroups that he had previously defined to correspond with the nine groups based on amino acid sequences (Kabat et al. 1991). As with the human V_H sequences, a more stringent rule was applied in the 1994 version of the Kabat database, creating a somewhat unwieldy 27 groups.

The number of V_H families was subsequently revised further, based on family members sharing >80% nucleotide sequence identity, and nonfamily members having 70–75% identity: first by Strohal et al. (1989) to 11, then by Honjo and Matsuda (1995) to 14, and finally by Mainville et al. (1996) to 15. This grouping into 15 subgroups is now recognized as standard and is used by the IMGT database (Lefranc 2001).

5.6.3.2 Mouse Light Chain Variable Genes (V_κ and V_λ)

Classification of mouse V_κ sequences into subgroups based on nucleotide sequence identity is not as unambiguous as it is with V_H sequences (Strohal et al. 1989). Using an 80% threshold resulted in 16 subgroups, though the existence of more was predicted (D'Hoostelaere and Klinman 1990); and the figure was increased to 19 by Kofler and Helmberg (1991). However, some subgroups shared >75% identity, members of different subgroups sometimes shared >80% identity, and there was not always a correspondence between these subgroups and V_κ protein groups. These awkward results suggest that while the arbitrary threshold of 80% might be useful, it is not necessarily significant in evolutionary terms (Kroemer et al. 1991).

In 1991, Kabat arranged the V_κ sequences into seven families (Kabat et al. 1991), with an additional "miscellaneous" family for sequences that were problematic. These groupings were revised in the 1994 database, creating 26 families. Lambda chains represent only around 5% of the total murine light chains, and the three genes can be classified into two families (Sitnikova and Su 1998), but such a classification is not particularly useful.

5.6.4 Correspondence Between Human and Mouse Families

5.6.4.1 Heavy Chain Variable Genes (V_H)

Genes of the same family in different species can be more alike than genes of different families in the same species (Lee et al. 1987). Such interspecies similarities could be explained by evolution from common ancestral genes or by shared requirements for structure and diversity (Berman et al. 1988).

As described above, early work by Kabat classified human V_H sequences into subgroups I, II, and III. More early work by Rechavi et al. (1982) suggested that the human V_H III subgroup underwent a significant gene expansion compared to the equivalent mouse subgroup. Surprisingly, however, they found through comparison of amino acid sequences, that the large set of human genes correspond to a small subset of mouse genes. Analysis of human and mouse germline V_H regions suggests that V_H families developed before speciation, and that they have been conserved by selection at the protein level (Brodeur and Riblet 1984).

More recent work has expanded the number of families based on analysis of complete genomes. Excluding pseudogenes and rearranged gene sequences, mouse V_H genes are generally classified into 15 families (Mainville et al. 1996) (termed "subgroups" in IMGT), while human V_H genes are classified into seven families (Schroeder et al. 1990).

However, human V_H genes have historically been clustered using more liberal criteria than those applied to mouse V_H genes, so to allow better comparisons mouse V_H sequences have been grouped into sets using the same criteria normally used for humans (de Bono et al. 2004). This produced eight rather than 15 "sets," each containing between one and five of the 15 conventional V_H subgroups (Table 5.4). Three of these sets have a one-to-one match with human V_H families; in two cases two mouse sets correspond to a single human family and in one case two human families correspond to a single mouse set. Phylogenetic analysis by de Bono et al. (2004) has confirmed the human family–mouse set relationships established by sequence comparison.

5.6.4.2 Light Chain Variable Genes (V_κ and V_λ)

As with heavy chains, V_κ sequences from corresponding families of different species can show greater similarity than sequences of different families within a species, indicating that V_κ family genes were fixed prior to mammalian speciation (Kroemer et al. 1991). Using nucleotide sequence identity, equivalent human and mouse families were ascertained, though the complexity of the mappings varied. The single gene in human family V_κ IV has at least 10 equivalents in mice, spread across three families; and a small mouse family is related to two human V_κ families, estimated to contain at least three times as many genes. A single member human family and large mouse family do not correspond to a family from the other species. Despite this variation, the maintenance of family specificity over 5070 million years suggests some degree of environmental selection pressure.

Table 5.4 Correspondence between mouse V_H sets (de Bono et al. 2004), the standard 15 families (Mainville et al. 1996) or subgroups as used by IMGT and the seven human V_H families (Schroeder et al. 1990).

Mouse V_H set	*IMGT subgroup*	*Human V_H family*
1	1	I
	14	
2	8	II
3a	4	III
	11	
3b	5	III
	6	
	7	
	10	
	13	
4a	3	IV
	12	
4b	2	IV
N/A	–	V
N/A	–	VI
7	9	VII
8	15	–

Data from de Bono et al. (2004).

It is difficult to establish interspecies correspondence for V_λ genes, since the number of mouse V_λ genes is small. The high diversity of mouse V_κ genes which compensates for this means that some of those families may have equivalents in the human V_λ families.

5.6.5 Tools for Assigning Subgroups

The Kabat Database website allows one to submit a sequence to determine the subgroup as specified in the 1991 Kabat book (Kabat et al. 1991).

Déret's SUBIM program (Déret et al. 1995) which may be downloaded from Paris Institute of Mineralogy FTP site (ftp://ftp.lmcp.jussieu.fr/pub/sincris/software/protein/subim/), allows the assignment of the variability subgroup of human sequences by comparison of the N-terminal 15 residues with consensus sequences determined by Kabat et al. (1991). The subgroup assignment function of Déret's program is also accessible via a web interface available on Andrew Martin's Subgroup website (http://www.bioinf.org.uk/abs/hsubgroup.html). The assignment algorithm is also built into the KabatMan software discussed above.

5.7
Screening new antibody sequences

Given a new antibody sequence, one can assign families and subgroups using the tools described above. In addition it may be interesting to look for unusual features in your sequence. These may indicate cloning artifacts, errors in the sequencing, or residues that are critical to the binding of this particular antibody. A server which compares your sequence against sequences in the Kabat database and reports amino acids occurring in less than 1% of the data in the database is available on Andrew Martin's Seqtest web page (http://www.bioinf.org.uk/abs/seqtest.html) and a typical sequence does have one or two "unusual" residues. To use the server, you simply enter the amino acid sequence of your Fv fragment (one or both chains). Optionally you may include the whole Fab fragment, but only the Fv portion will be tested. The method is described in detail on the web pages.

A new server from Raghavan and Martin on Andrew Martin's SHAB web page (http://www.bioinf.org.uk/abs/shab/) assesses the "humanness" of an antibody. This may be of value in selecting non-human antibodies which can be used successfully as chimerics in human therapy or for *in vivo* diagnostics. The method compares the antibody sequence with all known human sequences and calculates an average sequence identity. This is then plotted onto a distribution of scores achieved for every human antibody sequence in the Kabat database and a *Z*-score is assigned. Positive *Z*-scores indicate sequences that are more typically human than average while negative scores indicate sequences with less than average scores. Using this tool, it is observed that many mouse antibodies have positive *Z*-scores, indicating that they are more typically "human" than some human antibodies.

5.8
Antibody Structure Prediction

Since the 1980s there has been much interest in modeling the three-dimensional structure of antibodies. In part this is because antibodies provide a unique opportunity for protein modelers. The framework is so conserved that the problem is essentially reduced to one of modeling the six CDRs and Chothia's analysis has determined rules that make it straightforward to obtain generally accurate models for all but CDR-H3 in at least 75% of cases. However, modeling can also be valuable in understanding the binding of antibody and antigen with a view to modifying affinity or specificity, or in understanding cross-reactivity and autoimmune disease.

The problem of modeling the three-dimensional structure of an antibody can be broken down into two major steps: building the framework and building the CDRs.

5.8.1 Build the framework

Antibody crystal structures with the most similar light and heavy chains are identified from the Protein Databank (PDB, see RCSB website, http://www.rcsb.org/pdb/). Light and heavy chains may be identified separately and then must be combined. The combination of chains will inherit the packing between V_L and V_H from one of the parent structures. Thus, if one selects light chain, L*a* (paired with H*a*) and heavy chain, H*b* (paired with L*b*) then one can choose the V_L/V_H packing based on either antibody *a* or antibody *b*. To inherit the packing from antibody *b*, the structure of L*a* is fitted to L*b* and chains L*a* and H*b* are retained, discarding H*a* and L*b*. To inherit the packing from antibody *a*, H*b* is fitted to H*a*, and H*b* and L*a* are retained discarding H*a* and L*b*. Currently the choice of packing is arbitrary and it may be worth constructing two models. The sidechains of the framework are then replaced using automated processes available in molecular graphics programs, or software such as SCWRL (Canutescu et al. 2003).

5.8.2 Build the CDRs

Typically CDRs are built using the canonical classes described by Chothia. A server which will assign canonical classes automatically given the sequence of an antibody is available on Andrew Martin's Chothia web page (http://www.bioinf.org.uk/abs/chothia.html). The website also has a summary of the key residues described in the various papers by Chothia et al. and those resulting from an automated classification by Martin and Thornton (1996). Once canonical classes have been identified, examples from the Protein Databank are selected (typically on the basis of maximum sequence identity throughout the CDR) and fitted onto the framework.

In general, canonical classes can be identified for 4 or 5 of the 6 CDRs. CDR-H3 is too variable to be classified into canonical classes at the same level of detail although some work has been done classifying its conformations into groups (Martin and Thornton 1996; Shirai et al. 1996; Morea et al. 1998; Oliva et al. 1998).

CDRs which cannot be built using canonicals must be built using another modeling method. This can be conformational search using software such as CONGEN (Bruccoleri and Karplus 1987), by searching the PDB for loops of the same length and with similar distance between the attachment points to the framework, or by combined methods such as CAMAL (Martin et al. 1989).

5.8.3 Automated Modeling Tools

A number of automated tools are available for general protein modeling. These include MODELLER (http://www.salilab.org/modeller/) and SwissModel (http://

swissmodel.expasy.org/). They may both be used to generate a model in a quick and simple manner, but they do not take advantage of the special properties of antibodies and therefore are unlikely to produce a model of the quality that can be generated if a specialist program is used.

The commercial program AbM, designed specifically for antibody modeling, is, unfortunately, no longer available. However, a modified version of the software known as WAM (Whitelegg and Rees 2000, 2004) is accessible over the web (http://antibody.bath.ac.uk/). This server automates the generation of antibody models using the methods described above. Canonicals are used to build the hypervariable loops where possible and the remaining loops are built using the CAMAL method of Martin et al. (1989).

Another automated approach, ABGEN, has been proposed by Mandal et al. (1996), but is no longer available as a server.

5.9 Summary

This chapter has briefly reviewed antibody structure and discussed the different numbering schemes that have been used for antibody sequences. While the latest schemes may have their advantages, the Kabat and Chothia schemes are unlikely to be replaced by immunologists. The chapter then went on to look at the different definitions of regions around the CDRs which have been used by different groups for different purposes. Antibody sequence and structure databases were presented together with the tools and web resources that can be used to access them. The plethora of schemes for classifying antibody sequences were discussed highlighting some recent information on the equivalence between human and mouse groupings. Finally, tools for dealing with a new antibody sequence, both at the level of sequence analysis and structure prediction were presented.

It is the nature of the web that resources come and go or move location. Links to all the services mentioned here will be maintained on Andrew Martin's web pages.

References

Al-Lazikani, B., Lesk, A.M., Chothia, C. (1997) Standard conformations for the canonical structures of immunoglobulins. *J Mol Biol* 273: 927–948.

Allcorn, L.C., Martin, A.C.R. (2002) SACS – self-maintaining database of antibody crystal structure information. *Bioinformatics* 18: 175–181.

Almagro, J.C., Hernández, I., Ramírez, M. C., Vargas-Madrazo, E. (1998) Structural differences between the repertoires of mouse and human germline genes and their evolutionary implications. *Immunogenetics* 47: 355–363.

Alzari, P.M., Lascombe, M.-B., Poljak, R.J. (1988) Three-dimensional structure of antibodies. *Annu Rev Immunol* 6: 555–580.

Berman, J., Mellis, S., Pollock, R., Smith, C., Suh, H., Heinke, B., Kowal, C., Surti, U., Chess, L., Cantor, C., F.W., A. (1988)

Content and organization of the human Ig V_H locus: definition of three new V_H families and linkage to the Ig C_H locus. *EMBO J*, 7: 727–738.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., Bourne, P. E. (2000) The Protein Data Bank. *Nucleic Acids Res* 28: 235–242.

Brodeur, P., Riblet, R. (1984) The immunoglobulin heavy chain variable region (IgH-V) locus in the mouse. I. One hundred IgH-V genes comprise seven families of homologous genes. *Eur J Immunol* 14: 922–930.

Bruccoleri, R.E., Karplus, M. (1987) Prediction of the folding of short polypeptide segments by uniform conformational sampling. *Biopolymers*, 26: 137–168.

Canutescu, A.A., Shelenkov, A.A., Dunbrack, R.L. (2003) A graph-theory algorithm for rapid protein side-chain prediction. *Protein Sci* 12: 2001–2014.

Chothia, C., Lesk, A.M. (1987) Canonical structures for the hypervariable regions of immunoglobulins. *J Mol Biol* 196: 901–917.

Chothia, C., Lesk, A.M., Tramontano, A., Levitt, M., Smith-Gill, S.J., Air, G., Sheriff, S., Padlan, E.A., Davies, D., Tulip, W.R., Colman, P.M., Spinelli, S., Alzari, P.M., Poljak, R.J. (1989) Conformations of immunoglobulin hypervariable regions. *Nature (Lond)* 342: 877–883.

Cook, G., Tomlinson, I. (1995) The human immunoglobulin V_H repertoire. *Immun Today* 16: 237–242.

Davies, D.R., Padlan, E.A., Sheriff, S. (1990) Antibody-antigen complexes. *Annu Rev Biochem* 59: 439–473.

de Bono, B., Madera, M., Chothia, C. (2004) V_H gene segments in the mouse and human genome. *J Mol Biol* 342: 131–143.

Déret, S., Maissiat, C., Aucouturier, P., Chomilier, J. (1995) SUBIM: A program for analysing the Kabat database and determining the variability subgroup of a new immunoglobulin sequence. *Comput Appl Biosci* 11: 435–439.

D'Hoostelaere, L., Klinman, D. (1990) Characterization of new mouse v groups. *J Immunol* 145: 2706–2712.

Dildrop, R. (1984) A new classification of mouse V_H sequences. *Immunol Today* 5: 85–86.

Dildrop, R., Krawinkel, U., Winter, E., Rajewsky, K. (1985) V_H-gene expression in murine lipopolysaccharide blasts distributes over the nine known V_H-gene groups and may be random. *Eur J Immunol* 15: 1154–1156.

Frippiat, J.-P., Williams, S., Tomlinson, I., Cook, G., Cherif, D., Le Paslier, D., Collins, J., Dunham, I., Winter, G., Lefranc, M.-P. (1995) Organization of the human immunoglobulin lambda light-chain locus on chromosome 22q11.2. *Hum Mol Genet* 4: 983–991.

Giudicelli, V., Lefranc, M.-P. (1999) Ontology for immunogenetics: the IMGT-ONTOLOGY. *Bioinformatics* 15: 1047–1054.

Honegger, A., Plückthun, A. (2001) Yet another numbering scheme for immunoglobulin variable domains: an automatic modeling and analysis tool. *J Mol Biol* 309: 657–670.

Honjo, T., Matsuda, F. (1995) *Immunoglobulin Genes*, 2nd edn. London: Academic Press, pp. 145–171.

Kaas, Q., Ruiz, M., Lefranc, M.-P. (2004) IMGT/3Dstructure-DB and IMGT/StructuralQuery, a database and a tool for immunoglobulin, T cell receptor and MHC structural data. *Nucleic Acids Res* 32: D208–D210.

Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S., Foeller, C. (1991). *Sequences of Proteins of Immunological Interest*, 5th edn. Bethesda, MD: US Department of Health and Human Services, National Institutes for Health.

Kodaira, M., Kinashi, T., Umemura, I., Matsuda, F., Noma, T., Ono, Y., Honjo, T. (1986) Organization and evolution of variable region genes of the human immunoglobulin heavy chain. *J Mol Biol* 190: 529–541.

Kofler, R., Helmberg, A. (1991) A new Igk-V family in the mouse. *Immunogenetics* 34: 139–140.

Kroemer, G., Helmberg, A., Bernot, A., Auffray, C., Kofler, R. (1991) Evolutionary relationship between human and mouse immunoglobulin kappa light chain

variable region genes. *Immunogenetics* 33: 42–49.

Lee, K., Matsuda, F., Kinashi, T., Kodaira, M., Honjo, T. (1987) A novel family of variable region genes of the human immunoglobulin heavy chain. *J Mol Biol* 195: 761–768.

Lefranc, M.P. (1997) Unique database numbering system for immunogenetic analysis. *Immunol Today* 18: 509–509.

Lefranc, M.-P. (2001) Imgt, the international immunogenetics database. *Nucleic Acids Res* 29: 207–209.

MacCallum, R.M., Martin, A.C., Thornton, J.M. (1996) Antibody-antigen interactions: Contact analysis and binding site topography. *J Mol Biol* 262: 732–745.

Mainville, C., Sheehan, K., Klaman, L., Giorgetti, C., Press, J., Brodeur, P. (1996) Deletional mapping of fifteen mouse V_H gene families reveals a common organization for three Igh haplotypes. *J Immunol* 156: 1038–1046.

Mandal, C., Kingery, B.D., Anchin, J.M., Subramaniam, S., Linthicum, D.S. (1996) ABGEN: a knowledge-based automated approach for antibody structure modeling. *Nat Biotechnol* 14: 323–328.

Mariuzza, R.A., Phillips, S.E.V., Poljak, R.J. (1987) The structural basis of antigen-antibody recognition. *Annu Rev Biophys Bioeng* 139–159.

Martin, A.C.R. (1996) Accessing the Kabat antibody sequence database by computer. *Proteins Struct Funct Genet* 25: 130–133.

Martin, A.C.R., Thornton, J.M. (1996) Structural families in homologous proteins: automatic classification, modelling and application to antibodies. *J Mol Biol* 263: 800–815.

Martin, A.C.R., Cheetham, J.C., Rees, A.R. (1989) Modelling antibody hypervariable loops: A combined algorithm. *Proc Natl Acad Sci USA* 86: 9268–9272.

Morea, V., Tramontano, A., Rustici, M., Chothia, C., Lesk, A.M. (1998) Conformations of the third hypervariable region in the V_H domain of immunoglobulins. *J Mol Biol* 275: 269–294.

Oliva, B., Bates, P.A., Querol, E., Avilés, F. X., Sternberg, M.J. (1998) Automated classification of antibody complementarity determining region 3 of the heavy chain (H3) loops into canonical forms and its application to protein structure prediction. *J Mol Biol* 279: 1193–1210.

Orengo, C.A., Michie, A.D., Jones, S., Jones, D.T., Swindells, M.B., Thornton, J.M. (1997) CATH – a hierarchic classification of protein domain structures. *Structure* 5: 1093–1108.

Padlan, Eduardo, A. (1994) Anatomy of the antibody molecule. *Mol Immunol* 31: 169–217.

Padlan, E.A. (1977) The structural basis for the specificity of antibody-antigen reactions and structural mechanisms for the diversification of antigen-binding specificities. *Quant Rev Biophys* 10: 35–65.

Pech, M., Jaenichen, H., Pohlenz, H., Neumaier, P., Klobeck, H., Zachau, H. (1984) Organization and evolution of a gene cluster for human immunoglobulin variable regions of the kappa type. *J Mol Biol* 176: 189–204.

Poljak, R.J., Amzel, L.M., Avey, H.P., Chen, B.L., Phizackerley, R.P., Saul, F. (1973) The three-dimensional structure of the Fab fragment of a human immunoglobulin at 2.8Å resolution. *Proc Natl Acad Sci USA* 70: 3305–3310.

Porter, R.R. (1959) The hydrolysis of rabbit-globulin and antibodies with crystalline papain. *Biochem J* 73: 119–127.

Ramirez-Benitez, M.d.C., Moreno-Hagelsieb, G., Almagro, J.C. (2001) VIR.II: a new interface with the antibody sequences in the Kabat database. Biosystems, 61: 125–131.

Rechavi, G., Bienz, B., Ram, D., Ben-Neriah, Y., Cohen, J., Zakut, R., Givol, D. (1982) Organization and evolution of immunoglobulin V_H gene subgroups. *Proc Natl Acad Sci USA* 79: 4405–4409.

Rechavi, G., Ram, D., Glazer, L., Zakut, R., Givol, D. (1983) Evolutionary aspects of immunoglobulin heavy chain variable region (V_H) gene subgroups. *Proc Natl Acad Sci USA* 90: 855–859.

Schroeder, H.J., Hillson, J., Perlmutter, R. (1990) Structure and evolution of mammalian V_H families. *Int Immunol* 2: 41–50.

Searle, S.J., Pedersen, J.T., Henry, A.H., Webster, D.M., Rees, A.R. (1994) Antibody structure and function. In: Borreback, C.

A.K. (ed.) *Antibody Engineering*. Oxford: Oxford University Press, pp. 3–51.
Shen, A., Humphries, C., Tucker, P., Blattner, F. (1987) Human heavy-chain variable region gene family nonrandomly rearranged in familial chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 84: 8563–8567.
Shirai, H., Kidera, A., Nakamura, H. (1996) Structural classification of CDR-H3 in antibodies. *FEBS Lett* 399: 1–8.
Sitnikova, T., Nei, M. (1998) Evolution of immunoglobulin kappa chain variable region genes in vertebrates. *Mol Biol Evol* 15: 50–60.
Sitnikova, T., Su, C. (1998) Coevolution of immunoglobulin heavy- and light-chain variable-region gene families. *Mol Biol Evol* 15: 617–625.
Strohal, R., Helmberg, A., Kroemer, G., Kofler, R. (1989) Mouse V_κ gene classification by nucleic acid sequence similarity. *Immunogenetics* 30: 475–493.
Tomlinson, I. M., Walter, G., Marks, J. D., Llewelyn, M. B., Winter, G. (1992) The repertoire of human germline V_H sequences reveals about fifty groups of V_H segments with different hypervariable loops. *J Mol Biol* 227: 776–798.
Valdar, W.S., Thornton, J.M. (2001) Protein-protein interfaces: Analysis of amino acid conservation in homodimers. *Proteins Struct Funct Genet* 42: 108–124.
Valdar, W.S.J. (2002) Scoring residue conservation. *Proteins Struct Funct Genet* 48: 227–241.
Valentine, R.C., Green, N.M. (1967) Electron microscopy of an antibody-hapten complex. *J Mol Biol* 27: 615–617.
Whitelegg, N.R., Rees, A.R. (2000) WAM: an improved algorithm for modelling antibodies on the WEB. *Protein Eng* 13: 819–824.
Whitelegg, N., Rees, A.R. (2004) Antibody variable regions: Toward a unified modeling method. *Methods Mol Biol* 248: 51–91.
Williams, S., Frippiat, J.-P., Tomlinson, I., Ignatovich, O., Lefranc, M.-P., Winter, G. (1996) Sequence and evolution of the human germline V lambda repertoire. *J Mol Biol* 264: 220–232.
Wilson, I.A., Stanfield, R.L. (1993) Antibodyantigen interactions. *Curr Opin Struct Biol* 3: 113–118.
Wu, T.T., Kabat, E.A. (1970) An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J Exp Med* 132: 211–250.

Websites

ABG Database: http://www.ibt.unam.mx/vir/V_mice.html
Andrew Martin: http://www.bioinf.org.uk/abs/
Andrew Martin Chothia: http://www.bioinf.org.uk/abs/chothia.html
Andrew Martin Kabatman: http://www.bioinf.org.uk/abs/kabatman.html and http://www.bioinf.org.uk/abs/simkab.html
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Andrew Martin Subgroup: http://www.bioinf.org.uk/abs/hsubgroup.html
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EBI IMGTdata: ftp://ftp.ebi.ac.uk/pub/databases/imgt/ligm/
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EBI Kabat data: ftp://ftp.ebi.ac.uk/pub/databases/kabat/
IMGT3D: http://imgt3d.igh.cnrs.fr/
IMGT Database: http://imgt.cines.fr/
IMGT Database Warranty: http://imgt.cines.fr/textes/Warranty.html
Kabat Database: http://www.kabatdatabase.com/

MODELLER: http://www.salilab.org/modeller/
NCBI Kabat data: ftp://ftp.ncbi.nlm.nih.gov/repository/kabat/
Paris Institute of Mineralogy FTP site: ftp://ftp.lmcp.jussieu.fr/pub/sincris/software/protein/subim/
RCSB: http://www.rcsb.org/pdb/
SACS Database: http://www.bioinf.org.uk/abs/sacs/
SwissModel: http://swissmodel.expasy.org/
VBase Database: http://vbase.mrc-cpe.cam.ac.uk/
VIR Database: http://www.ibt.unam.mx/vir/VIR/vir_index.html
VIR Structures: http://www.ibt.unam.mx/vir/structure/structures.html
WAM: http://antibody.bath.ac.uk/

Note added in proof

The search and analysis tools are no longer available on the Kabat database website at http://www.kabatdatabase.com/ but the data and tools may be obtained for a fee.

A new compilation of germline sequences in human and mouse (soon to be extended to other species) has become available at http://www.vbase2.org/

6
Molecular Engineering I: Humanization

José W. Saldanha

6.1
Introduction

Humanization, also referred to as reshaping, complementarity determining region (CDR) grafting, veneering, resurfacing, specificity determining residue (SDR) transfer or DeImmunization, comprises strategies for reducing the immunogenicity of monoclonal antibodies (mAbs) from animal sources and for improving their activation of the human immune system. There are now many humanized mAbs in late-phase clinical trials and several have been given approval to be used as biopharmaceuticals (www.fda.gov). The source of the donor antibodies is usually mouse or rat, but rabbit (Steinberger et al. 2000) and chicken (Tsurushita et al. 2004) have also been used, the former because their CDR-H3 length is closer to human than mouse, the latter because they are useful for raising antibodies against conserved mammalian antigens. Although the mechanics of producing engineered mAbs using the techniques of molecular biology are relatively straightforward, the design of the humanized antibody sequence is critical for reproducing the affinity, specificity, and function of the original molecule whilst minimizing human anti-mouse antibody (HAMA) responses elicited in patients. In some cases, humanization has even led to an increase in the affinity of the antibody (Kolbinger et al. 1993; Brams et al. 2001; Luo et al. 2003).

There are many strategies leading to the design of the humanized variable regions (Fvs) and thus various choices are open to the antibody designer. These strategies and choices are the subject matter of this chapter. However, it is worth noting that some animal mAbs have proved difficult to humanize using current protocols (Pichla et al. 1997) and there is a need to experimentally verify the various approaches. The design and engineering of humanized mAbs are still interesting areas of research, as much for the light they shed on protein structure and function as for the potential therapeutic and diagnostic benefits.

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6.2 History of Humanization

Over a century ago, Paul Ehrlich proposed that antibodies could be used as magic bullets to target and destroy human diseases. This vision is still being pursued today since antibodies combine the properties of specificity and affinity with the ability to recruit effector functions of the immune system such as complement-dependent cytolysis (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). Alternatively, a toxic payload (such as a radioactive isotope, protein, or small molecule toxin) attached to the antibody can be accurately delivered to the target. Historically, antibodies have been produced from the serum of animals containing a cocktail of polyclonals, but the advent of hybridoma technology (Kohler and Milstein 1975) allowed monoclonals to become useful research and diagnostic tools, even though their use as therapeutics has been hindered by the elicitation of the HAMA response. Despite this problem, several animal mAbs have been approved by the US Food and Drug Administration (FDA). The obvious solution to this problem would be to raise human mAbs to the therapeutic targets, but this is difficult both practically and ethically using hybridoma technology. Nevertheless, production of fully human mAbs from transgenic mice and phage display has been possible since the early 1990s and Humira (adalimumab), isolated by phage display, was approved for rheumatoid arthritis in 2002. However, it does incur immunogenicity in 12% of the treated population when used alone (Hwang and Foote 2005).

Scientists are now using the techniques of molecular biology to design, engineer, and express mAbs from hybridoma technology to produce humanized mAbs. These approaches are suitable because of the domain structure of antibody molecules that allows functional domains carrying antigen binding or effector functions to be exchanged (Fig. 6.1). The first step was to produce a chimeric antibody (Morrison et al. 1984; Boulianne et al. 1984) where the xenogeneic variable light (V_L) or variable heavy (V_H) and human constant (Fc) domains were constructed by linking together the genes encoding them and expressing the engineered, recombinant antibodies in myeloma cells. In particular, the Fc was chosen to provide an isotype relevant to the desired biological function. However, when these antibodies were used therapeutically in humans, some still generated human antichimeric antibody (HACA) responses directed against the V regions. Since the level of HACA varies depending on the chimeric antibody, several have still been approved by the FDA.

6.3 CDR Grafting

The next step was to replace only the antigen-binding site from the human antibody by that of the source antibody. The first reported CDR graft was performed using the heavy-chain CDRs of a donor antihapten antibody B1-8 from a murine

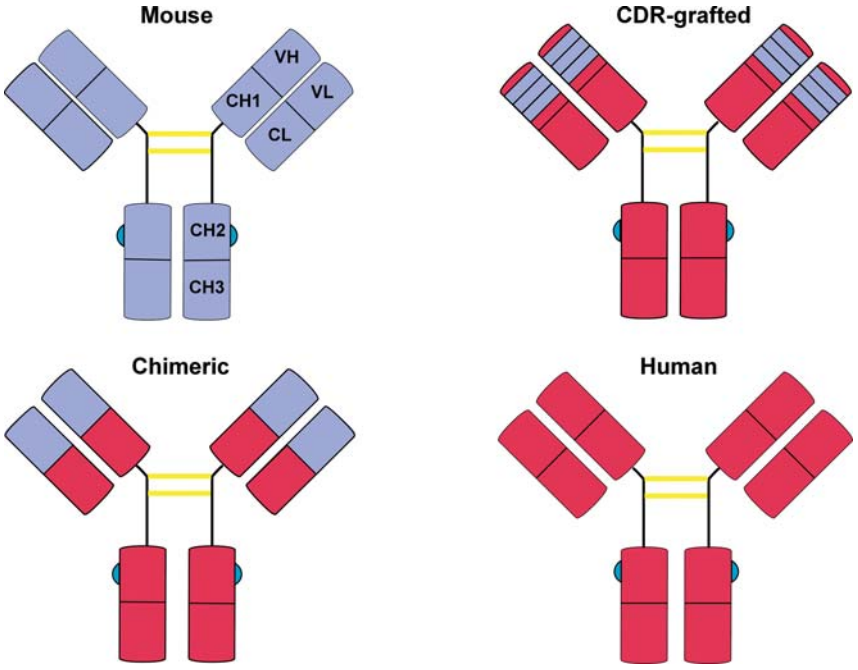

Fig. 6.1 Antibody engineering for chimeric and CDR-grafted mAbs. Blue – mouse protein domain or CDRs; red – human protein domain; green – carbohydrate; yellow – disulfide bridge.

source grafted into human acceptor V_H NEWM frameworks (Jones et al. 1986) to determine "whether the frameworks represent a simple beta-sheet scaffold on which new binding sites may be built and whether the structure of the CDRs (and antigen binding) is therefore independent of the framework context." Although the affinity of the (hemi-) CDR-grafted antibody was 2/3-fold lower than the mouse, proof of principle was established. However, the assumption was made that mutations in the frameworks do not affect the conformation of the CDR loops. This first experiment was followed a couple of years later by a similar CDR graft using the heavy-chain CDRs of murine antilysozyme antibody D1.3 (Verhoeyen et al. 1988). The results were considered remarkable, despite the binding being 10-fold less than the source antibody, given that CDR loops are not standalone structures and framework mutations actually do affect their conformation.

In the same year, the complete CDR graft of the first antibody of therapeutic interest was reported (Riechmann et al. 1988). All six CDRs from both the V_L and V_H of rat antibody Campath-1R were grafted into the V_L frameworks of human antibody REI and the V_H frameworks of human antibody NEWM. These frameworks were chosen since structural data was available for these human antibodies. The light chain of NEW was not used because there is a deletion at

the beginning of the third framework region. In addition, framework reversions (backmutations from human to rat) were made, for the first time, in the V_H domain to enhance the affinity. The reshaped antibody showed 3-fold lower affinity, but comparable activity to the source antibody in functional assays. This CDR-grafted antibody is now on the market as Campath (alemtuzumab) for the treatment of B-cell chronic lymphocytic leukemia (B-CLL).

The following year, the first completely CDR-grafted mouse antibody was reported (Queen et al. 1989). All six CDRs were grafted into human EU frameworks (V_H and V_L). In this case, the human V_H framework was chosen based on similarity to the mouse, and the complementary V_L from the same antibody was also used. Several backmutations were introduced, based on analysis of a computer model of the mouse Fv. The engineered antibody had 3-fold lower affinity but is now marketed as Zenapax (daclizumab) for the prophylaxis of acute organ rejection in patients receiving renal transplants. Later work in cynomolgus monkeys (Schneider et al. 1993) showed the immune response to the CDR-grafted anti-Tac antibody was mainly directed against idiotopes rather than to the modified human framework regions. In general, chimerization reduces the immunogenicity of therapeutic mAbs, and CDR grafting reduces it further (Hwang and Foote 2005).

These early experiments illustrated that choices were necessary in the design of CDR-grafted antibodies, for instance in the selection of human frameworks.

6.4 The Design Cycle

The design of CDR-grafted antibodies often involves an iterated approach where sequence designs are generated and tested in binding and/or functional assays. An outline of the general approach to this design cycle is presented below:

1. Analyze the source donor amino acid sequences.
2. Construct a 3D computer model of the Fv.
3. Find suitable human acceptor framework sequences.
4. Identify putative backmutations (reversions) in the chosen frameworks.
5. Reconsider the framework choice and design the humanized antibody sequence.
6. Construct humanized and (if possible) chimeric antibody sequences.
7. Test constructs (humanized light chain can be tested independently by combining with heavy chimeric chain (hemi-chimeric) and vice versa).
8. Success? If "No" then continue, If "Yes" consider how to reduce backmutations by returning to "Identify putative backmutations . . .".
9. Return to "Find suitable human acceptor . . .".

6.4.1 Analysis of the Source (Donor) Sequence

6.4.1.1 Complementarity Determining Regions (CDRs)

These are six highly variable regions in the Fv, three in V_L, three in V_H. It is worth noting that the preponderance of backmutations at position 73 in V_H suggests that the loop encompassing this residue may be a fourth CDR. The CDRs contain the residues most likely to bind antigen and are therefore usually retained in the humanized antibody. They can be defined by sequence according to Kabat (Wu and Kabat 1970; Kabat et al. 1987, 1991) or by structure according to Chothia (Chothia and Lesk 1987; Chothia et al. 1989) (see Chapter 5). These definitions may be mixed in a humanization experiment. The advantage of using the Chothia definitions is that the CDRs are shorter and therefore the humanized antibody should have less xenogeneic fragments in it. Since Kabat et al. (1987, 1991) place CDR-H1 from positions 31–35, whereas crystal structures show the loop to be from positions 26–32 (Chothia et al. 1986), and murine residues 28–30 have been reported to exacerbate the immunogenic response in humans (Tempest et al. 1995), this advantage may be true.

However, the experience of Rodrigues et al. (1992) has shown that the shorter Chothia definition of CDR-H2 required several backmutations, mainly in the region covered by the Kabat definition. Conversely, the Kabat definition of CDR-H1 often requires backmutations in the region covered by the Chothia definition. Therefore, some strategies have combined the Kabat and Chothia definitions of CDR-H1, increasing its length (Presta et al. 1997; Vajdos et al. 2002). Others have kept some CDR residues human (Presta et al. 1993; Hsiao et al. 1994), tried to match CDR lengths between the source and human frameworks (Sims et al. 1993) and even found the mouse and human CDRs to be identical in sequence (as was the case with CDR-L2, Park et al. 1996). Pulito et al. (1996) tried to reduce the number of murine residues in the humanized antibody OKT4A, but found that CDR residues that do not contact antigen directly are also essential for antigen binding. In fact, Vajdos et al. (2002) subsequently discovered, through alanine scanning, that residues in the CDRs that contribute to antigen binding fall into two groups: solvent exposed residues that make direct contact with the antigen, and buried sidechains that can pack against other CDR residues and act as a scaffold. The ideal situation would be to have the crystal structure of the source antibody in complex with its antigen so that only the antigen-binding CDRs would be grafted. The structure of antibody 26-2F FAB was solved with its antigen (Chavali et al. 2003) and the authors predict the humanization would require the graft of only four of the six CDRs, since they are solely in contact with the antigen.

6.4.1.2 Canonical Residues

Originally defined by Chothia and Lesk (1987), the canonical residues have now been revised by Martin and Thornton (1996). The web page www.bioinf.org.uk/abs/chothia.html allows input of variable region sequences and automatic

identification of the canonical structure class and important residues. Canonical residues are key residues in the CDR and/or framework that determine the conformation of the CDR loop. They can be hydrophobic residues that pack in the body of the loop, polar residues which form important stabilizing hydrogen bonds, or residues which can assume abnormal phi/psi conformations. Canonical residues should be retained in the humanized antibody if they are different to those in the human acceptor frameworks. However, it should also be noted that sometimes, backmutation of canonical residues has no effect or even decreases binding (see Section 6.4.4). The cause of these anomalies might be (1) the particular amino acid at that canonical position in the donor sequence is unimportant or (2) the acceptor residue at that position is better – a case of *in vitro* affinity maturation.

6.4.1.3 **Interface Packing Residues**

Originally investigated by Novotny and colleagues (Novotny et al. 1983; Novotny and Haber 1985) and defined by Chothia et al. (1985) (Table 6.1), interface packing residues occur at the interface between the V_L and V_H domains. These residues govern the packing of the variable domains, thus affecting the binding site. The main reason for the selection of human frameworks for V_L and V_H from the same antibody clone is to maintain the integrity of the interface between the variable domains (Section 6.4.3.2). Generally, unusual packing residues should be retained in the humanized antibody if they differ from those in the human frameworks. Their influence on affinity is illustrated in the humanization of 1B4 (Singer et al. 1993). Interestingly, their importance might also be functional. In antibody KM966, Nakamura et al. (2000) retained murine residues at V_L/V_H packing positions 38 and 40 in V_H. These residues had little effect on binding, but did improve the CDC of the humanized antibody.

6.4.1.4 **Rare Framework Residues**

Rare or atypical residues can be located by determining the Kabat subgroup (Kabat et al. 1987, 1991) and identifying the residue positions which differ from the consensus sequence (Gussow and Seemann 1991). These donor-specific differences may point to somatic mutations that enhance activity, atypical residues near to the binding site possibly contacting antigen (e.g. antibody BMA 031, Shearman et al. 1991). Humanizing these rare residues can cause the loss of binding affinity (Chan et al. 2001). However, if they are not important for binding, then it is desirable to get rid of them because they may create immunogenic neoepitopes in the humanized antibody. Note that unusual residues in the donor sequence are sometimes actually common residues in the acceptor (Queen et al. 1989). Atypical residues in the acceptor frameworks are not desirable because of the possibility of immunogenicity, unless of course they correspond to unusual residues in the donor and thus may be important functionally. Rarely occurring amino acids in the human frameworks have been mutated to human consensus residues (Co et al. 1991; Baker et al. 1994).

Table 6.1 Residues at the V_L/V_H interface[a].

	Kabat number[b]	*Mouse*[c]	*Human*[c]
V_K	34	H678 N420 A408 Y147 E114	A531 N147 D66
	36	Y1653 F198 L96	Y748 F80
	38	Q1865 H47	Q799 H22
	44(+)	P1767 V132 I40	P839 L5
	46	L1381 R374 P97	L760 V37
	87	Y1457 F448	Y795 F41
	89	Q1170 L206 F144	Q687 M107
	91	W376 S374 G356 Y295 H182	Y404 R115 S105 A84
	96(+)	L537 Y380 W285	L134 Y215 F78 W73 I71
	98(+)	F1724	F654
V_H	35	H1001 N636 S402	E184 S527 H340 G167 A143
	37	V2336 I200	V1037 I477 L27
	39	Q2518 K67	Q1539 R16
	45(+)	L2636 P16	L1531 P24
	47	W2518 L64 Y50	W1534 Y21
	91	Y2149 F479	Y1429 F116
	93	A2202 T222 V102	A1346 T90 V71
	95	Y399 G375 S340 D340 R226	D268 G266 R109 E100
	100k(+)	F1285 M450	F540 M109 L33
	103(+)	W1469	W323

a The positions of interdomain residues were as defined by Chothia et al. (1985).
b Numbering is according to Kabat et al. (1991). Residues underlined are in the framework, other residues are in the CDRs. (+) residues are the six that form the core of the V_L/V_H interface according to Chothia et al. (1985).
c The number following the one-letter amino acid code is the frequency taken from the Kabat database (November 1997 dataset).

6.4.1.5 *N*- or *O*-Glycosylation Sites

Potential *N*-glycosylation sites are specific to the consensus pattern asparagine-X-serine/threonine. It must be noted that the presence of the consensus tripeptide is not sufficient to conclude that an asparagine residue is glycosylated, due to the fact that the folding of the protein plays an important role in the regulation of *N*-glycosylation. It has been shown that the presence of proline between asparagine and serine/threonine will inhibit *N*-glycosylation and about 50% of the sites that have a proline C-terminal to serine/threonine are not glycosylated. It should also be noted that there are a few reported cases of glycosylation sites with the pattern asparagine-X-cysteine. Potential *N*-glycosylation sites can be located with the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) (www.expasy.ch). It was expected that addition,

removal or modification of glycosylation sites in the humanized antibody might affect the binding or immunogenicity. However, removal of potential *N*-glycosylation sites in either the V_L or V_H domain has not destroyed the binding affinity of a humanized antibody thus far (Leger et al. 1997) and in the case of M195 (Co et al. 1993) and LL2 (Leung et al. 1995) it actually increased. In another example, an *N*-glycosylation site was found at canonical residue 30 in V_H, although its removal through backmutation did not influence binding (Sato et al. 1996). Glycosylation sites can also be used as conjugation sites for drug or radionuclides as has been the case in antibody constant domains (Qu et al. 1998).

O-Glycosylation sites are usually found in helical segments, meaning that they are uncommon in the beta-sheet structure of antibodies. They have no consensus pattern. Couto et al. (1994) ruled out the removal of an *N*-linked glycosylation site in their humanized antibody (BrE-3) as being responsible for increased binding, but were open to the possibility of differences in *O*-linked glycosylation.

6.4.2 Three-Dimensional Computer Modeling of the Antibody Structure

Early humanizations by CDR grafting utilized human frameworks for which the crystal structures were available (Riechmann et al. 1988). Analysis of these structures was useful in the design of the humanized antibody. Although some solely used sequence analysis (Poul et al. 1995), later approaches relied on a carefully built model of the Fv regions of the source antibody and in some cases also the humanized antibody (Nakatani et al. 1994; Hsiao et al. 1994). Superposition of the source and humanized antibody models and analysis of size, charge, hydrophobicity, or hydrogen bond potential between equivalent residues highlighted important residues in the frameworks for maintaining the conformation of the CDRs or contacting antigen. The identification of these residues was useful in suggesting putative backmutations (Section 6.4.4).

In some cases, where possible, a model of the antigen was also built (Nisihara et al. 2001). A model of the source antibody docked to the antigen would be ideal for the design of the humanized antibody, in the absence of a crystal structure for the complex, and has been achieved in some cases by computer-guided docking (Zhang et al. 2005). The first reported use of a model in the design of a humanized antibody was by Queen et al. (1989) where a molecular model of the anti-Tac Fv was constructed with the ENCAD (Levitt 1983) computer program and examined with the MIDAS (Ferrin et al. 1991) visualization software on an IRIS (Silicon Graphics Inc., Mountain View, CA, USA) graphics workstation. This model was used to identify several framework amino acids in the mouse antibody that might interact with the CDRs or directly with the antigen.

Using a computer model of the donor mouse Fv, Kettleborough et al. (1991) analyzed the influence of certain framework residues in antigen binding. This model was built using QUANTA (Accelrys, San Diego, CA, USA) and the CHARMm (Brooks et al. 1983) force field on a Silicon Graphics IRIS workstation.

Carter et al. (1992) constructed a model using seven Fab crystal structures from the Protein Data Bank (PDB) (Deshpande et al. 2005). Their modeling program was Discover & Insight (Accelrys, San Diego, CA, USA) with the AMBER force field (Weiner et al. 1984). They acknowledged the crucial role of molecular modeling illustrated by the designed antibody binding antigen 250-fold more tightly than the simple CDR loop swap.

The modeling of the source or humanized antibody usually begins with a search over the PDB (Deshpande et al. 2005) to find template structures on which to build the model. The search is performed with standard packages (BLASTP (Altschul et al. 1997) or FASTA (Pearson 2000)) and the selection of template structures for V_L and V_H takes into account such parameters as sequence identity, overall sequence conservation and the resolutions of the structures. If different structures are chosen for V_L and V_H, then invariant residues at the V_L/V_H interface (Novotny and Haber 1985) must be superposed to model the interaction between the protein domains. Sometimes, several structures are used as templates (Carter et al. 1992) and structurally conserved regions (SCRs) are determined. The model can be built by splicing together regions with the highest sequence identity between the SCRs and the sequence to be modeled or modeling on average α-carbon coordinates in the SCRs. Identical residues in the framework regions are retained while nonidentical residues are substituted with the modeling package.

Modeling of the CDR loop regions poses a greater challenge. Searches can be performed to find an antibody structural loop of the same length and similar stem or overall canonical structure as tabulated by Martin and Thornton (1996). The matching structural loop can then be grafted into the model using the chosen modeling package. For loop regions of unknown canonical structure, a search can be performed using the positions of residues flanking the loop as anchors, over all known structures to find a loop region of the same length and similar base structure. The best match can be grafted into the model. Alternatively, *ab initio* methods can be employed using a conformational search program such as CONGEN (Bruccoleri and Karplus 1987), or a combination of database search for the base stem structure of the loop and *ab initio* conformational search for the central portion of the loop (Martin et al. 1989).

Of particular importance is the CDR-H3 loop. Shirai et al. (1996, 1999) showed that in many cases these loops exhibit "kinked" or "extended" C-terminal regions predicted by sequence-based rules. These rules can be applied to determine additional features of CDR-H3, thus aiding the modeling of its conformation.

The entire model is finally energy minimized to relieve unfavorable atomic contacts and to optimize nonbonded interactions. Stereochemical verification of the model is generally performed using ProCheck (Laskowski et al. 1993), while VERIFY3D (Eisenberg et al. 1997) and PROSA-II (Sippl 1993) can be used to measure model quality in terms of packing and solvent exposure.

Nowadays, it is entirely possible to build a model completely automatically using programs such as Swiss PDB Viewer and academic servers such as Swiss-Model (Guex and Peitsch 1997). However, the danger of allowing a computer to make

all the decisions is highlighted in the humanization of antibody AT13/5 (Ellis et al. 1995) where the interaction between residues at positions 29 and 78 in the heavy chain was not modeled correctly. The experience of an expert in protein structure modeling is always welcomed. Additionally, it may be better to also model the constant regions of the antibody, since interactions at the variable/constant domain interface are likely to affect the affinity and/or activity of the molecule. Landolfi et al. (2001) found that altering framework position 11 in the V_H although only slightly affecting affinity, severely diminished the activity of humanized antibody AF2. This residue had been identified as being involved in a "ball-and-socket" joint between the V and C domains of the immunoglobulin Fab (Lesk and Chothia 1988).

Modeling can only be an interim measure on the way to determining the structure of the source antibody by X-ray crystallography or NMR. Increasingly, antibody structures are being determined, sometimes in complex with antigen, which can help the design process. Redwan et al. (2003) used the structure of a cocaine-binding antibody to humanize it using real structure-based design to incorporate human residues that would not affect the binding pocket or key cocaine-contacting residues. Yazaki et al. (2004) used the program VAST (Gibrat et al. 1996) to identify a human (or humanized Fv) acceptor for the CDR graft of antibody T84.66, whose crystal structure had already been determined. Interestingly, Herceptin (trastuzumab) (Eigenbrot et al. 1993), a successfully humanized antibody, was chosen for its high degree of overlap of α-carbon atoms and overall sequence identity.

6.4.3 Choice of Human Framework Sequences

This is the most critical area of the humanization design cycle, yet there are no hard-and-fast rules for choosing the human acceptor frameworks into which to graft the donor CDRs. This is because the benefits of the various choices in terms of recovery of affinity, specificity, and activity with the fewest backmutations, and also immunogenicity in the patient, have not been clearly proven in the clinic. Therefore, there are only sets of approaches that need to be combined with the collective experience of previous humanizations, although the antibodies gaining FDA approval are giving clues to which approach may be best (Table 6.2).

6.4.3.1 Fixed Frameworks or Best Fit?

Some groups prefer to use fixed frameworks (usually NEW for the heavy chain and REI for the light chain since their structures are solved, sometimes KOL for the heavy chain) for all their humanized antibodies. This was the case for the first therapeutically CDR-grafted antibody, Campath-1 (Riechmann et al. 1988) and Actemra (tocilizumab) (Sato et al. 1993) that has also reached the market. Other groups try to use the most similar frameworks to their donor sequence (homology matching, also called "best-fit" by Gorman et al. 1991) by searching over large sequence databases – nowadays the nonredundant (nr) database at the

Table 6.2 CDR-grafted biopharmaceuticals approved by the Food and Drug Administration (FDA).

Drug name	*Year FDA*	*Active ingredient*	*Therapeutic area*[a]	*Antigen*[b]	*Model*[c]	*Frameworks*[d]	*CDRs*[e]	*Back*[f]	*Affinity with source mAb*	*% patients with AAR*[g]	*Reference*
Zenapax	1997	daclizumab	immune	CD25	m	best-fit	kab	yes	3-fold less	8.4	Queen et al. 1989
Herceptin	1998	trastuzumab	oncology	HER2	m/h	consen	mix	yes	3-fold more	0.1	Carter et al. 1992
Synagis	1998	palivizumab	infectious disease	RSVF	m	germ V_L expr V_H	kab	yes	similar	0.7–1.8	Johnson et al. 1997
Mylotarg	2000	gemtuzumab, ozogamicin	oncology	CD33	?	fixed	mix	yes	?	2.9†	Hamann et al. 1999
Campath	2001	alemtuzumab	oncology	CD52	strs	fixed	kab	yes	3-fold less	2–63	Riechmann et al. 1988
Raptiva	2003	efalizumab	immune	CD11a	m/h	consen	chot	yes	similar	6.3	Werther et al. 1996
Xolair	2003	omalizumab	immune	IgE	m/h	consen	mix	yes	similar	<0.1	Presta et al. 1993
Avastin	2004	bevacizumab	oncology	VEGF	m/h	consen	mix	yes	2-fold less	?	Presta et al. 1997

a Immune, immune disorders.
b VEFG, vascular endothelial growth factor; HER2, human epidermal growth factor receptor 2; RSV F, respiratory syncytial virus F protein; IgE, immunoglobulin E.
c m, mouse; h, human; strs, structures available for human frameworks.
d Consen, human consensus; fixed, fixed framework approach; germ, human germline; expr, functionally expressed human antibody; best-fit, best-fit framework approach.
e Mix, mixture of Kabat sequence (kab) and Chothia structure (chot) definitions.
f Rodent backmutations incorporated into human frameworks in either V_L, V_H or both.
g AAR, anti-antibody response (data from Tabrizi 2005, except † Hwang and Foote 2005).

National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). This was the approach of Queen et al. (1989) for the V_H of the anti-Tac antibody, but the V_L frameworks were chosen to match the V_H (i.e. the same human antibody for both chains). Others used V_L/V_H frameworks from different human antibodies (Singer et al. 1993). The best-fit strategy requires other choices to be made. Should the sequence similarity extend over the whole V-region, only the framework regions, or a mix of matching CDR lengths and framework identity? A subtle comparison of the fixed frameworks and homology matched (best-fit) methods, in terms of the ease of producing a functional humanized antibody, can be found in the humanization of antibody M22 (Graziano et al. 1995). The preferential choice appears to be the latter, where the more homologous KOL frameworks gave better binding than NEWM.

Hamilton et al. (1997) questioned the best-fit approach, arguing that the advantage of choosing homologous frameworks might be outweighed by the advantage of using fixed frameworks for which a database of experience had been assembled. Certainly, if the sequences of the fixed frameworks have a low homology to the original source sequences, then there is increased likelihood of low binding (Sha and Xiang 1994) but knowledge of which backmutations to make should restore binding. The crystal structures of two humanized forms of antibody AF2, which differed in the sequence identity of the donor heavy chain to the human frameworks, provided evidence supporting the best-fit approach (Bourne et al. 2004). The humanized form with the greater identity between donor V_H and human frameworks was significantly more structurally similar to the mouse antibody.

Note that it is possible to mix and match frameworks. Qu et al. (1999) for antibody Immu31 mixed human EU frameworks 1, 2, and 3, with NEW framework 4 in an essentially fixed framework approach. In the best-fit approach, it is possible to choose frameworks 1–4 from different human antibodies according to sequence similarity. Additionally, Ono et al. (1999) ensured that all the framework regions came from naturally processed human antibodies. In their case framework 3 of the heavy chain came from a different human antibody to frameworks 1 and 2, and framework 4 was the human JH6 germline.

6.4.3.2 V_L/V_H Frameworks from the Same or Different Clone?

In general, light and heavy chains from the same antibody are more likely to associate to form a functional binding site than light and heavy chains from different antibodies. Frameworks from the same clone were used for antibodies FD79 and FD138-80 by Co et al. (1991) to reduce the possibility of incompatibility in assembly of the heavy and light chains. However, since the interface between the chains is so well conserved, this is not usually a problem. A comparison of the two approaches was made with antibody anti-B4 (Roguska et al. 1994, 1996). Once again, the preferential choice appears to be the latter, full restoration of antigen-binding affinity being achieved when the most identical, but clonally different, human V_L and V_H acceptor frameworks were selected.

6.4.3.3 Human Subgroup Consensus or Expressed Framework?

Being limited to expressed frameworks from particular human antibodies runs the risk of their somatic mutations creating immunogenic epitopes, even though the frameworks are human. An alternative approach is to use the frameworks from human consensus sequences where idiosyncratic somatic mutations will have been removed – first suggested by Shearman et al. (1991). The two approaches have been compared; in one case showing no difference in binding affinities (Kolbinger et al. 1993) and in the other case showing better binding with individual frameworks (Sato et al. 1994). The fixed framework approach versus using consensus human frameworks was tested by Maeda et al. (1991). The fixed framework humanized antibodies showed loss of binding whilst the consensus framework antibodies did not. However, since no model was used in this case, judicious choice of backmutations might have led to different results. In several instances, homologous human frameworks have been chosen, only to then change some residue positions to their consensus amino acid (e.g. Hakimi et al. 1993) (see also Section 6.4.1.4).

6.4.3.4 Germline Frameworks

Consensus sequences are artificial and although they have no idiosyncratic residues, may create unnatural sequence motifs that are immunogenic. An alternative is to use human germline sequences that have been compiled in various databases (IMGT (Lefranc et al. 2005), VBASE2 (Retter et al. 2005)). Originally suggested by Shearman et al. (1991), several groups have reported using germline frameworks (Rosok et al. 1996; Caldas et al. 2003; DiJoseph et al. 2005). In fact, in our laboratory consensus sequences were first considered because it was reasoned that they would most closely resemble the germline sequences when the latter were still unavailable. Also, somatic mutations from the germline may indicate residues that contact antigen. This was the case in antibody MOC-31 (Beiboer et al. 2000) where modeling predicted that one of the germline mutations might bind antigen. Germline frameworks can be chosen based on similarity to the donor sequences, including amino acid identity at important residue positions (Brams et al. 2001) or multiple germline frameworks can be used (Gonzales et al. 2004).

Tan et al. (2002) matched the canonical templates of the donor antibody with human genomic V gene sequences and CDR grafted into these frameworks. No backmutations were included, and the resultant "superhumanized" antibody showed a 30-fold reduction in affinity but maintained biological activity. A later experiment with anti-lysozyme murine antibody D1.3 showed only a 6-fold reduction in affinity (Hwang et al. 2005).

6.4.3.5 Database Search

Having decided on an approach to take in order to choose the human frameworks; which particular human antibody, consensus or germline sequence should be used? This is simple in the fixed framework approach since the choice is always

NEW for the heavy chain and REI for the light. The approach generally taken is to perform a search for the most similar human acceptor sequence over the appropriate database. Choice of the particular human frameworks for the light and heavy chain variable regions should be made by trying to match the length of the CDRs, the canonical residues and the interface packing residues (Section 6.4.1) as well as trying to find the highest percentage identity between the donor and acceptor sequences. Try to find human frameworks that are similar (in terms of percentage identity) to the source sequences and also require the least number of backmutations. The consensus approach utilizes V_L and V_H frameworks derived from the most common amino acid found at each position within a given human subgroup from the Kabat database and can be chosen based on similarity (Kettleborough et al. 1991) to the mouse V-region sequences using search programs as above. Another approach is to use the same human consensus frameworks for each design, regardless of sequence similarity. The most abundant human subgroups are V_H subgroup III, V_L kappa subgroup I. These have been used in several humanization experiments (Carter et al. 1992; Presta et al. 1993; Presta et al. 1997; Luo et al. 2003; Adams et al. 2005).

6.4.4
Identify Putative Backmutations

Although straight CDR grafts can recover both the affinity and specificity of the source antibody (Thomas et al. 1996), usually backmutations (reversions) to the donor residues are required in the human acceptor frameworks. This is the most difficult and unpredictable procedure in any humanization strategy, sometimes requiring many different versions of the humanized antibody to be made (Presta et al. 1993). It is also the area that throws much light on protein structure and function. A solid body of data for helping to identify strategic alterations is available on the "Humanization bY Design" website (www.cryst.bbk.ac.uk/~ubcg07s). Riechmann et al. (1988) were the first to employ backmutations reverting two residues in the heavy chain at positions 27 and 30, and found an increase in binding to antigen. It is from this work that we gain the term "reshaped" to indicate a CDR-grafted antibody with backmutations. Queen et al. (1989) incorporated seven backmutations in the heavy chain and two in the light chain that either influenced CDR conformation or interacted directly with antigen for their anti-Tac humanization. This work also muddied the waters of what a backmutation actually was, since they additionally changed some human framework residues to mouse, arguing that the human residues were unusual and the mouse residues were more typically human. Taken together this meant that the humanized anti-Tac antibody had 13 "backmutations" in the heavy chain and three in the light.

By introducing ordered steps of additional backmutations, Tempest et al. (1991) minimized the number of changes required to restore affinity and specificity. One simple approach to identifying backmutations is to keep all source residues within four positions of every CDR (Shearman et al. 1991). Alternatively, the

structural model can be used to analyze residues within 5Å (Graves et al. 1999) of any CDR residue. This will ensure the integrity of the Vernier zone (Foote and Winter 1992) (a platform on which the CDRs rest) and identify residues in the framework that may bind antigen.

However, the main method for identifying backmutations is to study the differences between donor and acceptor frameworks and analyze them on the structural model. Most of the differences are not important, lying on the surface and far from the CDRs. Those not on the surface and/or close to the CDRs are worthy of greater attention. Putative point reversions from the acceptor residue back to the original donor residue will already have been identified from the analysis of the sequences for canonical residues, interchain packing residues and rare residues. Experience has shown that it is especially important to retain the source's canonical and interchain packing residues, though not in all cases. In antibody HMFG1 (Krauss et al. 2004) position 71 in the heavy chain, although being a canonical residue, was found to stabilize the scFV while having only a minor effect on the binding. Sometimes, position 71 was found to have no effect (Hsiao et al. 1994; Tempest et al. 1995) while Sato et al. (1993) reported that the backmutation at position 71 actually caused worse binding.

Due to the extreme variability in sequence and length of CDR-H3, there are no canonical residues defined for this loop although certain positions are known to interact and maintain some conformational stability. For instance, a salt-bridge between the positively charged position 94 and the usually invariant aspartic acid at 101 in CDR-H3 is seen in many antibody structures (Tempest et al. 1991) and arginine 94 also forms interactions with positions 31 and 74 in the heavy chain (Chuntharapai et al. 2001). In addition, residue 101 is known to form a cooperative (possibly indirect) interaction with position 49 in the light chain of an anti-CD40 antibody (Wu et al. 1999). Therefore, special attention should be paid to this loop, analyzing the structural model for residues that may potentially affect its conformation.

Backmutations are not transferable between different antibodies, even if they have high sequence homology and similar antigen specificity (Rosok et al. 1996). Ohtomo et al. (1995) introduced a new method to identify important backmutations when the first version of their humanized V_L did not bind. They constructed "hybrid variable" regions, joining together mouse and CDR-grafted framework regions. In this way, they found that proline 46 in framework 2 was required to recreate the functional binding site, and this was not even among the five backmutations that they had originally considered.

Having decided on the residues to backmutate, the question of human acceptor frameworks should be reconsidered. It is not unlikely that an overlooked human framework may actually contain the backmutations that are to be retained. If this is the case, then there is no need to introduce residues from the source sequence, thus making the humanized antibody more "human." However, not all backmutations are necessary, and there is a need for experimental validation. In some cases, the acceptor residue can be better than the donor (Gonzales et al. 2003; Caldas et al. 2000) either decreasing the immunogenicity or increasing the

affinity of a humanized antibody. Backmutations can also have an effect on antibody expression, a 5-fold increase being found with a backmutation at position 75 in V_H of antibody ABL364 (Co et al. 1996). There are also surprises in store. Caldas et al. (2003) through a systematic analysis of related structures unexpectedly identified position 37 in the light chain as a putative site for backmutation. This led to a more effective humanized antibody in cell binding assays, although in this case the affinity was not measured.

6.5 Other Approaches to Antibody Humanization

6.5.1 Resurfacing/Veneering

The backmutations required in CDR-grafted antibodies may introduce new antigenic epitopes or lose the advantage over chimeric antibodies if a great many are required. A solution to this is to maintain the core and CDRs of the murine variable regions, but replace the surface residues with those from a human sequence by a strategy known as "resurfacing." This strategy originates from a systematic analysis of known antibody structures to determine the relative solvent accessibility distributions of residues in human and mouse variable regions (Pedersen et al. 1994). A description of the differences in the presentation of surface residues in a small number of mouse and human antibody variable regions had already been published in a process known as "veneering" (Padlan 1991). The analysis showed that the sequence alignment positions of surface residues were conserved 98% of the time between the two species. Also, the pattern of amino acid substitution was conserved within a species, but not between the species (i.e. no mouse framework displayed the exact pattern of surface residues found in any human framework). Thus, it was possible to convert a murine surface pattern to that of human with relatively few mutations. However, a choice was still required for selecting a characteristic human surface pattern.

Two methods were compared for two different murine antibodies (Roguska et al. 1994). For anti-B4 (an anti-CD19 mAb) a database of clonally derived human V_L/V_H sequence pairs was used. For N901 (an anti-CD56 mAb), sequences for V_L and V_H were independently selected from the Kabat database. Both resurfaced antibodies presented apparent affinities for their antigens identical to those of their source murine antibodies. Further versions of these antibodies (where the number of murine surface residues was reduced) were compared with CDR-grafted versions (Roguska et al. 1996) (Section 6.4.3.2). The goal of generating humanized antibodies that retained the affinity and specificity of the source murine antibody, but with as few murine residues as possible in the variable domain framework was achieved by selecting the most similar human V_L and V_H frameworks, without regard to clonal origin. This was the case for both CDR-grafted and resurfaced antibodies. However, the CDR-grafted anti-B4 antibodies

had more murine residues at surface positions than the resurfaced antibodies, and were more difficult to engineer requiring seventeen attempts. The conclusion was that resurfaced antibodies are easier to produce and are conceivably less immunogenic, although this ignores the possibility of T-cell epitopes presented from the murine core.

Resurfacing technology has been developed by Immunogen (Cambridge, MA, USA) and two antibodies humanized using this strategy are currently in clinical trials.

6.5.2 SDR Transfer

CDR-grafting of a xenogeneic antibody does not necessarily eliminate the immunogenicity of the molecule because of idiotypic responses directed against the xenogeneic CDRs, particularly when given in multiple doses. CDR-grafted mAbs have been shown to be immunogenic in both primate animal models (Schneider et al. 1993; Stephens et al. 1995) and in humans (Richards et al. 1999; Ritter et al. 2001). Antigen binding usually involves between 20% and 33% of the CDR residues (Padlan 1994) that have been labeled "specificity determining residues" (SDRs). Padlan et al. (1995) using the PDB determined the boundaries of the potential SDRs in different CDRs and called these segments "abbreviated CDRs." The SDRs are commonly located at positions of high variability and are possibly unique to each mAb. However, they can be identified by site-directed mutation or determination of the 3D structure of the Fv or, in the absence of this information, the variability of positions within the abbreviated CDRs can be used to suggest which residues are SDRs.

Transfer of SDRs has only been used successfully in the humanization of anti-carcinoma mAb CC49 which specifically recognizes tumor-associated glycoprotein (TAG)-72 (Tamura et al. 2000). SDR transfer has also been utilized in the humanization of murine mCOL-1 that specifically recognizes carcinoembryonic antigen (CEA). In this case, the SDR-transfer antibody had comparable binding activity to the CDR-grafted equivalent and significantly higher activity compared with the abbreviated CDR-grafted antibody. It also showed decreased reactivity for anti-V-region antibodies present in the sera of patients treated with mCOL-1 (Gonzales et al. 2004).

6.5.3 DeImmunization Technology

This technology applied by Biovation (an associate of Merck KGaA, Germany) combines veneering (based on Padlan's approach) to effectively humanize surface residues (thus removing B-cell epitopes) with the identification and removal of potential helper T-cell epitopes from antibody biopharmaceuticals. Helper T-cell epitopes are short peptide sequences within proteins that bind to MHC class II molecules. These epitopes can be created by somatic mutations occurring

naturally in human antibodies or by the veneering process. The peptide – MHC class II complexes are recognized by T cells and trigger the activation and differentiation of helper T cells, thus stimulating a cellular immune response. Helper T cells initiate and maintain immunogenicity by interacting with B cells, resulting in the production of antibodies that bind specifically to the administered antibody. In DeImmunization, helper T cell epitopes are identified within the primary sequence of the antibody using prediction software and these sequences are altered by amino acid substitution to avoid recognition by T cells.

The prediction software is principally based on modeling work with the crystal structures of MHC class II allotypes combined with a database search of known T-cell epitopes. As a result, the modified antibody should no longer trigger T cell help. In this way immunogenicity may be eliminated or substantially reduced. However, particular peptides are not necessarily processed and presented by MHC class II, so some unnecessary epitope deletion is possible. Furthermore, there is the issue of tolerance, and this is handled by ignoring peptides present in human immunoglobulin sequences. Currently, two products are in clinical trials following DeImmunization.

6.5.4 Phage Libraries

Since the relative importance of backmutations varies between different mAbs, identifying important positions and determining the optimal amino acid at those positions has proven difficult. With the advent of bacterial expression and phage display of antibody fragments (Fvs, Fabs, etc.) (McCafferty et al. 1990) combined with efficient screening methods, large numbers of variants can be rapidly characterized for activity. This permits antibody function to be optimized or even evolved *in vitro*, as opposed to using successive iterations in the design cycle. Thus, combinatorial antibody libraries have been used for the humanization of mAbs from murine, chicken, or rabbit sources. Rosok et al. (1996) grafted murine CDRs into homologous human germline frameworks and, keeping surface residues human, determined buried positions to be randomized with all possible combinations of murine/human amino acids in a phage library. Thus, the screening simultaneously selected the best binders out of many different humanized Fabs, differing only in their backmutations. Baca et al. (1997) took a similar approach, but differed in details. They CDR grafted into consensus human frameworks and only selected those positions empirically found to be important for antigen binding. Thus the randomized set of backmutations were almost entirely different. This approach can also be used to optimize CDR residues (Wu et al. 1999), combining humanization and *in vitro* affinity maturation in the same procedure.

A different strategy termed guided selection or chain shuffling has been used to isolate human mAbs from phage display libraries in a two-step process. In the first stage, the source V_H is paired with a repertoire of human V_Ls. The resulting Fabs are displayed on filamentous phage and the selected human V_L isolated from

the screening process is paired in the second stage with a human V_H repertoire. Thus the source variable domains are sequentially replaced by human variable domains to derive high affinity human (Jespers et al. 1994) or mainly human (Rader et al. 1998) mAbs. The approach of Rader et al. (1998) has also been called framework shuffling where the CDR-L3 of the source antibody is grafted into human V_L domains and the selected V_L paired in the second step with a library of human V_H domains grafted with the source CDR-H3. This process thus obviates the necessity of information from a structural model, while the resulting humanized mAbs can maintain the specificity and affinity of the original source mAb.

References

Adams, C.W., Allison, D.E., Flagella, K., Presta, L., Clarke, J., Dybdal, N., McKeever, K., Sliwkowski, M.X. (2005) Humanization of a recombinant monoclonal antibody to produce a therapeutic HER dimerization inhibitor, pertuzumab. *Cancer Immunol Immunother* 55: 717–727.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25: 3389–3402.

Baca, M., Presta, L.G., O'Connor, S.J., Wells, J.A. (1997) Antibody humanization using monovalent phage display. *J Biol Chem* 272: 10678–10684.

Baker, T.S., Bose, C.C., Caskey-Finney, H. M., King, D.J., Lawson, A.D., Lyons, A., Mountain,A., Owens, R.J., Rolfe, M.R., Sehdev, M., Yarranton, G.T., Adair, J.R. (1994) Humanization of an anti-mucin antibody for breast and ovarian cancer therapy. *Adv Exp Med Biol* 353: 61–82.

Beiboer, S.H., Reurs, A., Roovers, R.C., Arends, J.W., Whitelegg, N.R., Rees, A.R., Hoogenboom, H.R. (2000) Guided selection of a pan carcinoma specific antibody reveals similar binding characteristics yet structural divergence between the original murine antibody and its human equivalent. *J Mol Biol* 296: 833–849.

Boulianne, G.L., Hozumi, N., Shulman, M.J. (1984) Production of functional chimaeric mouse/human antibody. *Nature* 312: 643–646.

Bourne, P.C., Terzyan, S.S., Cloud G., Landolfi, N.F., Vasquez, M., Edmundson, A.B. (2004) Three-dimensional structures of a humanized anti-IFN-gamma Fab (HuZAF) in two crystal forms. *Acta Crystallogr D Biol Crystallogr* 60: 1761–1769.

Brams, P., Black, A., Padlan, E.A., Hariharan, K., Leonard, J., Chambers-Slater, K., Noelle, R.J., Newman, R. (2001) A humanized anti-human CD154 monoclonal antibody blocks CD154-CD40 mediated human B cell activation. *Int Immunopharmacol* 1: 277–294.

Brooks, B.R., Bruccoleri, R.E., Olafson, B.D., States, D.J., Swaminathan, S., Karplus, M. (1983) CHARMm: A program for macromolecular energy, minimization, and dynamics calculations. *J Comp Chem* 4: 187–217.

Bruccoleri, R.E., Karplus, M. (1987) Prediction of the folding of short polypeptide segments by uniform conformational sampling. *Biopolymers* 26: 137–168.

Caldas, C., Coelho, V.P., Rigden, D.J., Neschich, G., Moro, A.M., Brigido, M.M. (2000) Design and synthesis of germline-based hemi-humanized single-chain Fv against the CD18 surface antigen. *Protein Eng* 13: 353–360.

Caldas, C., Coelho V., Kalil J., Moro, A.M., Maranhao, A.Q., Brigido, M.M. (2003) Humanization of the anti-CD18 antibody 6.7: an unexpected effect of a framework residue in binding to antigen. *Mol Immunol* 39: 941–952.

Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B., Henner, D., Wong, W.L., Rowland, A.M., Kotts, C., Carver, M.E., Shepard, H.M. (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc Natl Acad Sci USA* 89: 4285–4289.

Chan, K.T., Cheng, S.C., Xie, H., Xie, Y. (2001) A humanized monoclonal antibody constructed from intronless expression vectors targets human hepatocellular carcinoma cells. *Biochem Biophys Res Commun* 284: 157–167.

Chavali, G.B., Papageorgiou, A.C., Olson, K.A., Fett, J.W., Hu, G., Shapiro, R., Acharya, K.R. (2003) The crystal structure of human angiogenin in complex with an antitumor neutralizing antibody. *Structure* 11: 875–885.

Chothia, C., Lesk, A.M. (1987) Canonical structures for the hypervariable regions of immunoglobulins. *J Mol Biol* 196: 901–917.

Chothia, C., Novotny, J., Bruccoleri, R., Karplus, M. (1985) Domain association in immunoglobulin molecules. The packing of variable domains. *J Mol Biol* 186: 651–663.

Chothia, C., Lesk, A.M., Levitt, M., Amit, A. G., Mariuzza, R.A., Phillips, S.E., Poljak, R.J. (1986) The predicted structure of immunoglobulin D1.3 and its comparison with the crystal structure. *Science* 233: 755–758.

Chothia, C., Lesk, A.M., Tramontano, A., Levitt, M., Smith-Gill, S.J., Air, G., Sheriff, S., Padlan, E.A., Davies, D., Tulip, W.R., Colman, P.M., Spinelli, S., Alzari, P.M., Poljak, R.J. (1989) Conformations of immunoglobulin hypervariable regions. *Nature* 342: 877–883.

Chuntharapai, A., Lai, J., Huang, X., Gibbs, V., Kim, K.J., Presta, L.G., Stewart, T.A. (2001) Characterization and humanization of a monoclonal antibody that neutralizes human leukocyte interferon: a candidate therapeutic for IDDM and SLE. *Cytokine* 15: 250–260.

Co, M.S., Deschamps, M., Whitley, R.J., Queen, C. (1991) Humanized antibodies for antiviral therapy. *Proc Natl Acad Sci USA* 88: 2869–2873.

Co, M.S., Scheinberg, D.A., Avdalovic, N.M., McGraw, K., Vasquez, M., Caron, P.C., Queen, C. (1993) Genetically engineered deglycosylation of the variable domain increases the affinity of an anti-CD33 monoclonal antibody. *Mol Immunol* 30: 1361–1367.

Co, M.S., Baker, J., Bednarik, K., Janzek, E., Neruda, W., Mayer, P., Plot, R., Stumper, B., Vasquez, M., Queen, C., Loibner, H. (1996) Humanized anti-Lewis Y antibodies: in vitro properties and pharmacokinetics in rhesus monkeys. *Cancer Res* 56: 1118–1125.

Couto, J.R., Blank, E.W., Peterson, J.A., Kiwan R., Padlan, E.A., Ceriani, R.L. (1994) Engineering of antibodies for breast cancer therapy: construction of chimeric and humanized versions of the murine monoclonal antibody BrE-3. *Adv Exp Med Biol* 353: 55–59.

Deshpande, N., Addess, K.J., Bluhm, W.F., Merino-Ott, J.C., Townsend-Merino, W., Zhang, Q., Knezevich, C., Xie, L., Chen, L., Feng, Z., Green, R.K., Flippen-Anderson, J.L., Westbrook, J., Berman, H.M., Bourne, PE. (2005) The RCSB Protein Data Bank: a redesigned query system and relational database based on the mmCIF schema. *Nucleic Acids Res* 33(Database issue): D233–237.

DiJoseph, J.F., Popplewell, A., Tickle, S., Ladyman, H., Lawson, A., Kunz, A., Khandke, K., Armellino, D.C., Boghaert, E.R., Hamann, P., Zinkewich-Peotti, K., Stephens, S., Weir, N., Damle, N.K. (2005) Antibody-targeted chemotherapy of B-cell lymphoma using calicheamicin conjugated to murine or humanized antibody against CD22. *Cancer Immunol Immunother* 54: 11–24.

Eigenbrot, C., Randal, M., Presta, L., Carter, P., Kossiakoff, A.A. (1993) X-ray structures of the antigen-binding domains from three variants of humanized anti-p185HER2 antibody 4D5 and comparison with molecular modeling. *J Mol Biol* 229: 969–995.

Eisenberg, D., Luthy, R., Bowie, J.U. (1997) VERIFY3D: assessment of protein models with three-dimensional profiles. *Methods Enzymol* 277: 396–404.

Ellis, J.H., Barber, K.A., Tutt, A., Hale, C., Lewis, A.P., Glennie, M.J., Stevenson, G.T., Crowe, J.S. (1995) Engineered anti-CD38 monoclonal antibodies for immunotherapy

of multiple myeloma. *J Immunol* 155: 925–937.

Ferrin, T.E., Couch, G.S., Huang, C.C., Pettersen, E.F., Langridge, R. (1991) An affordable approach to interactive desktop molecular modeling. *J Mol Graph* 9: 27–32, 37–38.

Foote, J., Winter, G. (1992) Antibody framework residues affecting the conformation of the hypervariable loops. *J Mol Biol* 224: 487–499.

Gibrat, J.F., Madej, T., Bryant, S.H. (1996) Surprising similarities in structure comparison. *Curr Opin Struct Biol* 6: 377–385.

Gonzales, N.R., Padlan, E.A., De Pascalis, R., Schuck, P., Schlom, J., Kashmiri, S.V. (2003) Minimizing immunogenicity of the SDR-grafted humanized antibody CC49 by genetic manipulation of the framework residues. *Mol Immunol* 40: 337–349.

Gonzales, N.R., Padlan, E.A., De Pascalis, R., Schuck, P., Schlom, J., Kashmiri, S.V. (2004) SDR grafting of a murine antibody using multiple human germline templates to minimize its immunogenicity. *Mol Immunol* 41: 863–872.

Gorman, S.D., Clark, M.R., Routledge, E.G., Cobbold, S.P., Waldmann, H. (1991) Reshaping a therapeutic CD4 antibody. *Proc Natl Acad Sci USA* 88: 4181–4185.

Graves, S.S., Goshorn, S.C., Stone, D.M., Axworthy, D.B., Reno, J.M., Bottino, B., Searle, S., Henry, A., Pedersen, J., Rees, A. R., Libby, R.T. (1999) Molecular modeling and preclinical evaluation of the humanized NR-LU-13 antibody. *Clin Cancer Res* 5: 899–908.

Graziano, R.F., Tempest, P.R., White, P., Keler, T., Deo, Y., Ghebremariam, H., Coleman, K., Pfefferkorn, L.C., Fanger, M. W., Guyre, P.M. (1995) Construction and characterization of a humanized anti-gamma-Ig receptor type I (Fc gamma RI) monoclonal antibody. *J Immunol* 155: 4996–5002.

Guex, N., Peitsch MC. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 18: 2714–2723.

Gussow, D., Seemann, G. (1991) Humanization of monoclonal antibodies. *Methods Enzymol* 203: 99–121.

Hakimi, J., Ha, V.C., Lin, P., Campbell, E., Gately, M.K., Tsudo, M., Payne, P.W., Waldmann, T.A., Grant, A.J., Tsien, W.H., Schneider, W.P. (1993) Humanized Mik beta 1, a humanized antibody to the IL-2 receptor beta-chain that acts synergistically with humanized anti-TAC. *J Immunol* 151: 1075–1085.

Hamann, P.R., Hinman, L., Hollander, I., Holcomb, R., Hallett, W., Tsou, H-R., Weiss, M.J. (1999) Process for preparing conjugates of methyltrithio antitumor agents. U.S. patent 5, 877, 296.

Hamilton, A.A., Manuel, D.M., Grundy, J.E., Turner, A.J., King, S.I., Adair, J.R., White, P., Carr, F.J., Harris, W.J. (1997) A humanized antibody against human cytomegalovirus (CMV) gpUL75 (gH) for prophylaxis or treatment of CMV infections. *J Infect Dis* 176: 59–68.

Hsiao, K.C., Bajorath, J., Harris, L.J. (1994) Humanization of 60.3, an anti-CD18 antibody; importance of the L2 loop. *Protein Eng* 7: 815–822.

Hwang, W.Y., Foote, J. (2005) Immunogenicity of engineered antibodies. *Methods* 36: 3–10.

Hwang, W.Y., Almagro, J.C., Buss, T.N., Tan, P., Foote, J. (2005) Use of human germline genes in a CDR homology-based approach to antibody humanization. *Methods* 36: 35–42.

Jespers, L.S., Roberts, A., Mahler, S.M., Winter, G., Hoogenboom, H.R. (1994) Guiding the selection of human antibodies from phage display repertoires to a single epitope of an antigen. *Biotechnology* 12: 899–903.

Johnson, S., Oliver, C., Prince, G.A., Hemming, V.G., Pfarr, D.S., Wang, S.C., Dormitzer, M., O'Grady, J., Koenig, S., Tamura, J.K., Woods, R., Bansal, G., Couchenour, D., Tsao, E., Hall, W.C., Young, J.F. (1997) Development of a humanized monoclonal antibody (MEDI-493) with potent in vitro and in vivo activity against respiratory syncytial virus. *J Infect Dis* 176: 1215–1224.

Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S., Winter, G. (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* 321: 522–525.

Kabat, E.A., Wu, T.T., Reid-Miller, M., Perry, H., Gottesman, K. (1987) *Sequences of Proteins of Immunological Interest*, 4th edn. US Govt. Printing Off. No. 165-492.

Kabat, E.A., Wu, T.T., Perry, H., Gottesman, K., Foeller, C. (1991) *Sequences of Proteins of Immunological Interest*, 5th edn. NIH Publication No. 91-3242.

Kettleborough, C.A., Saldanha, J., Heath, V. J., Morrison, C.J., Bendig, M.M. (1991) Humanization of a mouse monoclonal antibody by CDR-grafting: the importance of framework residues on loop conformation. *Protein Eng* 4: 773–783.

Kohler, G., Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.

Kolbinger, F., Saldanha, J., Hardman, N., Bendig, M.M. (1993) Humanization of a mouse anti-human IgE antibody: a potential therapeutic for IgE-mediated allergies. *Protein Eng* 6: 971–980.

Krauss, J., Arndt, M.A., Zhu, Z., Newton, D.L., Vu, B.K., Choudhry, V., Darbha, R., Ji X., Courtenay-Luck, N.S., Deonarain, M.P., Richards, J., Rybak, S.M. (2004) Impact of antibody framework residue V_H-71 on the stability of a humanised anti-MUC1 scFv and derived immunoenzyme. *Br J Cancer* 90: 1863–1870.

Landolfi, N.F., Thakur, A.B., Fu, H., Vasquez, M., Queen, C., Tsurushita, N. (2001) The integrity of the ball-and-socket joint between V and C domains is essential for complete activity of a humanized antibody. *J Immunol* 166: 1748–1754.

Laskowski, R.A., MacArthur, M.W., Moss, D.S., Thornton, J.M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures *J Appl Cryst* 26: 283–291.

Lefranc, M.P., Giudicelli, V., Kaas, Q., Duprat, E., Jabado-Michaloud, J., Scaviner, D., Ginestoux, C., Clement, O., Chaume, D., Lefranc, G. (2005) IMGT, the international ImMunoGeneTics information system. *Nucleic Acids Res* 33: D593–D597.

Leger, O.J., Yednock, T.A., Tanner L., Horner, H.C., Hines, D.K., Keen, S., Saldanha, J., Jones, S.T., Fritz, L.C., Bendig, M.M. (1997) Humanization of a mouse antibody against human alpha-4 integrin: a potential therapeutic for the treatment of multiple sclerosis. *Hum Antibodies* 8: 3–16.

Lesk, A.M., Chothia, C. (1988) Elbow motion in the immunoglobulins involves a molecular ball-and-socket joint. *Nature* 335: 188–190.

Leung, S.O., Goldenberg, D.M., Dion, A.S., Pellegrini, M.C., Shevitz, J., Shih, L.B., Hansen, H.J. (1995) Construction and characterization of a humanized, internalizing, B-cell (CD22)-specific, leukemia/lymphoma antibody, LL2. *Mol Immunol* 32: 1413–1427.

Levitt, M. (1983) Molecular dynamics of native protein. I. Computer simulation of trajectories. *J Mol Biol* 168: 595–617.

Luo, G.X., Kohlstaedt, L.A., Charles, C.H., Gorfain, E., Morantte, I., Williams, J.H., Fang, F. (2003) Humanization of an anti-ICAM-1 antibody with over 50-fold affinity and functional improvement. *J Immunol Methods* 275: 31–40.

Maeda, H., Matsushita, S., Eda, Y., Kimachi, K., Tokiyoshi, S., Bendig, M.M. (1991) Construction of reshaped human antibodies with HIV-neutralizing activity. *Hum Antibodies Hybridomas* 2: 124–134.

Martin, A.C., Cheetham, J.C., Rees, A.R. (1989) Modeling antibody hypervariable loops: a combined algorithm. *Proc Natl Acad Sci USA* 86: 9268–9272.

Martin, A.C., Thornton, J.M. (1996) Structural families in loops of homologous proteins: automatic classification, modelling and application to antibodies. *J Mol Biol* 263: 800–815.

McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J. (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348: 552–554.

Morrison, S.L., Johnson, M.J., Herzenberg, L.A., Oi, V.T. (1984) Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. *Proc Natl Acad Sci USA* 81: 6851–6855.

Nakamura, K., Tanaka, Y., Fujino, I., Hirayama, N., Shitara, K., Hanai, N. (2000) Dissection and optimization of immune effector functions of humanized anti-ganglioside GM2 monoclonal antibody. *Mol Immunol* 37: 1035–1046.

Nakatani, T., Lone, Y.C., Yamakawa, J., Kanaoka, M., Gomi, H., Wijdenes, J., Noguchi, H. (1994) Humanization of mouse anti-human IL-2 receptor antibody B-B10. *Protein Eng* 7: 435–443.

Nisihara, T., Ushio, Y., Higuchi, H., Kayagaki, N., Yamaguchi, N., Soejima, K., Matsuo, S., Maeda, H., Eda, Y., Okumura, K., Yagita, H. (2001) Humanization and epitope mapping of neutralizing anti-human Fas ligand monoclonal antibodies: structural insights into Fas/Fas ligand interaction. *J Immunol* 167: 3266–3275.

Novotny, J., Haber, E. (1985) Structural invariants of antigen binding: comparison of immunoglobulin V_L-V_H and V_L-V_L domain dimers. *Proc Natl Acad Sci USA* 82: 4592–4596.

Novotny, J., Bruccoleri, R., Newell, J., Murphy, D., Haber, E., Karplus, M. (1983) Molecular anatomy of the antibody binding site. *J Biol Chem* 258: 14433–14437.

Ohtomo, T., Tsuchiya, M., Sato, K., Shimizu, K., Moriuchi, S., Miyao, Y., Akimoto, T., Akamatsu, K., Hayakawa, T., Ohsugi, Y. (1995) Humanization of mouse ONS-M21 antibody with the aid of hybrid variable regions. *Mol Immunol* 32: 407–416.

Ono, K., Ohtomo, T., Yoshida, K., Yoshimura, Y., Kawai, S., Koishihara, Y., Ozaki, S., Kosaka, M., Tsuchiya, M. (1999) The humanized anti-HM1.24 antibody effectively kills multiple myeloma cells by human effector cell-mediated cytotoxicity. *Mol Immunol* 36: 387–395.

Padlan, E.A. (1991) A possible procedure for reducing the immunogenicity of antibody variable domains while preserving their ligand-binding properties. *Mol Immunol* 28: 489–498.

Padlan, E.A. (1994) Anatomy of the antibody molecule. *Mol Immunol* 31: 169–217.

Padlan, E.A., Abergel C., Tipper, J.P. (1995) Identification of specificity-determining residues in antibodies. *FASEB J* 9: 133–139.

Park, S.S., Ryu, C.J., Gripon, P., Guguen-Guillouzo, C., Hong, H.J. (1996) Generation and characterization of a humanized antibody with specificity for preS2 surface antigen of hepatitis B virus. *Hybridoma* 15: 435–441.

Pearson, W.R. (2000) Flexible sequence similarity searching with the FASTA3 program package. *Methods Mol Biol* 132: 185–219.

Pedersen, J.T., Henry, A.H., Searle, S.J., Guild, B.C., Roguska, M., Rees, A.R. (1994) Comparison of surface accessible residues in human and murine immunoglobulin Fv domains. Implication for humanization of murine antibodies. *J Mol Biol* 235: 959–973.

Pichla, S.L., Murali, R., Burnett, RM. (1997) The crystal structure of a Fab fragment to the melanoma-associated GD2 ganglioside. *J Struct Biol* 119: 6–16.

Poul, M.A., Ticchioni, M., Bernard, A., Lefranc, M.P. (1995) Inhibition of T cell activation with a humanized anti-beta 1 integrin chain mAb. *Mol Immunol* 32: 101–116.

Presta, L.G., Lahr, S.J., Shields, R.L., Porter, J.P., Gorman, C.M., Fendly, B.M., Jardieu, P.M. (1993) Humanization of an antibody directed against IgE. *J Immunol* 151: 2623–2632.

Presta, L.G., Chen, H., O'Connor, S.J., Chisholm, V., Meng, Y.G., Krummen, L., Winkler, M., Ferrara, N. (1997) Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res* 57: 4593–4599.

Pulito, V.L., Roberts, V.A., Adair, J.R., Rothermel, A.L., Collins, A.M., Varga, S. S., Martocello, C., Bodmer, M., Jolliffe, L.K., Zivin, R.A. (1996) Humanization and molecular modeling of the anti-CD4 monoclonal antibody. OKT4A. *J Immunol* 156: 2840–2850.

Qu, Z., Sharkey, R.M., Hansen, H.J., Shih, L.B., Govindan, S.V., Shen J., Goldenberg, D.M., Leung, S.O. (1998) Carbohydrates engineered at antibody constant domains can be used for site-specific conjugation of drugs and chelates. *J Immunol Methods* 213: 131–144.

Qu, Z., Losman, M.J., Eliassen, K.C., Hansen, H.J., Goldenberg, D.M., Leung, S.O. (1999) Humanization of Immu31: an alpha-fetoprotein-specific antibody. *Clin Cancer Res* 5: 3095s–3100s.

Queen, C., Schneider, W.P., Selick, H.E., Payne, P.W., Landolfi, N.F., Duncan, J.F., Avdalovic, N.M., Levitt M., Junghans, R.P., Waldmann, T.A. (1989) A humanized

antibody that binds to the interleukin 2 receptor. *Proc Natl Acad Sci USA* 86: 10029–10033.

Rader, C., Cheresh, D.A., Barbas, C.F. III (1998) A phage display approach for rapid antibody humanization: designed combinatorial V gene libraries. *Proc Natl Acad Sci USA* 95: 8910–8915.

Redwan, el-, R.M., Larsen, N.A., Zhou, B., Wirsching, P., Janda, K.D., Wilson, I.A. (2003) Expression and characterization of a humanized cocaine-binding antibody. *Biotechnol Bioeng* 82: 612–618.

Retter, I., Althaus, H.H., Munch, R., Muller, W. (2005) VBASE2: an integrative V gene database. *Nucleic Acids Res* 33: D671–D674.

Richards, J., Auger, J., Peace, D., Gale, D., Michel, J., Koons, A., Haverty, T., Zivin, R., Jolliffe, L., Bluestone, J.A. (1999) Phase I evaluation of humanized OKT3: toxicity and immunomodulatory effects of hOKT3gamma4. *Cancer Res* 59: 2096–2101.

Riechmann, L., Clark, M., Waldmann, H., Winter, G. (1988) Reshaping human antibodies for therapy. *Nature* 332: 323–327.

Ritter, G., Cohen, L.S., Williams, C, Jr., Richards, E.C., Old, L.J., Welt, S. (2001) Serological analysis of human anti-human antibody responses in colon cancer patients treated with repeated doses of humanized monoclonal antibody A33. *Cancer Res* 61: 6851–6859.

Rodrigues, M.L., Shalaby, M.R., Werther, W., Presta, L., Carter, P. (1992) Engineering a humanized bispecific F(ab')2 fragment for improved binding to T cells. *Int J Cancer Suppl* 7: 45–50.

Roguska, M.A., Pedersen, J.T., Keddy, C.A., Henry, A.H., Searle, S.J., Lambert, J.M., Goldmacher, V.S., Blattler, W.A., Rees, A. R., Guild, B.C. (1994) Humanization of murine monoclonal antibodies through variable domain resurfacing. *Proc Natl Acad Sci USA* 91: 969–673.

Roguska, M.A., Pedersen, J.T., Henry, A.H., Searle, S.M., Roja, C.M., Avery, B., Hoffee, M., Cook S., Lambert, J.M., Blattler, W.A., Rees, A.R., Guild, B.C. (1996) A comparison of two murine monoclonal antibodies humanized by CDR-grafting and variable domain resurfacing. *Protein Eng* 9: 895–904.

Rosok, M.J., Yelton, D.E., Harris, L.J., Bajorath, J., Hellstrom, K.E., Hellstrom, I., Cruz, G.A., Kristensson, K., Lin, H., Huse, W.D., Glaser, S.M. (1996) A combinatorial library strategy for the rapid humanization of anticarcinoma BR96 Fab. *J Biol Chem* 271: 22611–22618.

Sato, K., Tsuchiya, M., Saldanha, J., Koishihara, Y., Ohsugi, Y., Kishimoto, T., Bendig, M.M. (1993) Reshaping a human antibody to inhibit the interleukin 6-dependent tumor cell growth. *Cancer Res* 53: 851–856.

Sato, K., Tsuchiya M., Saldanha J., Koishihara Y., Ohsugi Y., Kishimoto T., Bendig MM. (1994) Humanization of a mouse anti-human interleukin-6 receptor antibody comparing two methods for selecting human framework regions. *Mol Immunol* 31: 371–381.

Sato, K., Ohtomo, T., Hirata, Y., Saito, H., Matsuura, T., Akimoto, T., Akamatsu, K., Koishihara, Y., Ohsugi, Y., Tsuchiya, M. (1996) Humanization of an anti-human IL-6 mouse monoclonal antibody glycosylated in its heavy chain variable region. *Hum Antibodies Hybridomas* 7: 175–183.

Schneider, W.P., Glaser, S.M., Kondas, J.A., Hakimi, J. (1993) The anti-idiotypic response by cynomolgus monkeys to humanized anti-Tac is primarily directed to complementarity-determining regions H1: H2: and L3. *J Immunol* 150: 3086–3090.

Sha, Y., Xiang, J. (1994) A heavy-chain grafted antibody that recognizes the tumor-associated TAG72 antigen. *Cancer Biother* 9: 341–349.

Shearman, C.W., Pollock, D., White, G., Hehir, K., Moore, G.P., Kanzy, E.J., Kurrle, R. (1991) Construction, expression and characterization of humanized antibodies directed against the human alpha/beta T cell receptor. *J Immunol* 147: 4366–4373.

Shirai, H., Kidera, A., Nakamura, H. (1996) Structural classification of CDR-H3 in antibodies. *FEBS Lett* 399: 1–8.

Shirai, H., Kidera, A., Nakamura, H. (1999) H3-rules: identification of CDR-H3 structures in antibodies. *FEBS Lett* 455: 188–197.

Sims, M.J., Hassal, D.G., Brett, S., Rowan, W., Lockyer, M.J., Angel, A., Lewis, A.P., Hale, G., Waldmann, H., Crowe, J.S.

(1993) A humanized CD18 antibody can block function without cell destruction. *J Immunol* 151: 2296–2308.

Singer, I.I., Kawka, D.W., DeMartino, J.A., Daugherty, B.L., Elliston, K.O., Alves, K., Bush, B.L., Cameron, P.M., Cuca, G.C., Davies, P., Forrest, M.J., Kazazis, D.M., Law, M-F., Lenny, A.B., MacIntyre, D.E., Meurer, R., Padlan, E.A., Pandya, S., Schmidt, J.A., Seamans, T.C., Scott, S., Silberklang, M., Williamson, A.R., Mark, G.E. (1993) Optimal humanization of 1B4: an anti-CD18 murine monoclonal antibody, is achieved by correct choice of human V-region framework sequences. *J Immunol* 150: 2844–2857.

Sippl, M.J. (1993) Recognition of errors in three-dimensional structures of proteins. *Proteins* 17: 355–362.

Steinberger, P., Sutton, J.K., Rader, C., Elia, M., Barbas, C.F. III (2000) Generation and characterization of a recombinant human CCR5-specific antibody. A phage display approach for rabbit antibody humanization. *J Biol Chem* 275: 36073–36078.

Stephens, S., Emtage, S., Vetterlein, O., Chaplin, L., Bebbington, C., Nesbitt, A., Sopwith, M., Athwal, D., Novak, C., Bodmer, M. (1995) Comprehensive pharmacokinetics of a humanized antibody and analysis of residual anti-idiotypic responses. *Immunology* 85: 668–674.

Tabrizi, M. (2005) Immunogenicity of therapeutics: monoclonal antibodies (mAbs). Presentation at the IBC Antibody Engineering Conference, San Diego, USA, December 2005.

Tamura, M., Milenic, D.E., Iwahashi, M., Padlan, E., Schlom, J., Kashmiri, S.V. (2000) Structural correlates of an anticarcinoma antibody: identification of specificity-determining residues (SDRs) and development of a minimally immunogenic antibody variant by retention of SDRs only. *J Immunol* 164: 1432–1441.

Tan, P., Mitchell, D.A., Buss, T.N., Holmes, M.A., Anasetti, C., Foote, J. (2002) 'Superhumanized' antibodies: reduction of immunogenic potential by complementarity-determining region grafting with human germline sequences: application to an anti-CD28. *J Immunol* 169: 1119–1125.

Tempest, P.R., Bremner, P., Lambert, M., Taylor, G., Furze, J.M., Carr, F.J., Harris, W.J. (1991) Reshaping a human monoclonal antibody to inhibit human respiratory syncytial virus infection in vivo. *Biotechnology (NY)* 9: 266–271.

Tempest, P.R., White, P., Buttle, M., Carr, F.J., Harris, W.J. (1995) Identification of framework residues required to restore antigen binding during reshaping of a monoclonal antibody against the glycoprotein gB of human cytomegalovirus. *Int J Biol Macromol* 17: 37–42.

Thomas, T.C., Rollins, S.A., Rother, R.P., Giannoni, M.A., Hartman, S.L., Elliott, E.A., Nye, S.H., Matis, L.A., Squinto, S.P., Evans, M.J. (1996) Inhibition of complement activity by humanized anti-C5 antibody and single-chain Fv. *Mol Immunol* 33: 1389–1401.

Tsurushita, N., Park, M., Pakabunto, K., Ong, K., Avdalovic, A., Fu, H., Jia, A., Vasquez, M., Kumar, S. (2004) Humanization of a chicken anti-IL-12 monoclonal antibody. *J Immunol Methods* 295: 9–19.

Vajdos, F.F., Adams, C.W., Breece, T.N., Presta, L.G., de Vos, A.M., Sidhu, S.S. (2002) Comprehensive functional maps of the antigen-binding site of an anti-ErbB2 antibody obtained with shotgun scanning mutagenesis. *J Mol Biol* 320: 415–428.

Verhoeyen, M., Milstein, C., Winter, G. (1988) Reshaping human antibodies: grafting an antilysozyme activity. *Science* 239: 1534–1536.

Weiner, S.J., Kollman, P.A., Case, D.A., Singh, U.C., Ghio, C., Alagona, G., Profeta, S., Weiner, P. (1984) A new force field for molecular mechanical simulation of nucleic acids and proteins. *J Am Chem Soc* 106: 765–784.

Werther, W.A., Gonzalez, T.N., O'Connor, S. J., McCabe, S., Chan, B., Hotaling, T., Champe, M., Fox, J.A., Jardieu, P.M., Berman, P.W., Presta, LG. (1996) Humanization of an anti-lymphocyte function-associated antigen (LFA)-1 monoclonal antibody and reengineering of the humanized antibody for binding to rhesus LFA-1. *J Immunol* 157: 4986–4995.

Wu, T.T., Kabat, EA. (1970) An analysis of the sequences of the variable regions of

Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J Exp Med* 132: 211–250.
Wu, H., Nie, Y., Huse, W.D., Watkins, J.D. (1999) Humanization of a murine monoclonal antibody by simultaneous optimization of framework and CDR residues. *J Mol Biol* 294: 151–162.
Yazaki, P.J., Sherman, M.A., Shively, J.E., Ikle, D., Williams, L.E., Wong, J.Y., Colcher, D., Wu, A.M., Raubitschek, A.A. (2004) Humanization of the anti-CEA T84.66 antibody based on crystal structure data. *Protein Eng Des Sel* 17: 481–489.
Zhang, W., Feng, J., Li, Y., Guo, N., Shen, B. (2005) Humanization of an anti-human TNF-alpha antibody by variable region resurfacing with the aid of molecular modeling. *Mol Immunol* 42: 1445–1451.

7
Molecular Engineering II: Antibody Affinity

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7.1
Introduction

Antibody affinity is a quantitative measurement of the strength of association between a single antibody-binding site and a single antigen-binding site. For any given concentration of antibody and antigen, the affinity determines the number of antigen molecules that will be bound by antibody. Affinity, therefore, is a critical variable affecting the potency of an antibody therapeutic. Potency will affect the dose and possibly the dosing interval. Since therapeutic doses (Roskos et al. 2004) and costs of goods of antibodies are frequently high, an adequate affinity might ultimately determine the clinical and commercial success of an antibody therapeutic. In this chapter the process of affinity maturation, the relationship between affinity and potency, and analytical approaches to the measurement of affinity are reviewed.

7.2
Affinity Maturation

7.2.1
Maturation *In Vivo*

Antibody diversity, which allows the antibodies of the primary repertoire to bind and to modulate a vast number of different antigens and antigen epitopes, is generated through sequential processes that occur at different stages of B cell development (Wu et al. 2003). VDJ rearrangement of gene segments in the immunoglobulin heavy chain locus, VJ rearrangements in the κ and λ immunoglobulin light chain loci, addition and deletion of nucleotides at the joints between the gene segments, and random pairing of the rearranged V_HDJ_H and $V_\kappa J_\kappa$ and $V_\lambda J_\lambda$ segments leads to enormous diversity in the order of 10^{11} B-cell receptors (BCRs) expressed by naive cells in humans. However, antigen binding affinity to

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the BCRs of the primary repertoire is usually weak. With appropriate signaling upon encountering antigen, additional antibody diversity is introduced by class switch DNA recombination, which changes the antibody constant region and alters antibody effector functions, and by somatic hypermutation, which drives the affinity maturation process.

The clonal selection of B cells producing high-affinity antibodies to an antigen is an essential part of the humoral response to an antigen challenge. Affinity maturation is the result of two processes of positive selection that take place in different compartments of the germinal center in secondary lymphoid organs (Wabl et al. 1999; Defrance et al. 2002; Wu et al. 2003). Somatic V(D)J hypermutation occurs by introduction of point mutations, with occasional insertions and deletions, in the variable region gene of antigen-activated B cells residing in the dark zone of the germinal center (Defrance et al. 2002). The somatic mutation rate in these cells is about one million times higher than the spontaneous somatic mutation rate of the genome at large (Wu et al. 2003). Only those cells expressing BCRs with sufficiently high affinity to compete with antigen trapped as IgM immune complexes on follicular dendritic cells receive a survival signal by antigen binding to the BCR. The antigen-activated B cells receive a second survival signal in the light zone of the germinal center, where positive selection of mutant B cell clones with high antigen binding affinity relies on efficient binding and presentation of antigen to helper T cells (Defrance et al. 2002). If the hypermutation mechanism creates a BCR that has lost antigen binding or has become self-reactive, a negative selection process can trigger either receptor editing, a "last-ditch" effort of reactivated V gene rearrangement to rescue antigen binding, or apoptosis. This natural process of affinity maturation, hypermutation combined with positive and negative selection for improvements in antibody affinity, is iterative, and can lead to one million fold or greater improvements in monoclonal antibody (mAb) affinity compared to the primary mAb with the germline V(D)J sequence from the naive repertoire.

Foote and Eisen have proposed a ceiling for affinities that can be obtained through *in vivo* selection (Foote and Eisen 1995, 2000). The basis for the affinity ceiling is that the association rate of the antigen with the BCR will eventually be rate limited by the diffusion rate to the BCR (rather than the association rate constant for binding), and that the dissociation rate of the antigen bound to BCR cannot be slower than the internalization rate of the antigen–BCR complex into the B cell (the net dissociation rate constant will be the sum of the internalization rate constant and the antigen–BCR dissociation rate constant). The maturation ceiling for the equilibrium dissociation constant has been estimated to be approximately 10^{-10} mol L^{-1}. Although the kinetic basis for an affinity ceiling is sound, somatic hypermutation is a stochastic process; therefore, there would be large variation in antibody affinity on both sides of a mean affinity ceiling. Three conditions can allow for the *in vivo* generation of antibodies with affinities exceeding the theoretical ceiling: (1) iterative rounds of positive selection generate BCRs that can evolve towards the theoretical affinity plateau; (2) the hypermutation process stochastically generates a BCR that exceeds the plateau for selection,

evolved either from a BCR near the ceiling or from a "jackpot" rearrangement or mutation; and (3) the absence of negative selection against BCRs with affinity $<10^{-10}$ mol L^{-1} allows for the continued survival of the B cell. Thus, though B cells expressing a BCR with an affinity $<10^{-10}$ mol L^{-1} would have no more or no less advantage than those with an affinity of 10^{-10} mol L^{-1}, they can be created and maintained. Indeed, rigorous screening of large pools of diverse mAbs recovered from hyperimmunized mice can yield antibodies with picomolar and even femtomolar affinity, which is many fold higher than the proposed *in vivo* ceiling (Rathanaswami et al. 2005).

Somatic hypermutation has evolved to favor mutations in the complementarity determining regions (CDRs) of antibodies; the framework regions (FRs) are mutated at a lower frequency (Wu et al. 2003). The FR mutations are usually less important for affinity and specificity than the CDR mutations. The V_H CDRs in general, and the V_H and V_L CDR3 in particular, tend to dominate the antibody–antigen interactions (Sundberg and Mariuzza 2002). For these reasons, random mutations frequently exist in the FRs and CDRs that do not contribute to antibody affinity and specificity. In the generation of therapeutic antibodies, some antibody generation groups routinely identify the nonessential mutations (usually in the FRs) by alanine scanning mutagenesis and convert these mutations back to the germline sequence (a process called "germlining"). The rationale is that nonessential, random mutations may increase the risk of immunogenicity relative to an antibody with the germline sequence. To date, the risk of immunogenicity as related to the number of CDR and FR mutations remains only a theoretical concern.

7.2.2 Maturation *In Vitro*

In vitro antibody display technologies can be used to generate high-affinity, antigen-specific antibodies (Hoogenboom 2005). Generally, DNA for rearranged V_H and either V_κ or V_λ derived either from naive B cells or selected V gene framework regions with synthetic CDRs are molecularly coupled and then expressed recombinantly in systems such as bacteriophage, yeast, or via translation *in vitro*. Because the protein product is engineered to remain physically linked to the nucleic acid encoding it, *in vitro* screening for binding of the displayed variable region repertoire to the antigen allows for recovery of the DNA or RNA encoding the antibody. Antibodies with moderate affinities on the order of 10^{-8}–10^{-9} are usually recovered after 2–3 rounds of standard selection, enrichment and screening for antigen binding. In theory, the *in vitro* nature of the process allows for generation of libraries of V_H/V_L combinations on the order of 10^{10}–10^{13}, sizes that greatly exceed the size of the B-cell compartment *in vivo*, which would therein provide superior diversity in the antibody repertoire. In practice, however, the individual dynamics of the various systems for *in vitro* display often can bias the repertoire and diminish the theoretical diversity by orders of magnitude.

Because most therapeutic mAbs would require affinities better than those of antibodies recovered directly from *in vitro* antibody display systems, large efforts have been undertaken to develop efficient *in vitro* means to mimic the *in vivo* affinity maturation process. Typically, the processes currently in use employ error prone processes such as polymerase chain reaction (PCR) and/or reverse transcription focused in and adjacent to the CDRs of the V_H and V_L coupled with stringent selection for higher affinity binding *in vitro*. The screening procedures may be manipulated to recover variable regions with selective improvements in k_{on} and/or in k_{off}, something not feasible with affinity maturation *in vivo*. As typical for the *in vivo* process, iterative rounds of *in vitro* mutagenesis and selection are employed to recover and incrementally improve selected variants. In this way, antibodies with affinities $<10^{-9}$–10^{-10} mol L^{-1} and occasionally greater can be routinely generated through *in vitro* processes. These processes, and with advancements such as utilization of combinations of *in vitro* procedures that complement deficiencies inherent in each (phage plus either ribosome display or yeast display) and improvements in operational efficiencies that allow for both parallel-processing of multiple starting V region templates and screening of larger pools of variants, have yielded antibodies with picomolar and femtomolar affinities (Schier et al. 1995; Boder et al. 2000; Hanes et al. 2000; Zahnd et al. 2004; Hoet et al. 2005; Rathanaswami et al. 2005).

7.3 Effect of Affinity on Antigen Binding and Antibody Potency

Affinity describes the strength of reversible association between antibody and antigen:

$$\mathrm{Ab} + \mathrm{Ag} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \mathrm{Ab \cdot Ag}$$

The strength of antibody-antigen binding is enhanced by a fast association rate, which is proportional to the *association rate constant* (k_{on} or k_a), and by a slow dissociation rate, which is proportional to the *dissociation rate constant* (k_{off} or k_d). The value of affinity is most frequently described by the *equilibrium dissociation constant* (K_D). The K_D, which is readily calculated by k_{off} divided by k_{on}, is the concentration of antibody-binding sites that will bind 50% of the antigen-binding sites when the concentration of antigen is much less than the K_D. This simple definition of K_D assumes that all antibody-binding sites are accessible to all antigen-binding sites and that no avid interactions occur. *Avidity* reflects the strength of binding when multivalent binding results in a cooperative antigen–antibody interaction. An example of an avid interaction is when both antibody-binding sites simultaneously bind an antigen on a surface, or form cyclic or lattice immune complexes. In such cases, the avidity may be much stronger (by several orders of magnitude) than reflected by the 1:1 site binding K_D. The values of K_D,

k_{on}, and k_{off} can be determined experimentally; the most robust methods are reviewed later in this chapter.

An understanding of the basic relationship between affinity, antibody concentration, antigen concentration, and the fraction of antigen bound is essential to the understanding of the relationship between antibody affinity and potency. The fraction of antigen bound ($F_{b,Ag}$) can be readily calculated as a function of the K_D, the antibody-binding site concentration (Ab_s), and the antigen binding site concentration (Ag_s):

$$F_b^{Ag} = \frac{Ab_s + Ag_s + K_D - \sqrt{Ab_s^2 + Ag_s^2 + K_D^2 - 2Ab_sAg_s + 2Ab_sK_D + 2Ag_sK_D}}{2Ag_s}$$

This relationship is illustrated by simulation in Fig. 7.1. The fraction of antigen bound is plotted as a function of the ratio of antibody concentration (each mole of antibody is assumed to bind 2 moles of antigen) to the K_D for different multiples of antigen concentration relative to K_D (antigen concentration varying from $K_D/100$ to $100K_D$). When the concentration of antigen is less than or equal to $K_D/10$, 50% of the antigen is bound when the antibody concentration is one-half the K_D (i.e. when the total antibody-binding site concentration is equal to the K_D). Under these conditions, the binding is said to be *K_D-dependent*. When the con-

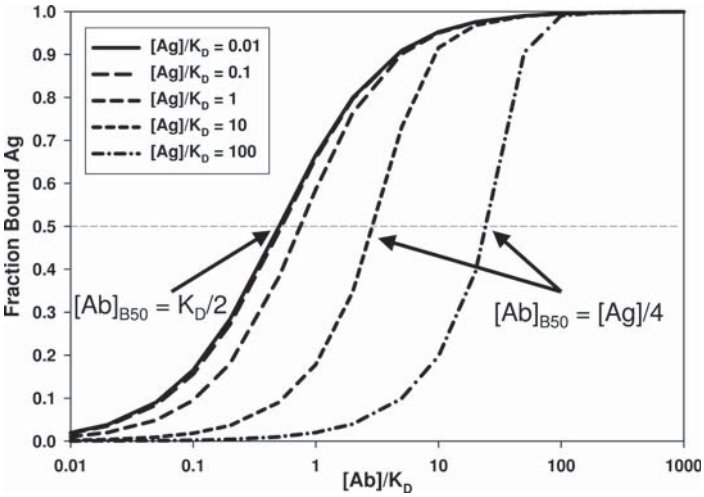


Fig. 7.1 Theoretical fraction of antigen bound by antibody as a function of antigen concentration (Ag) and antibody concentration (Ab) relative to the K_D. One antibody molecule was assumed to be capable of binding two antigen molecules. When Ag is 1/10th the K_D or less, the concentration of antibody required to bind 50% of Ag ($[Ab]_{B50}$) is K_D-dependent. When Ag is 10 times or more greater than the K_D, $[Ab]_{B50}$ is stoichiometric (antigen concentration-dependent).

centration of antigen exceeds the K_D by a multiple of 10 or more, then 50% of the antigen is bound when the antibody concentration is a quarter the antigen concentration (i.e., the antibody-binding site concentration is equal to one-half the antigen concentration). Under these conditions, the binding is said to be *stoichiometric*, since antigen is bound in approximately a 1:1 molar ratio to the available antibody-binding sites, and the binding is not dependent on the K_D. Likewise, for any fixed concentration of antigen, improvements in antibody affinity will eventually result in a transition from K_D-dependent binding conditions to stoichiometric conditions.

From these kinetic observations, a simple relationship between affinity and binding potency emerges. *For any given antigen concentration, an antibody affinity exists beyond which further improvements in affinity will not enhance antigen binding.* This potency ceiling for affinity occurs when the K_D of the antibody falls to approximately 1/10th the antigen concentration. As shown in the following section, this relationship holds *in vitro* and *in vivo*.

7.3.1 Binding and Potency *In Vitro*

Cell-based bioassays are routinely used to compare the functional potency of various mAbs to a given target. By varying antibody concentration in the presence of a fixed concentration of antigen (cell membrane or soluble antigen), the antibody potency can be expressed as a maximum effect in presence of a large excess of antigen (I_{max} or E_{max}) and the concentration of antibody producing the half-maximal effect (IC_{50} or EC_{50}). Since the affinity of the antibody under K_D-dependent conditions affects the fraction of antigen bound, the affinity might be a very influential variable in determination of the IC_{50} and EC_{50}. But interpretation of the results must be taken in context of the antibody affinities and experimental and *in vivo* antigen concentrations. In many cases, supraphysiological antigen concentrations may be needed in bioassays to allow for adequate analytical quantitation limits.

Examples of the effect of affinity on antibody potency are illustrated in Fig. 7.2. In Fig.7.2a, the IC_{50} for neutralizing mAbs to a soluble cytokine (present in cell culture at a 4 pmol L^{-1} concentration, reasonably reflective of *in vivo* concentrations) generated by immunization of XenoMouse animals (Mendez et al. 1997) is plotted as a function of antibody affinity, ranging from 100 fmol L^{-1} to 10 nmol L^{-1}. Since the concentration of antigen was less than the antibody K_D (with the exception of the femtomolar affinity antibody), a strong correlation existed between potency and affinity. In Fig. 7.2b, a supraphysiological concentration (2 nmol L^{-1}) of another soluble cytokine was required for the bioassay. In this case, the affinities of all the antibodies (ranging from 1 to 200 pmol L^{-1}), also generated in XenoMouse animals, were less than 1/10th the antigen concentration; thus the assay was conducted under stoichiometric rather than K_D-dependent conditions. As expected, no relationship existed between affinity and the IC_{50}. Variation in the IC_{50} reflected the intrinsic variability of assay. In this case, rank ordering

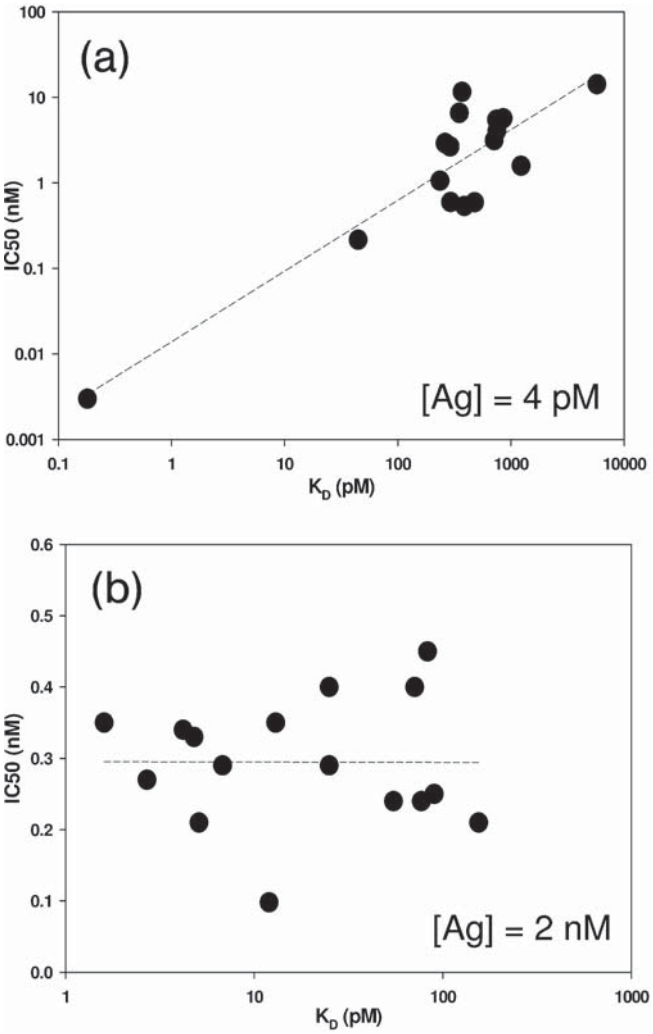


Fig. 7.2 Effect of antibody affinity and antigen concentration on antibody potency *in vitro* for two panels of monoclonal antibodies targeting cytokines. (a) In an experiment conducted under K_D-dependent binding conditions ([Ag] << K_D of most antibodies), a strong correlation of IC_{50} with K_D was observed. (b) In an experiment conducted under stoichiometric conditions ([Ag] >> K_D for all antibodies), no correlation of IC_{50} with K_D was observed because IC_{50} is dependent on the antigen concentration, not the K_D.

antibodies by IC_{50} might erroneously prioritize an antibody with low affinity and low *in vivo* potency.

In vitro assays should also be conducted under equilibrium conditions. In these assays, antigen and antibody are added prior to conduct of the assay, with a pre-incubation to allow antibody and antigen to reach equilibrium. However, if the

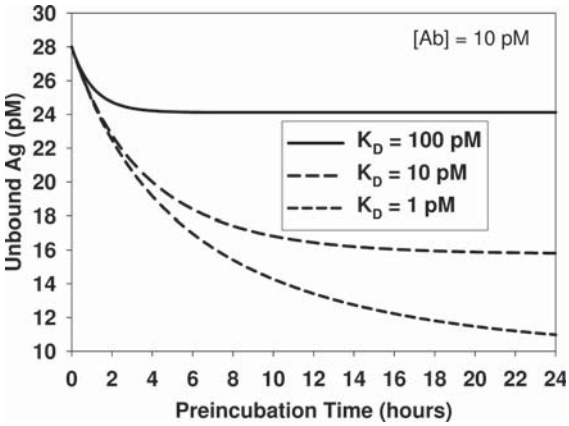


Fig. 7.3 Simulated effect of affinity on time to equilibrium for an antibody–antigen mixture. Under the particular conditions of this *in vitro* experiment ([Ag] = 28 pmol L^{-1}, [Ab] = 10 pmol L^{-1}), the time to reach equilibrium would be over 18 h for a 1 pmol L^{-1} affinity antibody.

k_{off} and the antigen concentration are very low, a long preincubation time may be required to reach equilibrium. If equilibrium for the high-affinity antibodies is not achieved during preincubation, the observed differences in potency might be diminished. Figure 7.3 demonstrates a simulation that was conducted to determine the optimum preincubation time for a bioassay comparing the potency of a panel of antibodies of known affinity in the presence of a fixed concentration of antigen. The time-course of unbound antigen concentration is shown as a function of incubation time and affinity. Over the range of antibody affinities, a minimum preincubation time of 18 h was determined to be necessary for binding to approach equilibrium when the antibody affinity was high. The simulations were supported by potency assays run with 1-h and 18-h preincubation times; potency differentiation between the antibodies was observed only after the 18-h preincubation.

7.3.2 Binding and Potency *In Vivo*

Most *in vitro* potency experiments are conducted under conditions where there is negligible production/input and degradation of antibody and antigen during the course of the experiment. In the *in vivo* situation, antibody is eliminated continuously and is usually dosed repeatedly, and antigen is continuously produced and degraded. Immune complexation of antigen will usually alter the pharmacokinetics of the antibody and/or the antigen (Tabrizi et al. 2006). When a soluble antigen binds to an antibody *in vivo*, the bound antigen will generally take on the elimination kinetics of the antibody. If an antibody binds to a cell membrane antigen, the bound antibody can be eliminated at the internalization

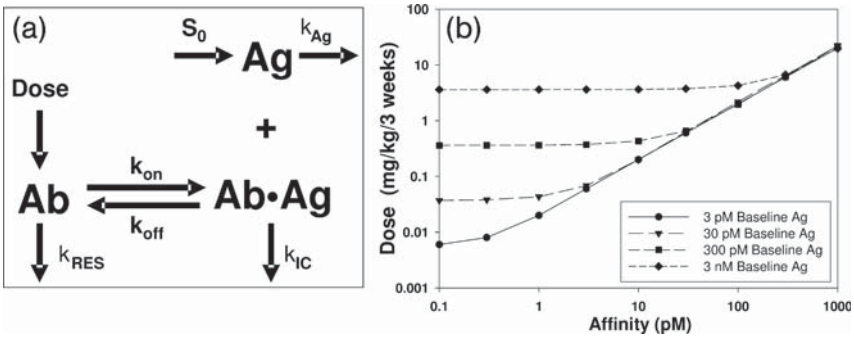


Fig. 7.4 Simulation of the maintenance dose of antibody required to suppress unbound concentrations of a soluble antigen in serum *in vivo* by 90% at steady-steady state prior to the next maintenance dose. (a) Kinetic model of Ag–Ab interaction *in vivo* used to simulate the interaction. S_0, production rate of Ag; k_{Ag} (rate constant for Ag elimination); k_{RES}, rate constant antibody elimination by the reticuloendothelial system; k_{IC}, rate constant for elimination of the immune complex. (b) The simulation, conducted as a function of affinity and [Ag], predicts that increased affinity will improve antibody potency until K_D falls below 1/10th the baseline [Ag].

rate of the antibody–antigen complex; if the antigen is widely expressed and the internalization rate is rapid, the antigen may create a saturable sink for antibody elimination.

Despite the complexity of the antibody and antigen kinetics *in vivo*, the effect of affinity on antibody potency is similar to that observed *in vitro*. Figure 7.4a illustrates a simple kinetic model of antibody and antigen interaction within the plasma pool. For simplicity, a "one-compartment" pharmacokinetic model is assumed for antibody and antigen. The model in Fig. 7.4a can be described by differential equations for unbound antibody, unbound antigen, and the antibody–antigen complex. In Fig. 7.4B, these equations have been used to calculate the dose of antibody, administered every three weeks, needed to decrease unbound concentrations of antigen at predose, steady-state levels of antibody, by 90%. Simulations were conducted assuming a soluble, intermediate clearance rate antigen with steady-state baseline concentrations ranging from $3\,\text{pmol}\,\text{L}^{-1}$ to $3\,\text{nmol}\,\text{L}^{-1}$ in plasma. Clearance of the immune complex was assumed to equal the reticuloendothelial clearance of antibody in absence of an antigen interaction. As illustrated in Fig. 7.4b, a point is reached where further improvements in affinity do not produce additional improvements in potency. Similar to the *in vitro* results described previously, this potency ceiling occurs when the affinity is reduced to about 1/10th the concentration of antigen. Therefore, for any antibody design goal aimed at maximizing the binding potency of a therapeutic antibody *in vivo*, the pathophysiological concentrations of antigen in the relevant biophase should be considered.

When a saturable antigen sink is present, high-affinity antibodies, under certain conditions, can be cleared at a faster rate than low-affinity antibodies. To

further illustrate the importance of the antigen concentration when considering the required affinity of the antibody for the antigen, a kinetic model was established (similar to the one illustrated in Fig. 7.4a) that described the bimolecular interaction of an mAb with a membrane-bound antigen, where the antibody exhibits two-compartment distribution kinetics and linear elimination through the reticuloendothelial system (k_{res}) and nonlinear elimination (k_{IC}) through a sink provided by a cell membrane antigen. Simulations were conducted after administering a single intravenous dose of antibody, and the model assumed a rate constant k_{int} of $0.017\,min^{-1}$ (internalization half-life of 40 min) for receptor internalization and a total receptor concentration of $1\,nmol\,L^{-1}$. The model was used to simulate antibody pharmacokinetics (Fig. 7.5a) and the concentration of unbound antigen (Fig. 7.5b) in serum for antibodies with different affinities. As seen in Fig. 7.5a, the serum half-life of the high-affinity antibodies becomes shorter than that of the low-affinity antibody when the suppression of unbound antigen is less than ~90% (around 1.5 days after administration of the single dose). However, the high-affinity antibodies produced greater suppression of unbound antigen when the antibody was present at saturating levels. As expected, the unbound antibody and unbound antigen profiles became nearly identical when the K_D of the antibody fell to 1/10th the antigen concentration or below. If subsaturating concentrations of antibody are required clinically, as might occur for some agonist antibodies or antibodies with a dose-limiting toxicity, then a lower affinity antibody might theoretically present a more favorable pharmacokinetic/pharmacodynamic profile. Under conditions of multiple dosing achieving saturating levels of antibody, high-affinity antibodies are generally expected to be advantageous with respect to dose potency.

7.4 High-Throughput Selection of Hybridomas Secreting High-Affinity Antibodies

The immunization and hybridoma selection strategies for antibodies derived from hyperimmunized animals vary depending on antigen type and antibody design criteria. A commonly used antibody screening process is described below for soluble and cell surface antigens.

7.4.1 Soluble Antigens

For soluble protein and peptide antigens, animals are immunized with the antigen or keyhole limpet hemocyanin (KLH)-conjugated antigen; hybridomas are generated and screened using an enzyme-linked immunosorbent assay (ELISA). The antigen is captured on the surface of ELISA microtiter plates. Antibodies in hybridoma supernatants bound to the antigen on the plates are detected with horseradish peroxidase (HRP)-conjugated antibodies against the immunoglobulin constant region. The hybridomas secreting antibodies that generate a

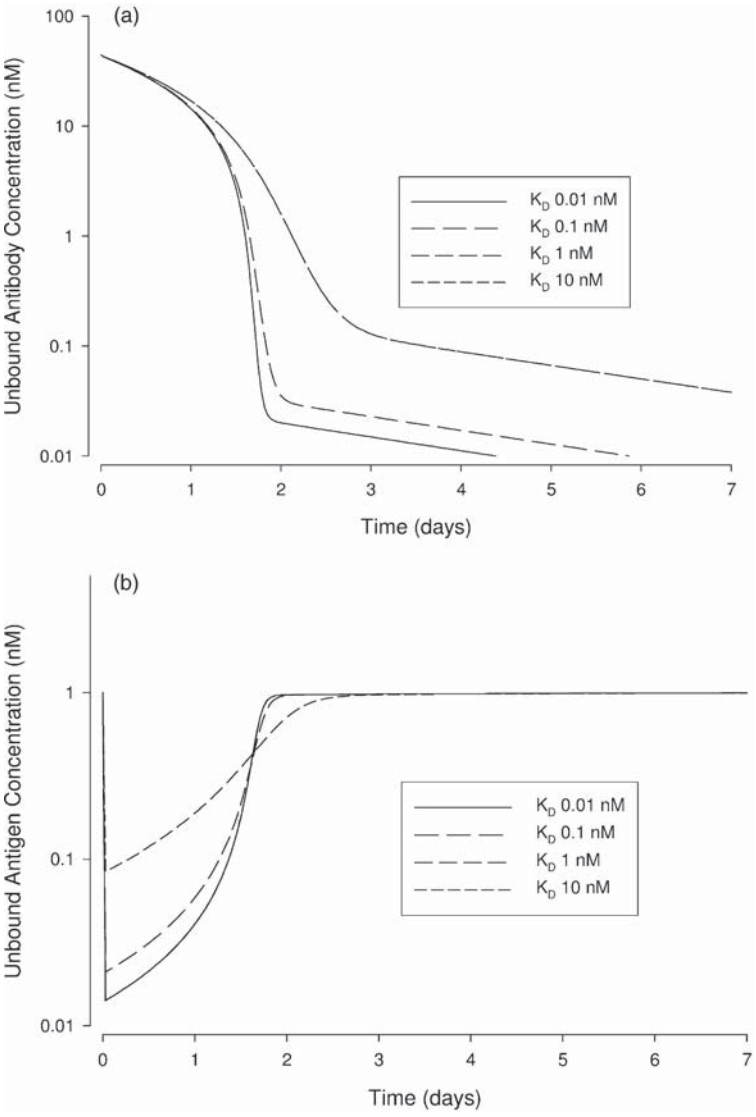


Fig. 7.5 Simulated effect of antibody affinity on (a) antibody pharmacokinetics and (b) unbound antigen levels for an antibody targeting an internalizing, cell membrane receptor. The simulations predict, under the conditions of these simulations, that higher affinity antibodies will have a shorter half-life at subsaturating concentrations, but higher receptor occupancy at saturating concentrations.

positive OD signal in ELISA are cultured continuously and the supernatants are tested again using the same ELISA format to confirm the antibody secretion. In addition, an ELISA assay using a closely related protein or an irrelevant protein, such as bovine serum albumin (BSA), is also performed in order to assess the specificity of antigen binding. Antibodies that bind to the antigen only, not to other proteins, are considered specific for the antigen.

A sophisticated immunization procedure and a significantly improved hybridoma technology can routinely result in few hundreds of antigen-specific, high-affinity mAbs. Therefore, more *in vitro* assays are needed to select the top antibodies for affinity measurement using Biacore or KinExA technologies (discussed in the next section) and ultimately to select the lead antibody candidates for *in vivo* efficacy determination. Typically, epitope binning, affinity ranking, and activity-based assays are utilized for this purpose.

Antibodies with different antigen-binding epitopes may show different potency and specificity profiles *in vivo*. Therefore, it is advantageous to select the mAb candidates that represent various antigen-binding specificities for *in vivo* testing. The conventional method to determine the antigen-binding epitope for an antibody is epitope mapping. However, this method is very time-consuming. To overcome this drawback, an epitope-binning methodology was developed to efficiently group antibodies with distinct antigen-binding specificity (Jia et al. 2004). This method utilizes Luminex bead-based multiplexing technology (Fulton et al. 1997) to detect antibody competition for antigen binding and enables sorting of a large panel of mAbs into different bins based on cross-competition. In this assay, each mAb is captured by an anti-IgG mAb coupled to a unique spectrally encoded bead from 100 commercially available Luminex beads. A Luminex 100 instrument quantified the extent of binding competition of any given mAb to an antigen against up to 99 other antibodies in a single assay. Although this assay does not identify the amino acid sequence in an antigen for an antibody binding as determined by epitope mapping, it provides valuable information to ensure selection of antibodies with different antigen-binding specificities for further testing.

Although Biacore and KinExA assays provide accurate affinity measurements, the complexity of these assays limits the number of antibodies that can be tested. To overcome these hurdles, an affinity ranking methodology using Luminex technology was developed. In this assay, three concentrations of a biotinylated antigen are each captured on a unique streptavidin-coupled spectrally encoded Luminex bead. MAb bound at four concentrations to each of these antigen beads are labeled with phycoerythrin (PE)-conjugated antibody against the IgG constant region, and the fluorescence associated with each of the beads is quantified with a Luminex 100 instrument. The relative fluorescence on each bead is proportional to the amount of bound antibodies to the antigen in the linear range of the binding curve. For a panel of mAbs tested in the same assay, the rank order of the amount of bound antibodies at a selected concentration in the linear range of the curve to a given concentration of antigen is generally correlated to the rank order of their affinities. This method provides a medium-throughput procedure to rank a large panel of mAbs based on their affinities and enables a quick

selection of the antibody candidates for affinity measurement by Biacore or KinExA.

7.4.2 Cell Surface Antigens

A number of cell surface proteins have proven to be valid therapeutic targets. However, antibody generation for this class of proteins, especially membrane multi-spanning proteins, can be more challenging than soluble antigens. Challenges exist for both immunogens and hybridoma selection processes. For this type of protein, immunogens can be a purified extracellular domain of an antigen, cell membrane preparations, or whole cells. A purified extracellular domain (ECD) of an antigen is an ideal immunogen to use; however, the ECD is not always available and may not have the native conformation. Although cell membranes or whole cells generally present cell surface proteins in their native conformations, specificity issues may arise with the use of membranes and cells as immunogens due to the existence of other membrane proteins. If the protein of interest was not the most abundant or the most immunogenic on the cell surface, generation of antibodies against these antigens may be difficult.

A conventional hybridoma screening method, ELISA, requires a purified ECD of the antigen, which may not be available or may not be conformationally similar to the cell membrane antigen. Therefore, a conventional ELISA might not be optimal for hybridoma selection for cell surface antigens. An ELISA assay using cell membranes or whole cells often gives high background, high coating variability, and low signal, resulting in a suboptimal assay. Fluorescence-activated cell sorting (FACS) analysis provides advantages compared to ELISA for detecting antibody binding to cell surface antigens. However, this methodology requires multiple washing steps and a complicated data collection/analysis process, not suitable for analyzing large number of hybridomas in a high-throughput manner. To overcome these drawbacks, a high-throughput cell-based antibody–antigen binding assay using fluorometric microvolume assay technology (FMAT) (Miraglia et al. 1999) has been developed. This assay provides high detection sensitivity that is comparable to FACS and is amenable to high-throughput hybridoma screening.

In the FMAT assay, antibodies in hybridoma supernatants bound to an antigen on the surface of the antigen-expressing cells are detected by a Cy5-conjugated F(ab′)2 anti-IgG, Fc-specific antibody. A FMAT 8200 HTS instrument is used to measure the fluorescence associated with the cells. In order to distinguish antibodies that bind to the antigen from those that bind nonspecifically to cells, the antibody binding is assessed using both antigen-expressing and parental cells. The antibodies binding specifically to antigen are identified as those that bind to antigen-expressing cells only, and nonspecific antibodies are identified as those that bind to both antigen-expressing and parental cells.

The key factor for a good FMAT assay is the antigen-expression level on the cells. Although FMAT assay can detect antibody binding to cells expressing an antigen at 9000 copies per cell, the assay performance is improved with a cell line

expressing an antigen at 30 000 copies per cell or above. One important step in FMAT assay optimization is to reduce the background. A high background is generally due to low cell viability or nonspecific internalization leading to antibody trapping inside cells. Cell viability can be improved by modifying cell culture and cell detaching conditions. Internalization can be minimized by treatment of cells with sodium azide.

A hook effect is observed with FMAT assay due to high concentration of antibodies in solution saturating Cy5-anti-IgG antibodies. This can potentially cause failure to identify antigen-binding antibodies when their concentrations in supernatants are extremely high. A strategy to address this issue is to test supernatants in more than one dilution. However, due to the broad, detectable range of antibody concentrations by a FMAT assay, the false negative rate estimated using a panel of known positive antibodies is very low and might not be a significant issue in hybridoma screening.

Incorporation of cell-based antibody binding screening using FMAT in a hybridoma selection process for cell-surface antigens has demonstrated significant improvement in finding antibodies that show antigen neutralization. For multispanning membrane proteins, FMAT generally identifies significantly more neutralizing antibodies than ELISA.

One disadvantage observed with FMAT assays is a higher false positive rate compared with FACS due to a no wash format of FMAT assays. To eliminate these false positives, the positive hybridomas identified by FMAT are confirmed by a FACS assay. Hybridoma supernatants are incubated with antigen-expressing cells and parental cells. The cell-associated antibodies are detected with Cy5-conjugated anti-IgG antibody. The fluorescence associated with the cells is analyzed with a FACSCalibur instrument. Antigen-specific antibodies are identified as those that only bind to antigen-expressing cells, but not to parental cells. Nonspecific antibodies are those that bind to both antigen-expressing and parental cells.

Antibodies that bind to cell surface antigen specifically are then assessed for their affinities using a FACS-based affinity determination methodology described in the next section. In addition, the functional activities of these antibodies are also determined in a panel of activity assays. Functional activity and affinity *in vitro* are used to select the top antibodies for *in vivo* testing.

7.5
Kinetic and Equilibrium Determinations of Antibody Affinity

7.5.1
Biacore Technology

Biacore technology is based on a surface plasmon resonance biosensor and is the premier biophysical method currently used for measuring antigen–antibody affinity. With Biacore one reactant is covalently immobilized to a biosensor surface

and the other binding partner is flowed across the surface, and the binding of the reactants is followed in real time by surface plasmon resonance (Karlsson and Falt 1997; Morton and Myszka 1998; Drake et al. 2004). Frequently, Biacore is criticized for not giving accurate or "solution-phase" rate constants and hence an inaccurate equilibrium constant for a biomolecular interaction since one binding partner is immobilized to a biosensor surface while the other reactant is flowed across the surface. This criticism may be warranted if the most optimal and advanced experimental design and data-processing techniques are not utilized in the conduct of a Biacore experiment, which is often the case observed in the Biacore literature. Many published articles do not give the reader enough information to reproduce the given experiment or even to critically evaluate whether the experiment was conducted appropriately (Myszka, 1999b; Rich and Myszka 2000, 2005). However, as nicely pointed out by Van Regenmortel (2003), Biacore results are often artifactual because the scientists conducting the experiment do not use the most advanced experimental design and processing techniques to start with. As a result, the Biacore instrument is blamed for giving inaccurate kinetic results when the real cause of the erroneous data is the "unprofessional" use of the technology.

One of the pitfalls of performing a Biacore experiment results from the highly automated nature and user-friendliness of the Biacore instrument, which has a tendency to lull users into never learning or forgetting the basic principles of physical chemistry that apply to the chemical binding reaction taking place in the flow cell (Van Regenmortel 2003). Understanding the basic principles of physical chemistry and how they relate specifically to the particular biophysical technique being undertaken is not unique to Biacore, but really applies to any physiochemical methodology, even KinExA technology as will be discussed later. In addition, the physical chemistry of the particular biomolecular interaction, in this case a bivalent monoclonal antibody and a monovalent or multivalent antigen, also needs to be considered in setting up a Biacore or KinExA experiment in order to yield accurate kinetic and thermodynamic results. Biacore does, indeed, give accurate kinetic rate constants and equilibrium constants that are very similar to values derived from solution-phase kinetic and equilibrium methods when optimal and advanced Biacore data collection and processing techniques are implemented (Day et al. 2002; Drake et al. 2004).

Biacore also is useful in screening mAbs for relative and absolute affinities even from crude mixtures like hybridoma supernatants (Canziani et al. 2004). The K_D values resulting from these Biacore experiments, where monoclonal antibody was captured on the biosensor surface from hybridoma supernatant and one concentration of purified antigen was flowed over the surface, were accurate and reliable because the same kinetics were observed with purified monoclonal antibody using standard Biacore methodology (Canziani et al. 2004).

In general, many therapeutic antibodies have equilibrium dissociation constants (K_D) that are less than 100 pmol L^{-1}. Measurement of on-rates (k_{on}), off-rates (k_d), and K_D values for mAbs possessing very high affinities (<100 pmol L^{-1}) can be challenging for three unique reasons: (1) the time for the antigen–antibody

mixture to reach equilibrium can be very long, on the order of days; (2) usually, the k_d for such a tight complex is extremely low, requiring long periods of data collection to discern enough information to predict complex stability; and (3) in cases where the k_d is easily measurable ($>5 \times 10^{-4}\,s^{-1}$), the k_a can be very fast, greater than 1×10^7 ($L\,mol^{-1}$) s^{-1}. When performing a Biacore experiment with any multivalent molecule like a mAb it is important to immobilize the bivalent antibody to the surface and to flow the monovalent antigen as shown in Fig. 7.6a. Many times Biacore experiments are performed in the incorrect orientation shown in Fig. 7.6b where antigen is immobilized to the biosensor surface and bivalent monoclonal antibody is flowed. This orientation quite often leads to sensorgrams that are described by complex kinetic interaction models.

In designing a Biacore experiment, it is absolutely crucial to stay away from orientations that may introduce complexity into the sensorgram data. Interpretation of complex sensorgram data and selection of the correct complex interaction mechanism that describes the complex kinetic data can be painstakingly difficult and take an inordinate amount of time and resources. The third scenario shown in Fig. 7.6c is a plausible Biacore orientation with which to measure monoclonal antibody binding kinetics, but as observed in reality quite frequently, it is difficult to achieve a low enough surface capacity of antigen to completely rid the sensorgrams of complexity and still observe a reasonable binding signal when antibody is flowed.

One interesting dilemma that occurs when measuring antigen–antibody kinetics by Biacore is when the antigen is multivalent like the antibody. What is the best Biacore experimental orientation for a situation like this? When both binding reactants are multivalent, the chance of observing complex kinetic sensorgrams increases dramatically owing to the possible multiple binding steps involved in the interaction as shown in Fig. 7.6b. The best way to optimize the orientation for a Biacore experiment involving two multivalent molecules is to set the experiment up as given in Fig. 7.6c. As discussed earlier, immobilizing a small amount

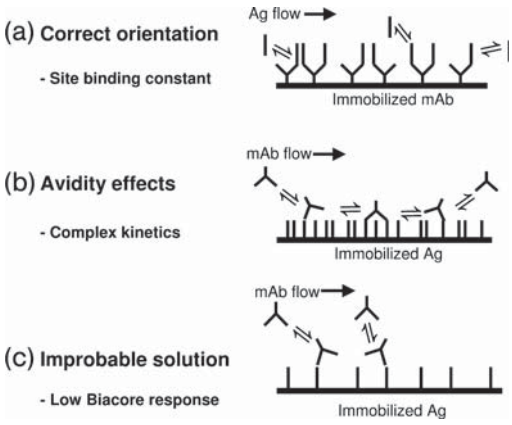


Fig. 7.6 Biacore experimental designs for measuring antigen–antibody interactions.

of multivalent antigen, or for that matter even bivalent monoclonal antibody, to the biosensor surface in no way necessarily guarantees sensorgrams that can be described by a simple 1:1 binding mechanism when both binding partners are multivalent. However, a low surface capacity greatly hedges the probability of collecting sensorgrams that fit a basic 1:1 model. Even in an experiment involving two multivalent binding partners, like in the case of a multivalent antigen and a bivalent antibody, in which sensorgrams are collected that fit a 1:1 interaction model, it is still ambiguous whether the K_D determined corresponds to a stoichiometric (macroscopic) binding constant or an intrinsic site (microscopic) binding constant. For example, with two bivalent molecules two binding sites of one molecule binding to two binding sites of the other molecule fit a 1:1 binding model potentially as well as one site of one molecule binding to one site of the other. The same argument applies to higher order complexes too (i.e. a bivalent antibody binding to a trivalent antigen, etc.).

From a biophysical standpoint, it is always best to determine the site binding dissociation equilibrium constant since this is an intrinsic binding constant that is inherent to the complex. Macroscopic or stoichiometric constants do not pertain to any particular binding site, but rather only provide information from a thermodynamic viewpoint. If the flowing bivalent binding partner is crosslinking two different multivalent immobilized ligands on the biosensor surface, then the stoichiometric binding constant really becomes an avidity constant. Obviously, avidity binding constants can change as a function of the amount of immobilized ligand on the biosensor surface, which in a sense yields an equilibrium "inconstant" since this so-called binding "constant" potentially could change with different surface densities. While it is attractive to argue from a biological standpoint that the avidity constant is the more meaningful equilibrium constant to measure since it may mimic the *in vivo* interaction, this argument breaks down quickly when scrutinized. For example, how is it possible to know experimentally that the biosensor surface created has the same antigen molecular density as found on the target tissue *in vivo*? It would appear that any avidity constant that was desired could be measured by making a biosensor surface with varying multivalent antigen capacities.

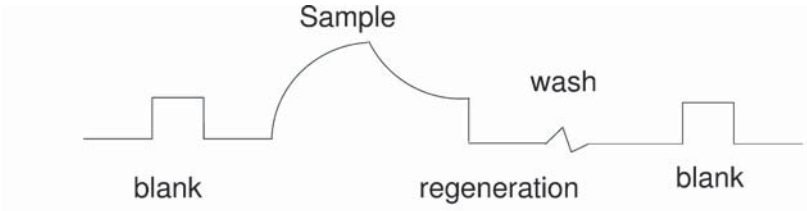
One other type of complex, in contrast to the "crosslinked" variety mentioned above, could occur between a multivalent antigen (immobilized on the surface) with antibody flowing wherein both sites of the antibody bind to one distinct antigen molecule. This type of interaction would result in a more legitimate avidity constant in a biophysical sense, though not necessarily from a biological perspective, because this avidity constant would not vary as a function of antigen capacity on the biosensor surface.

In summary, when both binding molecules are multivalent, the binding parameters determined by Biacore (from sensorgrams that truly are described by a simple 1:1 interaction model and are not complex) become ambiguous since two possible binding constants, site and stoichiometric (avidity), may be measured depending on the structure of the complex formed on the biosensor surface. In cases where sensorgrams with antigen–antibody complexes cannot be collected

that fit a simple 1 : 1 interaction model (after eliminating all other human-induced causes of kinetic complexity, *vide infra*), it is advisable to change biophysical methodology and turn to an equilibrium method like KinExA (to be discussed in the next section).

Regardless of whether the K_D measured is a site or avidity K_D, most researchers in the therapeutic antibody field find this measurement crucial because it allows selection of the monoclonal antibody candidates with the required affinities for further development.

Myszka (1999a) has written an excellent tutorial on how to rigorously design a Biacore experiment and how to optimally process biosensor data to ensure the highest quality kinetic results. Figure 7.7 gives selected highlights from Myszka's paper (Myszka 1999a) on improving biosensor analysis. To ensure the highest quality biosensor results it is important to perform microfluidic washing steps before and after each antigen injection. It is also of utmost importance that the Biacore is scrupulously cleaned at regular intervals and before beginning each experiment (Myszka 1999a). Antibody should always be immobilized at a surface capacity that yields a maximum resonance unit response (R_{max}) that is no greater than 50–100 RUs to avoid mass transport artifacts. High antigen flow rates are also preferable to minimize any mass transport artifacts that may be present during the experiment. A wide concentration range (at least 10- to 100-fold above and below the K_D of the interaction is ideal) of antigen should be injected randomly in duplicate or triplicate. It is worth noting that pragmatically antigen concentrations that are 10- to 100-fold above and below the K_D are often impossi-



- Always inject sample buffer at the start and end of a cycle in order to wash out the microfludics system.
- Ligand surface density should give an R_{max} less than 50 – 100 RU to avoid or minimize mass transport artifacts, steric hindrance, or aggregation.
- If the ligand is an antibody, this should always be immobilized or captured on the biosensor surface to avoid avidity effects.
- Always use a high flow rate like 100 µl/min to minimize any mass transport considerations, and to deliver a more consistent analyte plug across the biosensor chip.
- Always replicate in duplicate, at least, and preferably, in triplicate all sample injections over a wide concentration range. Also, randomize the order of injection of all samples.
- Double reference all sensorgrams to correct for non-specific binding, bulk refractive index changes, and systematic instrument noise.
- Globally fit nonlinearly all kinetic data utilizing any specialized biosensor software.

Fig. 7.7 Suggested steps for rigorous biosensor methodology (adapted from Myszka 1999a).

ble to achieve owing to nonspecific binding interactions at high concentration and loss of signal at very low antigen concentration, especially for antigen–antibody complexes possessing picomolar affinities. In these cases, the antigen concentration range should be as wide as experimentally possible. In addition, if a small amount of decay is seen in the dissociation phase of the sensorgrams for a tight antigen–antibody complex (K_D <100 pmol L^{-1}), indicating the complex has a slow k_{off} (<5×10^{-4} s^{-1}), several additional relatively high antigen concentrations and buffer blanks should be injected and the dissociation phase followed for ~4 h or longer (Drake et al. 2004). Using this "long k_{off}" dissociation methodology, it is possible to measure a k_{off} ~10^{-5} s^{-1}, and if longer dissociation phases are collected (4–24 h), a k_{off} ~10^{-6} can be determined. It has been shown that this long k_{off} methodology results in reliable binding parameters for antigen–antibody complexes when compared with solution-phase equilibrium methods (Drake et al. 2004).

If the association phase of a biomolecular interaction is extremely slow or the K_D is extremely tight (being in the picomolar range, thus requiring very low analyte concentrations to be flowed in order to be near the K_D of the interaction), it is also possible to follow the association phase for hours, instead of the normal few minutes, when specialized Biacore procedures are invoked (Navratilova et al. 2005).

Sensorgram data should always be double-referenced. Double-referencing many times transforms a singly referenced sensorgram data set that is not capable of being fit into a data set that fits easily to a simple 1 : 1 interaction model. The importance of double-referencing sensorgram data cannot be emphasized enough, especially when low surface capacities are used. Lastly, all processed sensorgram data should be globally fit nonlinearly using any readily available specialty biosensor software program. It should also be noted that if complex sensorgrams are observed, it is not scientifically justified to fit the data to a complex interaction model (referred to as "model surfing") without a plethora of additional biosensor and extra-biosensor kinetic and equilibrium data that supports the complex binding mechanism. Many artifacts can cause complex sensorgram data such as: impure monoclonal antibody, impure antigen, heterogeneity introduced by immobilization chemistry, badly processed data (poorly y- or x-aligned sensorgrams or non-double-referenced sensorgrams), nonspecific binding to the actual immobilized molecule or to the carboxylmethyldextran surface matrix that was not properly corrected for in data processing, or even a dirty or contaminated Biacore instrument.

7.5.2 KinExA Technology

KinExA (kinetic exclusion assay) technology differs from Biacore in that it is a solution-based method and requires a secondary fluorescent reporter molecule. The KinExA instrument is a flow spectrofluorimeter in which equilibrated solutions of an antigen–antibody complex are flowed through a bead pack with

immobilized antigen (Blake et al. 1999; Darling and Brault 2004). Detection of free monoclonal antibody from the equilibrated solution once flowed through the resin is accomplished with a secondary fluorescently labeled species-specific polyclonal antibody. The percentage of free monoclonal antibody versus the total antigen titrated into each equilibrated solution is fit to a standard 1:1 equilibrium model to yield a K_D for the given interaction. KinExA is ideally suited for measuring antigen–antibody affinities since it requires a secondary fluorescent reporter molecule of which many exist for mAbs. The sensitivity of fluorescence detection is also desirable for measuring interactions with very tight equilibrium dissociation constants.

The KinExA instrument is also capable of directly measuring $k_{on,}$ by following the decrease of free antibody as a function of time as an antigen–antibody complex approaches equilibrium, referred to as the "direct" method. Another alternative procedure for measuring the k_{on} with KinExA is to determine free antibody concentration as a function of added antigen known as the "injection method." The k_{off} is not measured directly in KinExA but rather is calculated from the product of $K_D \times k_{on}$. The timescale in which k_{on} is measured by KinExA (during a "direct" injection method) versus Biacore is drastically different. The longest injection time usually possible on a Biacore is about 2.5–10 min whereas with KinExA, kinetic data points are usually only collected every 5–15 min for a total time of hours until equilibrium is reached. For more specifics on these equilibrium and kinetic methods, please refer to the articles by Blake et al. and Darling and Brault (Blake et al. 1999; Darling and Brault 2004).

The shape of KinExA equilibrium titration curves is not dependent on the absolute antibody-binding site concentration but rather on the ratio of antibody-binding site concentration to the K_D of the interaction. If the antibody-binding site concentration is much higher than the K_D, then the curve is insensitive to the K_D and changes shape as a function of the active antibody-binding site concentration and is called an antibody-controlled curve. If the antibody-binding site concentration is near or slightly above the K_D, then the resulting curve is sensitive to the K_D and has little active antibody-binding site concentration information present in it and is referred to as a K_D-controlled curve. Both types of equilibrium titration curves can be fit simultaneously in a dual curve analysis (or *n*-curve analysis with "*n*" multiple curves) to yield reliable values of the active antibody-binding site concentration and the K_D. The most rigorous values for K_D and active antibody-binding site concentration result from an *n*-curve analysis since, obviously, it is more difficult to fit *n* curves globally than it is to fit each curve locally. In addition, there is more data with which to calculate the binding parameters from an *n*-curve analysis. Multiple (*n*) curve analysis also provides extra assurance that the equilibrium titration data truly do, indeed, fit a simple 1:1 equilibrium model.

With both types of titration curves being fit simultaneously, it is easier to see deviations from the theoretical model than in a single curve analysis. However, if the active antibody-binding site concentration is chosen correctly (discussed below) for a K_D-controlled titration, then a single curve analysis, in general,

results in an acceptable value for K_D that is near identical to the K_D determined from an *n*-curve analysis. This single curve titration approach can be useful for obtaining replicate measurements quickly for the K_D of an antigen–antibody interaction so accurate 95% confidence limits can be calculated. It is important to realize that the 95% confidence intervals calculated for the fitted parameters by the KinExA software reflect the precision of the parameters derived from the data fitting, and do not represent the 95% confidence intervals for replicate measurements.

Several major considerations need to be realized to design and perform high-quality KinExA equilibrium and kinetic experiments. First, antigen–antibody mixtures must be given ample time to equilibrate. For antigen–antibody complexes with picomolar K_D values (usually corresponding to complexes with extremely slow k_{off}) this means days to weeks are needed to allow solutions to equilibrate (Drake et al. 2004). Secondly, solution volumes in KinExA are much larger than those in Biacore experiments so "old-fashioned" analytical volumetric technique is required for the highest quality equilibrium and kinetic results from KinExA. Thirdly, an active antibody-binding site concentration should be used that is no more than 3-fold above the K_D of the interaction for collection of a K_D-controlled titration. With equilibrium titration data of exceptional quality, it is possible to have the active antibody-binding site concentration be set at 10-fold above the K_D and still determine a satisfactory K_D. Fourth, for antibody-controlled curves it is best to work at antibody-binding site concentrations greater than or equal to 10-fold above the K_D. Fifth, for "direct" kinetic experiments, select antigen and antibody-binding site concentrations so that ~80% of the antibody-binding sites are bound at equilibrium. Also, in a "direct" kinetic experiment choose antigen and antibody-binding site concentrations that will allow enough exponential decay of % free antibody-binding site concentration as a function of time for accurate determination of k_{on}. Sixth, for an "inject" kinetic experiment, use a range of initial antigen concentrations that gives 20–100% antibody complexation with a given constant antibody-binding site concentration for the particular mixing time that has been chosen for the KinExA instrument.

One point important to consider is that even with KinExA technology, as mentioned earlier for Biacore, the same ambiguity could potentially exist in the interpretation of an equilibrium constant for a multivalent antigen–bivalent antibody complex. However, the advantage of KinExA over Biacore in this particular situation is that binding data are collected after equilibrium has been reached, thus avoiding following any complex kinetic steps that can be difficult to model and fit in Biacore. KinExA experiments usually result in a titration curve that fits a 1:1 equilibrium model satisfactorily, allowing determination of a K_D for both monovalent and multivalent antigen–antibody complexes. Once again, as to what type of complex the determined K_D refers to in the case of a multivalent antigen–bivalent antibody complex is not readily known without other structural biological studies. KinExA technology has also been used to measure on-cell affinities directly, as described in the next section (Xie et al. 2005).

Certainly, it is not totally correct to imply that all rate constants and equilibrium constants determined by biosensors for antigen–antibody complexes will match exactly with solution-phase measurements, especially for interactions having picomolar K_D values as many therapeutic antibodies do. However, if optimized and advanced biosensor techniques are followed, reliable binding parameters can be determined for very tight interactions. Agreement between biosensor data and solution-phase methodology like KinExA is often found satisfactory when rigorous experimental protocols are utilized for both methodologies (Day et al. 2002; Drake et al. 2004). Biacore and KinExA complement each other nicely as biophysical methods for the development of therapeutic antibodies. This is illustrated by the higher throughput capability of Biacore and its ability to measure picomolar or less tight affinities, and the ability of KinExA to measure picomolar to subpicomolar affinities (Rathanaswami et al. 2005) more easily in certain circumstances than Biacore. It is also important to bear in mind when characterizing antigen–antibody interactions that analytical and biophysical techniques determine results only as reliable as their experimental design and data analysis methods.

7.5.3 Cell-based K_D Titrations

Obviously, biophysical techniques like Biacore and KinExA routinely require purified antigens in order to obtain high-quality kinetic and thermodynamic data. However, antigens like purified membrane receptors with transmembrane domains may not be amenable to easy purification, while a purified extracellular domain of a transmembrane receptor may still have the significant problem of losing native conformation and function. Fortunately, cell-based *in vitro* affinity determination methods can overcome the above-mentioned difficulties. One cell-based affinity method that has appeared recently uses KinExA technology to titrate an increasing concentration of cells expressing the antigen of interest into a constant concentration of antibody. By doing a dual curve titration and implementing an "antigen unknown" fitting process, the K_D of the interaction of the antigen–antibody interaction can be determined (Xie et al. 2005).

Another technique that is quite useful for determining K_D values for antibody binding to cell surface antigen is fluorescence-activated cell sorting (FACS) (Cardarelli et al. 2002). The FACS methodology usually consists of titrating monoclonal antibody into a constant amount of cells and allowing the binding reaction to come to equilibrium. Subsequently, the bound monoclonal antibody is labeled with a fluorescently-labeled secondary polyclonal antibody and the fluorescence signal, which is proportional to bound monoclonal antibody, is followed as a function of total monoclonal antibody concentration added. Analysis of the data is accomplished most often by a Scatchard plot.

Recently, Drake and Klakamp (2007) have developed a new four-parameter nonlinear equation and methodology based on the multiple, independent binding site equation that fits cell-based binding data much more rigorously than cur-

rently existing methods. This new equation and methodology allows a titration curve, whose shape is sensitive to either the active cell-based antigen concentration or the K_D of the interaction, to be fit accurately no matter what condition is controlling the shape of the titration curve.

With routine FACS-based Scatchard analysis, the K_D determined is not necessarily always accurate. The accuracy depends on the experimental conditions the titration is run under since this type of analysis always assumes total monoclonal antibody concentration titrated into the cells is equal to free monoclonal antibody concentration at equilibrium. Often this is true, however, for very tight interactions ($<100\,pmol\,L^{-1}$) this is sometimes not the case (Drake and Klakamp 2007).

Cell-based methodologies should only be utilized when the antigen is incapable of being highly purified for traditional biophysical characterization, or when it is known based on strong scientific arguments that the purified antigen is not as functional or potent as in its native state. One drawback of all cell-based methodologies is that the K_D determined could be a site-binding constant or avidity constant as discussed above for Biacore (to the first approximation the cell surface can be thought of as being analogous to the biosensor surface for this avidity discussion). For example, if a cell line expressing more antigen on its surface is used versus a cell line expressing less antigen, two different K_D values might be measured for the same antibody. What is the correct K_D and which cell line mimics most closely the real *in vivo* situation? Obviously, there are no easy answers to these questions. Hence, it is almost always more advantageous to measure antigen–antibody interactions with purified reagents in a well-defined system, unless as stated above, it is impossible to obtain pure or functional antigen preparations.

KinExA cell-based and FACS cell-based titrations, when used properly, add indispensable tools to the arsenal of biophysical methodologies available for studying the binding of mAbs to cell surface antigens.

7.6 Conclusions

Antibodies of high affinity are usually required to effectively neutralize or modulate antigens. Thus, affinity maturation has evolved into a critical element of the humoral response to antigen exposure. When dosing therapeutic antibodies, affinity is an important characteristic affecting the potency and dose requirement for a mAb directed against a given antigen target. The affinity and potency requirement for a therapeutic antibody should be considered on a case-by-case basis: in all cases, an affinity threshold exists beyond which further improvements in affinity will not produce further improvements in potency. Conversely, for a given target, selection of an antibody with inadequate affinity will result in clinical or commercial failure of the therapeutic product. In the selection process for a lead therapeutic antibody, the appropriate application of analytical methods

for biophysical characterization of antibody affinity is essential. Rational application of basic, kinetic principles governing antigen–antibody interactions can greatly facilitate the development of therapeutic antibodies.

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References

Blake, R.C., Pavlov, A.R., Blake, D.A. (1999) Automated kinetic exclusion assays to quantify protein binding interactions in homogeneous solution. *Anal Biochem* 272: 123–134.

Boder, E.T., Midelfort, K.S., Wittrup, K.D. (2000) Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc Natl Acad Sci USA* 97: 10701–10705.

Canziani, G.A., Klakamp, S., Myszka, D.G. (2004) Kinetic screening of antibodies from crude hybridoma samples using Biacore. *Anal Biochem* 325: 301–307.

Cardarelli, P.M., Quinn, M., Buckman, D., Fang, Y., Colcher, D., King, D.J., Bebbington, C., Yarranton, G. (2002) Binding to CD20 by anti-B1 antibody or F(ab′)(2) is sufficient for induction of apoptosis in B-cell lines. *Cancer Immunol Immunother* 51: 15–24.

Darling, R.J., Brault, P.A. (2004) Kinetic exclusion assay technology: characterization of molecular interactions. *Assay Drug Dev Technol* 2: 647–657.

Day, Y.S., Baird, C.L., Rich, R.L., Myszka, D.G. (2002) Direct comparison of binding equilibrium, thermodynamic, and rate constants determined by surface- and solution-based biophysical methods. *Protein Sci* 11: 1017–1025.

Defrance, T., Casamayor-Palleja, M., Krammer, P.H. (2002) The life and death of a B cell. *Adv Cancer Res* 86: 195–225.

Drake, A.W., Klakamp, S.L. (2007) A rigorous multiple independent binding site model for determining cell-based equilibrium dissociation constants. J. Immunol Methods, in press.

Drake, A.W., Myszka, D.G., Klakamp, S.L. (2004) Characterizing high-affinity antigen/antibody complexes by kinetic- and equilibrium-based methods. *Anal Biochem* 328: 35–43.

Foote, J., Eisen, H.N. (1995) Kinetic and affinity limits on antibodies produced during immune responses. *Proc Natl Acad Sci USA* 92: 1254–1256.

Foote, J., Eisen, H.N. (2000) Breaking the affinity ceiling for antibodies and T cell receptors. *Proc Natl Acad Sci USA* 97: 10679–10681.

Fulton, R.J., McDade, R.L., Smith, P.L., Kienker, L.J., Kettman, J.R., Jr. (1997) Advanced multiplexed analysis with the FlowMetrix system. *Clin Chem* 43: 1749–1756.

Hanes, J., Schaffitzel, C., Knappik, A., Pluckthun, A. (2000) Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display. *Nat Biotechnol* 18: 1287–1292.

Hoet, R.M., Cohen, E.H., Kent, R.B., Rookey, K., Schoonbroodt, S., Hogan, S., Rem, L., Frans, N., Daukandt, M., Pieters, H., van Hegelsom, R., Neer, N.C., Nastri, H.G., Rondon, I.J., Leeds, J.A., Hufton, S. E., Huang, L., Kashin, I., Devlin, M., Kuang, G., Steukers, M., Viswanathan, M., Nixon, A.E., Sexton, D.J., Hoogenboom, H.R., Ladner, R.C. (2005) Generation of high-affinity human antibodies by combining donor-derived and synthetic complementarity-determining-region diversity. *Nat Biotechnol* 23: 344–348.

Hoogenboom, H.R. (2005) Selecting and screening recombinant antibody libraries. *Nat Biotechnol* 23: 1105–1116.

Jia, X.C., Raya, R., Zhang, L., Foord, O., Walker, W.L., Gallo, M.L., Haak-Frendscho, M., Green, L.L., Davis, C.G. (2004) A novel method of multiplexed competitive antibody binning for the characterization of monoclonal antibodies. *J Immunol Methods* 288: 91–98.

Karlsson, R., Falt, A. (1997) Experimental design for kinetic analysis of protein-protein interactions with surface plasmon resonance biosensors. *J Immunol Methods* 200: 121–133.

Mendez, M.J., Green, L.L., Corvalan, J.R., Jia, X.C., Maynard-Currie, C.E., Yang, X. D., Gallo, M.L., Louie, D.M., Lee, D.V., Erickson, K.L., Luna, J., Roy, C.M., Abderrahim, H., Kirschenbaum, F., Noguchi, M., Smith, D.H., Fukushima, A., Hales, J.F., Klapholz, S., Finer, M. H., Davis, C.G., Zsebo, K.M., Jakobovits, A. (1997) Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. *Nat Genet* 15: 146–156.

Miraglia, S., Swartzman, E.E., Mellentin-Michelotti, J., Evangelista, L., Smith, C., Gunawan, I., Lohman, K., Goldberg, E.M., Manian, B., Yuan, P.M. (1999) Homogeneous cell- and bead-based assays for high throughput screening using fluorometric microvolume assay technology. *J BiomolScreen* 4: 193–204.

Morton, T.A., Myszka, D.G. (1998) Kinetic analysis of macromolecular interactions using surface plasmon resonance biosensors. *Methods Enzymol* 295: 268–294.

Myszka, D.G. (1999a) Improving biosensor analysis. *J Mol Recognit* 12: 279–284.

Myszka, D.G. (1999b) Survey of the 1998 optical biosensor literature. *J Mol Recognit* 12: 390–408.

Navratilova, I., Eisenstien, E., Myszka, D.G. (2005) Measuring long association phases using Biacore. *Anal Biochem* 344: 295–297.

Rathanaswami, P., Roalstad, S., Roskos, L., Su, Q.J., Lackie, S., Babcook, J. (2005) Demonstration of an *in vivo* generated sub-picomolar affinity fully human monoclonal antibody to interleukin-8. *Biochem Biophys Res Commun* 334: 1004–1013.

Rich, R.L., Myszka, D.G. (2000) Survey of the 1999 surface plasmon resonance biosensor literature. *J Mol Recognit* 13: 388–407.

Rich, R.L., Myszka, D.G. (2005) Survey of the year 2004 commercial optical biosensor literature. *J Mol Recognit* 18: 431–478.

Roskos, L.K., Davis G.C., Schwab, G.M. (2004) The clinical pharmacology of therapeutic monoclonal antibodies. *Drug Dev Res* 61: 108–120.

Schier, R., Marks, J.D., Wolf, E.J., Apell, G., Wong, C., McCartney, J.E., Bookman, M.A., Huston, J.S., Houston, L.L., Weiner, L.M. (1995) *In vitro* and *in vivo* characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. *Immunotechnology* 1: 73–81.

Sundberg, E.J., Mariuzza, R.A. (2002) Molecular recognition in antibody-antigen complexes. *Adv Protein Chem* 61: 119–160.

Tabrizi, M.A., Tseng, C.M., Roskos, L.K. (2006) Elimination mechanisms of therapeutic monoclonal antibodies. *Drug DiscovToday* 11: 81–88.

Van Regenmortel, M.H. (2003) Improving the quality of BIACORE-based affinity measurements. *Dev Biol (Basel)* 112: 141–151.

Wabl, M., Cascalho, M., Steinberg, C. (1999) Hypermutation in antibody affinity maturation. *Curr Opin Immunol* 11: 186–189.

Wu, X., Feng, J., Komori, A., Kim, E.C., Zan, H., Casali, P. (2003) Immunoglobulin somatic hypermutation: double-strand DNA breaks, AID and error-prone DNA repair. *J Clin Immunol* 23: 235–246.

Xie, L., Mark, J.R., Glass, T.R., Navoa, R., Wang, Y., Grace, M.J. (2005) Measurement of the functional affinity constant of a monoclonal antibody for cell surface receptors using kinetic exclusion fluorescence immunoassay. *J Immunol Methods* 304: 1–14.

Zahnd, C., Spinelli, S., Luginbuhl, B., Amstutz, P., Cambillau, C., Pluckthun, A. (2004) Directed *in vitro* evolution and crystallographic analysis of a peptide-binding single chain antibody fragment (scFv) with low picomolar affinity. *J Biol Chem* 279: 18870–18877.

8
Molecular Engineering III: Fc Engineering

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8.1
Mechanisms of Action of Monoclonal Antibodies

8.1.1
Introduction

Monoclonal antibodies have gained increasing importance as therapeutic reagents in clinical medicine over the last decade (Reichert et al. 2005), but many patients obtain only suboptimal responses to this expensive treatment. Since the market for antibodies is expected to become significantly more competitive over the next few years, there is an urgent need for optimized antibody constructs with excellent safety and high efficacy. For many years, efforts to improve antibodies concentrated on reducing their potential immunogenicity (Riechmann et al. 1988), leading to humanized or even fully human antibodies. Another approach aims to optimize antibodies by improving their effector functions. These effector functions are conceptionally divided into direct effects, which are mediated by the variable antigen binding region of the antibody, and indirect effects mediated by the constant Fc region. Although the fine specificity of monoclonal antibodies against the same target antigen (e.g. CD20) may control the immune effector mechanisms of individual antibodies (Cragg and Glennie 2004), efforts to improve effector functions concentrate on modulating the Fc region.

Sound knowledge of the relevant mechanisms of action of a particular antibody may have significant relevance for its clinical development, since this information may suggest ways to further enhance its efficacy (e.g. by antibody engineering). Thus, following a short discussion of candidate mechanisms, we describe modifications of the Fc region designed to improve specific effector functions triggered by therapeutic antibodies.

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8.1.2 Preclinical Evidence

For the majority of antibodies, a complex mixture of different mechanisms of action is supposed to cooperate *in vivo* (Glennie and van de Winkel 2003) (Fig. 8.1). *In vitro* assays have proved particularly helpful in demonstrating direct effects on tumor cells, such as apoptosis induction, inhibition or stimulation of cell signaling, blockade of growth factors and cell proliferation. However, Fc-mediated, indirect effects of antibodies, such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), can also easily be investigated *in vitro*. In addition, the relative contribution of different effector cell populations, such as mononuclear cells (MNC) and polymorphonuclear cells (PMN), can be assessed under these assay conditions. The relevance of these findings for clinical situations requires further study.

Several lines of evidence support the role of Fc-mediated effector functions for the efficacy of antibodies in animal models. Probably the most direct evidence for individual effector mechanisms is derived from genetically modified mice. For example, several therapeutic antibodies (rituximab, Herceptin (trastuzumab), Campath-1H, anti-CD2, anti-CD25) lost most of their efficacy in FcRγ chain knockout mice, which lack activating Fcγ receptors, while antibody efficacy was enhanced in mice lacking the inhibitory mouse FcγRIIb receptor (Clynes et al. 2000; Nimmerjahn and Ravetch 2006). The contribution of individual Fcγ receptors and the influence of different murine IgG isotypes was elegantly investigated

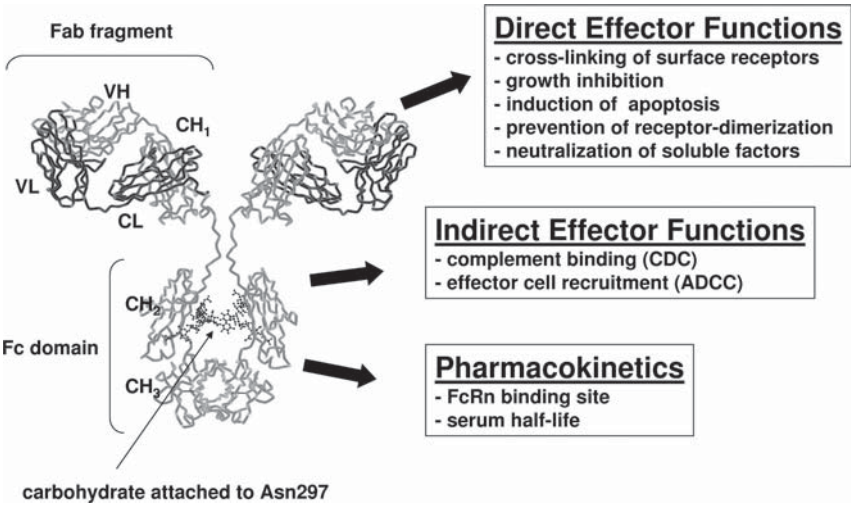

Fig. 8.1 Structure and function of IgG. Antibodies mediate different effector functions via distinct domains of the antibody molecule. Antibody model: computer-generated model structure of human IgG1 (Clark 1997). Dark gray: heavy chains; light gray: light chains.

using matched sets of antibody isotypes in mice deficient for select Fcγ receptors. (Nimmerjahn and Ravetch 2005). The role of the complement system for rituximab efficacy was assessed (e.g. in $C1q^{-/-}$ mice). Here, the response of a human CD20-transfected, complement-sensitive cell line to rituximab therapy was significantly diminished in $C1q^{-/-}$ mice compared to wildtype mice (Di Gaetano et al. 2003). However, when syngeneic B cell depletion by anti-mouse CD20 antibodies was investigated, antibody efficacy was diminished in $FcR\gamma^{-/-}$, $Fc\gamma RI^{-/-}$, or $Fc\gamma RIII^{-/-}$ mice, but not in $C3^{-/-}$, $C4^{-/-}$, or $C1q^{-/-}$ mice, indicating that under these conditions Fc receptor-mediated mechanisms predominate (Uchida et al. 2004). Induction of a tumor-directed immune response after antibody therapy has been reported in animal models (Clynes et al. 1998), but clinical examples of this probably ideal mechanism of action have been rare so far.

8.1.3 Clinical Evidence

Our knowledge about the clinically relevant mechanisms of action for monoclonal antibodies is rather limited. Early clinical studies with isotype switch variants of the rat CD52 antibody Campath-1H indicated that the rat IgG2b version of the antibody was more effective in depleting human B cells than its rat IgM and IgG2a counterparts. Interestingly, all three isotypes similarly activated human complement, but only the rat IgG2b version efficiently triggered ADCC (Dyer et al. 1989). Most of the more recent information is derived from studies with rituximab. Here, several lines of evidence point to an important role of Fc receptors. For example, clinical response to rituximab therapy correlated with the number of natural killer (NK) cells in the peripheral blood of lymphoma patients (Janakiraman et al. 1998). Furthermore, response to rituximab correlated with the expression of certain Fc receptor alloforms. Preclinical studies have demonstrated that donors expressing a histidine in position 131 of the Fcγ receptor IIa (FcγRIIa-131H) bound human IgG stronger and triggered higher levels of ADCC than donors with an arginine in this position (FcγRIIa-131R) (Parren et al. 1992). Corresponding results were observed for the FcγRIIIa receptor, where the FcγRIIIa-158V alloform was more active than the FcγRIIIa-158F alloform. In clinical trials, patients had significantly higher clinical and molecular response rates to rituximab if they expressed the FcγRIIIa-158V rather than the FcγRIIIa-158F alloform (Cartron et al. 2002; Treon et al. 2005). Another study reported a correlation between both the FcγRIIa and FcγRIIIa allotypes and response to rituximab (Weng and Levy 2003).

In addition to supporting the role of Fc receptor-mediated mechanisms of action, these studies also suggest particular effector cell populations to be clinically relevant. While the FcγRIIIa receptor is almost exclusively expressed by human NK cells and tissue macrophages, FcγRIIa is widely expressed by cells of the myeloid lineage, including monocytes and PMNs (Table 8.1). Interestingly, the same FcγRIIa and FcγRIIIa polymorphisms were not correlated with response to rituximab or Campath-1H in patients with chronic lymphocytic leukemia

Table 8.1 Human Fcγ receptors.

	Receptor function	*IgG affinity (K_a) (L mol^{-1})*	*Expression*		*Polymorphisms*	*Ligand specificity (human IgG)*
			Constitutive	*Inducible*		
FcγRI (CD64)	Activating	High (10^8–10^9)	Monocytes, macrophages	Neutrophils, eosinophils (?)		3 > 1 > 4 >>> 2
FcγRIIa (CD32a)	Activating	Low (<10^7)	Monocytes, macrophages, neutrophils, basophils, eosinophils, Langerhans cells, B cells, platelets, endothelial cells		131R 131H	3 > 1 >>> 2,4 3 > 1 = 2 >>> 4
FcγRIIb (CD32b)	Inhibitory	Low (<10^7)	Monocytes, macrophages, neutrophils, basophils, eosinophils, Langerhans cells, B cells, platelets, endothelial cells		187I/T	3 ≥ 1> 4 >> 2
FcγRIIIa (CD16a)	Activating	Medium (3×10^7)	NK cells, macrophages, subtypes of monocytes and T cells	Monocytes	158V/F affecting IgG1	1 = 3 >>> 2,4
FcγRIIIb (CD16b)	(GPI-linked)	Low (<10^7)	Neutrophils	Eosinophils (?)	NA1/NA2	1 = 3 >>> 2,4

(CLL), suggesting that in CLL patients systems other than Fc receptor-mediated effector mechanisms may predominate (Farag et al. 2004; Lin et al. 2005).

Another potential Fc-mediated mechanism is CDC. While evidence for systemic complement activation after rituximab therapy has been observed in several studies (Kennedy et al. 2004), its contribution to the killing of lymphoma cells *in vivo* is more controversial. While some investigators reported a correlation between the *in vitro* sensitivity to CDC and clinical responses to rituximab (Manches et al. 2003), this was not observed in other studies (Weng and Levy 2001). Since systemic complement activation after rituximab application may play a key role in the side effects of this treatment (van der Kolk et al. 2001), potential benefits of modified antibody constructs with increased capacity to activate complement need to be balanced against the risk of more severe toxicity. Interestingly, a novel human CD20 antibody, which proved particularly effective in activating human complement *in vitro*, did not demonstrate unexpected toxicities in early clinical trials (Coiffier et al. 2005).

Today, the clinically relevant mechanisms of action for therapeutic antibodies against solid tumors are even more elusive. Here, available information suggests a stronger impact of tumor cell-related factors rather than antibody-mediated functions (Hirsch and Bunn 2005). Data on the influence of Fcγ receptor polymorphisms for the response to solid tumor antibodies have, to our knowledge, not been reported, but are eagerly expected.

8.2 Modifying Effector Functions

8.2.1 Antibody Isotype

Among the human antibody isotypes (IgG1–4, IgA1 and 2, IgM, IgD, and IgE), IgG1 is therapeutically the most widely used isotype today. In this section, we will discuss the rationale for using this isotype, and discuss potential alternatives, the selection of which will depend on the required effector functions and on pharmacokinetic characteristics.

8.2.1.1 IgG Antibodies

Murine IgG subclasses (IgG1, IgG2a, IgG2b, IgG3) display substantial differences in their ability to mediate effector functions. Recently, subclass-dependent differences in the affinities for specific activating or inhibitory murine Fcγ receptors were described. The ratio of activation to inhibition (A/I ratio) differed by several orders of magnitude between the murine IgG subclasses, and predicted the *in vivo* activity of antibodies in two different models (Nimmerjahn and Ravetch 2005). However, these models cannot be directly transferred to the human situation, because mouse and human IgG subclasses do not directly correspond, and the human FcγR system is more complex than the murine one (Woof 2005).

In the human system, four IgG subclasses (IgG1, IgG2, IgG3, and IgG4) have been distinguished serologically (Jefferis 1986) and genetically (Rabbitts et al. 1981). Serum half-lives of 21 days have been reported for IgG1, IgG2, and IgG4 antibodies, whereas IgG3 antibodies have a half-life of only 7 days (Waldmann and Strober 1969) (Table 8.2). Furthermore, human IgG subclasses also display differential specificity and affinity to activating and inhibiting FcγR (see Table 8.1), indicating that the selection of the appropriate isotype is an important consideration for clinical applications. Further variation in the human γ heavy chain genes and subsequent heterogeneity in the IgG proteins has been described in what is called the Gm allotype system. Today, at least 18 different Gm allotypes have been described (van Loghem 1986; de Lange 1989). The functional relevance of these allotypes is widely unclear, but at least two IgG3 allotypes were reported to differ in their capacity to activate complement (Bruggemann et al. 1987; Bindon et al. 1988).

Concerning their structure, the main differences between the IgG subclasses reside in the amino acid composition of the C_H2 domain in the lower hinge region. This region also contains the binding sites for complement and Fcγ receptors, and determines the flexibility of the molecule (Burton 1985) (Table 8.2).

The initial event in the activation of the classical complement pathway is binding of C1q. The capacity of the four human IgG subclasses to bind C1q was determined using matched sets of chimeric antibodies with identical variable regions (Bruggemann et al. 1987). Surprisingly, despite higher C1q binding capacity of IgG3, the final complement killing activity of IgG1 was higher (Bruggemann et al. 1987; Dechant et al. 2002). Furthermore, IgG1 proved to be the most effective subclass at high antigen concentrations, whereas IgG3 was superior at lower concentrations (Lucisano Valim and Lachmann 1991; Michaelsen et al. 1991). Interestingly, IgG2 was demonstrated to mediate effective complement lysis at very high antigen densities by additional activation of the alternative pathway, which is regarded to be especially important for opsonization and killing of bacteria (Lucisano Valim and Lachmann 1991). IgG4 was consistently inactive in complement activation.

Table 8.2 Common features of human IgG subclasses.

	IgG1	*IgG2*	*IgG3*	*IgG4*
Molecular weight (kDa)	146	146	170	146
% of serum IgG	70	20	6	4
Serum half-life (days)	21	21	7	21
Gm allotypes	4	1	13	–
Inter-heavy chain disulfide bonds	2	4	11	2
Hinge length (aa)	15	12	62	12
Protein A binding	+++	+++	(+)	+++
Protein G binding	+++	+++	+++	+++
CDC	+++	+	++	–
ADCC	+++	+/–	++	–

Antibodies interact with effector cells via binding of their Fc region to cellular Fcγ receptors (reviewed in van de Winkel and Capel 1993). This process results in either activation or inhibition of effector cells, depending on which Fcγ receptors are predominantly engaged. In humans, three activating FcγR have been characterized: FcγRI, FcγRIIa and FcγRIIIa, which demonstrate distinct cellular expression patterns (Table 8.1). FcγRIIb, the only inhibitory cellular FcγR, has low affinity and contains an intracytoplasmatic immunoreceptor tyrosine-based inhibitory motif (ITIM). Human FcγRs have been demonstrated to have differential IgG subclass specificity (Table 8.1). Importantly, FcγRI is the only high-affinity IgG receptor, while the other Fcγ receptors have low or intermediate affinity. FcγRIIa is the only FcγR that binds human IgG2. This binding affinity for IgG2 is critically affected by a genetic polymorphism of the FCGRIIA gene, resulting in two distinct allotypes: FcγRIIa-131H has significantly higher affinity for human IgG2 than the FcγRIIa-131R allotype. NK cell expressed FcγRIIIa binds IgG1 and IgG3, while no binding of IgG2 and IgG4 has been found. For FcγRIIIa an important biallelic polymorphism has been well characterized: the FcγRIIIa-158V allele binds human IgG1 significantly stronger than the FcγRIIIa-158F allele. Neutrophil-expressed FcγRIIIb is a GPI-linked molecule, which displays the functionally relevant NA1/NA2 polymorphism. The inhibitory FcγRIIb receptor has similar affinity for human IgG1 and IgG3, lower affinity for IgG4 and does not bind IgG2 (reviewed in van Sorge et al. 2003). Functionally, ADCC induction by matched sets of chimeric antibodies was determined to be in the following order: IgG1 > IgG3 >> IgG2 and IgG4 (Bruggemann et al. 1987; Dechant et al. 2002). Analyses of different effector cell populations revealed that IgG1 antibodies triggered NK cells very effectively via FcγRIIIa, whereas neutrophils were only poorly activated via FcγRIIa.

In conclusion, human IgG1 is the preferred IgG subclass, if activating effector mechanisms are required, because IgG1 effectively triggers complement and NK cells. On the other hand, human IgG4 or IgG2 appear to be candidate isotypes if interactions with the host immune system are undesired. However, IgG4 antibodies have a tendency to form half-molecules, which may even be exchanged between two IgG4 molecules (reviewed in Aalberse and Schuurman 2002). This instability of human IgG4 could be corrected by a single S228P mutation in the hinge region (Angal et al. 1993). For therapeutic purposes, IgG4 molecules were further engineered by a E235S mutation, resulting in complete incapacity to interact with cellular Fcγ receptors (Reddy et al. 2000). Human IgG2 also has low complement-activating capacity and poorly interacts with most cellular Fc receptors. However, this later interaction is strongly influenced by the FcγRIIa-131R/H polymorphism, suggesting that human IgG2 should also be modified for therapeutic applications (see below).

8.2.1.2 **IgA Antibodies**

IgA represents the most abundantly produced antibody isotype *in vivo* (Kerr 1990), is critically involved in the host defense at mucosal surfaces (Monteiro and van de Winkel 2003), and activates human neutrophils more effectively than IgG

antibodies (Dechant and Valerius 2001). Neutrophils are the most numerous phagocytic cell population *in vivo*, and constitute the first line of defense against bacteria and fungi. Furthermore, neutrophils can kill a broad spectrum of tumor cells and have been shown to be critically involved in tumor rejection in animal models (Di Carlo et al. 2001). As outlined in the following paragraphs, human IgA has additional properties, which make IgA an attractive antibody isotype for immunotherapy.

Two subclasses – IgA1 and IgA2 – are distinguished, with IgA2 having a shorter hinge region, and an increased resistance against enzymatic degradation by bacterial proteases. Both isotypes contain *N*- and *O*-linked carbohydrates (Yoo and Morrison 2005). After covalent binding to plasma cell-produced joining (J) chain, IgA antibodies form natural dimers. Binding of these dimers to the polymeric immunoglobulin receptor (pIgR) leads to the directed transcellular secretion of IgA onto mucosal surfaces. At the luminal surface, secretory IgA (sIgA) is released, which consists of the IgA dimer, the J chain, and the proteolytically cleaved extracellular part of the pIgR (Fig. 8.2). Thus, monomeric, dimeric and secretory IgA are distinguished, with a predominance of monomeric IgA in plasma, and sIgA at mucosal surfaces.

The pharmacokinetic properties of IgA are fundamentally different from those of IgG. In contrast to IgG, IgA does not bind to FcRn, is therefore not protected from degradation, and its serum half-life (approx. 5 days) is significantly shorter than that of IgG. On the other hand, IgA, but not IgG, is actively transported to mucosal surfaces of the gut, the airways, and the urogenital tract. This offers the potential advantage that intravenously applied IgA could target pathogens or common tumors, such as lung or colon cancers, from the luminal surface, which is often enriched in neutrophilic effector cells.

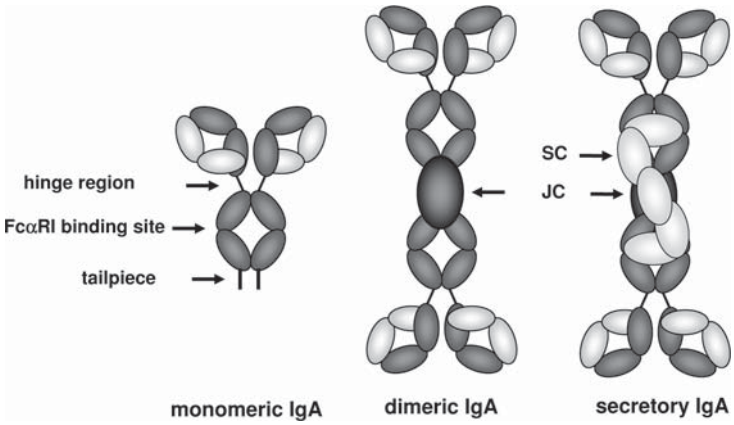

Fig. 8.2 Schematic representation of the three IgA isoforms. Dark gray: heavy chain; light gray: light chain. JC, J chain; SC, secretory component.

Functions of IgA include direct neutralization of pathogens on mucosal surfaces, intracellular neutralization of viruses during transepithelial transport, as well as activation of immune effector cells, which is triggered by the myeloid IgA receptor (FcαR; CD89) (Woof and Mestecky 2005). In contrast to IgG antibodies, both human IgA isotypes bind to their receptor at the C_H2/C_H3 interface, and activate human complement poorly. Interestingly, tumor cell killing by neutrophils was significantly enhanced in the presence of human IgA compared with human IgG antibodies (Valerius et al. 1997; Huls et al. 1999; Dechant et al. 2002). IgA-mediated tumor cell killing was further enhanced when blood or isolated effector cells from myeloid growth factor (G-CSF or GM-CSF)-treated patients was analyzed, suggesting that the combination of IgA antibodies and myeloid growth factors may act synergistically. However, the inhibitory effects of IgA antibodies on phagocyte function have also been described (Pasquier et al. 2005).

Until recently, animal models to investigate the function of human IgA *in vivo* were limited, since mice do not express a human FcαRI homolog. Transgenic mice, which express the human receptor under its physiological regulatory elements, served to reassess the role of IgA *in vivo* (van Egmond et al. 2000). In the future, the therapeutic potential of human IgA in infectious diseases (van Spriel et al. 1999; Vidarsson et al. 2001; Pleass et al. 2003) and for tumor immunotherapy may be investigated in these animals. So far, no clinical experience with the systemic application of human IgA antibodies has been reported.

8.2.2 Altered Fc Receptor Binding

8.2.2.1 Introduction

While fundamental structural requirements for the effector functions of IgG antibodies were apparent from early studies, such as glycosylation at Asn297 for C1q binding and interaction with Fcγ receptors (Nose and Wigzell 1983; Tao and Morrison 1989; Jefferis and Lund 2002), more refined insights were obtained from crystal structures. Thus, analyses of the co-crystal structure of the IgG Fc with FcγRIII revealed that direct interaction sites were mainly located in the protein moiety of IgG, while only minor contacts between sugar residues of the Fc and FcγRIII were observed (Sondermann et al. 2000). Since the activation of leukocyte Fc receptors was dependent on Fc glycosylation, it was supposed that the oligosaccharide moiety excerted its influence indirectly, probably through modulation of the Fc conformation (Jefferis et al. 1995; Wright and Morrison 1997). Co-crystal structures from a series of Fc glycosylation variants with FcγRIII indicated that the sugars act both to increase the distance and to decrease the mobility of the receptor-interacting segments of the C_H2 domains (Krapp et al. 2003). From these observations, two approaches to modulate Fc functions became evident: altering the glycosylation profile (glyco-engineering), or mutation of selected amino acids in the IgG Fc portion (protein engineering).

8.2.2.2 Glyco-Engineered Antibodies

All human antibody isotypes contain carbohydrates at conserved positions in the constant regions of their heavy chains, with each isotype possessing a distinct array of *N*-linked carbohydrate structures, which variably affects protein assembly, secretion or functional activity. The structure of the attached *N*-linked carbohydrate varies considerably, depending upon the degree of posttranslational processing, and can include high-mannose, multiply branched as well as biantennary complex oligosaccharides (Wright and Morrison 1997). IgG, the most abundant antibody isotype in human serum, has a single *N*-linked biantennary complex type structure attached to Asn297, which shows considerable heterogeneity (Jefferis et al. 1995). A "core" heptasacharide can be defined with variable addition of outer arm sugar residues (Jefferis 2005) (Fig. 8.3). The attached oligosaccharide is approximately as large as the C_H2 domain itself, and is buried between the two C_H2 domains, forming extensive contacts with amino acid residues within C_H2 (Rademacher et al. 1988; Wright and Morrison 1997).

Glycosylation is a cotranslational/posttranslational modification that results in the attachment of a glucosylated high mannose oligosaccharide (GlcNac-2Man9Glu3). This sugar is then trimmed to a GlcNac2Man9Glu structure, which is bound by chaperones that aid and monitor folding fidelity (Jefferis 2005). The resulting glycoprotein, which is targeted for secretion, then transits the Golgi apperatus, where the oligosaccharide is initially trimmed back by glycosidases to a GlcNac2Man5 structure, before being processed by the successive action of glycosyltransferases to generate the complex type biantennary structure (Jefferis 2005) (Fig. 8.3).

Several factors can influence glycosylation. Therefore, the selection of appropriate expression cell lines for the production of therapeutic antibodies is critical to obtain a favorable glycosylation profile. Early studies established that CHO, NS0, and Sp2/0 cells are able to produce chimeric and humanized IgG antibodies with major glycoforms identical to glycoforms present in polyclonal human IgG (reviewed in Jefferis 2005). Campath-1H, a humanized IgG1 antibody directed against CD52, has been expressed in several mammalian cell lines, including YB2/0 rat myeloma, NS0 mouse myeloma and CHO cells. Results from these studies demonstrated that major differences in antibody glycosylation occurred between these cell lines. YB2/0-expressed Campath-1H contained an antibody fraction with a bisecting GlcNAc on the core oligosaccharide, as well as a fraction of nonfucosylated antibody. In ADCC assays, the YB2/0-expressed antibody reached similar activity as Campath-1H expressed in CHO and NS0 cells, but required significantly lower antibody concentrations (Lifely et al. 1995). Several reports suggested that a bisecting GlcNAc, lack of fucose, and the content of galactose in the antibody oligosaccharide all affect the levels of ADCC (Kumpel et al. 1995; Umana et al. 1999; Shields et al. 2002). In a comparative analysis, the relative contribution of these sugar residues to enhance ADCC was analyzed. In this study, galactose had no effect on ADCC, and a high content of bisecting GlcNAc had a relatively weak effect on enhancing ADCC. More importantly, nonfucosylated oligosaccharide was demonstrated to have a prominent effect in

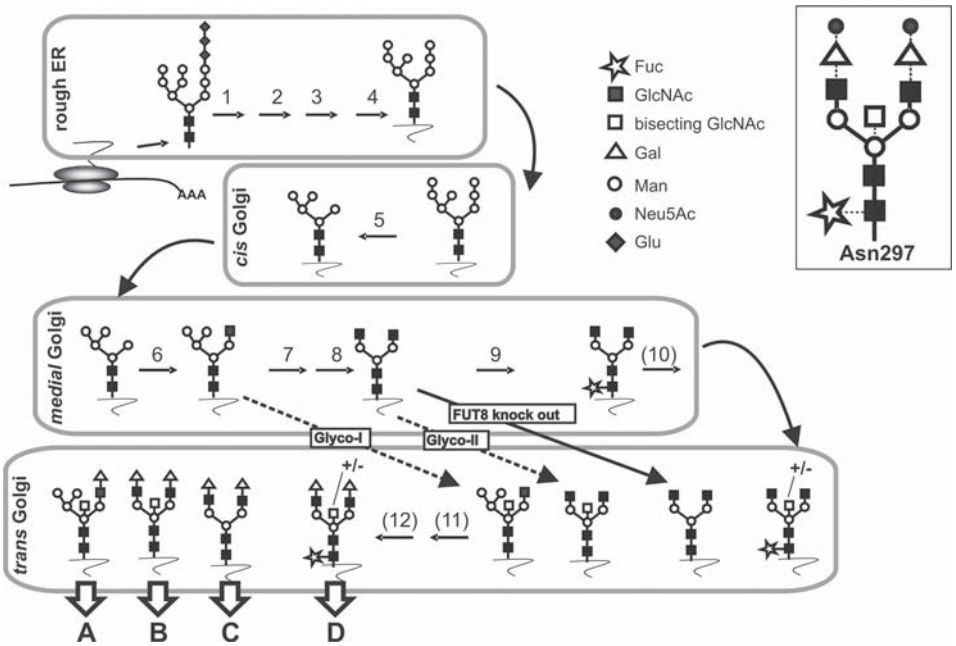

Fig. 8.3 Biosynthesis of *N*-glycans and bisected *N*-glycans and its manipulation to achieve reduced fucose content. Glyco-I approach: overexpression of engineered GnT-III with localization domain exchanges, leading to a high proportion of bisected, non-fucosylated, hybrid type glycans. Glyco-II approach: co-expression of GnT-III and Man-II, directing the pathway to bisected, complex type oligosaccharides (Ferrara et al. 2006). FUT8 knockout approach: no α1,6-FucT activity, leading to complex type nonfucosylated oligosaccharides. A dashed line indicates a certain balance between molecules modified in the modified and non-modified pathway. A: nonfucosylated, bisected, hybrid type; B: nonfucosylated, bisected, complex type; C: nonfucosylated, complex type; D: bisected or nonbisected complex type. 1: oligosaccharyltransferase; 2: α-glucosidase I; 3: α-glucosidase II; 4: α-1,2-mannosidase; 5: α-mannosidase I; 6: β1,2-*N*-acetylglucosaminyltransferase I; 7: Golgi α-mannosidase II (Man-II); 8: β1,2-*N*-acetylglucosaminyltransferase II; 9: core α1,6-fucosyltransferase (α1,6-FucT); 10: β1,4-*N*-acetylglucosaminyltransferase III (GnT-III); 11: β1,4-galactosyltransferase; 12: sialyltransferase. ER, endoplasmatic reticulum; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; Gal, galactose; Man, mannose; Neu5Ac, *N*-acetylneuramic acid; Glu, glucose. Box: Biantennary oligosaccharide of the IgG Fc portion. Full lines define the core oligosaccharide structure; dashed lines show additional sugar residues that may be attached.

enhancing ADCC compared with a bisecting GlcNAc-containing oligosaccharide in the IgG1 molecule (Shinkawa et al. 2003).

Following these observations, two strategies have been followed to alter the glycosylation profile of therapeutic antibodies in order to specifically improve their capacity to trigger ADCC. Both strategies aimed at reducing core fucosylation to enhance FcR binding, which could be achieved by direct or indirect mechanisms. In the indirect approach, overexpression of β1,4-*N*-acetylglucosami-

nyltransferase III (GnT-III) led to the addition of a bisecting GlcNAc residue, which had an important influence on multiple subsequent enzymatic glycosylation reactions in the Golgi complex of the cell. Once an oligosaccharide is bisected by the action of GnT-III, it cannot serve as a suitable substrate for several glycosylation enzymes, especially Golgi α-mannosidase II (Man-II) and, importantly, α1,6-fucosyltransferase (α1,6-FucT) (Schachter 2000). Several groups investigated the influence of a bisecting GlcNAc by overexpression of GnT-III in cell lines used for the production of therapeutic antibodies. The glycosylation pattern of an anti-neuroblastoma antibody (chCE7) was engineered in CHO cells by tetracycline-regulated expression of the GnT-III enzyme. The results demonstrated that the 15- to 20-fold enhancement in ADCC activity correlated with the level of bisected, nonfucosylated oligosaccharides, and that there was an optimal range of GnT-III overexpression for maximal *in vitro* ADCC (Umana et al. 1999). Similar results were reported for the CD20-directed antibody rituximab produced in CHO cells overexpressing GnT-III (Davies et al. 2001), and for a CD19-directed chimeric antibody produced in HEK-293 cells (Barbin et al. 2006).

Recently, a Lewis Y-specific antibody with 10-fold enhanced ADCC activity has been reported by overexpressing GnT-III (Schuster et al. 2005). In this report, two approaches were followed. In the first approach (Glyco-I), the GnT-III was expressed along the Golgi apparatus by fusing the α-mannosidase-II localization domain to the catalytic domain of GnT-III. The authors observed increased levels of bisected, non-fucosylated hybrid type oligosaccharides, and these forms seemed to be related to a moderate reduction of complement activation. In the second approach (Glyco-II), GnT-III with its autologous localization domain, directing the enzyme to the *trans*-Golgi cisternae together with α-mannosidase-II, the CDC activity could be recovered and even slightly enhanced without affecting ADCC activity (Fig. 8.3). The authors stated that, depending on the clinical requirements, fine-tuning of complement activation might be possible (Schuster et al. 2005).

Applying the same technology, two glycovariants of Campath-1H were produced: Cam-Glyco-I, enriched in nonfucosylated, hybrid-type Fc carbohydrates, and Cam-Glyco-II, carrying nonfucosylated oligosaccharides both of hybrid and complex type. Both glycovariants demonstrated markedly enhanced ADCC in comparison with regular Campath-1H. Cam-Glyco-I mediated lower CDC than Cam-Glyco-II. The *in vivo* activity of the two glycoforms was assessed in cynomolgus monkeys. The pharmacokinetics of both glycoforms were not significantly different from wildtype Campath-1H and both variants appeared to deplete lymphocytes from both blood and lymph nodes better than wildtype Campath-1H. (Dyer et al. 2005).

The impact of fucose on ADCC was initially analyzed using cell lines with low α1,6-FucT activity (CHO-Lec13 and rat YB2/0). This resulted in antibody preparations with varying fucose content, and up to 50-fold enhanced ADCC by CD20-directed antibodies. C1q binding was not influenced by the lack of fucose, suggesting no impact on CDC activity (Shields et al. 2002). Aiming for fully defucosylated antibody preparations, a direct approach has also been described. Thus,

α1,6-FucT was switched off by gene knockout of the *FUT8* gene, or by introducing small interfering RNA into the CHO producer cell line (Mori et al. 2004; Yamane-Ohnuki et al. 2004). α1,6-FucT is the key enzyme that catalyzes the transfer of fucose from GDP-fucose to the GlcNAc residue in an α1,6 linkage in the medial Golgi cisternae. Knockout of both *FUT8* alleles in CHO cells resulted in a complete loss of α1,6-FucT activity and enabled the production of fully defucosylated CD20 antibodies (Fig. 8.3). *FUT8*$^{-/-}$-produced CD20 antibodies were not altered with respect to antigen binding. However, they exhibited more than 100-fold higher ADCC activity compared with fully fucosylated antibodies, and were significantly more effective than their YB2/0-expressed counterparts (44% defucosylated). Importantly, elimination of α1,6-fucosylation activity by *FUT8* knockout did not alter other *N*-linked glycosylation patterns (Yamane-Ohnuki et al. 2004).

The increased ADCC activity of under-fucosylated IgG1 (and IgG2, IgG3, and IgG4) may be explained by enhanced binding to FcγRIIIa on NK cells, whereas binding to FcγRI and FcγRIIa/b was less influenced by the lack of fucose (Shields et al. 2002; Niwa et al. 2005). Both polymorphic forms of FcγRIIIa exhibited significantly improved binding to IgG1 that lacked fucose. Binding of dimeric nonfucosylated forms of Hu4D5 (a humanized anti p185HER2 antibody) to FcγRIIIa-158F or FcγRIIIa-158V revealed about 42-fold or 19-fold improvements, respectively (Shields et al. 2002). The structural basis for this affinity enhancement is not fully understood. Recent data suggest that the glycosylation of FcγRIIIa plays an important role and that the high affinity between glyco-engineered antibodies and FcγRIIIa is mediated by productive interactions formed between the receptor carbohydrate attached at N162 and regions of the Fc part that are only accessible when it is nonfucosylated (Ferrara et al. 2005). As FcγRIIIa and FcγRIIIb are the only human Fcγ receptors that are glycosylated at this position, the proposed interactions between the FcγRIIIa-attached carbohydrate and the Fc portion might explain the observed selective affinity increase of glyco-engineered antibodies for only this receptor (Ferrara et al. 2005). The effects of the FcγRIIIa gene polymorphism on ADCC mediated by fucosylated versus nonfucosylated antibodies were analyzed using different glycoforms of rituximab. Nonfucosylated forms were more potent in inducing ADCC than fucosylated rituximab, and this difference was independent of the FcγRIIIa polymorphism at position 158. Importantly, the use of nonfucosylated rituximab reduced the difference in ADCC activity between low-affinity and high-affinity Fc receptors. In contrast to V-carriers showing a 10-fold greater activity than F-carriers for fucosylated rituximab, there was no significant difference observed when nonfucosylated Rituximab was used (Niwa et al. 2004). Therefore, it is speculated that the use of low-fucose antibodies might improve the therapeutic effects of CD20-directed therapy for all patients independent of the FcγRIIIa phenotype.

8.2.2.3 **Protein-Engineered Antibodies**

In contrast to glyco-engineering, which so far has only been employed to enhance antibody binding to activating Fcγ receptors, engineering the protein backbone

of antibodies was used to generate antibodies with either enhanced or diminished binding to individual Fcγ receptors. Decreased Fc receptor binding appears particularly attractive for immunotoxins, but also naked antibodies have been developed which should not bind to leukocyte Fc receptors.

Protein-engineered antibodies with diminished interactions with Fcγ receptors For antibodies which should not bind to cellular Fc receptors, $F(ab)_2$ fragments would be a logical step forward, but $F(ab)_2$ fragments are expensive to produce and have a short plasma half-life due to their lack of binding to FcRn. Therefore, whole IgG antibodies with reduced binding to Fcγ receptors have been engineered. Considering the different binding affinities of various human IgG isotypes to cellular Fc receptors (Table 8.1), human IgG4 or human IgG2 backbones appear as logical starting platforms for non-Fc receptor binding variants. For example, unmodified human IgG4 was selected to target toxins such as calicheamicin to either CD33 (Gemtuzumab)- or CD22 (CMC-544)-expressing tumor cells (Damle and Frost 2003). However, variants of human IgG1 with diminished Fc receptor binding have also been generated (see below).

Antibodies against CD3 are prototypic examples where Fc receptor engagement triggers significant clinical toxicity, probably without contributing to therapeutic efficacy (Chatenoud 2005). So far, OKT-3 (muromonab, mIgG2a) is the only approved CD3 antibody that is associated with severe clinical toxicity due to activation of resting T cells. This T-cell activation was demonstrated to be triggered by antibody-mediated crosslinking of T cells with Fcγ receptor-bearing bystander cells such as monocytes/macrophages. T-cell receptor crosslinking results in T-cell mitogenesis and in massive cytokine release, which limits the clinical applicability of mitogenic CD3 antibodies.

However, CD3-directed antibodies have significant potential as immunosuppressive agents for the prevention and treatment of transplant rejections, in the treatment of severe T cell-mediated autoimmune diseases and may even be employed to induce antigen-specific tolerance (Chatenoud 2003). Therefore, several approaches have been followed to generate less mitogenic CD3 antibodies by reducing Fcγ receptor binding. For example, a rat anti-human CD3 antibody (YTH 12-5) was humanized and expressed as human IgG (including all four subclasses), IgA, or IgE. All isotypes could elicit cytokine release *in vitro* (Bolt et al. 1993). Mutating amino acid 297 (N297A) in the humanized IgG1 version prevented antibody glycosylation, resulting in a CD3 antibody with impaired binding to all FcγRs and with significantly reduced complement-activating capacity. This antibody proved nonmitogenic *in vitro*, and demonstrated low toxicity and signs for immunosuppressive activity in a phase I clinical study (Friend et al. 1999). In a randomized phase II trial in patients with new-onset type 1 diabetes, a 6-day course of an aglycosylated chimeric CD3 antibody (human IgG1, ChAglyCD3) was effective in preserving residual beta cell function. However, all antibody-treated patients experienced infusional side effects, and the majority reported symptoms from transient Epstein–Barr virus (EBV) reactivation (Keymeulen et al. 2005).

hOKT3γ1(Ala-Ala) is a humanized IgG1 version of OKT3, in which the amino acids in positions 234 and 235 have been mutated to alanine. Thereby, hOKT3γ1(Ala-Ala) was reported to lose complement-activating capacity, Fcγ receptor binding, and mitogenicity. A subsequent phase I study with hOKT3γ1(Ala-Ala) demonstrated efficacy similar to that of conventional OKT3 in the treatment of renal allograft rejection with markedly fewer side effects (Woodle et al. 1999). hOKT3γ1(Ala-Ala) was also tested in patients with psoriatic arthritis (Utset et al. 2002) or type I diabetes (Herold et al. 2002). In both patient populations, no significant cytokine release was observed, infusion-related toxicity was low and, importantly, these phase II trials suggested clinical efficacy.

Another approach used human IgG2 as a template for the introduction of mutations, because human IgG2 interacts only with FcγRIIa, and CD3-directed human IgG2 antibodies required 10- to 100-fold higher antibody concentrations to induce T-cell proliferation. Binding to FcγRII receptors was further reduced by two engineered mutations in the constant regions of HuM291 (V234A and G237A) (Cole et al. 1997). As expected, this construct was nonmitogenic *in vitro*. As HuM291 dissociated quickly from cell surface CD3 molecules, only minimal internalization, but sustained signaling by the T-cell receptor was observed. Thereby, HuM291 effectively triggered apoptosis in activated human T cells (Carpenter et al. 2002). In clinical phase I studies in renal allograft or allogeneic bone marrow transplantation patients, the majority of patients did not demonstrate measurable cytokine levels after antibody application, infusion-related toxicity was low, and immunosuppressive activity was observed (Norman et al. 2000; Carpenter et al. 2002).

Protein-engineered antibodies with improved Fcγ receptor binding Clinical observations and animal studies indicate that efficient recruitment of immune effector cells and triggering of cellular effector functions such as ADCC are major mechanisms of action for some therapeutic antibodies (see above). The therapeutic efficacy of these antibodies may be improved by increasing binding of their Fc domains to activating FcγRs. For this strategy, the balance between activating and inhibitory receptors is an important consideration (Nimmerjahn and Ravetch 2005), and optimal effector functions may result from Fc parts with enhanced affinity for activating Fcγ receptors relative to the inhibitory FcγRIIb isoform (Clynes et al. 2000; Nimmerjahn and Ravetch 2005). Several different strategies have been followed to identify critical amino acids that could be altered to modify FcR binding and enhance immune effector mechanisms.

In a comprehensive study, all solvent-exposed residues in the Fc part of human IgG1 were individually changed to alanine, and binding to the different FcγRs was analyzed (Shields et al. 2001). These studies identified several groups of mutants that discriminated between binding to FcγRI, FcγRIIb, and FcγRIIIa. Interestingly, several Fc variants, in which two or more amino acids were simultaneously altered to alanine, exhibited additive binding characteristics. For example, the triple Fc mutant S298A/E333A/K334A demonstrated improved binding to FcγRIIIa and diminished binding to FcγRIIb (Shields et al. 2001)

(Fig. 8.4). Several variants exhibited significant improvements in ADCC with effector cells from either FcγRIIIa-V/V or FcγRIIIa-F/F homozygous donors. Using this triple mutant with FcγRIIIa-F/F donor cells, maximal ADCC levels were increased by more than 100%, and 1–2 logs improvement in potency were observed, as reflected by shifts in the EC_{50} values to lower concentrations (Shields et al. 2001; Lazar et al. 2006).

In a second approach using a yeast display system (Stavenhagen et al. 2004), mutated human IgG1 Fc regions were screened for altered binding affinity to different Fcγ receptors. For this purpose, a mutant Fc library was generated by error-prone PCR, and the mutated sequences were fused to the Aga2p cell wall protein, which allowed display on the yeast cell wall. This library was screened by soluble tetrameric FcγR complexes (FcγRIIIa or FcγRIIb) and cell sorting. Different screening strategies were applied, combining positive selection and depletion cycles. A variety of Fc mutants with higher affinity for FcγRIIIa and enhanced ADCC activity were isolated (see Table 8.3 and Fig. 8.4 for a selection of mutants). Interestingly, several variants with enhanced ADCC activity were identified, in

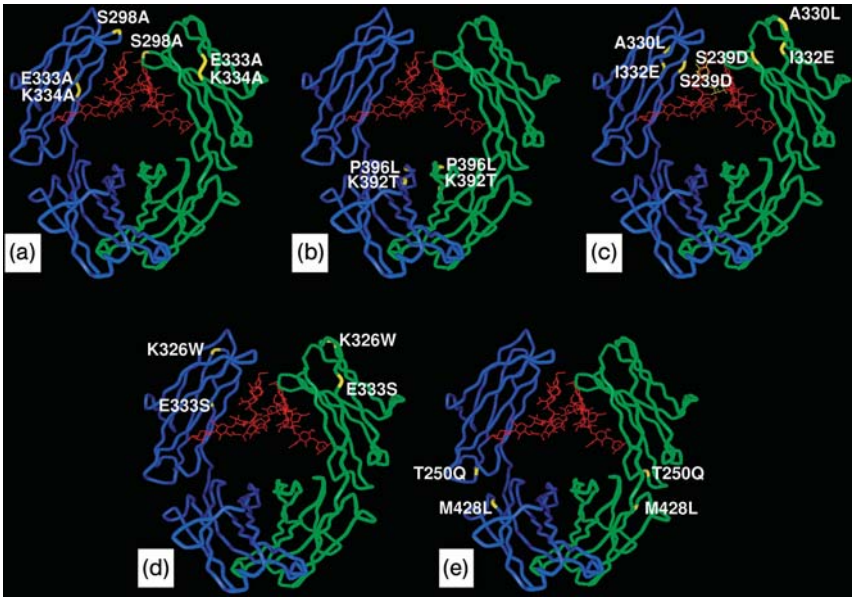

Fig. 8.4 Engineered Fc portions with altered effector functions. (a–c) Enhanced ADCC; (d) altered complement activation; (e) prolonged half-life. (a) S298A/E333A/K334A variant (Shields et al. 2001). (b) K392T/P396L variant (Stavenhagen et al. 2004). (c) S239D/I332E/A330L variant (Lazar et al. 2006). (d) K326W/E333S variant (Idusogie et al. 2001). (e) T250Q/M428L variant (Hinton et al. 2006). Altered amino acid positions resulting in modified effector functions are indicated. Human IgG1 Fc portion: crystal structures adapted from Krapp et al. (2003). Blue and green: C_H2-C_H3 domains; red: carbohydrate; yellow: modifed amino acid position.

Table 8.3 Characteristics of selected engineered Fc variants.

Variant	*FcγRIIIa binding*	*FcγRIIb binding*	*IIIa/IIb ratio*	*ADCC induction*	*Fold reduction in EC_{50} value*	*Complement activation*	*Reference*
Wildtype	↑	↑	1	↑	–	+	
S298A-E333A-K334A	↑↑	↓	10	↑↑↑	10–100	No data available	Shields et al. 2001
S239D-I332E	↑↑↑	↑↑	4	↑↑↑	100–1000	+	Lazar et al. 2006
S239D-I332E-A330L	↑↑↑↑	↑↑↑	9	↑↑↑	100–1000	–	Lazar et al. 2006
K288N-A330S-P396L	↑↑	↑↑	2	↑↑↑		No data available	Stavenhagen et al. 2004
K392T-P396L	↑↑	↑↑	2	↑↑↑	10–100	No data available	Stavenhagen et al. 2004

IIIa/IIb: Fold FcγRIIIa binding/FcγRIIb binding; EC_{50}: effective concentration 50%; ↑ enhanced activity/binding compared with wildtype; ↓ reduced activity/binding compared with wildtype.

which the mutated amino acids were located outside of the Fc receptor binding region in the C_H3 domain of the antibody (see Fig. 8.4). These data suggest that some mutants increase Fc receptor binding indirectly (e.g. by altering the structural properties of the Fc domain or by influencing the glycosylation of the Fc region) (Stavenhagen et al. 2004).

Lazar and colleagues used computational design algorithms and high-throughput screening to engineer Fc variants with optimized FcγR affinity and specificity (Lazar et al. 2006). When structural information was available (Fc/FcγRIII complex), affinity was directly optimized by designing substitutions that provided more favorable interactions at the Fc/FcγR interface. When structural information was incomplete or lacking (as for the Fc/FcγRIIb complex), calculations provided a set of variants enriched for stability and solubility. A number of engineered Fc variants demonstrated significant enhancements in binding affinity to both human FcγRIIIa-158V and FcγRIIIa-158F alleles along with an improved FcγRIIIa/IIb ratio (Table 8.3; Fig. 8.4). In the context of Campath-1H, both variants S239D/I332E and S239D/I332E/A330L displayed more than two orders of magnitude enhanced ADCC potency compared with wildtype Campath-1H, and were 10-fold more active than the S298A/E333A/K334A triple mutant described by Shields and colleagues. Interestingly, in the rituximab background variant S239D/I332E elicited CDC comparable to wildtype rituximab, while the addition of the A330L mutation ablated CDC. Thus, the set of S239D/I332E and S239D/I332E/A330L variants provides the option for enhancing ADCC with or without triggering CDC as additional mechanism of action. To demonstrate the superior cytotoxic potential of the Fc variants *in vivo*, B-cell depletion using CD20-directed antibodies was analyzed in cynomolgus monkeys. The approximate dose required

for 50% B-cell depletion by wildtype antibody was approximately 10 µg kg^{-1} per day. For the S239D/I332E variant, a dose of 0.2 µg kg^{-1} per day was sufficient to achieve 50% depletion – an apparent 50-fold increase in potency (Lazar et al. 2006).

In conclusion, protein-engineered Fc variants demonstrated substantial improvements in Fc receptor-mediated effector functions *in vitro* and in primate models. Whether these types of second-generation antibodies will improve clinical outcomes needs to be evaluated in future clinical trials.

8.2.3 Altered Complement Activation

Since the contribution of complement to the therapeutic efficacy of antibodies is controversial, and since complement activation may contribute to the side effects of antibodies in patients, approaches to either enhance or to diminish complement activation have been proposed. Activation of the classical complement pathway requires binding of the C1q serum protein to the Fc portion of IgG. This C1q binding is completely abolished in Asn297 mutated, aglycosylated IgG, but these mutants also lack Fc receptor binding (see above). Therefore, more complement-specific approaches were required.

Initial studies with a mouse IgG2b antibody mapped the core C1q-binding site to amino acids E318, K320, and K322 (Duncan and Winter 1988). More recent mutational analyses of human IgG1 revealed that the C1q-binding region of human IgG1 is centered around D270, K322, P329, and P331 in the C_H2 domain. Thus, two mutants – D270A and P329A – were particularly ineffective in binding C1q and activating human complement, but still retained some complement-activating capacity at higher complement concentrations. Interestingly, the P329A mutant also demonstrated significantly impaired ADCC activity compared to wildtype, while the ADCC activity of the D270A mutant was similar to the control antibody (Idusogie et al. 2000).

Further studies revealed that amino acids K326 and E333 – located at the edges of the C1q-binding region – profoundly influenced complement activation by IgG1 mutants (Idusogie et al. 2001). Thus, a K326W substitution provided the highest increase in C1q binding (3-fold) and complement-mediated killing (2-fold). In amino acid position 333, the E333S substitution resulted in the highest increase in C1q binding (2-fold) and CDC activity (1.6-fold). The K326W/E333S double mutant demonstrated additive increases in C1q binding (5-fold), but CDC activity was not further increased compared to the single mutants. Interestingly, the K326W and the K326W/E333S mutants were completely inactive in ADCC assays (Fig. 8.4). These observations make them very interesting tools to elucidate the effector mechanism of antibodies *in vivo*, as they were supposed to act only by complement, but not by ADCC.

8.3
Modifying the Pharmacokinetics of Antibodies

8.3.1
Introduction

Studies in rituximab-treated patients have indicated that clinical responses were correlated with favorable pharmacokinetics in patients (Berinstein et al. 1998). Therefore, improving the serum half-life of therapeutic antibodies is an attractive approach, which may reduce the amount of required antibodies, and may increase their convenience for patients by prolonging treatment intervals. For most human IgG antibodies, serum half-life is in the range of 3 weeks. Elegant studies have demonstrated that this prolonged half-life of IgG compared with other serum molecules or antibody isotypes is not merely a function of protein size, but requires interaction with a specific Fc receptor. This receptor (FcRn) is a heterodimer of β_2 microglobulin and an HLA class I-related α-chain, which – in humans – is mainly expressed by endothelial cells (Ghetie and Ward 2000). After internalization by fluid phase pinocytosis, IgG is routed to acidic endosomes, where binding to FcRn is believed to occur at low pH. This binding prevents lysosomal degradation of IgG, and triggers sorting and transport to the cell surface, where IgG is released from FcRn at near neutral pH. Thereby, FcRn actively protects IgG from lysosomal degradation, and controls serum half-life of endogenous and exogenous antibodies.

Recent studies have demonstrated that antibody consumption (e.g. by widely expressed and rapidly internalized antigens) may also dramatically influence their serum half-life (Lammerts van Bueren *et al.* 2006; Shih et al. 1994). The relative contribution of FcRn binding and target antigen-mediated antibody consumption for the pharmacokinetic profile of therapeutic antibodies is unknown. Whether antibody consumption is amenable to molecular engineering – without impeding antibody efficacy – has not been addressed.

8.3.2
Modifying Binding to FcRn

Analysis of the crystal structure of FcRn with Fc has mapped their interaction site to the interface between C_H2 and C_H3 in IgG molecules (Burmeister et al. 1994), while leukocyte Fc receptors bind to IgG in the lower hinge region. This region between C_H2 and C_H3 contains several histidine residues, which may account for the sharply pH-dependent interaction between FcRn and IgG. Considering the potential clinical relevance of this interaction, approaches to modify binding of IgG to FcRn were obvious. However, considering the biology of FcRn, mutations should probably not interfere with the pH dependency of binding.

Initial mutagenesis studies identified 10 human IgG1 mutants with higher affinity toward murine and human FcRn at pH 6.0. However, these mutants

exhibited parallel increases in binding to murine FcRn at pH 7.4, which may explain why their serum half-lives in mice were not prolonged (Dall'Acqua et al. 2002). In another report, modeling the binding of human IgG2 to human FcRn guided the selection of amino acid positions 250, 314, and 428 of the human IgG2 heavy chain for further mutagenesis studies (Hinton et al. 2004). Random mutagenesis identified IgG variants at position 250 and 428 with increased binding to FcRn, while none of the 314 mutants resulted in increased FcRn binding. *In vitro*, the optimal mutants – T250Q, M428L, and T250Q/M428L – demonstrated approximately 4-, 8-, or 27-fold higher binding to FcRn at pH 6.0. In rhesus monkeys, the M428L and the T250Q/M428L mutants showed an approx. 2-fold increase in serum half-lives compared with the wildtype antibody. Furthermore, both amino acid substitutions proved to be transferrable to human IgG1 in a recent report, since amino acids 250 and 428 are conserved between the four human IgG isotypes. The serum half-life in rhesus monkeys was prolonged by 2.5-fold. It may be expected that these mutations also improve the pharmacokinetics of IgG isotypes in humans, provided that the data from rhesus monkeys can be transferred, and assuming that these mutations do not increase the immunogenicity in clinical studies (Hinton et al. 2006) (Fig. 8.4).

8.4 Summary and Conclusions

Despite considerable efforts to elucidate the mechanisms of action for therapeutic monoclonal antibodies, our current understanding of these mechanisms is still rather incomplete. Furthermore, the contribution of individual mechanisms may significantly vary between different target antigens, but also between antibodies targeting the same molecule. At present, it is not determined which preclinical assay will optimally predict clinical responses in patients, but with more antibodies coming along the line, our knowledge about relevant mechanisms will certainly increase. Meanwhile, molecular engineering will provide solid platforms for second-generation antibodies, which allow particular aspects of therapeutic antibodies to be selectively improved. Hopefully, these novel reagents will then translate into further improvements in antibody therapy.

References

Aalberse, R.C., Schuurman, J. (2002) IgG4 breaking the rules. *Immunology* 105: 9–19.

Angal, S., King, D.J., Bodmer, M.W., Turner, A., Lawson, A.D., Roberts, G., Pedley, B., Adair, J.R. (1993) A single amino acid substitution abolishes the heterogeneity of chimeric mouse/human (IgG4) antibody. *Mol Immunol* 30: 105–108.

Barbin, K., Stieglmaier, J., Saul, D., Stieglmaier, K., Stockmeyer, B., Pfeiffer, M., Lang, P., Fey, G.H. (2006) Influence of variable *N*-glycosylation on the cytolytic potential of chimeric CD19 antibodies. *J Immunother* 29: 122–133.

Berinstein, N.L., Grillo-Lopez, A.J., White, C.A., Bence-Bruckler, I., Maloney, D.,

Czuczman, M., Green, D., Rosenberg, J., McLaughlin, P., Shen, D. (1998) Association of serum rituximab (IDEC-C2B8) concentration and anti-tumor response in the treatment of recurrent low-grade or follicular non-Hodgkin's lymphoma. *Ann Oncol* 9: 995–1001.

Bindon, C.I., Hale, G., Bruggemann, M., Waldmann, H. (1988) Human monoclonal IgG isotypes differ in complement activating function at the level of C4 as well as C1q. *J Exp Med* 168: 127–142.

Bolt, S., Routledge, E., Lloyd, I., Chatenoud, L., Pope, H., Gorman, S.D., Clark, M., Waldmann, H. (1993) The generation of a humanized, non-mitogenic CD3 monoclonal antibody which retains in vitro immunosuppressive properties. *Eur J Immunol* 23: 403–411.

Bruggemann, M., Williams, G.T., Bindon, C. I., Clark, M.R., Walker, M.R., Jefferis, R., Waldmann, H., Neuberger, M.S. (1987) Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. *J Exp Med* 166: 1351–1361.

Burmeister, W.P., Huber, A.H., Bjorkman, P.J. (1994) Crystal structure of the complex of rat neonatal Fc receptor with Fc. *Nature* 372: 379–383.

Burton, D.R. (1985) Immunoglobulin G: functional sites. *Mol Immunol* 22: 161–206.

Carpenter, P.A., Appelbaum, F.R., Corey, L., Deeg, H.J., Doney, K., Gooley, T., Krueger, J., Martin, P., Pavlovic, S., Sanders, J., Slattery, J., Levitt, D., Storb, R., Woolfrey, A., Anasetti, C. (2002) A humanized non-FcR-binding anti-CD3 antibody, visilizumab, for treatment of steroid-refractory acute graft-versus-host disease. *Blood* 99: 2712–2719.

Cartron, G., Dacheux, L., Salles, G., Solal-Celigny, P., Bardos, P., Colombat, P., Watier, H. (2002) Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcγRIIIa gene. *Blood* 99: 754–758.

Chatenoud, L. (2003) CD3-specific antibody-induced active tolerance: from bench to bedside. *Nat Rev Immunol* 3: 123–132.

Chatenoud, L. (2005) CD3-specific antibodies restore self-tolerance: mechanisms and clinical applications. *Curr Opin Immunol* 17: 632–637.

Clark, M.R. (1997) IgG effector mechanisms. *Chem Immunol* 65: 88–110.

Clynes, R., Takechi, Y., Moroi, Y., Houghton, A., Ravetch, J.V. (1998) Fc receptors are required in passive and active immunity to melanoma. *Proc Natl Acad Sci USA* 95: 652–656.

Clynes, R.A., Towers, T.L., Presta, L.G., Ravetch, J.V. (2000) Inhibitory Fc receptors modulate in vivo cytoxicity against tumor targets. *Nat Med* 6: 443–446.

Coiffier, B., Tilly, H., Pedersen, L.M., Plesner, T., Frederiksen, H., van Oers, M. H.J., Wooldridge, J., Kloczko, J., Holowiecki, J., Hellmann, A., Walewski, J. J., Flensburg, M.F., Petersen, J., Robak, T. (2005) HuMax CD20 fully human monoclonal antibody in chronic lymphocytic leukemia. Early results from an ongoing phase i/ii clinical trial. *Blood* 160: 448A.

Cole, M.S., Anasetti, C., Tso, J.Y. (1997) Human IgG2 variants of chimeric anti-CD3 are nonmitogenic to T cells. *J Immunol* 159: 3613–3621.

Cragg, M.S., Glennie, M.J. (2004) Antibody specificity controls in vivo effector mechanisms of anti-CD20 reagents. *Blood* 103: 2738–2743.

Dall'Acqua, W.F., Woods, R.M., Ward, E.S., Palaszynski, S.R., Patel, N.K., Brewah, Y. A., Wu, H., Kiener, P.A., Langermann, S. (2002) Increasing the affinity of a human IgG1 for the neonatal Fc receptor: biological consequences. *J Immunol* 169: 5171–5180.

Damle, N.K., Frost, P. (2003) Antibody-targeted chemotherapy with immunoconjugates of calicheamicin. *Curr Opin Pharmacol* 3: 386–390.

Davies, J., Jiang, L., Pan, L.Z., LaBarre, M.J., Anderson, D., Reff, M. (2001) Expression of GnTIII in a recombinant anti-CD20 CHO production cell line: Expression of antibodies with altered glycoforms leads to an increase in ADCC through higher affinity for FcγRIII. *Biotechnol Bioeng* 74: 288–294.

de Lange, G.G. (1989) Polymorphisms of human immunoglobulins: Gm, Am, Em and Km allotypes. *Exp Clin Immunogenet* 6: 7–17.

Dechant, M., Valerius, T. (2001) IgA antibodies for cancer therapy. *Crit Rev Oncol Hematol* 39: 69–77.

Dechant, M., Vidarsson, G., Stockmeyer, B., Repp, R., Glennie, M.J., Gramatzki, M., van De Winkel, J.G., Valerius, T. (2002) Chimeric IgA antibodies against HLA class II effectively trigger lymphoma cell killing. *Blood* 100: 4574–4580.

Di Carlo, E., Forni, G., Lollini, P., Colombo, M.P., Modesti, A., Musiani, P. (2001) The intriguing role of polymorphonuclear neutrophils in antitumor reactions. *Blood* 97: 339–345.

Di Gaetano, N., Cittera, E., Nota, R., Vecchi, A., Grieco, V., Scanziani, E., Botto, M., Introna, M., Golay, J. (2003) Complement activation determines the therapeutic activity of rituximab in vivo. *J Immunol* 171: 1581–1587.

Duncan, A.R., Winter, G. (1988) The binding site for C1q on IgG. *Nature* 332: 738–740.

Dyer, M.J., Hale, G., Hayhoe, F.G., Waldmann, H. (1989) Effects of CAMPATH-1 antibodies in vivo in patients with lymphoid malignancies: influence of antibody isotype. *Blood* 73: 1431–1439.

Dyer, M.J.S., Moser, S., Brünker, P., Bird, P., Almond, N., Püentener, U., Wheat, L.M.W., Bolam, E., Berrie, E., Grau, R., Buckby, E., Kennedy, B., Stebbings, R., Hale, G., Umana, P. (2005) Enhanced potency of glycoengineered anti-CD52 monoclonal antibodies (MAbs). *Blood* 160: 2958A.

Farag, S.S., Flinn, I.W., Modali, R., Lehman, T.A., Young, D., Byrd, J.C. (2004) Fcγ RIIIa and FcγRIIa polymorphisms do not predict response to rituximab in B-cell chronic lymphocytic leukemia. *Blood* 103: 1472–1474.

Ferrara, C., Stuart, F., Sondermann, P., Brunker, P., Umana, P. (2005) The carbohydrate at FcγRIIIa ASN162: An element required for high affinity binding to non-fucosylated IgG glycoforms. *J Biol Chem* 281: 5032–5036.

Ferrara, C., Brunker, P., Suter, T., Moser, S., Puntener, U., Umana, P. (2006) Modulation of therapeutic antibody effector functions by glycosylation engineering: influence of Golgi enzyme localization domain and co-expression of heterologous beta1:4-*N*-acetylglucosaminyltransferase III and Golgi alpha-mannosidase II. *Biotechnol Bioeng* 93: 851–861.

Friend, P.J., Hale, G., Chatenoud, L., Rebello, P., Bradley, J., Thiru, S., Phillips, J.M., Waldmann, H. (1999) Phase I study of an engineered aglycosylated humanized CD3 antibody in renal transplant rejection. *Transplantation* 68: 1632–1637.

Ghetie, V., Ward, E.S. (2000) Multiple roles for the major histocompatibility complex class I- related receptor FcRn. *Annu Rev Immunol* 18: 739–766.

Glennie, M.J., van de Winkel, J.G. (2003) Renaissance of cancer therapeutic antibodies. *Drug Discov Today* 8: 503–510.

Herold, K.C., Hagopian, W., Auger, J.A., Poumian-Ruiz, E., Taylor, L., Donaldson, D., Gitelman, S.E., Harlan, D.M., Xu, D., Zivin, R.A., Bluestone, J.A. (2002) Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. *N Engl J Med* 346: 1692–1698.

Hinton, P.R., Johlfs, M.G., Xiong, J.M., Hanestad, K., Ong, K.C., Bullock, C., Keller, S., Tang, M.T., Tso, J.Y., Vasquez, M., Tsurushita, N. (2004) Engineered human IgG antibodies with longer serum half-lives in primates. *J Biol Chem* 279: 6213–6216.

Hinton, P.R., Xiong, J.M., Johlfs, M.G., Tang, M.T., Keller, S., Tsurushita, N. (2006) An engineered human IgG1 antibody with longer serum half-life. *J Immunol* 176: 346–356.

Hirsch, F.R., Bunn, P.A., Jr. (2005) Epidermal growth factor receptor inhibitors in lung cancer: smaller or larger molecules, selected or unselected populations? *J Clin Oncol* 23: 9044–9047.

Huls, G., Heijnen, I.A., Cuomo, E., van der Linden, J., Boel, E., van de Winkel, J.G., Logtenberg, T. (1999) Antitumor immune effector mechanisms recruited by phage display-derived fully human IgG1 and IgA1 monoclonal antibodies. *Cancer Res* 59: 5778–5784.

Idusogie, E.E., Presta, L.G., Gazzano-Santoro, H., Totpal, K., Wong, P.Y., Ultsch, M., Meng, Y.G., Mulkerrin, M.G. (2000) Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc. *J Immunol* 164: 4178–4184.

Idusogie, E.E., Wong, P.Y., Presta, L.G., Gazzano-Santoro, H., Totpal, K., Ultsch, M., Mulkerrin, M.G. (2001) Engineered antibodies with increased activity to recruit complement. *J Immunol* 166: 2571–2575.

Janakiraman, N., McLaughlin, P., White, C. A., Maloney, D.G., Shen, D., Grillo-Lopez, A.J. (1998) Rituximab: correlation between effector cells and clinical activity in NHL. *Blood* 92: 337A.

Jefferis, R. (1986) Polyclonal and monoclonal antibody reagents specific for IgG subclasses. *Monogr Allergy* 19: 71–85.

Jefferis, R. (2005) Glycosylation of recombinant antibody therapeutics. *Biotechnol Prog* 21: 11–16.

Jefferis, R., Lund, J. (2002) Interaction sites on human IgG-Fc for FcγR: current models. *Immunol Lett* 82: 57–65.

Jefferis, R., Lund, J., Goodall, M. (1995) Recognition sites on human IgG for Fcγ receptors: the role of glycosylation. *Immunol Lett* 44: 111–117.

Kennedy, A.D., Beum, P.V., Solga, M.D., DiLillo, D.J., Lindorfer, M.A., Hess, C.E., Densmore, J.J., Williams, M.E., Taylor, R.P. (2004) Rituximab infusion promotes rapid complement depletion and acute CD20 loss in chronic lymphocytic leukemia. *J Immunol* 172: 3280–3288.

Kerr, M.A. (1990) The structure and function of human IgA. *Biochem J* 271: 285–296.

Keymeulen, B., Vandemeulebroucke, E., Ziegler, A.G., Mathieu, C., Kaufman, L., Hale, G., Gorus, F., Goldman, M., Walter, M., Candon, S., Schandene, L., Crenier, L., De Block, C., Seigneurin, J.M., De Pauw, P., Pierard, D., Weets, I., Rebello, P., Bird, P., Berrie, E., Frewin, M., Waldmann, H., Bach, J.F., Pipeleers, D., Chatenoud, L. (2005) Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. *N Engl J Med* 352: 2598–2608.

Krapp, S., Mimura, Y., Jefferis, R., Huber, R., Sondermann, P. (2003) Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. *J Mol Biol* 325: 979–989.

Kumpel, B.M., Wang, Y., Griffiths, H.L., Hadley, A.G., Rook, G.A. (1995) The biological activity of human monoclonal IgG anti-D is reduced by beta-galactosidase treatment. *Hum Antibodies Hybridomas* 6: 82–88.

Lammerts van Bueren, J.J., Bleeker, W.K., Bøgh, H.O., Houtkamp, M., Schuurman, J., van de Winkel, J.G.J., Parren, P.W.H.I. (2006) Effect of target dynamics on pharmacokinetics of a novel therapeutic antibody against the epidermal growth factor receptor: implications for the mechanisms of action. *Cancer Res* 66: 7630–7638.

Lazar, G.A., Dang, W., Karki, S., Vafa, O., Peng, J.S., Hyun, L., Chan, C., Chung, H. S., Eivazi, A., Yoder, S.C., Vielmetter, J., Carmichael, D.F., Hayes, R.J., Dahiyat, B.I. (2006) Engineered antibody Fc variants with enhanced effector function. *Proc Natl Acad Sci USA* 103: 4005–4010.

Lifely, M.R., Hale, C., Boyce, S., Keen, M.J., Phillips, J. (1995) Glycosylation and biological activity of CAMPATH-1H expressed in different cell lines and grown under different culture conditions. *Glycobiology* 5: 813–822.

Lin, T.S., Flinn, I.W., Modali, R., Lehman, T. A., Webb, J., Waymer, S., Moran, M.E., Lucas, M.S., Farag, S.S., Byrd, J.C. (2005) FCGR3A and FCGR2A polymorphisms may not correlate with response to alemtuzumab in chronic lymphocytic leukemia. *Blood* 105: 289–291.

Lucisano Valim, Y.M., Lachmann, P.J. (1991) The effect of antibody isotype and antigenic epitope density on the complement-fixing activity of immune complexes: a systematic study using chimaeric anti-NIP antibodies with human Fc regions. *Clin Exp Immunol* 84: 1–8.

Manches, O., Lui, G., Chaperot, L., Gressin, R., Molens, J.P., Jacob, M.C., Sotto, J.J., Leroux, D., Bensa, J.C., Plumas, J. (2003) In vitro mechanisms of action of rituximab on primary non-Hodgkin lymphomas. *Blood* 101: 949–954.

Michaelsen, T.E., Garred, P., Aase, A. (1991) Human IgG subclass pattern of inducing complement-mediated cytolysis depends on antigen concentration and to a lesser extent on epitope patchiness, antibody affinity and complement concentration. *Eur J Immunol* 21: 11–16.

Monteiro, R.C., van de Winkel, J.G. (2003) IgA Fc receptors. *Annu Rev Immunol* 21: 177–204.

Mori, K., Kuni-Kamochi, R., Yamane-Ohnuki, N., Wakitani, M., Yamano, K., Imai, H., Kanda, Y., Niwa, R., Iida, S., Uchida, K., Shitara, K., Satoh, M. (2004) Engineering Chinese hamster ovary cells to maximize effector function of produced antibodies using FUT8 siRNA. *Biotechnol Bioeng* 88: 901–908.

Nimmerjahn, F., Ravetch, J.V. (2005) Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science* 310: 1510–1512.

Nimmerjahn, F., Ravetch, J.V. (2006) Fcγ receptors: old friends and new family members. *Immunity* 24: 19–28.

Niwa, R., Hatanaka, S., Shoji-Hosaka, E., Sakurada, M., Kobayashi, Y., Uehara, A., Yokoi, H., Nakamura, K., Shitara, K. (2004) Enhancement of the antibody-dependent cellular cytotoxicity of low-fucose IgG1 Is independent of FcγRIIIa functional polymorphism. *Clin Cancer Res* 10: 6248–6255.

Niwa, R., Natsume, A., Uehara, A., Wakitani, M., Iida, S., Uchida, K., Satoh, M., Shitara, K. (2005) IgG subclass-independent improvement of antibody-dependent cellular cytotoxicity by fucose removal from Asn297-linked oligosaccharides. *J Immunol Methods* 306: 151–160.

Norman, D.J., Vincenti, F., de Mattos, A.M., Barry, J.M., Levitt, D.J., Wedel, N.I., Maia, M., Light, S.E. (2000) Phase I trial of HuM291: a humanized anti-CD3 antibody, in patients receiving renal allografts from living donors. *Transplantation* 70: 1707–1712.

Nose, M., Wigzell, H. (1983) Biological significance of carbohydrate chains on monoclonal antibodies. *Proc Natl Acad Sci USA* 80: 6632–6636.

Parren, P.W., Warmerdam, P.A., Boeije, L.C., Arts, J., Westerdaal, N.A., Vlug, A., Capel, P.J., Aarden, L.A., van de Winkel, J.G. (1992) On the interaction of IgG subclasses with the low affinity FcγRIIa (CD32) on human monocytes, neutrophils, and platelets. Analysis of a functional polymorphism to human IgG2. *J Clin Invest* 90: 1537–1546.

Pasquier, B., Launay, P., Kanamaru, Y., Moura, I.C., Pfirsch, S., Ruffie, C., Henin, D., Benhamou, M., Pretolani, M., Blank, U., Monteiro, R.C. (2005) Identification of FcαRI as an inhibitory receptor that controls inflammation: dual role of FcRγ ITAM. *Immunity* 22: 31–42.

Pleass, R.J., Ogun, S.A., McGuinness, D.H., van de Winkel, J.G., Holder, A.A., Woof, J. M. (2003) Novel antimalarial antibodies highlight the importance of the antibody Fc region in mediating protection. *Blood* 102: 4424–4430.

Rabbitts, T.H., Forster, A., Milstein, C.P. (1981) Human immunoglobulin heavy chain genes: evolutionary comparisons of Cμ, Cδ and Cγ genes and associated switch sequences. *Nucleic Acids Res* 9: 4509–4524.

Rademacher, T.W., Parekh, R.B., Dwek, R. A., Isenberg, D., Rook, G., Axford, J.S., Roitt, I. (1988) The role of IgG glycoforms in the pathogenesis of rheumatoid arthritis. *Springer Semin Immunopathol* 10: 231–249.

Reddy, M.P., Kinney, C.A., Chaikin, M.A., Payne, A., Fishman-Lobell, J., Tsui, P., Dal Monte, P.R., Doyle, M.L., Brigham-Burke, M.R., Anderson, D., Reff, M., Newman, R., Hanna, N., Sweet, R.W., Truneh, A. (2000) Elimination of Fc receptor-dependent effector functions of a modified IgG4 monoclonal antibody to human CD4. *J Immunol* 164: 1925–1933.

Reichert, J.M., Rosensweig, C.J., Faden, L. B., Dewitz, M.C. (2005) Monoclonal antibody successes in the clinic. *Nat Biotechnol* 23: 1073–1078.

Riechmann, L., Clark, M., Waldmann, H., Winter, G. (1988) Reshaping human antibodies for therapy. *Nature* 332: 323–327.

Schachter, H. (2000) The joys of HexNAc. The synthesis and function of N- and O-glycan branches. *Glycoconj J* 17: 465–483.

Schuster, M., Umana, P., Ferrara, C., Brunker, P., Gerdes, C., Waxenecker, G., Wiederkum, S., Schwager, C., Loibner, H., Himmler, G., Mudde, G.C. (2005) Improved effector functions of a therapeutic monoclonal Lewis Y-specific antibody by glycoform engineering. *Cancer Res* 65: 7934–7941.

Shields, R.L., Namenuk, A.K., Hong, K., Meng, Y.G., Rae, J., Briggs, J., Xie, D., Lai, J., Stadlen, A., Li, B., Fox, J.A., Presta, L.G. (2001) High resolution mapping of the

binding site on human IgG1 for FcγRI, FcγRII, FcγRIII, and FcRn and design of IgG1 variants with improved binding to the FcγR. *J Biol Chem* 276: 6591–6604.

Shields, R.L., Lai, J., Keck, R., O'Connell, L.Y., Hong, K., Meng, Y.G., Weikert, S.H., Presta, L.G. (2002) Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcγRIII and antibody-dependent cellular toxicity. *J Biol Chem* 277: 26733–26740.

Shih, L.B., Lu, H.H., Xuan, H., Goldenberg, D.M. (1994) Internalization and intracellular processing of an anti-B-cell lymphoma monoclonal antibody, LL2. *Int J Cancer* 56: 538–545.

Shinkawa, T., Nakamura, K., Yamane, N., Shoji-Hosaka, E., Kanda, Y., Sakurada, M., Uchida, K., Anazawa, H., Satoh, M., Yamasaki, M., Hanai, N., Shitara, K. (2003) The absence of fucose but not the presence of galactose or bisecting N-acetylglucosamine of human IgG1 complex-type oligosaccharides shows the critical role of enhancing antibody-dependent cellular cytotoxicity. *J Biol Chem* 278: 3466–3473.

Sondermann, P., Huber, R., Oosthuizen, V., Jacob, U. (2000) The 3.2-A crystal structure of the human IgG1 Fc fragment-FcγRIII complex. *Nature* 406: 267–273.

Stavenhagen, J., Vijh, S., Rankin, C., Gorlatov, S., Huang, L. (2004) Identification and engineering of antibodies with variant Fc regions and methods of using same US 2005/0064514 A1 MacroGenics, Inc.

Tao, M.H., Morrison, S.L. (1989) Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J Immunol* 143: 2595–2601.

Treon, S.P., Hansen, M., Branagan, A.R., Verselis, S., Emmanouilides, C., Kimby, E., Frankel, S.R., Touroutoglou, N., Turnbull, B., Anderson, K.C., Maloney, D.G., Fox, E.A. (2005) Polymorphisms in FcγRIIIA (CD16) receptor expression are associated with clinical response to rituximab in Waldenstrom's macroglobulinemia. *J Clin Oncol* 23: 474–481.

Uchida, J., Hamaguchi, Y., Oliver, J.A., Ravetch, J.V., Poe, J.C., Haas, K.M., Tedder, T.F. (2004) The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J Exp Med* 199: 1659–1669.

Umana, P., Jean-Mairet, J., Moudry, R., Amstutz, H., Bailey, J.E. (1999) Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat Biotechnol* 17: 176–180.

Utset, T.O., Auger, J.A., Peace, D., Zivin, R.A., Xu, D., Jolliffe, L., Alegre, M.L., Bluestone, J.A., Clark, M.R. (2002) Modified anti-CD3 therapy in psoriatic arthritis: a phase I/II clinical trial. *J Rheumatol* 29: 1907–1913.

Valerius, T., Stockmeyer, B., van Spriel, A. B., Graziano, R.F., van den Herik-Oudijk, I.E., Repp, R., Deo, Y.M., Lund, J., Kalden, J.R., Gramatzki, M., van de Winkel, J.G. (1997) FcαRI (CD89) as a novel trigger molecule for bispecific antibody therapy. *Blood* 90: 4485–4492.

van de Winkel, J.G., Capel, P.J. (1993) Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. *Immunol Today* 14: 215–221.

van der Kolk, L.E., Grillo-Lopez, A.J., Baars, J.W., Hack, C.E., van Oers, M.H. (2001) Complement activation plays a key role in the side-effects of rituximab treatment. *Br J Haematol* 115: 807–811.

van Egmond, M., van Garderen, E., van Spriel, A.B., Damen, C.A., van Amersfoort, E.S., van Zandbergen, G., van Hattum, J., Kuiper, J., van de Winkel, J.G. (2000) FcαRI-positive liver Kupffer cells: reappraisal of the function of immunoglobulin A in immunity. *Nat Med* 6: 680–685.

van Loghem, E. (1986) Allotypic markers. Monogr Allergy 19: 40–51.

van Sorge, N.M., van der Pol, W.L., van de Winkel, J.G. (2003) FcγR polymorphisms: Implications for function, disease susceptibility and immunotherapy. *Tissue Antigens* 61: 189–202.

van Spriel, A.B., van den Herik-Oudijk, I.E., van Sorge, N.M., Vile, H.A., van Strijp, J. A., van de Winkel, J.G. (1999) Effective phagocytosis and killing of *Candida albicans* via targeting FcγRI (CD64) or

FcαRI (CD89) on neutrophils. *J Infect Dis* 179: 661–669.

Vidarsson, G., van Der Pol, W.L., van Den Elsen, J.M., Vile, H., Jansen, M., Duijs, J., Morton, H.C., Boel, E., Daha, M.R., Corthesy, B., van De Winkel, J.G. (2001) Activity of human IgG and IgA subclasses in immune defense against *Neisseria meningitidis* serogroup B. *J Immunol* 166: 6250–6256.

Waldmann, T.A., Strober, W. (1969) Metabolism of immunoglobulins. *Prog Allergy* 13: 1–110.

Weng, W.K., Levy, R. (2001) Expression of complement inhibitors CD46: CD55: and CD59 on tumor cells does not predict clinical outcome after rituximab treatment in follicular non-Hodgkin lymphoma. *Blood* 98: 1352–1357.

Weng, W.K., Levy, R. (2003) Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol* 21: 3940–3947.

Woodle, E.S., Xu, D., Zivin, R.A., Auger, J., Charette, J., O'Laughlin, R., Peace, D., Jollife, L.K., Haverty, T., Bluestone, J.A., Thistlethwaite, J.R., Jr. (1999) Phase I trial of a humanized, Fc receptor nonbinding OKT3 antibody, huOKT3gamma1(Ala-Ala) in the treatment of acute renal allograft rejection. *Transplantation* 68: 608–616.

Woof, J.M. (2005) Immunology. Tipping the scales toward more effective antibodies. *Science* 310: 1442–1443.

Woof, J.M., Mestecky, J. (2005) Mucosal immunoglobulins. *Immunol Rev* 206: 64–82.

Wright, A., Morrison, S.L. (1997) Effect of glycosylation on antibody function: implications for genetic engineering. *Trends Biotechnol* 15: 26–32.

Yamane-Ohnuki, N., Kinoshita, S., Inoue-Urakubo, M., Kusunoki, M., Iida, S., Nakano, R., Wakitani, M., Niwa, R., Sakurada, M., Uchida, K., Shitara, K., Satoh, M. (2004) Establishment of FUT8 knockout Chinese hamster ovary cells: an ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity. *Biotechnol Bioeng* 87: 614–622.

Yoo, E.M., Morrison, S.L. (2005) IgA: an immune glycoprotein. *Clin Immunol* 116: 3–10.

Part II
The Way into the Clinic

9
Production and Downstream Processing

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9.1
Introduction

Biopharmaceuticals such as monoclonal antibodies (mAbs) are complex molecules that, today, can only be produced economically by using mammalian cells. As mAbs are highly susceptible to physical and chemical stress, supply for clinical studies and therapy requires the development of a suitable production process. This process should ensure high quality and stability of the product in accordance with the quality obligations of the regulatory authorities at an acceptable cost of goods. The development of a suitable production process for mAbs requires the concerted and coordinated activities of a number of disciplines such as molecular and cell biology, upstream and downstream processing, formulation development, filling operations, quality testing, and quality control.

Molecular and cell biology techniques are involved in cloning the antibody genes into an appropriate expression vector followed by the generation of a production cell line. The goal is to develop a production cell line possessing a high specific productivity which is capable of growing to high cell densities in serum-free and chemically defined media.

Upstream processing consists of all operations that are involved in the generation of the crude mAb. This is generally achieved by cultivation of the production cells, mostly by means of fermentation using bioreactors. Development of an optimal cell culture medium and process design are important elements for an efficient process capable of delivering the product in high quantities. After separation of the cells from the culture medium, which contains the crude product, the purification of the mAb from contaminants originating from the cells and the cell culture medium is addressed in the downstream processing steps. These consist mainly of chromatographic and filtration methods. High recovery of the protein, efficient removal of contaminants and the demonstration of an efficient inactivation and removal of potential viruses, while retaining the molecule's correct structure, are crucial at this stage of development.

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The resulting drug substance undergoes final formulation prior to filling into the primary packaging container, which can be either a glass vial or a syringe. The development of the final formulation has to take the physicochemical and biological characteristics of the product, as well as the intended application route, into account in order to secure the defined product quality specifications during the shelf-life of the product.

The detailed description of the molecular characteristics of the product represents the basis for the definition of the quality parameters to ensure the safety and biological activity of the product. Quality parameters such as identity, purity, potency, strength, and stability are closely monitored on a lot-by-lot basis prior to release of the product for human use.

As the market for biopharmaceuticals continues to grow rapidly, key factors for successful development of mAbs are the so-called platform technologies. These technologies represent a company's know-how and have proven efficient and successful in previous development programs. Use of these technologies may significantly speed up development times.

Another important factor for a successful process development program is the coordination and optimal timing of the various activities and work packages of the different disciplines. This can be achieved by the implementation of an interdisciplinary project team with clearly defined responsibilities, roles and tasks for the team members and the project manager. This team is responsible for all different aspects of a product development program in different organizational units, which includes the monitoring of timelines and costs and the establishment of tools for further improvements.

9.2 Upstream Processing

The upstream process consists of all operations that are involved in the generation of the crude therapeutic antibody product, up to or even including the harvest as separation of the cells from the product-containing culture fluid.

Antibody products are generally generated by fermentation (cell cultivation) mostly in bioreactors, for all kinds of purposes, extending from product for research and development through product for clinical studies and up to the market supply for approved therapeutic antibody products.

9.2.1 Expression System

Currently, development of production processes for biopharmaceuticals is undergoing significant changes as the technology matures. Recombinant proteins can be produced in various expression systems such as bacterial, mammalian, insect, plant, and *in vitro* translation systems. A fundamental prerequisite for successful

production of biologics from any of these expression systems is, of course, efficient transcription and translation. However, the choice of system is mainly driven by the overall yield of the production process and the biological activity and efficacy of the therapeutic entity.

Expression of recombinant proteins from *E. coli* cells is a well-established technology (Swartz 2001). *E. coli* cells can produce proteins in large quantities, fast growth rates enable short fermentation times, and the system has been successfully scaled up to supply the market demand for important drugs such as insulin (Lee 1996). However, *E. coli* cells are not capable of glycosylating proteins. Therefore, expression of glycoproteins such as mAbs requires other expression systems. The vast majority of such proteins are currently being expressed in mammalian cells, mostly Chinese hamster ovary (CHO) and mouse myeloma cells (NS0) cells. The two most prominent challenges for mammalian cell-based systems relate to (i) product titer in the cell culture fluid at the end of cultivation and (ii) development times from final drug candidate selection to an established process to produce clinical grade material. On both fronts recent developments indicate that the newest generations of mammalian cell culture production processes evolve rapidly to overcome these limitations (Wurm 2004). Many improvements, including novel or modified genetic elements to improve transcription rate, high-throughput screening concepts to obtain highly productive clones reliably, and host cell lines that grow to high densities in serum-free chemically defined media, have achieved specific productivities of values above 50 pg per cell and day (pg/c*d) for mAbs. Figure 9.1 shows the productivity profile for a CHO DG-44 high-producer cell line. Serial cultivation in spinner flasks (typical for industrial inoculum settings) displays a constant specific productivity of 55 pg per cell and day for 120 days in culture. In addition to high productivities, cell lines need to have stable phenotypic and genotypic product expression profiles to enable economically attractive production campaigns with many fed-batch runs initiated from one inoculum culture.

Furthermore, sophisticated design of clone-screening platforms, including platform media and well-characterized down-scale models, is crucial to ensure that high titers can be achieved with little or no time for process optimization. Such a high-expression concept can result in titers above 1 g L^{-1} without the need for optimizing the upstream process subsequent to clone screening. Due to the biochemical complexity of biologics and the influence of host cell, media, and other factors on the microheterogeneity of the product, comparability issues are of great importance. It is therefore essential to any fast-track cell line generation concept that it results in a production cell line that has the potential to form the basis for a high titer process as it is needed to generate material all the way to the market. A well-characterized media platform is crucial to minimize changes throughout further process optimization. Many recent approaches have demonstrated the great potential of host cell engineering, suggesting that future mammalian host cells will comprise optimized molecular pathways that control growth, apoptosis, transport, and metabolic fluxes (Kaufmann and Fussenegger 2003).

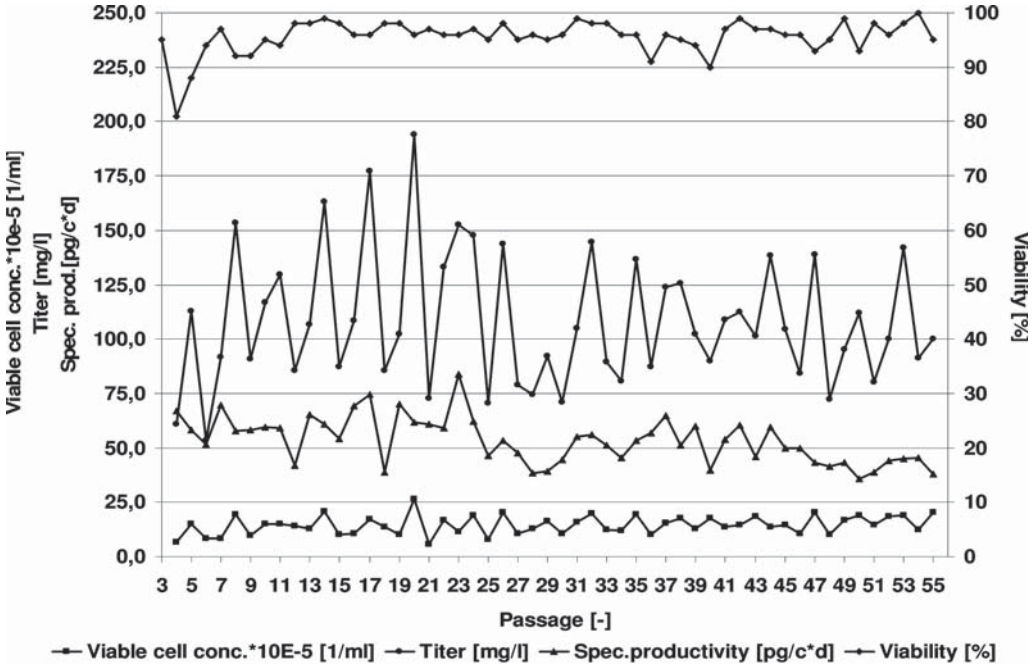

Fig. 9.1 BI HEX CHO (Boehringer Ingelheim's proprietary high-expression platform). Stable high expression of a monoclonal antibody in a clonal CHO cell line.

Although, for mAbs, the current expression platform of choice is mammalian cells, in the future, other hosts may represent interesting alternatives in the production of new biopharmaceuticals. Processing of yeast glycoproteins within the endoplasmic reticulum and the Golgi apparatus is different from the processing of their mammalian counterparts. This results in distinct nonmammalian glycopatterns of yeast proteins. In particular, high-mannose type *N*-glycosylation precludes the use of recombinant glycoproteins produced from yeasts for therapeutic use in humans as it leads to a short circulatory half-life *in vivo* and possibly altered activity, thereby compromising drug efficacy. Recently a lot of work has been focused on generating genetically engineered yeast strains that confer a synthetic *N*-glycosylation pathway designed to enable production of proteins with a more mammalian-like glycosylation pattern (Wildt and Gerngross 2005). Although these approaches are promising, many problems such as the lack of terminal sialylation need to be solved before yeast cells can be viewed as an alternative expression platform for producing glycoproteins for use in humans.

Another fundamental question that needs to be answered in the future concerns the robustness, growth performance and general phenotype of yeasts cells that have been genetically modified to substitute entire biological pathways such as glycosylation.

Another interesting field with regard to future expression systems is the evaluation of the production of biopharmaceuticals from plants. A variety of different plant-based systems may provide interesting options, including production within the leaves of transgenic plants, secretion from roots or leaves, or growth of plant cells in a bioreactor (Fischer et al. 2004). However, several fundamental problems need to be overcome before recombinant proteins produced from plants can be approved for therapeutic use in humans. Yields for antibodies or other proteins are commonly low, protein stability is often poor and in general downstream processing of plant-derived recombinant proteins is cumbersome. Recent studies point to directions this field may take in order to overcome these limitations. Strategies include targeting of heterologous proteins to the secretory pathway of plant cells, product expression in seeds and the use of strong inducible promoters (Fischer et al. 2004).

9.2.2 Cell Culture Media

A second key element for the upstream process is the development and use of suitable media for the fermentation. Media for microbial cells or yeast cells consist of a carbon and energy source, nutrient salts and trace elements, and sometimes a limited number of vitamins or amino acids. Media for mammalian cells are much more complex. About 30–80 ingredients are dissolved in purified (deionized) water and a proper balance of buffering and osmoregulating substances, trace elements, precursors for the primary and secondary cell metabolism (e.g. amino acids, vitamins, lipids) and nutrients is critical for a successful long-term cultivation of mammalian cells *in vitro*. Until recently it was industry standard to supplement the basal medium consisting of defined components with 5–20% serum (mostly from calf or cattle). Preparations of animal sera consist of a complex nondefined mixture of growth factors and other biomolecules that mediate cell survival and growth. The major disadvantages of using sera in cell culture processes are the residual risk of human pathogens present in animal sera, limited supply, high cost and high batch-to-batch variability (Allison et al. 2005).

During the last 25 years large efforts were devoted to the removal and substitution of serum. In the first generation of serum-free culture media, serum was replaced by defined substances still from animal sources added at defined concentrations such as bovine serum albumin (BSA), bovine insulin, bovine transferrin, lipoproteins, and cholesterol (Hewlett 1991).

Today's state-of-the-art culture media are free of bovine components and chemically defined. Here animal components are substituted by chemically defined mixtures or single substances such as recombinant insulin or IGF (insulin-like growth factor), lipid concentrates, iron salts or complexes, precursors, or stimulating substances. Media development over the past decades has led to a reduction in the protein content of cell culture media from about $20\,g\,L^{-1}$ to about $0.01\,g\,L^{-1}$ or even to zero. Currently commercial media suppliers offer a variety of basic

media as powders or granulates or liquid formulations optimized for specific cell lines and the needs of serum-free cell culture processes (Kempken et al. 2006). Today's cell culture media provide an excellent platform for high-performance cell culture processes for the generation of therapeutic antibodies.

9.2.3 Cell Culture Process Design

The third key element is the development of an efficient, robust and reproducible cell culture process itself. Mammalian cells grow either anchorage-dependent or in suspension. *In vitro* cultivation systems for anchorage-dependent cells (Fig. 9.2a) range from T-flasks and roller bottles through fluidized bed bioreactors, where the cells are attached to microcarriers (i.e. massive or porous beads approx. 50–300 µm diameter) suspended in a bioreactor, up to fixed bed bioreactors, where the cells are attached in the capillaries of solid surfaces (e.g. in hollow-fiber membrane modules, Fig. 9.2b) or in ceramic modules. In all cases, culture medium and aeration is provided continuously or at intervals by exchange of medium and gas, or by perfusion (see below) of the bioreactors (Murakami et al. 1991).

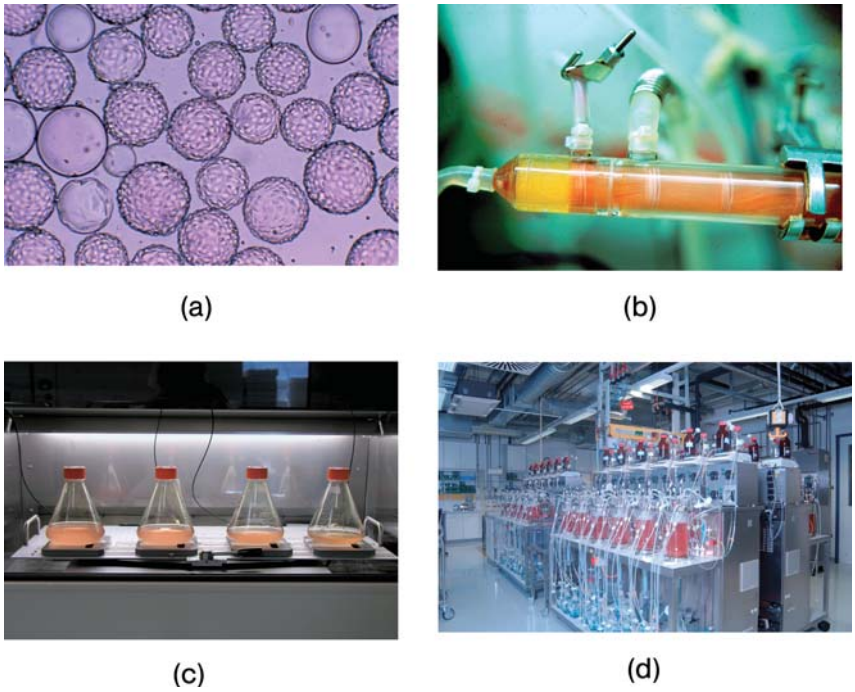

Fig. 9.2 *In vitro* cultivation systems: (a) microcarrier culture; (b) hollow fiber culture; (c) shake flask culture; (d) small-scale bioreactor culture.

Despite of the very high cell densities in fluidized and fixed bed cultivation systems, there are numerous disadvantages such as limited scalability, complex process technology, necrotic cells within multilayer cell populations, instability or degradation of multilayer cells in long-term cultivation, difficulties in cleaning for reuse of the systems and the need for numerous product harvests at short intervals (Preissmann et al. 1997).

Upstream processes with suspension cultures are the preferred format for industrial applications, such as the production of therapeutic antibodies in large quantities. For culturing mammalian cells in milliliter or low liter quantities, shake flasks (Fig. 9.2c) or spinner vessel systems are used. At a larger scale (5–20 000 L) continuous stirred tank reactors (CSTR) are widely used (Fig. 9.2d). This type of fermenter is standardized, easy to scale-up, allows efficient cleaning-in-place (CIP) and sterilization-in-place (SIP) procedures, and can be flexibly used for a variety of different processes (Chu and Robinson 2001).

In addition to the fermentation equipment, the cell cultivation method has to be developed and optimized. The standard cultivation process is the "batch" process. A batch process is initiated by adding medium and a seed cell suspension of defined cell density to the bioreactor and subsequently cells are cultivated for a defined period of time under suitable conditions without further manipulations (Fig. 9.3).

A more sophisticated process format is the "fed-batch" process. Here, concentrated solutions of specific cell culture additives are added (fed) during the cultivation period (e.g. nutrients, growth factors, inductors or enhancers for product generation). Enhanced cell growth, higher cell densities, fewer nutrient limitations, and prolongation of culture viability are the key characteristics of a fed-batch process, generally leading to high product yields (Fig. 9.3).

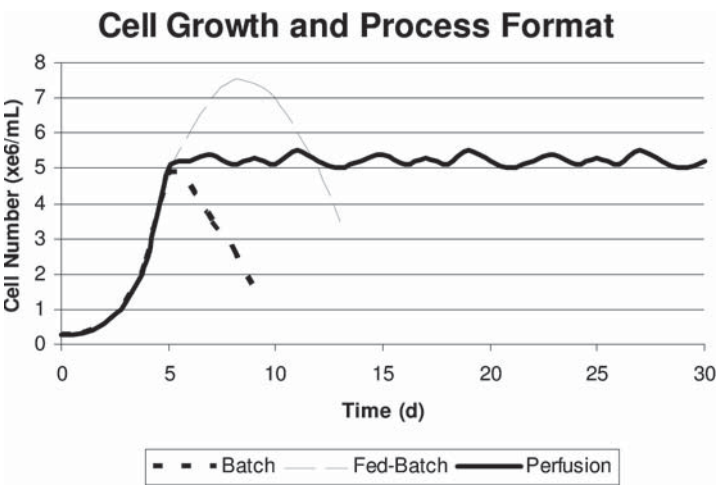

Fig. 9.3 Cell culture processes: batch process (thick dashed line), fed-batch process (thin dashed line), perfusion process (solid line).

In "continuous" processes such additions (feeds) are made continuously or periodically while an equal volume of culture fluid is removed simultaneously. Thereby a steady state can be established, leading to constant cultivation conditions and allowing continuous removal of cells, cell debris, and cytotoxic substances derived from apoptotic cells. Hence the run time of continuous fermentations can be very long, up to several months (Storhas 1994).

If a cell-free supernatant is removed from the bioreactor, this mode of continuous cultivation is called "perfusion" (Fig. 9.3). Very high cell densities can be obtained in perfusion cultures as well as high volumetric productivities (due to the large volume of perfused medium, while product concentrations themselves are rather low).

To date, the majority of manufacturing processes for therapeutic antibodies is based on the fed-batch format as they are characterized by short process development times, short generation time for individual product batches, robust and easily scalable mode of cultivation, fast and easy process validation and registration, and flexible production schedules in multipurpose manufacturing facilities (i.e. facilities in which several products are manufactured in campaigns concurrently, overlapping or sequentially).

9.2.4 Cell Culture Process Optimization

The key to high-performing upstream processes lies in a good combination of high-expression vector systems, robust cell lines capable of secreting high quantities of product per time, optimized media and additions (feeds), state-of-the art cell culture hardware, the appropriate cultivation method and optimized process design and operating conditions.

Technology platforms for all of these key elements can serve as a good basis for successful fast-track development of upstream processes for individual therapeutic antibodies. The developmental work leading to an optimized process is generally performed in small-scale systems. Crucial factors for optimization can be divided into cell parameters (such as seed density), physical parameters (such as temperature, pH, dissolved oxygen content, osmolality), biochemical parameters (such as concentrations of nutrients), process technology parameters (such as stirrer speed, characteristics of controllers), and mode of additions (such as times and quantities of feeds, compositions and concentrations of feeds).

Factorial design of experiments (DOE) is a valuable experimental and statistical tool for successful process optimization (Eriksson et al. 2000).

Recently, the described high-specific productivities were successfully translated into product titers of up to $6\,g\,L^{-1}$ for both CHO and NS0 cells. Figure 9.4 shows an example of how such high titers could be achieved through a process optimization program for a BI HEX CHO (Boehringer Ingelheim's proprietary high-expression platform) production cell. In this particular case, 11-day fed-batch cultivation led to approximately $4\,g\,L^{-1}$ of a monoclonal IgG1 subtype antibody.

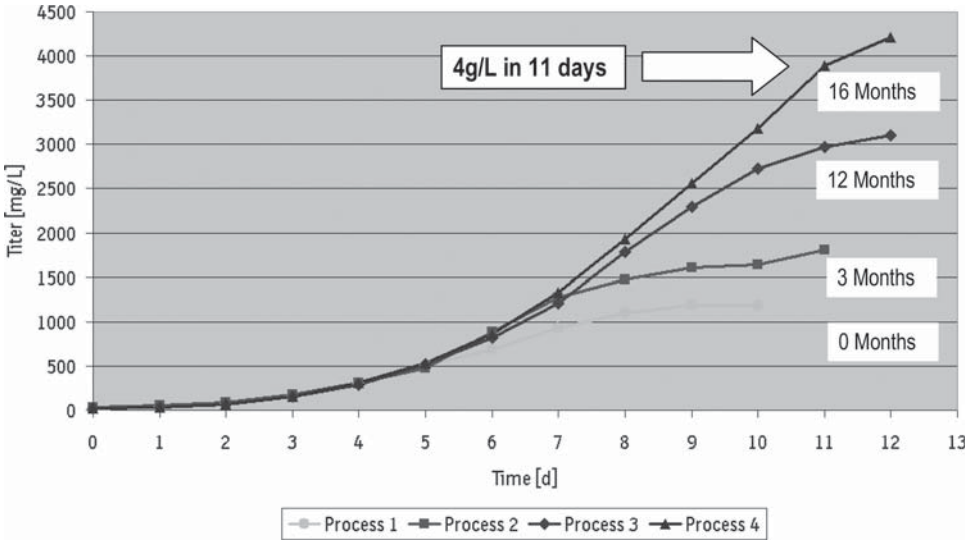

Fig. 9.4 High-yield processes through process optimization (0–16 months): 4 g L^{-1} in 11-day fed-batch CHO BI HEX process (Boehringer Ingelheim's proprietary high expression platform).

9.2.5 Scale-up, Economy of Scale

Another challenge during process development is the scalability of results obtained in small-scale cultivations (e.g. 1 L scale) to the manufacturing scale (e.g. 10 000 L scale). Due to physical and technical reasons, not all characteristics and parameters from a 10 000-L bioreactor can be mimicked exactly in a 1-L bioreactor. But with technical expertise and experience and solid technical characterization of the bioreactor systems, results from the most relevant process parameters can be transformed from small scale to large scale and vice versa (Zlokarnik 2002). This means that the experiments at laboratory scale are meaningful for the large-scale systems and that the optimal small-scale process can be transferred successfully to the final manufacturing scale.

Nearly all therapeutic antibodies are applied in high doses. Treatments with antibodies also require multiple doses and/or long-term administrations, especially when targeted at tumor growth in cancerous diseases. Such proteins are therefore needed in large quantities, and consequently large manufacturing capacities are bound for the annual market supply of therapeutic antibodies. Cell culture facilities with large bioreactors (e.g. 10 000 L working volume) provide substantial cost benefits due to their economy of scale. Table 9.1 illustrates how over recent years the increase in titer moved production of antibodies from CHO cells towards the metric tonne-scale (yields in kilograms may be lower for proteins of small molecular weight). With the assumption that antibody product from a

Table 9.1 Producing antibodies from mammalian cells: towards the tonne scale.

Product	*mAb1*	*mAb2*	*mAb3*	*mAb4*
Titer ($mg\,L^{-1}$)	500	1000	2000	4000
Yield (%)	60	70	70	70[a]
Batch (g)	3750	8750	17500	35000
Yield p.a. (kg)	375	875	1750	3500

Basis: 1 batch = 12500 L. 100 batches/year.
a Assumption.

commercial $4\,g\,L^{-1}$ process could be purified with a yield of 70%, a production site that runs 100 successful batches of such a process annually at 12500-L scale could supply the market with more than 3 tonnes of antibody each year (Table 9.1). However, it is important to note that the high titers of current state-of-the-art mammalian cell culture processes remain a challenge for establishing economical downstream (purification) processes at large scale.

9.2.6 Harvest

As antibodies expressed from mammalian cells contain signal peptides within their primary amino acid sequence they are secreted into the culture medium during the process. Therefore the product harvest at the end of the cultivation process occurs by simply separating cell culture fluid from the cells. In general, the cell separation method should be gentle to minimize cell disruption to avoid the release of proteases and other molecules that could affect the quality of the antibody product (Berthold and Kempken 1994).

In principle, three methods are used for harvest of products from mammalian cell cultures: filtration, centrifugation, and EBA (expanded bed adsorption).

Filtration can be done in a dead-end modus (static filtration) or in a tangential flow modus (dynamic filtration). If the cell suspension is filtered directly through a dead-end filtration system, a cascade of several filter steps is needed. The preferred system involves pre-filters with sieving and adsorption effects such as lenticular filters used to separate the cells, cell debris, and large particles. This is followed by clarification filters and optionally by sterilizing-grade filters to remove the particle load for the subsequent downstream processing. The latest developments of such systems have resulted in a combination of all these steps in a single filter cassette.

The separation of cells and large particles can be enhanced by tangential flow filtration (TFF). Here, the cell suspension is fed into hollow-fiber or flat plate microfiltration membrane modules and recirculated into the bioreactor (Fig. 9.5a).

Fig. 9.5 Harvest systems for mammalian cell cultures: (a) tangential flow filtration system, (b) disk stack centrifugation system.

Cells are pumped at high flow rates through the TFF system and concentrated approx. 10-fold, while the product-containing culture fluid can pass the membranes and is collected in a harvest hold tank (van Reis et al. 1991). To remove cell debris and small particles from the TFF filtrate, an additional dead-end clarification step is needed.

An attractive alternative to TFF membrane filtration is centrifugation. Standard disk stack centrifuges can be modified to allow gentle handling of the fragile mammalian cells and protein products (Tebbe et al. 1996). Centrifuges can be cleaned in place (CIP) and can be used for multiple products. The centrifugation step is followed by dead-end clarification which is similar to the clarification step behind a TFF system. The major advantage of centrifugation over TFF-based harvest regimes is the avoidance of problems such as membrane clogging and fouling (Fig. 9.5b).

EBA offers another alternative to filter-based separation. It is a chromatographic method which combines three unit operations (the separation of cells, the subsequent ultrafiltration/diafiltration of the cell culture fluid, the first chromatography step for product capture and purification) into one single step (Blank et al. 2001). The cell suspension is floated into the bottom of a chromatographic column which is operated as a fluidized bed (instead of a fixed bed as in regular chromatography processes). The product binds to the chromatographic matrix while the cells and particles pass the EBA column. The product can be eluted with high purity in the same way as in conventional fixed-bed chromatographic columns.

9.3
Downstream Processing

Most critical issues in downstream processing are the overall yield, sustained product quality, and feasibility in pilot- and large-scale production (Werner 2005). Therefore, a sophisticated purification strategy has to be developed in order to meet predefined criteria for purity, quality, efficacy, and safety of a therapeutic antibody as well as for process economy.

In general, the protein of interest represents only 1–40% of the total protein content of a mammalian production cell, the rest being a complex mixture of unwanted compounds including protein impurities, lipids, carbohydrates, nucleotides, fermentation ingredients (growth hormones, vitamins, trace elements), cell particulates, and, to a large extent, water. Moreover, adventitious agents such as virus and bacterial endotoxins may contaminate the target antibody solution and must be eliminated for therapeutic application by specific steps for removal and inactivation. Today, product purity and safety is achieved by a combination of various chromatography and dia- and ultrafiltration principles in conjunction with appropriate buffer systems and chemical or physical virus inactivation methods.

From a biochemical point of view any treatment during purification may exert stress on the overall three-dimensional structure of the protein, due to drastic changes in pH values, protein or salt concentrations, buffers or solvents, and by shear forces at liquid stream and surface interfaces. Such stress conditions may result in denaturation or aggregation of the antibody with losses in yield and efficacy. It is therefore necessary to monitor product quality and functionality during downstream processing by appropriate and fast analytical tools.

With regard to economy a limited number of robust process steps are focused on and the design of the process must enable up-scaling to pilot and production scale, that means processing of 400–2000 L and 12 000 L fermentation volumes, concomitant with suitable process turnover times. Finally, a crucial aspect of process development is that product quality and the production process have to comply with regulatory requirements.

The following sections describe and discuss challenges and solutions for the purification of efficacious, safe therapeutic antibodies with emphasis on requirements for up-scaling, economy, and regulatory acceptance using platform technologies. Future trends, such as affinity ligands, will be mentioned at the bottom of the article.

9.3.1
Platform Technologies for Downstream Processing of Monoclonal Antibodies

Therapeutic antibodies are frequently used at high doses and, therefore, pressure for development of robust and economic purification processes is an increasing demand. Currently, downstream processing of mAbs focuses mainly on the application of platform technologies such as represented in Fig. 9.6. Four main sections can be distinguished: primary recovery, viral clearance, purification and

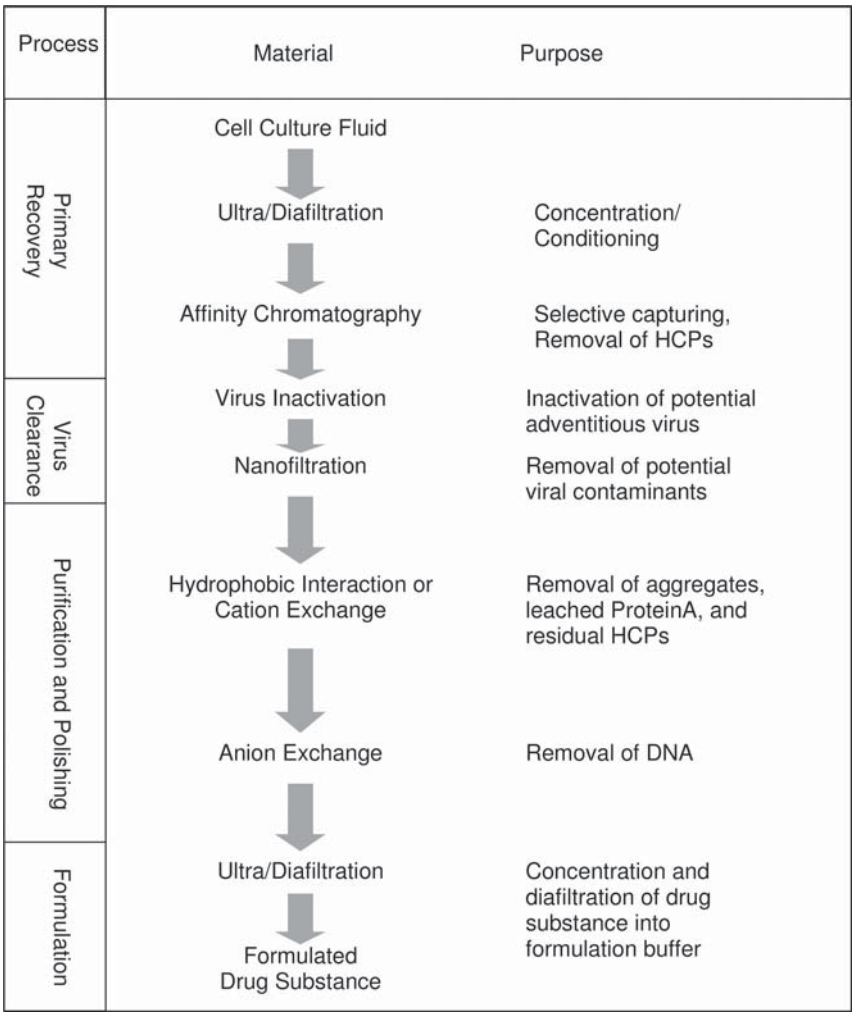

Fig. 9.6 Scheme of a standard platform purification process for therapeutic monoclonal antibodies.

polishing, and formulation of the drug substance. Neither the order in which the individual steps are carried out nor the applied principles used have to be applied as shown; each mAb requires a unique treatment for optimum results.

9.3.2 Primary Recovery

9.3.2.1 Ultra-/Diafiltration (UF/DF)

For the purposes of concentration and conditioning crossflow units are commonly used. Large fermentation volumes are readily reduced by ultrafiltration (UF), and

during diafiltration (DF) ideal and constant starting conditions for the following capture step can be adjusted with regard to pH, conductivity, or buffer strength. Membranes can be operated quickly and with no significant loss of product. Time is an issue in this regard since high turnover rates of proteases can degrade significant amounts of product during the comparably slow loading procedure to the capture column.

9.3.2.2 **Affinity Chromatography**

Affinity chromatography is a very powerful capture technology. For purification of therapeutic antibodies, chromatography resins with Protein A or variants thereof are widely spread. Protein A has strong affinity to the Fc part of antibodies with an affinity constant K_D of 70 nmol L^{-1} (Li et al. 1998). Product purities of more than 95% can be achieved in one step (Follman and Fahrner 2004). The highly specific interaction takes place in a broad pH range and is nearly independent of the conductivity of the loading buffer. However, Protein A affinity chromatography is the most expensive operation unit in downstream processing with up to 50% of total costs (Fig. 9.7).

9.3.3 **Virus Clearance**

Virus clearance methods positioned downstream of the bioreactor are a regulatory requirement for product release and generally comprise two steps: virus inactivation and virus removal. Viral contaminants can enter production from a variety of sources. Rodent cell lines, such as Chinese hamster ovary (CHO) or mouse (NS0) cell lines, are routinely used in the production of mAbs, raising the possibility of contamination with human and rodent viruses, for example parainfluenza or reovirus. According to International Conference on Harmonisation of

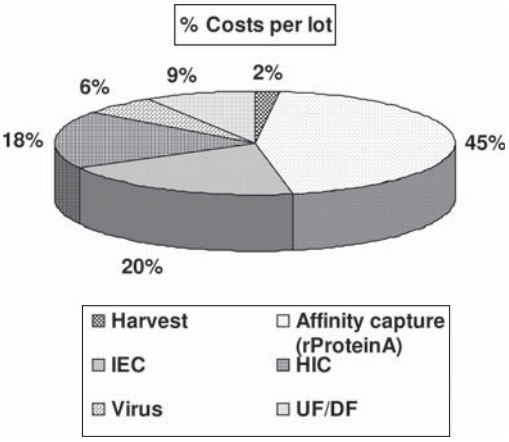

Fig. 9.7 Major cost drivers of raw materials used in downstream processing are chromatography resins.

Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guideline Q5A (http://www.emea.eu.int/index/indexh1.htm), processes must be validated to remove or inactivate four to six orders of magnitude more virus than is estimated to be present in the starting material. Additionally it is recommended that a purification scheme harbors at least two chromatography steps, both having the capability of reducing potential virus load.

Effective virus inactivation can be achieved by:

- Chemical inactivation (extreme pH; pH < 3.9 or pH > 13)
- Heat (e.g. by microwave)
- Photochemical inactivation (UV irradiation)
- Solvent-detergent inactivation.

Methods for virus removal are:

- Filtration (nanofiltration)
- Chromatographic separation.

Effective virus inactivation and removal has to be proven experimentally using a validated scale-down model of the actual purification process. Virus solutions are spiked to the samples and analyzed before and after each process step. A selection of the following viruses is commonly used: murine leukemia virus (MuLV), pseudorabies virus (PRV), reovirus 3 or parvovirus (MVM, PPV, BPV).

9.3.4 Purification and Polishing

In many cases the antibody's purity and monomer content exceed 95% after affinity chromatography. However, additional purification steps and final formulation are necessary to ensure high and defined product quality as well as product safety to suit administration into humans. Polishing of the target antibody should make use of orthogonal chromatographic principles to remove any residual impurities or adventitious agents interfering with product quality. Defined low levels of residual HCP (host cell proteins), leached rProteinA and DNA must be achieved by appropriate downstream processing steps in order to be accepted by regulatory authorities.

9.3.4.1 Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) provides a powerful tool for depletion of protein aggregates, leached rProteinA and HCP. Gel filtration (size-exclusion chromatography (SEC)), often described in textbooks, is not supposed to be a feasible alternative for production scales of more than 1–2 g of the desired protein (Table 9.2). HIC is able to cope with over 30 g antibody per L resin with yields beyond 85%, high flow rates, 10-fold lower costs, moderate initial product concentrations, and no dilution of product. Even higher product throughput (processing time) is feasible with HIC if used in negative mode (i.e. selective binding of unwanted constituents while the antibody appears in the flowthrough).

Table 9.2 Comparison of process and economic aspects of hydrophobic interaction chromatography (HIC) and gel filtration or size-exclusion chromatography (SEC) for production of 100 g monoclonal antibody.

	HIC	*SEC*
Column size (L)	4	40
Column load	25 g L^{-1}	25 g per cycle
Initial product concentration (g L^{-1})	10	25
Column dimension (cm)	14 × 26	20 × 120
No of cycles	1	4
Resin cost (euros)	7200	80000
Flow rate		
cm h^{-1}	150	30
ml min^{-1}	400	167
Yield (%)	85	80
Buffer volume[a] (L)	18	160
Process time[a] (h)	0.75	17
Final product concentration (g L^{-1})	12	5

a Only bind/elution, no equilibration, wash, regeneration/sanitization.

One drawback of HIC is that high salt concentrations are necessary for protein binding, bearing the risk of aggregation.

9.3.4.2 Ion Exchange Chromatography

Ion exchange chromatography (IEC) is a very efficient method for numerous aspects of downstream processing. Today's IEC resins have high binding capacities of 50 mg antibody per mL resin or more and they can be run in either bind/elute (positive mode) or flowthrough mode (negative mode). Scale-up of column dimensions at equal resin performance is feasible and costs are moderate compared with affinity or hydrophobic resins. Sanitization can be achieved by strong acids and base solutions (e.g. 1 mol L^{-1} HAc and 1 mol L^{-1} NaOH). A feature of IEC is that samples have to be applied at low conductivity, often necessitating a UF/DF step in advance.

Cation exchange chromatography Since therapeutic antibodies often have basic pI values of 8–9.5, cation exchange (CIEX) chromatography is applied in positive mode purifications at physiological or mild acidic buffer conditions. CIEX chromatography is capable of depletion of HCPs, leached rProteinA, aggregates, and fermentation ingredients, giving rise to nonproduct-related precipitation. Due to its capabilities, CIEX chromatography is also attractive as a primary capture step; however, product enrichment and depletion of HCPs is normally less efficient than by affinity chromatography and depends strongly on the type of protein applied.

Anion exchange chromatography and removal of DNA Although suited for purification, binding of antibodies to anion exchange (AIEX) resins often requires pH values above 9–10 and therefore bears an elevated risk for deamidation of Asn residues (Creighton 1996).

Among the impurities to be eliminated during the downstream purification process, one component of major interest for safety is residual host cell DNA. It is necessary to guarantee that this impurity is reduced to a level of less than 100 pg/dose in the product administered to a patient. Due to its high content of phosphate, DNA is highly negatively charged at physiological pH and thus well suited to be removed quantitatively by binding to AIEX ligands.

Today's purification processes often make use of membranes functionalized with AIEX ligands on their surface. Membranes such as Sartobind Q are designed as single-use entities, making regeneration procedures superfluous. Pricing is moderate and further advantages are higher yields and savings in total labor time, buffer, and buffer tank capacities, which are usually needed for sanitization or for validation of a chromatography column. Furthermore, the risk of cross-contamination is excluded. The binding capacity of AIEX membranes is easily sufficient for quantitative removal of DNA, since capture and/or midstream purification steps normally leave only low DNA concentrations of 50–500 $pg\,mg^{-1}$ protein (Walter 1998). The filter area is dictated by requirements for flux rather than DNA-binding capacity. Finally, AIEX membranes are amenable to scale-up from lab to production scale.

In some cases AIEX chromatography will be preferred as a polishing step if removal of DNA and residual interfering impurities can advantageously be combined.

Validation of DNA removal Regulatory authorities, like the US Food and Drug Administration (FDA) or the European Agency for the Evaluation of Medicinal Products (EMEA), demand to monitor DNA depletion at various steps of the downstream process. For this purpose, radiolabeled DNA is spiked to protein samples and depletion factors are evaluated in a validated scale-down purification model. In addition, binding capacity of the main DNA removal step in the downstream purification scheme is determined to ensure that the residual cellular DNA content in the final product is reduced to a defined level of no more than 100 pg per dose. A major drawback of AIEX chromatography compared with single-use membranes is that repeated cycles have to be run in order to ensure safe reuse of a chromatography column.

9.3.5 Final Formulation

As a final step within downstream processing, formulation is conducted by an appropriate UF/DF system. Membranes with low protein binding, such as polyethersulfone or regenerated cellulose, are applied. Membranes are not resistant to extensive sterilization or sanitization with hot steam or NaOH. Therefore,

sterility of the formulated bulk is taken special care of by determining the levels endotoxins and bioburden.

9.3.6 Integrated Downstream Process Development

With regard to an ambitious product development timeline an integrated development strategy (Fig. 9.8) is mandatory, which means that overlapping rather than sequential development activities are realized in upstream, downstream, and pharma development units. Support and early feedback to upstream colleagues is important with regard to product and process quality. Clone selection finally giving rise to the valuable production clone is also assisted by the downstream department. Low milligram quantities of the target protein being obtained from small culture samples are indispensable for the assessment of biochemical and biophysical protein properties and initial studies on purification and filtration steps. A process is deemed feasible if a series of process steps jointly result in reasonable yield, product quality, and a scaleable format. Development during the feasibility study encompasses purification of milligram amounts up to 1 g of target protein.

Once a feasible downstream process has been developed, up-scaling is performed to challenge robustness, which means the reproducibility of the process in larger scale at constant product quality, as well as economic aspects, for example processing times and buffer volumes. A fine-tuning process is applied to purify several grams to 100 g quantities of therapeutic protein in order to support toxicology and preclinical studies or, under Good Manufacturing Practise (GMP) regulations, clinical phase I and II studies. Production scale normally aims for delivery of hundreds of grams to several kilograms in the case of therapeutic mAbs. Facility constraints, such as space or turnover times, become an important issue and often intensive development effort is encountered to fulfill the goals for economy, quality, and regulatory demands.

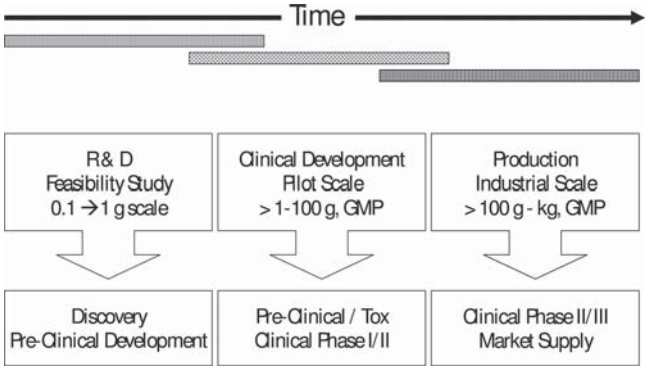

Fig. 9.8 Integrated process development strategy.

9.3.7
Future Perspectives

Time constraints and increasing demands on product quality require rapid and comprehensive methods for the development of downstream processes. The competitive market environment requires short- and long-term solutions to cope with these challenges. Currently, state-of-the-art techniques have almost been improved to their theoretical optima and possible alternatives have not yet been developed to a degree of maturity applicable to biopharmaceutical production scale.

Monolithic resins (Zmak et al. 2003) could overcome the diffusion limitations of traditional bead resins, but the cost and size of monoliths are currently out of scope for production scale (Branovic et al 2003). Simulated moving bed (SMB) is a powerful approach with respect to large-scale applications and low buffer consumption (Nicoud 1998). The drawbacks of this technology are the high number of valves and pumps involved, bearing an enhanced risk of malfunction and germ contamination during the production process. The power of continuous operation of SMB renders the definition of a product lot difficult.

Novel affinity ligands on resins or innovative tags on mAbs could overcome the main disadvantages of Protein A resins: high cost and moderate stability. The cost structure may change with competitive scaffold proteins or small chemical or peptidomimetic ligands having comparable properties to Protein A. The advantages of small ligands are that they are cheap and easy to synthesize in large quantities, they have high chemical stability, resistance against proteases and no leaching. Leaching is of relevance because the removal of these process-related impurities has to be demonstrated by biochemical assays prior to release for human use.

Protein tags, such as His-, Strep-, Intein- or GST-tags – to mention just a few – are well characterized and widespread in molecular biology, in research and diagnostic applications. Some of them, as short as six amino acids, can be inserted within a polypeptide chain to undergo chemically induced self-cleavage. However, these modifications raise concerns with regard to immunogenicity which has to be addressed during preclinical and clinical development.

Ambitious development timelines with increasing demands for throughput and product qualities require automated parallel screening of resins and chromatography conditions with implemented fast process analytics. Miniaturized robot platforms provide an excellent tool to address these needs.

9.4
Formulation Development

9.4.1
Challenges during Early Formulation Development Phase of Biopharmaceuticals

It is a prerequisite that a marketable protein formulation must be safe to administer, inducing no or minimal local irritation, meets the specific clinical and

delivery requirements, and that its physical, chemical, as well as biological stability is given during the recommended product shelf-life (LeHir 2001; Langguth et al. 2004). Therefore, the development of an optimal protein formulation requires a sound understanding of the protein's physicochemical and biophysical properties, evaluated by sensitive analytical tools.

During early process development, challenges with regard to protein stability are encountered with special emphasis on protein downstream processing, where protein degradation as well as physical instabilities (e.g. aggregation) have to be avoided. With this in mind this section focuses on protein stability in a liquid formulation and analytical characterization tools in the early development phase. Depending on the chosen formulation and environmental conditions for a certain process step during the manufacturing of biopharmaceuticals, stability of the protein may be affected leading to a loss of structure by conformational change, resulting in the formation of, for example, aggregates. The appearance of aggregates is commonly observed in highly concentrated protein formulations due to the higher probability of protein–protein interactions.

Most proteins adopt a secondary, tertiary, or even a quaternary structure which is essential for biological activity. Various forces are involved in determining protein-folding pathways and thus overall protein stability. The forces involved in the formation of higher protein structure include hydrophobic and electrostatic interactions, the formation of hydrogen bonding networks, covalent bonds, and van der Waals interactions. Any event (e.g. shear stress during sterile filtration) that upsets the sensitive balance between these forces and interactions can lead to conformational changes, for example the exposure of hydrophobic areas inducing reduced solubility and thus an increased tendency to form aggregates.

9.4.2
Strategies and Analytical Tools for Rapid and Economic Formulation Development

In the early development phase of an optimal protein formulation various test protocols are used to get a deeper understanding of the chemical and physical stability/instability of the investigated protein formulation. An overview of physicochemical test methods is shown in Table 9.3. A key parameter to be investigated is induced protein denaturation as a function of the temperature applied. Other stability-indicating studies include shaking, shear, and/or freeze/thaw stress studies in order to obtain information about potential degradation pathways.

Sensitivity to oxidation and light also needs to be considered. Important data are derived from accelerated stress stability studies at elevated temperatures, for example, storage for 4–12 weeks at 25 and/or 40 °C (Waterman and Adami 2005). To facilitate increased intramolecular protein interactions various excipients are screened using the methods summarized in Table 9.3 (class II) with the aim of developing formulations inducing highest protein stability.

Table 9.3 Analytical tools and physicochemical methods used for the development of an optimal protein (e.g. antibody) formulation.

Class I	*Class II*	*Class III*
Appearance, color, clarity	Infrared spectroscopy	X-ray
Rheology	Raman spectroscopy	Nuclear magnetic resonance
Surface tension	Circular dichroism	Atomic force microscopy
Analytical centrifugation	Fluorescence spectroscopy (IF, EF)	Electron microscopy
Turbidimetry	Scattering techniques (DLS, SLS, RALS, light, neutron)	Mass spectrometry (MALDI-TOF, MS-MS)
Optical microscopy	Electrophoresis (SDS-PAGE, IEF, CE)	Chemical analysis (peptide mapping, sequencing, AA analysis)
	Chromatography (RP-HPLC, HP-SEC, IEC)	
	Calorimetry	
	Surface plasmon resonance	

RP-HPLC, reverse-phase high-performance liquid chromatography; HP-SEC, high-performance size-exclusion chromatography; IEC, ion-exchange chromatography; IF, intrinsic fluorescence; EF, extrinsic fluorescence; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MS-MS, tandem mass spectrometry; DLS, dynamic light scattering; SLS, static light scattering; RALS, right angle light scattering; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IEF, isoelectric focusing; CE, capillary electrophoresis; AA, amino acid.

Such studies are aimed at developing a protein formulation suitable for toxicological studies and for early clinical studies. Further development programs have to be performed for the development of the final formulation appropriate for pivotal clinical studies and market supply due to the fact that the prerequisites for the formulation, namely dosage form, primary packaging, delivery device and sometimes even the application route may change during the clinical development. However, in order to reduce cost in the early development phase, physicochemical and biophysical test methods are used for rapid formulation screening and thus to identify formulation conditions and potential excipients which may stabilize the protein in solution. The analysis of protein properties in formulations requires often specific tailor-made analytical methods able to detect small differences against a more or less complex formulation background, and consequently provide information on the protein structure of various formulations.

Table 9.3 summarizes the most important analytical tools used for the development of an optimal protein formulation. These techniques can be separated into classes I, II, and III. The analytical tools in class I give information on protein macroscopic bulk properties (i.e. on the properties of protein populations, such as surface tension or viscosity) (Liu et al. 2005), whereas the techniques in class III (e.g. nuclear magnetic resonance, X-ray techniques) are more appropriate for

the elucidation of specific structural alterations and detailed information at atomic level resolution. Methods in class II are appropriate for the characterization of protein structures at the molecular/microscopic as well macroscopic level. For rapid formulation screening, class I and II methods are most appropriate (Rouessac and Rouessac 2004, Kellner et al. 2004).

The specific analytical techniques used for formulation screening depend on the protein behavior as well as on the dosage form and protein concentration in the formulation. The challenge is to use techniques that have the desired sensitivity, allowing the detection of even small changes of the protein, and also to ensure that the techniques do not affect the composition of the sample being investigated. As an example, depending on the analytical method used, the sample cannot be analyzed at its initial protein concentration. As a consequence, the protein solution has to be diluted, and this may directly interfere with, for example, the formation of particles in the formulation. Using fluorescence spectroscopic techniques for the analysis of protein formulations the protein concentration is usually between 0.05 and 0.2 mg mL^{-1}. This allows the investigation of formulation of extremely diluted samples. However, if stability information are requested for protein formulation with protein concentrations between 5 to at least 100 mg mL^{-1}, other techniques such as infrared spectroscopy can be used (Garidel and Schott 2006).

In early formulation development the avoidance of protein aggregation is a challenge, because it may result in significant product loss during downstream processing. In addition, protein aggregates present in the drug product may compromise the safety of the product (e.g. by the generation of immunological responses) (Chi et al. 2003). Therefore, various analytical tools summarized in Table 9.4 have been developed to investigate soluble as well as insoluble aggregates in solutions (Sine 2003; Tatford et al. 2004; Fraunhofer and Winter 2004). Spectroscopic techniques (Hollas 2003) such as circular dichroism and infrared spectroscopy are used to determine the microscopic causes for protein aggregation (Manning 2005).

It is important to get a deeper understanding of the protein's degradation pathway in order to be able to develop a strategy for its stabilization in solution. One example to illustrate this approach is shown in Fig. 9.9. In this example, an increase of beta-sheet secondary protein structure is observed in relation to protein aggregation.

There are indications that the aggregation mechanism of certain proteins involves two steps: first a transition from random coiled and helical secondary protein structures to beta-sheet structures and in a second step, aggregation of the beta-sheet structures. In order to avoid such an aggregation, environmental conditions including the presence of certain excipients have to be found, enabling the stabilization of the helical secondary protein structure to reduce and even avoid the transition to beta-sheet structures. However, it has also been shown that proteins (IgG1) may aggregate without obvious changes in the secondary structure (Schüle et al. 2004, 2005). Therefore, various test methods are used together to understand the aggregation mechanism. Furthermore, these exam-

Table 9.4 Analytical tools for the characterization of the formation and presence of particles/aggregates in protein solutions.

Method	*Individual technique*	*Remarks*
Visual	"Eye"	Visual particle
Particle counting	Electrical impedance	Particle number per volume unit, size, quantification, micrometer scale
Microscopy	LM	Shape and size determination, quantification, micrometer scale
	EM	Shape and size determination of insoluble aggregates, nanometer scale
	AFM	Shape and size determination of insoluble aggregates, morphology, roughness, nanometer scale
Light scattering	SLS	Size and shape
	DLS	Size and relative distribution of soluble aggregates
	LS/turbidimetry	Size estimation and relative distribution
	RALS	Aggregation process, soluble/insoluble particles
Rheology	DSR	Gelation characteristics, shear module, protein–protein interaction
Chromatography	HP-SEC	Size estimation and quantification, soluble, small particles, quantification
	RP-HPLC	Aggregate iso-forms, quantification
	FFF, AFFF	Size determination and quantification, small and large particles
Electrophoresis	SDS-PAGE	Size estimation and nature of aggregate formation, quantification
	Native PAGE	Aggregation process and mechanistic formation
Calorimetry	DSC	Thermally induced protein unfolding and aggregation
Centrifugation	AC	Size, mass, and shape estimation
Spectroscopy	FTIR	Aggregation process and mechanistic, change in protein secondary structure
	CD	Aggregation process, change in protein secondary and tertiary structure
	Fluorescence	Aggregation process
	UV-VIS	Detection of soluble and insoluble aggregates
	NMR	Aggregation formation

LM, light microscopy; EM, electron microscopy; AFM, atomic force microscopy; LS, light scattering; DSR, dynamic shear rheometry; FFF, field flow fractionation; AFFF, asymmetric field flow fractionation; DSC, differential scanning calorimetry; AC, analytical centrifugation; FTIR, Fourier transform infrared; CD, circular dichroism; UV-VIS, ultraviolet-visible; NMR, nuclear magnetic resonance.
For an overview and further details see Kellner et al. (2003).

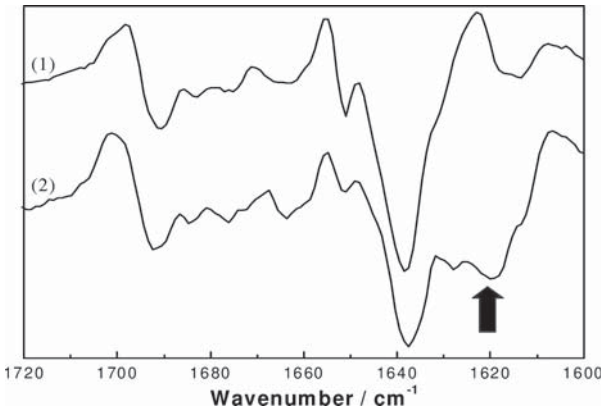

Fig. 9.9 Second derivative infrared spectroscopy of a liquid antibody (IgG1) formulation at a concentration of 10 mg mL^{-1}. (1) Infrared spectrum of the initial protein conformation. (2) Infrared spectrum of the protein formulation after a 3-day shaking stress at room temperature. The appearance of the formation of intermolecular antiparallel beta-sheet structure which is characterized by a low-frequency band around 1620 cm^{-1} (marked by an arrow) is indicative for the formation of protein particles and thus protein aggregates (Garidel 2004; Manning 2005).

ples show that the formation of protein aggregates may be induced by different pathways.

As mentioned above, one of the most common mechanisms leading to loss of bioactivity of a protein is the time-dependent formation of aggregates and loss of solubility (Arakawa and Timasheff 1985; Schein 1990). Protein solubility in a certain formulation is often unknown. Various methods are described in the literature to determine protein solubility but most of them have strong drawbacks and can only be employed for certain cases. It is a challenge to obtain highly concentrated protein solutions without inducing protein degradation. One approach is to slowly concentrate a given protein solution (e.g. by ultrafiltration) using membranes with low protein binding capacity. Using this technique it is possible to saturate the solution with the concomitant appearance of a solid phase which, in most cases, represents protein crystals. In this case, the determined protein solubility is that of the crystalline form of the protein which could differ substantially from that of the amorphous solid (Arakawa and Timasheff 1985).

Another approach for the determination of protein solubility uses an inert, extraneous excipient like polyethylene glycol to induce protein precipitation (Middaugh et al. 1979). Based on thermodynamic considerations, the protein solubility is determined by plotting the logarithm of protein solubility versus the precipitation excipient concentration, which is often linear. Solubility is derived by extrapolation to zero precipitation excipient. However, this method should be used with care and is best employed in a comparative manner. It should also be considered that the derived protein solubility data refer to the particular solid state of the protein obtained by excipient precipitation.

9.4.3 Stabilization of Liquid Protein Formulations by Excipients

Besides its concentration, other factors that have a large impact on the stability of a protein in solution are formulation conditions such as pH, ionic strength, presence or absence of solutes, and storage conditions. The best choice of pH for a protein formulation depends on the chemical structure and the amino acid sequence of the protein. The most common chemical degradation pathways in proteins are deamidation, isomerization, cyclic imide formation, cleavage, oxidation, pyroglutamate formation, beta-elimination, and crosslinking. The kinetics and activation energies of a number of degradations are pH dependent. The hydroxinium ion is directly involved in specific acid-catalyzed reactions (e.g. Asp-Pro cleavage, direct Asp-Gly hydrolysis, or succinimide formation at Asn-X residues). Deamidation of Asn is more pronounced at alkaline pH, because this reaction is base catalyzed (Ahern and Manning 1992). The degree of protonation of a protein also influences its overall polarity (e.g. changes in ionic and/or dipole–dipole interactions) and, thus, protein–protein interactions and solubility (pH dependent).

The choice of pH and formulation components also plays a critical role in the maintenance of glass container (primary packaging) integrity, and leaching of extractables from rubber stoppers. An alkaline pH could promote dissolution of silica from glass, for example, resulting in pH changes in the formulation during long-term storage.

Excipients for the prevention of protein degradation and aggregation of therapeutic antibodies seem to exert their effects via a number of different pathways. These include:

- Binding of the excipient to the protein and stabilization of the native conformation. Examples of such direct protein–excipient interactions have been observed in polysulfates and cyclodextrins.
- Preferential exclusion from the protein surface which allows a preferential protein hydration. According to this mechanism the protecting role of excipients such as polyethylene glycol, amino acids, polymers, polyols, sugars, or other substances with multiple hydroxyl groups, has been described.
- Prevention of protein–protein or protein–surface interactions or increasing solubility of the aggregates. Nonionic surfactants are believed to function according to this mechanism (Cleland et al. 1993).

Many of the abovementioned excipients (Ahern and Manning 1992) are able to increase protein stability and/or inhibit irreversible aggregation and thus maintain protein integrity. These effects can be explained by an increased difference in free energy between the native and the denatured states, stabilizing the native

state by making it even more energetically favorable than any unfolded state. Stabilizing excipients acting as cosolvent increase the protein stability by inducing a preferential hydration of the protein. This effect reduces the frequency of protein–protein interactions and limits their ability to initiate nucleation and so protein aggregation. If a protein is unstable in liquid formulation, the formulation can be frozen in order to minimize degradation kinetics or to develop a solid, dry formulation (e.g. freeze-dried, spray-dried formulation). However, other degradation pathways and stresses have then to be taken into account and addressed specifically.

In summary, the successful development of an optimal protein formulation depends on an intimate understanding of the protein's physicochemical and biological characteristics, including chemical and physical stability (Frokjaer and Otzen 2005). Because each protein molecule (and even mAbs) acts as a single individual with a large number of often underestimated specific properties, only general strategies for the development are available. The formulations of most biopharmaceuticals are therefore developed case by case, taking the individual demands for clinical studies and marketing as well as the application route into account.

9.5 Protein Characterization and Quality Control Testing

9.5.1 Protein Characterization

Initial protein characterization typically starts during preclinical development and is further intensified during clinical development phases. Characterization activities for investigational new drug applications generally are limited in their extent but should address the influence of product heterogeneity on biological activity. For example, the role of glycosylation concerning the efficacy of an mAb is product-dependent, thus the required depth of carbohydrate analysis will be dictated by the product. For marketing applications, all possible aspects of protein heterogeneity (protein variants) and possible influences of the manufacturing process on the physicochemical and biological properties in relation to the biological activity should be elucidated. The key to characterization is founded in the appropriate combination of the different methods that analyze the sample from substantially orthogonal and independent directions (Harris et al. 2001).

9.5.1.1 Protein Variants

Characterization of the protein variants comprises identification of the modification, including the respective site(s) and the mechanism of accumulation. Thus a variant may be categorized as a product-related substance or a product-related impurity, which results in an assessment of whether routine monitoring of the variant has to be performed and limits established. Variants with decreased bio-

logical activity would be classified as product-related impurities. Variants comparable to the unaltered protein in its potency would be regarded as a protein-related variants or product characteristics unless efficacy and safety of the protein is impaired (ICH S6 guideline; CPMP/3097/02 2003). Generation of stress samples to create variant-enriched material is a common approach to identify sites most susceptible to degradation. The most common product variants observed in antibody production are described below.

Aggregates and oligomers Aggregation is a key issue underlying multiple deleterious effects, including loss of efficacy and immunogenicity. Size exclusion chromatography (HP-SEC) is most suitable for routine quantification of soluble aggregates and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) detects covalent non-SDS dissociable aggregates. Orthogonal methods, such as sedimentation velocity analytical ultracentrifugation (AUC) (Laue and Stafford 1999), field flow fractionation (FFF, shortcomings, e.g. robustness; Schimpf et al. 2000), and light scattering (Liu and Chu 2002) are commonly used to ensure that HP-SEC is providing a complete characterization of aggregates, especially detecting very large species that might not enter the column and noncovalent, reversible, or weakly associated aggregates that might break up during analysis by HP-SEC. SDS-PAGE, and HP-SEC are commonly used for lot release, whereas AUC, FFF, and LS are carried out for characterization and cross-validation purposes, only. A more detailed characterization of aggregates the individual HP-SEC species (obtained by collection of the respective peaks from multiple injections) or AUC fractions could be analyzed by reduced and nonreduced SDS-PAGE in combination with liquid chromatography/mass spectrometry (LC-MS) techniques, N-terminal sequence analysis, denaturing HP-SEC, capillary electrophoresis (CE), Fourier transform infrared spectroscopy (FTIR), and circular dichroism (CD) spectroscopy (Andya et al. 2003).

Protein degradants/fragments Impurities such as degradants or fragments induced by physical stress (exposure to accelerated temperatures, low pH conditions and shear stress as a function of the processing conditions), for example, should also be investigated. For manifestation of internal cleavages, the protein might have to be converted into its reduced state since disulfide bonds may hold the fragments in place. Degradants/fragments can be analyzed by SDS-PAGE and further characterization of the respective bands by a combination of LC-MS and N-terminal protein sequence analysis (NTS).

HP-SEC under native and denaturizing conditions is suitable to determine the overall number of fragments present and to isolate them for further investigation. Resolution of fragments can be achieved by SDS-PAGE analysis and characterization can be performed by enzymatic cleavage of the respective bands in the polyacrylamide matrix or by blotting the bands from the gels onto PVDF (polyvinylidine difluoride) membranes, for example, and subsequent analysis by MS techniques (Eckerskorn et al. 1992; Patterson and Aebersold 1995).

Deamidation, oxidation, disulfide pairing, N- and C-terminal variants and other less common modifications Enzymatic cleavage (e.g. by trypsin) in combination by mass spectrometry (Nguyen et al. 1995; Perkins et al. 2000) is sensitive to primary and secondary protein structure and can detect the following product modifications:

- Perturbations due to the formation of disulfide mispairings and scrambling (additional peptides with specific molecular weight: Gorman et al. 2002) and formation of trisulfides (+32 Da: Andersson et al. 1996).
- Deamidation, induced by high pH, usually occurs at the sequences of Asn (Gln) followed by glycine (–1 Da: Stephenson and Clark 1989; Zhang et al. 2002) and results in the formation of a 3 : 1 ratio of isoaspartate/aspartate. Formation of succinimide variants (–18 Da) has been detected in proteins (Teshima 1991).
- Methionine oxidation (+16 Da).
- Cyclization of N-terminal glutamate and glutamine residues to pyroglutamate (–17 Da and –18 Da, respectively).
- Enzymatic cleavage of C-terminal lysines (–128 Da) by mammalian carboxypeptidases (Harris 1995) and possible subsequent enzymatic processing of the new C-terminal glycine (Prigge 1997) to result in amidation of the amino acid penultimate to the glycine residue (–186 Da).
- Posttranslational phosphorylation at serine, threonine, and tyrosine residues (+80 Da): PEGylation introduced at free amino groups to increase bioavailability (making MALDI-TOF without enzymatic digestion also feasible), carbamylation introduced during processing of the protein at high concentrations of urea and elevated temperatures (+43 Da), adducts as a result of protein processing (refolding buffer additives, e.g. cysteine), and drug conjugates with molecules such as maytansine (Wang et al. 2005).

Ion-exchange chromatography (IEC) is an especially simple and powerful technique to resolve and quantitate charge variants introduced, for example, by deamidation (cation-exchange chromatography is most suitable) or enzymatic C-terminal processing. Alternative methods are capillary electrophoresis-based methods (cIEF and CZE) (Good 2004) and isoelectric focusing (IEF) (Gianazza 1995).

Oxidation may be resolved by HIC and is also detected by IEC as a blurred profile or a shift of retention times. Structural integrity with respect to disulfide pairing can be analyzed by, for example, HIC, reverse-phase high-performance

liquid chromatography (RP-HPLC), and differential scanning calorimetry (DSC). Integrity of the N-terminus can be assessed by N-terminal sequencing.

Carbohydrate heterogeneity To assess glycosylation heterogeneity a combination of several chromatographic and mass spectrometric techniques is commonly used (Sheeley et al. 1997; Mechref and Novotny 2002). The chromatographic techniques include enzymatic or chemical release of the *N*-linked oligosaccharides followed by fluorescence derivatization and a subsequent chromatographic or capillary electrophoretic separation. The thus released oligosaccharides can be characterized by comparison to known standards, the structural composition might be confirmed by exoglycosidase digestion studies and molecular masses can be determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

To identify the site occupancy of the oligosaccharides, and determine their composition electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) is performed on the enzymatically digested protein. For this purpose, several proteases are available typically used enzymes are trypsin, lys-C, AspN, Papain (to generate Fab and Fc fragments) or a combination thereof.

9.5.1.2 Overall Structural Confirmation (Higher Order Structure)

The secondary, tertiary, or quaternary structure of a protein can be impaired by any of the aforementioned chemical changes, or under the influence of other factors (e.g. temperature and pH). In depth overall structural characterization is usually performed prior to filing for a marketing authorization application. For this purpose the following methods can be applied: CD spectroscopy (Perczel et al. 1991), DSC (Remmele et al. 2000), FTIR and Raman spectroscopy (Van de Weert et al. 2001) and nuclear magnetic resonance spectroscopy (NMR) (Fersht and Daggett 2002).

9.5.1.3 Relationship Between Physicochemical/Structural Properties and Biological Activity

Protein variants that are likely to affect potency can occur from, for example, disulfide scrambling, misfolding, oxidation of methionines, and mainly unspecific deamidation, aggregation, and clippings. Aggregates or fragments resolved by HP-SEC should also be analyzed for their *in vitro* potency to determine if the respective species is biologically active. The glycosylation pattern of mAbs is known to affect their biological performance (Wright and Morrison 1998; Jefferis 2001; Shields et al. 2002). Differences in the sialylation of a glycoprotein may cause attenuated pharmacokinetics and pharmacodynamics (Stockert 1995) and α-galactose motives are potentially immunogenic (it should be taken into account that there is a corresponding natural antibody that constitutes 1% of circulating IgG in humans; Galili 1992).

With the start of phase I clinical trials, a functional potency assay should be available, with "functional" meaning relevant to the mechanism of action of the

therapeutic antibody. Common cell-based potency assays include proliferation, inhibition of proliferation, and apoptosis.

9.5.2 Quality Control Testing

At the start of clinical development, regulatory test methods (specified analytical methods with defined acceptance criteria for the product) should have been established. The development and selection of regulatory test methods and criteria to characterize the drug product and establish standards of conformance for product release are guided by the principles and practices described in ICH guideline Q6B or are considered to be product specific. The set of testing methods and specifications for release of drug substance and drug product should be designed to monitor the overall quality, safety, identity, strength, purity, and potency as well as general characteristic properties. Development and justification of specification criteria is based on the overall manufacturing experience, with consideration of results from preclinical and clinical production lots and data from the validation of analytical methods. Release testing methods usually are a subset of the tests used for characterization of the molecule. A typical set of lot release and stability testing methods is given in Table 9.5.

In addition to lot release testing, process monitoring and in-process testing of process-related impurities has to be performed and is a key to product quality. Impurity levels allowed depend upon, for example, the dose administered, the schedule of administration, duration (chronic versus acute) etc. since these may comprise a risk potential with respect to immunogenicity, toxicity, genotoxicity, and transmission of transmissible spongiform encephalopathy (TSE) (see ICH Q3 and S6 guidelines). Potential process-related impurities are media components (e.g. transferrin, insulin), cell components (host cell proteins and DNA), chemical additives (e.g. antibiotics, methotrexate, antifoam agents), and leachables (e.g. protein A, heavy metals).

Protein impurities (e.g. protein A, insulin, host cell protein) are most often measured using immunoassays as well as SDS-PAGE or immunoblot assays, whereas lower molecular weight impurities (e.g. methotrexate, hydrocortisone) are usually analyzed by HPLC. For measuring host cell proteins it is a common strategy in many companies to develop a multiproduct ELISA for all products derived from a particular cell type, or to use a commercially available "generic" ELISA. Internationally accepted specifications for impurities are not established, except for DNA, for which specification is available (World Health Organization 1997).

Several approaches are possible for the control of an impurity: a specification may be established for routine release testing on the drug substance/drug product stage or a limit may be set for in-process samples for batch-to-batch testing without implementing a release specification. Most preferably, when approaching an application for marketing authorization, a manufacturer would demonstrate consistent removal of existing impurity components by comprehensive process

Table 9.5 Lot release and stability testing methods (excluding safety) and acceptance criteria for a typical monoclonal antibody for clinical phase I.

Test	*Method*	*Acceptance criterion*	*DS release*	*DP release*	*DP stability*	*Comments*
Characteristics	Degree of coloration	NMT Y6	x	x	x	Pharmacopeial method, limit depends on formulation
	Clarity and degree of opalescence	NMT Reference III	x	x	x	Pharmacopeial method, limit depends on concentration
	pH	value ± 0.2	x	x	x	Pharmacopeial method
	Osmolality	value ± 30 mOsm kg^{-1}	x	x	x	Pharmacopeial method
	Extractable Volume/ volume in container	NLT (sum of) nominal volume (according Ph. Eur./ USP)		x		Pharmacopeial method
	Appearance	according Ph. Eur./USP		x	x	May be difficult to evaluate
	Solubility	according Ph. Eur./USP		x	x	
	UV-scan	Value ± 10%	x	x	x	
Identity and heterogeneity (structural integrity)	IEF or IEC (CEC)	Pattern qualitatively comparable to standard material (+ for IEC: report result for acidic species)	x	x	x	IEC more suitable for evaluation (quantitatively) of deamidation
	Peptide map	Profile qualitatively comparable to standard material	(x)			

Table 9.5 *Continued*

Test	*Method*	*Acceptance criterion*	*DS release*	*DP release*	*DP stability*	*Comments*
	Oligosaccharide map	Profile qualitatively comparable to standard material	(x)			Often not required as specified assay depending on (biological functionality of the antibody) but may be performed for internal information to monitor cell culture processing
Purity	SDS-PAGE (Coomassie)	Reduced: Sum of heavy and light chains NLT 90% Non reduced: main band NLT 85% Reduced and non reduced: number, intensity and molecular weight of main bands comparable to reference standard	x	x	x	
	HP-SEC	IgG monomer: NLT 95%	x	x	x	
	Particulate matter/ subvisible particles	≥10 µm: NMT 6000 ≥25 µm: NMT 600		x	(x)	Pharmacopeial method, may not have to be performed, in case an on-line filter is used for application
Potency	Cell-based bioassay	60–140% of standard material	x	x	x	If justified, a binding ELISA can be used instead

NMT, not more than; NLT, not less than; DS, drug substance; DP, drug product; Y6, yellow 6; IEF, isoelectric focusing; IEC, ion exchange chromatography; CEC, cation exchange chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HP-SEC, high-performance size-exclusion chromatography; ELISA, enzyme-linked immunosorbent assay; Ph Eur, European Pharmacopoeia; USP, United States Pharmacopeia.

validation and characterization (e.g. by performing additional small-scale spiking studies and thus avoiding to set limits for routine in process or release testing or reduce the extent of in-process testing).

Improvement, adjustment, and establishment of analytical methods and related acceptance criteria for in-process and product release testing appropriate to the development stage of the product and in response to process changes during development is generally required.

Prior to release of GMP material for phase I clinical trials, an adequate validation of regulatory tests should have been completed. Typically, the scope of a phase I validation is limited compared with the full ICH validation performed prior to submission of a market application (ICH Q2A).

9.5.3 Stability Testing

The biochemical, physicochemical, and biological methods of the stability testing program should be designed to control the degradation profile and to monitor the potency of the product for the intended shelf-life. Typically, analytical methods employed for stability testing are a subset of the ones developed for release testing (Table 9.5). Since methods designed to test for identity, process-related impurities, and contaminants are not stability indicating, they do not need to be included in the stability program (ICH Q5C and ICH Q1A). If a different specification for release and expiration is used, this should be justified by sufficient data to demonstrate that the clinical efficacy is not affected. Stability studies may be economized by applying sample selection criteria based on ICH Q1D.

Changes in the manufacturing process and formulation of the product, either during clinical development or post marketing authorization are likely to initiate an appropriate stability program. With submission of an investigational new drug application, typically a minimum of 3 months real time stability data for the intended storage temperature as well as data from accelerated stability studies should be available to ensure the defined quality of the respective clinical lots. Ideally, preceding stability data stemming from developmental lots (toxicology studies) should be available.

The drug product should be stable at least for the duration of the planned clinical study at the intended storage temperature. Storage time of the product may therefore not be allowed to exceed real-time stability data unless supportive stability data justify an extension. With filing of an marketing authorization application, stability data stemming from three drug product lots, possibly from different drug substance lots, data stemming from a photo-stability study of at least one batch (ICH Q1B), and data demonstrating stability of relevant hold steps such as intermediates or drug substance should be available (for more details please refer to USP 1049 and Q5C, Q1E, Q1A).

9.6 Overall Development Strategy and Outlook

A growing demand during early product development is to shorten the timeline for the supply of material for toxicological studies in animals and clinical studies in humans. Usually the production process needs to be developed before material can be supplied, which can be very time consuming. Thus, supply of material appropriate for toxicological studies in animals and first clinical studies in humans is often on the critical path in early stages of development. For later clinical studies, however, resupply can be managed timely on the basis of the existing process.

State-of-the-art process development has to comply with the growing demand of shortening the timeline and, at the same time, the importance of a "do it right the first time" paradigm to prevent unnecessary costs and delays at later development stages. The design of such a (state-of-the-art) development concept is presented in Fig. 9.10. In this strategy, material for toxicological studies produced from CHO cells can be supplied as little as 15 months after the cloning of product-encoding genetic sequences into high-expression vectors. At the same time it ensures that the production cell generated during this program will have the high

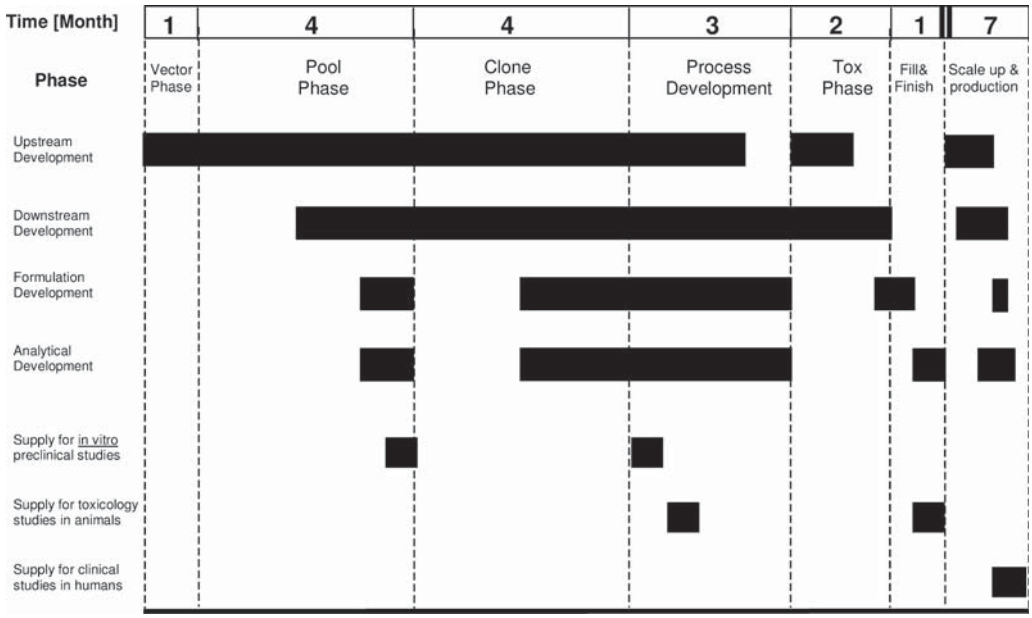

Fig. 9.10 Example of an integrated development strategy for supply of monoclonal antibody material appropriate for toxicological studies within 15 months and clinical material within 22 months.

expression potential needed to avoid a change in the production cell line at later stages in development.

Using the newly designed basic production process, sufficient quantities of material for clinical studies can be supplied after an additional 7 months, a total of 22 months after the start of development (i.e. cloning of the gene into the expression vector) (Fig. 9.10). Once the product has proven successful in early clinical trials (e.g. has shown a favorable safety profile) and proof-of-concept in humans has been demonstrated, the basic production process can be further developed. The final commercial process has to be capable of supplying sufficient material appropriate for pivotal studies and market at acceptable cost of goods. The optimal timing for development of the commercial process is in parallel with the ongoing clinical studies in order to secure product supply for clinical studies and market without any delay or gap.

The key to success for any such strategy is the well-coordinated early parallel development of the upstream, downstream, and formulation activities as well as of analytical methods for production of toxicological and phase I clinical material in order to generate a solid basis for development of the commercial process later on. The driving force for this development program is an interdisciplinary project team with defined roles, tasks, and responsibilities for the project manager and the team members. One of the project team's first tasks is the generation of the detailed project plan containing timeline, work packages, and the required capacities, milestones, and deliverables. This plan, which has to be approved by the senior management, is the basis for the execution of the project. Regular presentations of the project status to the senior management allows close monitoring of any deviations to the plan and ensures the quick approval of any major changes to scope, timing, and cost. Using these tools it is possible to run several development projects in parallel and to perform a portfolio management based on the resources available and on the success of the development candidates.

9.7 Outlook

One of the main obstacles to the development of successful therapeutic antibodies (recombinant proteins) has been the relatively low productivity of the production systems, which sometimes leads to limited availability, high production costs, and hence high costs for therapy. The current state-of-the-art production processes for mAbs in mammalian cells have shown titers up to 6 g/L, and at least 10-fold improvement compared with routine processes of the late 1990s. These achievements, as well as further improvements that can be expected in the near future, present an optimistic perspective for patients worldwide for an improved availability of mAbs for treatment of diseases with a highly unmet medical need at acceptable costs.

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References

Ahern, T.J., Manning, M.C. (1992) *Stability of Protein Pharmaceutical. Part A, Chemical and Physical Pathways of Protein Degradation*. New York: Plenum Press.

Allison, D.W., Aboytes, K.A., Fong, D.K., Leugers, S.L., Johnson, T.K., Loke, H.N., Donahue, L.M. (2005) Development and optimization of cell culture media. *BioProcess Int* (January 2005), 38–45.

Andersson, C., Edlund, P.O., Gellerfors, P., Hansson, Y., Holmberg, E., Hult, C., Johansson, S., Kordel, J., Lundin, R., Mendel-Hartvig, I.B., Noren, B., Wehler, T., Widmalm, G., Ohman, J. (1996) Isolation and characterization of trisulfide variant of recombinant human growth hormone formed during expression in *Escherichia coli*. *Int J Pept Protein Res* 47: 311–321.

Andya, J.D., Hsu, C.C., Shire, S.J. (2003) Mechanisms of aggregate formation and carbohydrate excipient stabilization of lyophilized humanized monoclonal antibody formulations. *AAPS PharmaSci* 5: 1–11.

Arakawa, T., Timasheff, S.N. (1985) Theory of protein solubility. *Methods Enzymol* 114: 49–77.

Berthold, W., Kempken, R. (1994) Interaction of cell culture with downstream purification: a case study. *Cytotechnology* 15: 229–242.

Blank, G.S., Zapata, G., Fahrner, R., Milton, M., Yedinak, C., Knudsen, H., Schmelzer, C. (2001) Expanded bed adsorption in the purification of monoclonal antibodies: a comparison of process alternatives. *Bioseparation* 10: 65–71.

Branovic, K., Buchacher, A., Barut, M., Strancar, A., Josic, D. (2003) *J Chromatogr B* 790: 175–182.

Chi, E.Y., Krishnan, S., Randolph, T.W., Carpenter, J.F. (2003) Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. *Pharm Res* 20: 1325–1336.

Chu, L., Robinson, D.K. (2001) Industrial choices for protein production by large-scale cell culture. *Curr Opin Biotechnol* 12: 180–187.

Cleland, J.L., Powell, M.F., Shire, S.J. (1993) The development of stable protein formulations: a close look at protein aggregation, deamidation, and oxidation. *Crit Rev Ther Drug Carrier Syst* 10: 307–377.

CPMP/3097/02 (2003) *Note for Guidance on Comparability of Medicinal Products containing Biotechnology-deriverd Proteins as Drug Substance – Non Clinical and Clinical Issues*. http://www.emea.eu.int/index/indexh1.htm

Creighton, T.E. (ed.) (1996) *Proteins*, 2nd edn. New York: Freeman & Company.

Eckerskorn, C., Strupat, K., Karas, M., Hillenkamp, F., Lottspeich, F. (1992) Mass spectrometric analysis of blotted proteins after gel- electrophoretic separation by matrix-assisted laser desorption ionization mass spectrometry. *Electrophoresis* 13: 664–665.

Eriksson, L., Johansson, E., Kettaneh-Wold, N., Wikström, C., Wold, S. (2000) *Design of Experiments: Principles and Applications*. Umeå: Umetrics Academy Book.

Fersht, A.R., Daggett, V. (2002) Protein folding and unfolding at atomic resolution. *Cell* 108: 573–582.

Fischer, R., Stoger, E., Schillberg, S., Christon, P., Twyman, R.M. (2004) Plant-based production of biopharmaceuticals. *Curr Opin Plant Biol* 7: 152–158.

Follman, D., Fahrner, R.L. (2004) *J Chromatogr A* 1024: 79–85.
Fraunhofer, W., Winter, G. (2004) The use of asymmetrical flow field-flow fractionation in pharmaceutics and biopharmaceutics. *Eur J Pharm Biopharm* 58: 369–383.
Frokjaer, S., Otzen, D.E. (2005) Protein drug stability: a formulation challenge. *Nature Rev* 4: 298–306.
Galili, U. (1992) The natural anti-Gal antibody: evolution and function. *Immunology Series* 55: 355–373.
Garidel, P. (2004) What can we learn from infrared spectroscopy for the development of protein formulations? In: *Formulation and Drug Delivery Strategies for Biopharmaceuticals*. Munich: IBC Life Sciences, pp. 1–20.
Garidel, P., Schott, H. (2006) Fourier transform mid-infrared spectroscopy for the analysis and screening of liquid protein formulations in pharmaceutical development. *Int BioProcess* 4: 40–46, 48–55.
Gianazza, E. (1995) Isoelectric focusing as a tool for the investigation of post-translational processing and chemical modifications of proteins. *J Chromatogr A* 705: 67–87.
Good, D.L. (2004) Capillary electrophoresis of proteins in a quality control environment. In: *Capillary Electrophoresis of Proteins and Peptides, Methods in Molecular Biology*, Vol. 276. New Jersey: Humana Press.
Gorman, J.J., Wallis, T.P., Pitt, J.J. (2002) Protein disulfide bond determination by mass spectromety. *Mass Spectrom Rev* 21: 183–216.
Harris, R.J. (1995) Processing of C-terminal lysine and arginine residues of proteins isolated from mammalian cell culture. *J Chromatogr A* 705: 129–134.
Harris, R.J., Kabakoff, B., Macchi, F.D., Shen, F.J., Kwong, M., Andya, J.D., Shire, S.J., Bjork, N., Totpal, K., Chen, A.B. (2001) Identification of multiple sources of charge heterogeneity in a recombinant antibody. *J Chromatogr B* 752: 233–245.
Hewlett G. (1991) Strategies for optimizing serum-free media. *Cytotechnology* 5: 3–14.
Hollas, J.M. (2003) *Spectroscopie*. Paris: Edition Dunod.
Jefferis, R. (2001) Glycosylation of human IgG antibodies: relevance to therapeutic applications. *Biopharm* 14: 19–26.
Kaufmann, H., Fussenegger, M. (2003) Metabolic engineering of mammalian cells for higher protein yield. In: Makrides, S.C. (ed.) *New Advances in Biochemistry, Gene Transfer and Expression in Mammalian Cells*. Amsterdam: Elsevier Science, pp. 457–469.
Kellner, R., Mermet, J.M., Otto, M., Valcárcel, Widmer, H.M. (2004) *Analytical Chemistry*. Weinheim: Wiley-VCH.
Kempken, R., Howaldt, M., Walz, F. (2006) Kultur von Tierzellen. In: Chmiel, H. (ed.) *Bioprozeßtechnik*. München: Elsevier GmbH.
Langguth, P., Fricker, G., Wunderli-Allenspach, H. (2004) *Biopharmazie*. Weinheim: Wiley-VCH.
Laue, T.M., Stafford, W.F. (1999) Modern application of analytical ultracentrifugation *Annu Rev Biophys Biomol Struct* 28: 75–100.
Lee, S.Y. (1996) High cell-density culture of *Escherichia coli*. *Trends Biotechnol* 14: 98–105.
LeHir, A. (2001) *Pharmacie galénique*. Paris: Edition Masson.
Li, R., Dowd, V., Stewart, D.J., Burton, S.J., Lowe, C.R. (1998) *Nat Biotechnol* 16: 190–195.
Liu, J., Nguyen, M.D.H., Andya, J.D., Shire, S.J. (2005) Reversible self-association increases the viscosity of a concentrated monoclonal antibody in aqueous solution. *J Pharm Sci* 94: 1928–1940.
Liu, T., Chu, B. (2002) Light-scattering by proteins. In: Somasundran P. (ed.) *Encyclopedia of Surface and Colloid Science*. New York: Marcel Dekker, pp. 3023–3043.
Manning, M.C. (2005) Use of infrared spectroscopy to monitor protein structure and stability. *Expert Rev Proteomics* 2: 731–743.
Mechref, Y., Novotny, M.V. (2002) Structural investigations of glycoconjugates at high sensitivity. *Chem Rev* 102: 321–369.
Middaugh, C.R., Tisel, W.A., Haire, R.N., Rosenberg, A. (1979) Determination of the apparent thermodynamic activities of saturated protein solutions. *J Biol Chem* 254: 367–370.

Murakami, S., Chiou, T.-W., Wang, D.I.C. (1991) A fiber-bed bioreactor for anchorage-dependent animal cell cultures: Part II. Scaleup potential. *Biotechnol Bioeng* 37: 762–769.

Nguyen, D.N. et al. (1995) Protein mass spectrometry: applications to analytical biotechnology. *J Chromatogr A* 705: 21–45.

Nicoud, R.M. (1998) In: Subramanian, G. (ed.) *Bioseparation and Bioprocessing*. Weinheim: Wiley-VCH, 3–38.

Patterson, S.D., Aebersold, R. (1995) Mass spectrometric approaches for the identification of gel-separated proteins. *Electrophoresis* 16: 1791–1814.

Perczel, A., Hollosi, M., Tusnady, G., Fasman, G.D. (1991) Convex constraint analysis: a natural deconvolution circular dichroism curves of proteins. *Protein Eng* 4: 669–679.

Perkins, M., Theiler, R., Lunte, S., Jeschke, M. (2000) Determination of the origin of charge heterogeneity in a murine monoclonal antibody. *Pharm Res* 17: 1110–1117.

Preissmann, A., Wiesmann, R., Buchholz, R., Werner, R.G., Noé, W. (1997) Investigations on oxygen limitations of adherent cells growing on macroporous microcarriers. *Cytotechnology* 24: 121–134.

Prigge, S.T. (1997) Amidation of bioactive peptides: the structure of peptidylglycine – hydroxylating monooxygenase. *Science* 278: 1300–1305.

Remmele, R.L. jr. et al. (2000) Differential scanning calorimetry: a practical tool for elucidating stability in liquid pharmaceuticals. *Biopharm Eur* 56: 58–60.

Rouessac, F., Rouessac, A. (2004) *Analyses chimique. Méthodes et techniques instrumentals modernes*. Paris: Edition Dunod.

Schein, C.H. (1990) Solubility as a function of protein structure and solvent components. *Bio/Technology* 8: 308–317.

Schimpf, M.E., Caldwell, K.D., Giddings, J.C. (eds) (2000) *Field-Flow Fractionation Handbook*. New York: Wiley-Interscience.

Schüle, S., Schultz-Fademrecht, T., Bassarab, S., Bechtold-Peters, K., Frieß, W., Garidel, P. (2004) Determination of the secondary protein structure of IgG1 antibody formulations for spray drying. *Resp Drug Delivery* 2: 377–380.

Schüle, S., Schultz-Fademrecht, T., Bassarab, S., Bechtold-Peters, K., Frieß, W., Garidel, P. (2005) Spray drying of IgG1 antibody formulations: a CD and FTIR investigation with respect to protein secondary structure. *Resp Drug Delivery* 1: 185–188.

Sheeley, D.M., Merrill, B.M., Taylor, L.C. (1997) Characterization of monoclonal antibody glycosylation: comparison of expression systems and identification of terminal α-linked galactose. *Anal Biochem* 247: 102–110.

Shields, R.L., Lai, J., Keck, R., O'Connell, L.Y., Hong, K., Meng, Y.G., Weikert, S.H., Presta, L.G. (2002) Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcγRIII and antibody-dependent cellular toxicity. *J Biol Chem* 277: 26733–26740.

Sine, J.P. (2003) *Séparation et analyse des biomolécules*. Paris: Edition Ellipses.

Stephenson, R.C., Clark, S. (1989) Succinimide formation from aspartyl and asparaginyl peptides as a model for spontaneous degradation of proteins. *J Biol Chem* 264: 6164–6170.

Stockert, R.J. (1995) The asialoglycoprotein receptor: relationships between structure, function, and expression. *Physiol Rev* 75: 591–609.

Storhas, W. (1994) *Bioreaktoren und periphere Einrichtungen*. Berlin: Springer Verlag, pp. 279–297.

Swartz, J.R. (2001) Advances in *Escherichia coli* production of therapeutic proteins. *Curr Opin Biotechnol* 12: 195–201.

Tatford, O.C., Gomme, P.T., Bertolini, J. (2004) Analytical techniques for the evaluation of liquid protein therapeutics. *Biotechnol Appl Biochem* 40: 67–81.

Tebbe, H., Lütkemeyer, D., Gudermann, F., Heidemann, R., Lehmann, J. (1996) Lysis-free separation of hybridoma cells by continuous disc stack centrifugation. *Cytotechnnology* 22: 119–127.

Teshima, G., Stults, J.T., Ling, V., Canova-Davis, E. (1991) Isolation and Characterization of a Succinimide Variant of Methionyl Human Growth Hormone. *J Biol Chem* 266: 13544–13547.

Van de Weert, M., Haris, P.I., Hennink, W.E., Crommelin, D.J. (2001) Fourier transform infrared spectrometric analysis

of protein conformation: effect of sampling method and stress factors. *Anal Biochem* 297: 160–169.

Van Reis, R., Leonard, L.C., Hsu, C.C., Builder, S.E. (1991) Industrial scale harvest of proteins from mammalian cell culture by tangential flow filtration. *Biotechnol Bioeng* 38: 413–422.

Walter, J.K. (1998) In: Subramanian, G. (ed.) *Bioseparation and Bioprocessing*. Weinheim: Wiley-VCH, 1998: 447–460.

Wang, L., Amphlett, G., Blattler, W.A., Lambert, J.M., Zhang, W. (2005) Structural characterization of the maytansinoid–monoclonal antibody immunoconjugate, huN901–DM1: by mass spectrometry. *Protein Sci* 14: 2436–2446.

Waterman, K.C., Adami, R.C. (2005) Accelerated aging: prediction of chemical stability of pharmaceuticals. *Int J Pharm* 293: 101–125.

Werner, R.G. (2005) *BioProcess International* September 2005: 6–15.

Wildt, S., Gerngross, T.U. (2005) The humanization of N-glycosylation pathways in yeast. *Nat Rev Microbiol* 3: 119–128.

World Health Organization (1997) *WHO Epidemiological Records* 72: 143–145.

Wright, A., Morrison, S.L. (1998) Effect of C2-associated carbohydrate structure on IgG effector function: Studies with chimeric mouse-human IgG1 antibodies in glycosylation mutants of Chinese hamster ovary cells. *J Immunol* 160: 3393–3402.

Wurm, F.M. (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotechnol* 22: 1393–1398.

Zhang, W., Czupryn, J.M., Boyle, P.T. Jr, Amari, J. (2002) Characterization of asparagine deamidation and aspartate isomerization in recombinant human interleukin-11. *Pharm Res* 19: 1223–1231.

Zlokarnik, M. (2002) *Scale-up in Chemical Engineering*. Weinheim: Wiley VCH.

Zmak, P.M. et al. (2003) *J Chromatogr A* 1006: 195–205.

10
Pharmaceutical Formulation and Clinical Application

Gabriele Reich

10.1
Introduction

The therapeutic application of monoclonal antibodies (mAbs) has increased dramatically in recent years. More than 20% of all biopharmaceuticals currently being evaluated in clinical trials are full-length antibodies, antibody fragments, or conjugates (Pavlon and Belsey 2005). The key advantage of this class of therapeutics is their high level of specificity for the relevant disease targets. This pinpoint specificity serves to prevent harm to healthy cells and, hence, typically results in fewer side effects compared with traditional drugs.

Their clinical breakthrough as targeted effector molecules is a direct result of the successful engineering, large-scale production, and purification of tailor-made antibodies or fragments with optimal *in vivo* behavior. Chimeric, humanized, and fully human mAbs with reduced immunogenicity and improved half-life show high clinical efficacy for the treatment of a variety of diseases such as organ transplant rejections, cancer, and autoimmune, inflammatory, and infectious diseases. Bispecific antibodies are useful constructs to enhance immunological effector functions. In addition, Fab and single-chain Fv fragments have gained increasing interest for indications where the recruitment of Fc functions is not essential.

Besides their targeted effector function, antibodies are versatile molecules that may be used as carriers and vectors for the site-specific delivery of drugs, toxins, enzymes, radionuclides, and genes. Antibody-mediated targeting strategies hold promise for a wide number of clinical applications, especially since advances in recombinant antibody technology allow formats other than whole antibodies to be engineered. Modified antibodies with increased effector functions (e.g. bispecific mAbs) and fragments (e.g. Fabs, scFvs) of reduced size and thus improved cell penetrability are set to play a significant role in future product developments.

The huge clinical potential and broad variability of mAb designs are clearly reflected by a certain number of approved or nearly approved products being in

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clinical use, and a rather large number of formulations being in the development stage. However, product development with mAbs is somewhat different from traditional low molecular weight drug substances. This is due to their large size and complex three-dimensional structure, which is mandatory for their functional performance. Major challenges are chemical and physical instability during manufacturing and storage, and delivery issues.

This article addresses pharmaceutical formulation, manufacturing, and delivery, as well as clinical applications of therapeutic antibodies.

10.2
Clinical Application

10.2.1
Therapeutic Areas of Antibody Drugs

The majority of antibody-based therapies in the clinic have exploited the activity of the antibody per se, namely to specifically bind to antigens and modulate several different pathways. Indeed, mAbs can function as targeted effector molecules for a wide number of indications. In the past, the pharmaceutical industry has heavily focused on oncology, arthritis, autoimmune, inflammatory and infectious diseases, and this trend is set to continue over the next years.

The major strategy for using antibodies in clinical oncology is a result of their ability to bind to tumor-associated antigens of primary and metastatic cancer cells, and to create antitumor effects by complement-mediated cytolysis, cell-mediated cytotoxicity, and/or signal-transduction leading to apoptosis or growth arrest (Ross et al. 2003). The clinical success of this approach is closely related to the selective binding of the antibody to tumor cells (i.e. a homogeneous overexpression of the target antigen on the tumor cell surface), and limited expression of the antigen by normal tissues. Appropriate tumor-associated target antigens are epidermal growth factor receptors (EGFR: HER1, HER2, HER3, and HER4) in carcinomas, CD19, CD20, and CD22 in B cell lymphomas, and CD25 or CD52 in T cell leukemia. Anticancer antibodies in clinical use include anti-CD20, anti-CD52, and anti-EGFR (HER1 and HER2) for the treatment of B cell lymphoma, lymphatic leukemia and carcinoma, respectively. Examples for approved commercial products are: rituxumab, a chimeric anti-CD20 B cell mAb for the treatment of relapsed or refractory non-Hodgkin lymphoma and related malignancies; alemtuzumab, a humanized anti-CD52 mAb for the treatment of refractory chronic lymphocytic leukemia; trastuzumab for the treatment of HER2-positive metastatic breast cancer; and cetuximab and bevazizumab for the treatment of HER1-positive colon cancer.

Another approach in solid tumor therapy is to inhibit new blood vessel formation and/or angiogenesis by targeting mAbs to antigens expressed on the tumor vasculature, rather than to tumor-associated antigens of solid tumors. Directing therapy to the vascular compartment reduces the impact of the physical barriers

of solid tumors such as heterogeneous blood flow and elevated interstitial pressure. Novel advances in this field are the development of antibodies against angiogenesis-associated factors and receptors such as vascular endothelial growth factor (VEGF) and $\alpha_v\beta_3$ integrin. Examples are bevacizumab approved for the treatment of colorectal cancers, and vitaxin, which is being assessed in clinical trials (McNeel et al. 2005).

Antibody-based therapies have also gained increasing interest in chronic inflammatory diseases that are characterized by high levels of cytokines. Anti-tumor necrosis factor alpha (TNF-α) mAbs, in particular, have proven to be powerful therapeutics in Crohn's disease and rheumatoid arthritis (RA). These antibodies complex soluble TNF-α, a critical mediator in RA and Crohn's disease, thereby inhibiting its functional activity. Approved products are infliximab, adalimumab, and etanercept. Monoclonal antibodies targeting interleukins are another approach in this field. Two products – anakinra and daclazizumab – based on anti-interleukin (IL)-1 and anti-IL-2, have gained approval. Quite a number of anti-inflammatory mAb products targeting TNF-α or various interleukin subtypes are in preclinical and clinical trials (Sacre et al. 2005). To reduce immunological side effects triggered by the Fc part, and to increase circulating half-life, some of these are PEGylated fragments (see Section 10.2.3).

An example of the successful clinical use of a humanized anti-IgE antibody in allergic asthma therapy is omalizumab. When delivered systemically, the antibody targets the Fc portion of the human IgE, thus inhibiting IgE interaction with mast cells and basophils (i.e. removing it as a mediator of allergic asthma).

The potential of mAbs for the treatment of infectious diseases has not been fully exploited yet, although in theory, antibody-based therapies could be developed against any existing pathogen. Given the multitude of pathogens, the pathogen-specific nature of antibody therapies, and the high costs for product development, it seems reasonable that antibody-based therapies can only provide a therapeutic option for selected pathogens that affect primarily immunocompromised patients and/or for which there is no other antimicrobial therapy available. In view of this, it is not surprising that the only approved product yet is palivizumab, a humanized mAb against Rous sarcoma virus infection.

10.2.2
Antibody-Mediated Drug Delivery

In addition to their direct effector function, antibodies can be used as carriers or vectors for targeted drug delivery. The so-called immunoconjugates may be achieved by direct or indirect covalent linkage of an antibody (whole, fragment, or bispecific) to either a drug, toxin, enzyme, and/or radioisotope (Payne 2003; Cao and Lam 2004; Greenwald et al. 2004; Wu and Senter 2005), or to drug-containing sterically stabilized liposomes (Park et al. 2002; Schnyder and Huwyler 2005) or nanoparticles (Brannon-Peppas et al. 2004). Nanoparticles usually consist of biodegradable polymers, while liposomes are small vesicles composed

of unilamellar or multilamellar phospholipid vesicles ranging from 20 to 10000 nm (Fig. 10.1). Immunoliposomes-nanoparticles are intended to improve the therapeutic efficacy by enabling a larger quantity of drug to be delivered per antibody molecule. Moreover, they protect the drug from metabolism and inactivation in the plasma. Attachment of high molecular weight poly(ethyleneglycols) (PEGs), leading to sterically stabilized so-called "stealth" liposomes or nanoparticles, may prevent recognition by phagocytotic cells, thus improving circulating half-life and distribution to peripheral tissues (see Section 10.2.3).

Immunoconjugates are an interesting approach for tumor cell and brain targeting. Antibody-mediated drug targeting to tumors is generated in the same manner as "naked" anticancer antibodies. Antibody conjugates with small cytotoxic drugs are intended to improve the therapeutic index of these drugs by prolonging the bioavailability of the drug, increasing drug uptake in the target cells, and reducing drug toxicity to nontarget cells. Successful approaches require internalization of the drug–antibody conjugate with subsequent intracellular drug release, or extracellular drug cleavage and subsequent cellular drug uptake by diffusion or active transport. To this end, pH- or enzyme-sensitive linkers are required. The use of antibody fragments (Fab, scFv), having a reduced size while retaining their target function, are well suited to increase tumor penetration of immunoconjugates. Various antineoplastic drugs such as methotrexate, 5-fluorouracil, doxorubicin, and maytansine have been developed as immunoconjugates. Gemtuzumab ozogamicin, a calicheamicin-conjugated humanized mouse anti-CD33 mAb, has been approved by the US Food and Drug Administration (FDA) for therapy of drug-refractory acute myloid leukemia.

The option of using liposomes or nanoparticles in targeted cancer therapy is based on the observation that discontinuities in the endothelium of the tumor vasculature favor extravasation and local accumulation at the tumor. Stealth immunoliposomes utilizing internalizing mAbs such as anti-HER2 or anti-CD19

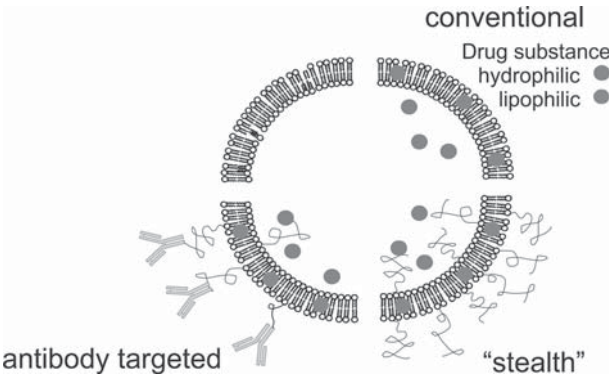

Fig. 10.1 Schematic representation of three different liposome types: conventional liposome; "stealth" liposome sterically stabilized by PEG attachment; "antibody targeted" stealth liposome (i.e. immunoliposome).

can be used to selectively deliver high drug concentrations into the cytoplasm of antigen-expressing tumor cells (Park et al. 2002). This approach has been shown to greatly enhance the therapeutic index of doxorubicin.

Radioimmunoconjugates are an interesting strategy for concentrating doses of radiation from isotopes such as iodine-131 or yttrium-90 to cancer tissues. It is supposed that even antigen-negative tumor cells may be eradicated, since these radionuclides are effective over a distance of several cell diameters. Two commercial products based on an anti-CD20 antibody are in clinical use for the treatment of patients with non-Hodgkin's lymphoma that have relapsed after chemotherapy and are no longer responding to rituximab.

Drug delivery to the brain is highly restricted by a tight vascular barrier, the blood–brain barrier (BBB). One option to enhance brain delivery is the use of an mAb to the extracellular domain of a BBB receptor which may trigger cellular uptake by the mechanism of receptor-mediated transcytosis. OX26, a anti-rat transferrin receptor mAb can be used for this purpose (Schnyder and Huwyler 2005). The mAb vector may be coupled to the drug via biotin–avidin conjugation. In case of sterically stabilized liposomes, the thiolated antibody or fragment is coupled to a thiol-reactive maleinimide-PEG-phospholipid. PEG-conjugated OX26 immunoliposomes are interesting carrier systems for brain targeting of small anticancer drugs or plasmids. Brain uptake of daunomycin was effectively increased in rats when it was entrapped in OX26 immunoliposomes. In a gene delivery approach based on OX26 immunoliposomes, gene expression in rat brain cells beyond the BBB was clearly demonstrated. Since alternative vectors, such as mAbs directed at the human insulin receptor, indicate the potential for higher targeting efficiency, it can be assumed that this approach will gain clinical relevance for human therapy in the near future.

10.2.3
PEGylated Antibodies and Antibody Fragments

Modification of proteins and colloidal carrier systems with PEG is a well-established technique (Caliceti and Veronese 2003) to

- reduce antigenicity, i.e. immunogenicity,
- increase circulating half-life by either evasion of renal clearance and/or cellular clearance mechanisms, and
- improve bioavailability and reduce overall drug toxicity.

Recent technological advances enabling humanized and fully human antibodies to be produced have largely overcome the problem of immunogenicity observed with murine antibodies. In addition, full-length antibodies generally have a long circulating half-life, since the Fc region binds to the neonatal Fc receptor (FcRn) providing a salvage mechanism (i.e. protection from *in vivo* degradation). In contrast, antibody fragments, lacking the Fc part and having a reduced size, usually suffer from relatively short half-lives (Weir et al. 2001). PEGylation of Fab or scFv fragments is therefore an attractive approach to increase the circulating

half-life, thus improving their therapeutic potential (Chapman 2002; Greenwald et al. 2004). The pharmocokinetic effect is mainly attributed to an increased hydrodynamic size of the molecules to above the normal limit for glomerular filtration, resulting in reduced renal clearance. In addition, PEGylation can shield the molecules from immunological recognition and subsequent clearance from the circulation, and/or affect biodistribution including improved tumor targeting. Several studies have clearly shown greater accumulation of antibodies and antibody fragments in tumors without higher levels in normal tissues following PEGylation (references are given in Chapman 2002).

Additional benefits of PEGylation to date are related to the solubility of antibodies and antibody fragments. PEGylated antibody fragments in aqueous solution can be concentrated to >200 mg mL^{-1} without aggregation. This technological advantage over non-PEGylated fragments opens up new opportunities for chronic immunotherapy with high dosing regimens by subcutaneous injection (see Section 10.5.1). Moreover, the ability of PEG to dissolve in many different solvents, ranging from water to hydrophobic organic solvents, can be exploited for the development of improved slow-release formulations (see Section 10.5.4).

Numerous functionalized PEG molecules with different structures, chain lengths, and conjugation chemistries are available. Moreover, the conjugation site and number of PEG chains attached per antibody molecule can be varied. However, in the context of antibodies and antibody fragments, the choice of an appropriate PEGylation approach requires various aspects to be considered. To avoid significant loss of antigen binding and/or immunological effector functions mediated by the Fc region, site-specific PEGylation should be preferred over random PEG conjugation (Chapman 2002). In case of Fab' fragments, the method typically involves the use of PEG maleimide to react with thiol groups of free cysteine residues in the hinge region of specifically engineered antibody molecules. Site-specific PEGylation in this region ensures that the PEG is located away from the antigen-binding region, thus retaining full antigen-binding activity.

10.2.4
Routes of Administration

Monoclonal antibodies, fragments, and conjugates thereof are large molecules with a complex and sensitive three-dimensional structure. Because of poor *in vivo* stability and permeability (i.e. poor bioavailability by other routes), parenteral delivery has been the most conventional route of administration for mAb therapy in humans. Due to high dosing (mg kg^{-1} per day) and greater control in clinical settings, intravenous (i.v.) infusion is the preferred dosage form for mAb-based cancer therapy in hospitals. Indeed, the majority of approved mAb therapeutics used in oncology, whether naked or conjugated to toxins or radionuclides, are formulated for i.v. administration. Depending on the stability and solubility profile of the antibody molecule, ready-to-use solutions, concentrates and freeze-dried powders for reconstitution prior to administration are in place.

In contrast to cancer therapy, where clinical safety control aspects are extremely important, indications such as inflammatory diseases and immune disorders require outpatient administration and home use of antibody drugs, since frequent and chronic dosing is mandatory. For these settings, alternative delivery routes are more appealing than i.v. infusions to facilitate administration and improve patient compliance. However, as indicated above, systemic delivery by oral, transdermal, nasal, or pulmonary routes of administration has been difficult to achieve. Thus, a small volume (<1.5 mL) subcutaneous (s.c.) injection is currently the preferred dosage form for chronic administration of antibody therapeutics in the physician's office or by the patient at home. Few products have been developed as stabilized high-concentration solutions. However, stability and delivery problems frequently associated with highly concentrated, high-viscosity mAb solutions (see Section 10.5.1) have stimulated the search for alternative dosage forms. Low-viscosity suspensions based on highly stable crystalline antibody formulations are a novel approach that became feasible following recent advances in antibody batch-crystallization techniques (see Section 10.5.3). Due to reduced stability problems, crystalline antibodies may also provide a better platform for future developments of carrier-based mAb delivery systems (see Section 10.5.4).

Local delivery of whole antibodies or fragments to the site of disease is an alternative approach to enhance therapeutic efficacy and improve patient compliance. One such strategy is the pulmonary administration of anti-IgE antibodies for the treatment of allergic asthma. Spray-dried and spray freeze-dried powders yielding particle sizes that are appropriate for inhalation and lung deposition have been reported as promising dosage forms for this purpose (Maa et al. 1999). Formulation strategies to overcome mAb stability problems and achieve good aerosol performance upon drying will be addressed in Section 10.6.1.

Topical application of antibodies recognizing and neutralizing the oral pathogen *Streptococcus mutans* might be attractive for control and prevention of dental caries (Kupper et al. 2005). Ocular administration of antibody fragments via eyedrops has been proposed for local treatment of eye infections. Intestine- or colon-specific mAb delivery following oral administration could be a future option for targeted local therapy of infectious diseases in the gut.

10.3 Pharmaceutical Product Development

To gain regulatory approval for human use of a new drug product, the applicant has to perform a number of investigations that confirm pharmaceutical product quality. In classical "drug discovery and development processes" a new chemical entity (NCE) is usually examined in some sort of preformulation study before it is further progressed to formulation development and subject to an ascending series of preclinical and clinical trials to ascertain potency, efficacy, and safety as a pharmaceutical agent for human use.

Generally, antibody-based drug substances are treated no differently from their low molecular weight counterparts (i.e. an assessment of physicochemical and biological properties has to be made to fulfill the quality acceptance criteria defined in the ICH harmonized tripartite guideline). However, there are some important a priori differences between antibodies and conventional NCEs. The macromolecular character and the specific secondary, tertiary and/or quartary structure may have a controlling influence over the physicochemical and biological properties of an antibody in solid and solution state. This implies more extensive studies during the drug development stage, including assessment of purity, stability, molecular size and structure, *in vitro* and *in vivo* biological activity, pharmacokinetics, delivery, and immunogenicity.

Since stability problems may be crucial for product performance in many respects, the way from drug discovery to an approved, marketed antibody drug product is rather challenging. In view of this, the following chapter is dedicated to stability issues. Formulation challenges and strategies for the development of parenteral and local delivery systems are discussed in detail in Sections 10.5 and 10.6.

10.4
Stability Issues

Therapeutic antibodies may be defined as whole monoclonal antibodies, specifically designed fragments or constructs of various composition. Understanding the degradation pathways of each of these molecules and how they relate to drug potency, efficacy, and safety is the key issue for the development of stable antibody drug products. In Section 10.4.1, the current understanding of the main degradation pathways of therapeutic antibodies will be presented. Regulatory requirements for the design of stability studies including analytical and kinetic aspects will be addressed in Section 10.4.2.

10.4.1
Degradation Pathways

The degradation pathways of antibody molecules may be classified as chemical and physical instability (Wang 1999). Chemical instability involves modification of the antibody molecule via covalent bond formation or cleavage, while physical instability refers to conformational changes, adsorption, aggregation, and/or precipitation. Depending on the sites of degradation, the functional consequences may be different. Degradation sites in the variable region are likely to result in loss of antigen binding capability. Degradation pathways in the constant (Fc) regions may affect the *in vivo* effector function and/or the metabolism of the antibody. Unfolding and aggregation can lead to increased immunogenicity.

10.4.1.1 Chemical Degradation

Chemical instability, as distinct from proteolysis, is generally associated with specific amino acid residues or sequences. Chemical reactions affecting antibody molecules often involve deamidation, oxidation, and formation of incorrect disulfide bonds.

Deamidation Deamidation is a common reaction that may occur in aqueous antibody solutions under a variety of *in vitro* conditions. Asparagine (Asn) and glutamine (Gln) residues are both susceptible to deamidation, although at a different rate (Asn > Gln). The side chain amide linkage may either be simply hydrolyzed to form a free carboxylic acid, or proceed through a five- or six-membered cyclic imide intermediate formed by intramolecular attack of the succeeding peptide nitrogen at the side chain carbonyl carbon of the Asn or Gln residue. Since the formation of a cyclic imide involves participation of the neighboring amino acid, the size and physicochemical characteristics of the succeeding amino acid side chain as well as the conformational chain mobility play a significant role in the deamidation rate. Generally, the sequence -Asn-Gly- is most susceptible to deamidation, especially under neutral and alkaline conditions, increased temperature, and/or ionic strength. Asn and Gln accessibility within the overall three-dimensional structure is another important parameter to be considered. Typically, only few Asn and Gln residues are located in highly flexible hydrophilic domains or on the surface of the molecule, such as a small number of reactive Asn residues in the Fc region of the heavy chain. Since their degradation leads to changes in the charge pattern of the molecule, the overall structural stability may be compromised or additional chemical reactions induced. Inhibition of deamidation by adjusting solution conditions (temperature, pH, ionic strength) or freeze-drying is therefore an important issue, even if *in vivo* consequences are not likely to be expected.

Oxidation Oxidation is a second major degradation pathway of antibody molecules. Potential reaction sites are the side chains of methionine, cysteine, histidine, tryptophan, and tyrosine residues, several of which are typically in the heavy and light chains of IgG molecules. As with other side chain reactions, their susceptibility is closely related to their accessibility, which in turn may be affected by temperature, pH, and solvent composition. Methionine (Met) residues are selectively oxidized under acidic conditions. Two pathways have been described, namely a temperature-induced oxidation via formation of free radicals from hydrogen peroxides, or a light-induced reaction with singlet oxygen (Lam et al. 1997). Both pathways have been reported to potentially occur in aqueous solutions of therapeutic antibodies formulated with non-ionic polyether surfactants such as Tween 80 or 20, since these excipients can undergo autoxidation to form peroxides or dissipate light energy by reacting with molecular oxygen to generate singlet oxygen. Consequently, pH adjustment, nitrogen flushing, light protection, and low-temperature storage are effective means to reduce Met oxidation rate.

The thiol group of cysteine (RSH) can be oxidized in various steps to sulfenic acid (RSOH), disulfide (RSSH), sulfinic acid (RSO_2H), and finally, sulfonic acid (RSO_3H). Cysteine (Cys) oxidation takes place in the presence of oxidizing agents such as hydrogen peroxide, or spontaneously via autoxidation by oxygen from the air. The reaction rate strongly depends on temperature, pH, buffer salts, presence of molecular oxygen, and/or metal ion catalysts. Typically, Cys oxidation is accelerated with increasing pH. Under favorable steric conditions, it can lead to intra- and/or even intermolecular disulfide formation, which in turn may lead to irreversible aggregation. The intrinsic number and alignment of intra- and interchain disulfide bonds is an integral structural element of each antibody subclass. Interchange of disulfide bonds can result in incorrect pairings, which may lead to an altered threedimensional structure and consequently to an altered solubility, biological activity, and/or immunogeneity.

Disulfide exchange Disulfide exchange is favored in solution, but may also occur in powder products with a certain molecular chain mobility resulting from residual moisture. The reaction mechanism depends on the pH of the medium. Under neutral and alkaline conditions, the reaction is catalyzed by thiols, which, in the form of thiolate ions, carry out a nucleophilic attack on the sulfur atom of the disulfide. In acidic media, the reaction takes place through a sulfenium cation, which carries out an electrophilic displacement on a sulfur atom of the disulfide.

10.4.1.2 Physical Degradation

Antibody molecules, as other proteins, fold to a specific three-dimensional superstructure that is essential for their biological function. Since the thermodynamic stability of the native conformation, resulting from a unique balance between large stabilizing and large destabilizing forces, is only small, the molecules can undergo a variety of structural changes independent of chemical modifications. The process can lead to a stepwise unfolding of the molecule, and may be reversible or irreversible.

In solution, rate and extent of the conformational changes are strongly affected by temperature, shear forces, pH, salt type and concentration, surfactants, and the presence of organic solvents or cosolutes (see Section 10.5.1). Since unfolding is typically favored at both high and low temperatures, freeze-thawing and freeze-drying may damage the native structure of antibody molecules to the same extent as elevated temperatures. Even in the solid state, conformational changes may occur at temperatures near or above the glass transition temperature (see Sections 10.5.2 and 10.6.1).

Conformational perturbations typically promote irreversible adsorption and nonnative aggregation, which in some cases may lead to subsequent precipitation. Recent publications have clearly demonstrated that partially unfolded molecules rather than fully unfolded molecules are the reactive species that form physically and/or covalently linked aggregates (Chi et al. 2003; Minton 2005). Antibody aggregates often retain a large amount of their secondary structure, while losing

their tertiary structure. Moreover, intermolecular disulfides are generally involved in the aggregation mechanism (Andya et al. 2003). Since aggregation of whole antibodies or fragments is inherently a nucleation and growth phenomenon, a lag phase resulting from an energy barrier to nucleation or assembly is often observed. Consequently, conditions that promote unfolding and/or the formation of soluble aggregates should be avoided, in order to obtain an acceptable long-term stability of the final antibody formulation.

10.4.2 Design of Stability Studies

Stability testing is intended to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity, and light, and enable recommended storage conditions, retest periods and shelf-lives to be established. Consequently, stability studies are an integral part of drug preformulation and formulation development, and subsequent quality control of the marketed product. The design of stability testing programs within the different stages of the drug development process strongly depends on regulatory issues, practical needs, scientific knowledge, economic aspects, and accessibility of appropriate analytical methods.

10.4.2.1 Regulatory Aspects

From a regulatory point of view, stability is defined as "the capacity of a drug to remain within product specifications that have been established to assure its identity, potency, purity, efficacy and safety." The regulatory stability testing requirements for a Registration Application within the areas of Europe, Japan, and the USA are generally summarized in the ICH harmonized tripartite guideline entitled "Stability Testing of New Drug Substances and Products" (ICH Guideline Q1A). The guideline provides information on test procedures for drug substance and finished product including storage conditions for long-term and accelerated testing, test criteria, selection of batches, testing frequency, packaging, and labeling. The guideline emphasizes that expiration dating should be based on real-time, real-temperature data. The length of the studies and the storage conditions should be sufficient to cover storage, shipment, and subsequent use. Validated analytical methods must be applied.

For biopharmaceutical drug substances, including antibodies with physicochemical properties that differ from those of classical low molecular weight drugs, the general tripartite ICH guideline on stability was supplemented by Annex Q5C (ICH Guideline Q5C). Q5C is intended to provide information on how to consider these distinguishing properties in a well-defined testing program for the development of appropriate stability data. Typically, Q5C implies that "there is no single stability-indicating assay or parameter that profiles the characteristics of a biotechnological product." Consequently, various physicochemical, biochemical and immunochemical methodologies must be applied to compre-

hensively characterize drug substance and drug product, and accurately detect any changes resulting from chemical or physical degradation during storage. In addition, accelerated and stress conditions described in Q1A may not be appropriate for biotechnological products. Thus, it is recommended in Q5C that accelerated and stress conditions should be carefully selected on a case-by-case basis. This aspect will be addressed in detail in Section 10.4.2.3.

10.4.2.2 **Analytical Tools**

Several analytical techniques are usually applied to gain a comprehensive insight into the stability profile of antibody drugs. These include electrophoretic methods, chromatographic and spectroscopic techniques, laser light scattering, ultracentrifugation, calorimetry and peptide mapping (Wang 1999). Selecting the appropriate analytical tool for antibody stability testing requires a thorough understanding of the power and limitations of each of these tools. This section briefly emphasizes the potential of the most frequently used analytical techniques.

The presence of deamidated forms of asparagine or glutamine can easily be detected by an acidic shift in isoelectric focusing (IEF) bands or shifts in ion-exchange high-performance liquid chromatography (HPLC) retention times. Peptide mapping by reverse-phase HPLC is usually the tool employed to identify sites of deamidation. Peptide mapping may also be used for detection of methionine sulfoxidation sites. Low molecular weight degradation products resulting from antibody proteolysis can be determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and size-exclusion chromatography (SEC), respectively.

Various spectroscopic techniques are intended to evaluate conformational changes including secondary and tertiary structural features. UV and fluorescence spectroscopy both rely on the susceptibility of aromatic amino acid side chains (Phe, Tyr, Trp) to their microenvironment, thus allowing tertiary structural changes to be monitored in solution. Circular dichroism (CD), Fourier transform infrared spectroscopy (FTIR), and Raman spectroscopy are sensitive to secondary structural changes allowing α-helix and β-sheet content to be determined. Like UV and fluorescence spectroscopy, CD measurements can only be performed in solution, while FTIR and Raman spectroscopy are well suited in solid and solution state. The latter two have therefore been used as powerful tools for the determination of secondary structural perturbations of various mAbs upon changes in solution pH, lyophilization, or spray drying (Sane et al. 2004; Chang 2005a). Since the extent of drying-induced structural perturbations have been reported to exhibit good correlation with the aggregation rate of various mAbs upon long-term storage, spectroscopic methods may be considered as quick and reliable tools to screen excipients and excipient concentrations in dry powder formulations.

Qualification and quantification of mAb aggregates are rather challenging. Theoretically, a great number of analytical techniques, including HPLC/SEC, ion-exchange chromatography (IEX), SDS-PAGE, turbidimetry, laser light scattering, ultracentrifugation, and filtration are intended to provide information on

the extent of aggregation. In practice, however, selective and reliable quantification of soluble and insoluble mAb aggregates requires advanced analytical methods such as asymmetric field flow fractionation (AFFF) combined with multiangle laser light scattering. Since operation is possible with dissolved and dispersed molecules, soluble and insoluble aggregates may be determined simultaneously. If only soluble aggregates are present, SEC and SDS-PAGE may provide a rough estimate of dimer, trimer etc. formation including information on the nature of aggregate formation (covalent versus noncovalent). Reliable quantification of soluble aggregates is feasible by sedimentation velocity in an ultracentrifuge.

Functional changes of "naked" whole antibodies or fragments can be analyzed by binding assays to purified antigens and/or defined regions of antigens. *In vitro* potency testing of drug–antibody conjugates is more sophisticated, since it requires an appropriate surrogate test that allows drug release under *in vivo* conditions to be considered.

10.4.2.3 **Practical Approach**

Stability studies during preformulation are intended to:

- Define stress parameters relevant for dosage form screening including manufacturing, shipping and storage conditions
- Provide information on degradation sites and pathways
- Evaluate degradation kinetics
- Screen potential stabilizers.

Since data generated at this stage serve as the basis for subsequent formulation development, efficient testing programs enabling fast assessment of stability characteristics with less expenditure of material are required. Procedures known as "accelerated stability testing" based on "classical isothermal" approaches require a large number of samples stored at different constant temperatures over a prolonged period of time. Moreover, their applicability to accelerated protein stability tests is still a matter of debate, since Arrhenius behavior of chemical protein degradation is only valid at temperatures where unfolding is not an issue. In view of this, accelerated "non-isothermal" kinetic methods in combination with analytical tools that allow conformational changes to be monitored, have recently been described as an alternative approach for the early-stage development phase of protein formulations (Reithmeier and Winter 2002). Degradation rate constants obtained from non-isothermal studies in the temperature range below T_m of the model protein showed good comparability to isothermal data. The resulting Arrhenius plots were linear and enabled shelf-life of various formulations to be estimated by extrapolating the stability data to the storage temperature. In addition, nonlinear behavior was detectable in a much shorter time. Since miniaturization and automation of non-isothermal experiments makes high-throughput screening of potential stress parameters and stabilizing excipients feasible, the non-isothermal approach is an interesting means to make

preformulation stability testing of antibody drugs more efficient and less time consuming.

Contrary to preformulation studies, the design of long-term and accelerated stability testing programs of the final formulation (i.e. the finished product) is well described in the ICH tripartite guidelines Q1A and Q5C, allowing test conditions for antibody products to be defined on a case-by-case basis. Consequently, storage conditions for real-time/real-temperature stability studies are usually confined to the proposed storage temperature which is often precisely defined. In the case of freeze-dried products, the stability of the product after reconstitution should be demonstrated in addition to dry powder stability.

10.5 Formulation and Manufacturing of Parenteral Delivery Systems

10.5.1 Ready-to-Use Solutions and Concentrates

The formulation and manufacturing design of parenteral antibody solutions requires general quality attributes and more specific stability, delivery, and compliance aspects to be considered. Typically, aqueous antibody solutions intended for intravenous application have to be sterile, particle-free, and isotonic, and in case of high volume infusions, free of endotoxins. Osmolarity is not a major issue when formulating subcutaneous injections; however, isotonic solutions are desirable to allow painless injections. The pH value of the solution should provide sufficient solubility, optimal chemical and physical long-term stability, and biocompatibility. Viscosity should be as low as possible to allow economic processing and reduce pain upon injection. If the product is intended for multi-use, it is essential to include preservatives in the formulation.

To maintain chemical and physical stability during manufacturing, shipping, and/or storage, potential stress parameters such as high and low temperature, oxygen, light, pH changes, shear stress and adsorption to interfaces should be reduced to a minimum. Protection from oxygen and light to improve chemical stability are usually easy to perform by using nitrogen flushing during the manufacturing process, and selecting a packaging material that protects the solution from oxygen and light. Temperature control during manufacturing is also feasible. As an example, pathogen inactivation in antibody solutions is usually performed by sterile filtration at room temperature instead of using heat. The method is gentle provided that the filter material does not induce antibody adsorption and/or aggregation. Unlike the manufacturing process, precise control of storage conditions during shipping is not always feasible.

Additional concerns to be considered are shear stress and contact with interfaces. Adsorption is always an issue in syringes, filters, and packaging materials and can lead to unrecoverable product loss. Shear stress-induced mAb unfolding, adsorption, aggregation, and/or precipitation can occur in filter and pumping

systems. For example, piston-driven pumps usually tend to generate more shear stress than rolling diaphragm pumps. This should be taken into consideration when filling shear sensitive antibody solutions, not only since unrecoverable product loss increases cost of goods and reduces potency, but also because non-native aggregates may have a major impact on the pharmacokinetic and safety profile of the drug product due to increased immunogenicity.

Viscosity implications are a major issue when developing small-volume, high-dose antibody solutions for subcutaneous injection (Liu et al. 2005). Reversible multivalent antibody self-association mediated by electrostatic interactions of charged residues on the molecule surface may result in unusually high solution viscosity. This nonlinear viscosity increase with increasing mAb concentration has been reported to complicate manufacturing processes, stabilization, and administration. High solution viscosity may result in reduced membrane flux and high pressure-drops during sterile filtration and tangential flow filtration (TFF), the main technology for large-scale buffer exchange (i.e. concentration and formulation of antibody solutions). Depending on the propensity of the antibody molecules to interact and unfold at the membrane surface, economically unacceptable losses may occur as a result of membrane clogging. Moreover, application is aggravated, since high-viscosity solutions require the use of large-bore needles, which may result in more painful injections. Furthermore, if the *in vivo* dissociation rate upon dilution is slow, reversible self-association may have an impact on antibody potency and overall immunogenicity.

In conclusion, formulation development requires an integrated approach whereby a stable formulation is developed that can be successfully administered and economically manufactured. Further to PEGylation, the proper choice of excipients such as buffers, tonicifiers, and various types of stabilizers is a primary concern (Wang 1999). Two examples of antibody formulations are described in Table 10.1.

10.5.1.1 Appropriate Excipients

Buffers Buffers serve to adjust the solution pH to optimal solubility, chemical and physical stability, and physiological compatibility. Various buffers such as

Table 10.1 Examples of excipients used in antibody solutions.

Bevacizumab concentrate (Avastin)	*Adalimumab concentrate (Humira)*
Na_2HPO_4/NaH_2PO_4	Na_2HPO_4/NaH_2PO_4
α,α-Trehalose $2H_2O$	Citric acid/sodium citrate
Polysorbate 20	Sodium chloride
Water for injection	Mannitol
	Sodium hydroxide
	Polysorbate 80
	Water for injection

citrate, histidine, Tris, etc. are in use. However, buffer choice can be critical, since salt type as well as ionic strength may have a major impact on the solution viscosity. Histidine is often the first choice in highly concentrated solutions, since it has been found to effectively reduce reversible self-association of monoclonal antibodies (Chen et al. 2003).

Tonicifiers Excipients that are often used as tonicifiers are mannitol, glycine, or sodium chloride. The latter is preferred when solution viscosity might be a concern, since sodium chloride has been observed to reduce electrostatic protein–protein interactions leading to antibody self-association. However, formulations containing sodium chloride should avoid being stored in stainless-steel vessels to prevent generation of iron ions that may catalyze oxidative degradation of antibody molecules (Lam et al. 1997).

Osmolytes To increase the thermodynamic stability of the native antibody structure (i.e. reduce temperature-, shear- or pH-induced antibody unfolding and nonnative aggregation), molar concentrations of osmolytes such as sugars and polyols have been applied. Since these compounds are preferentially excluded from the native antibody molecule surface, they increase the protein's chemical potential by preferential hydration of the native antibody structure. Despite this advantage, the use of osmolytes in antibody solutions may be limited, since they also add to the viscosity and osmolality of the formulation which in turn may render it impractical for subcutaneous delivery (Liu et al. 2005). High concentration of sugar can lead to hypertonic solutions and enhance self-association of native antibody molecules in high-concentration formulations, both of which will make injections painful.

Surfactants The use of surfactants is essential to reduce the intrinsic and agitation-induced adsorption tendency of mAbs to interfaces. Selection of the appropriate surfactant type and concentration may be critical, since surfactants usually have a certain tendency to bind to mAbs thereby compromising their conformation. In practise, stabilizing concentrations are very low and should be discerned experimentally. Regulatory approval for parenteral use is an issue to be considered when selecting the surfactant type. Nonionic polyether surfactants such as Tween 80 and 20 are usually applied.

Preservatives Multidose formulations must contain preservatives to protect them from microbial contamination upon multiple withdrawals. Selection of the optimal preservative depends on a number of factors including solubility, efficacy, and compatibility with the formulation and the route of administration (Gupta and Kaisheva 2003). In this respect, stability issues of the antibody are a major concern, since certain preservatives such as phenolic compounds are known to cause precipitation of humanized monoclonal antibodies in aqueous solution. Benzyl alcohol, one of the least toxic and most widely used parenteral preserva-

tives, has also been reported to trigger mAb aggregation, although in a concentration-dependent manner. Combinations of benzyl alcohol/chlorobutanol and benzyl alcohol/methylparaben have been screened as potential candidates for the preservation of antibody solutions containing histidine buffer, Tween 80, and sodium chloride as surfactant and tonicifier.

10.5.2 Freeze-Dried Powders

If parenteral administration is intended, a ready-to-use solution is the most convenient dosage form for the end user. However, chemical and physical stability issues of therapeutic antibodies are generally more pronounced in solution than in solid state, since increased mobility of dissolved molecules facilitates conformational changes and accessibility of reactive sites. In fact, whenever preformulation studies indicate that sufficient stability cannot be achieved in solution, dry powder formulations for reconstitution prior to administration are an attractive alternative. Freeze-drying of sterile filtered solutions under aseptic conditions is the most conventional way to obtain antibody products with the following characteristics:

- Elegant cake structure without any collapse upon storage
- Long-term chemical and physical stability
- Sterility upon storage, reconstitution and optional multi-use
- Fast reconstitution
- Isotonicity upon reconstitution
- Maintainance of all chemical and physical characteristics of the original dosage form upon reconstitution.

To achieve these quality attributes, a comprehensive process and formulation understanding, including potential stress parameters upon freezing, drying, storage, and reconstitution, is essential (Carpenter et al. 1997).

The freeze-drying process consists of three stages, namely freezing, primary drying, and secondary drying. The freezing step is intended to form pure ice crystals and solidify the remainder of the solution in an amorphous state. The temperature that allows the latter to be achieved is usually between –20 °C and –60 °C, i.e. well below the collapse temperature (T_c) of the formulation. In the drying step, the product should be kept as well below T_c to remain in the solid amorphous state. During the primary drying step, ice is removed by sublimation at subambient temperatures (usually between –40 °C and –10 °C) under vacuum (40–400 mTorr). In the secondary drying stage, small amounts of bound water may be removed by increasing the temperature to between 5 °C and 20 °C (i.e. below the glass transition temperature (T_g) of the product, but high enough to promote adequate desorption rates). Precise control of cooling rate, drying temperature, and residual moisture content is essential for final product performance (e.g. cake structure and antibody stability).

Critical stability issues in the freezing process are: (1) exposure of the antibody to the ice/water interface, and (2) pH and/or salt effects in the highly concentrated undercooled solution. Antibody exposure to the ice/water interface is mainly affected by the cooling rate which determines the number and size of ice crystals, and the antibody concentration which determines the percentage of molecules being in contact with the ice/water interface. A fast cooling rate leads to a large number of small ice crystals (i.e. a large ice/water interface). Since ice crystals may be considered as solid interfaces with the potential risk of protein unfolding, adsorption and/or aggregation, fast cooling and a low overall protein concentration are unfavorable conditions in this respect. Fast cooling, however, can be desirable to reduce the exposure time of the antibody drug to unfavorable pH conditions that may occur as a result of gradual buffer salt crystallization upon freezing, and lead to chemical and/or physical instabilities. The most drastic changes have been observed with sodium phosphate and potassium phosphate buffer salts, leading to acidic or alkaline pH values after crystallization of one of the buffer salt components.

A potential stress parameter during dehydration is the removal of the protein hydration shell, which causes significant conformational changes in the absence of appropriate stabilizers. In many cases, the extent of antibody unfolding during freeze-drying was found to directly correlate with the subsequent rate of non-native aggregation and/or chemical degradation upon storage (Sane et al. 2004). In fact, the chemical and physical long-term stability of a lyophilized antibody formulation strongly depends on the extent of structural perturbations during freezing and dehydration, and the molecular mobility of the protein within the dry powder matrix (i.e. the formulation composition, the residual moisture content, and the storage conditions) (Chang et al. 2005a/b). Product storage below the glass transition temperature (T_g) is recommended to enhance long-term stability (e.g. improve shelf-life and retain reconstitution properties at ambient room conditions to allow 100% recovery of intact antibody molecules).

In conclusion, formulation design of freeze-dried antibody powder products aims to provide (1) minimal pH change upon freezing, (2) structural preservation upon freezing and dehydration, (3) product solidification in the amorphous state, (4) a high glass transition temperature of the final product, thus reducing the molecular mobility of the protein upon storage, and (5) minimal unfolding and/or aggregation upon reconstitution. Appropriate excipients (Table 10.2) may be classsified as buffers, tonicifiers, stabilizers, and bulking agents (Carpenter et al. 1997).

Table 10.2 Examples of excipients used in freeze-dried antibody products.

Infliximab (Remicade)	*Trastuzumab (Herceptin)*	*Omalizumab (Xolair)*
Na_2HPO_4/NaH_2PO_4	Histidine/histidine HCl	Histidine/histidine HCl
Sucrose	α,α-Trehalose $2H_2O$	Sucrose
Polysorbate 80	Polysorbate 20	Polysorbate 20

10.5.2.1 Appropriate Excipients

Buffer salts Selection of buffer salt type and concentration is an important issue, since drastic pH changes upon freezing may increase the risk of deamidation, unfolding, and/or aggregation of antibodies. Buffers that have minimal pH change upon freezing include citrate, histidine, and Tris. Quite a number of lyophilized antibody formulations have been formulated using noncrystallizing histidine as a buffer at pH from 5.5 to 6.5. Besides functioning as a buffer, histidine has also been reported to protect antibodies such as rhuMAb HER2 and ABX-IL8 upon freezing as evidenced by lower levels of aggregation after multiple freeze/thaw cycles (Chen et al. 2003).

Tonicity modifiers Tonicity modifiers such as mannitol, glycine, sucrose, glycerol, and/or sodium chloride can be included either in the reconstitution medium or in the powder formulation. In the latter case, their crystallization tendency and their effect on the glass transition temperature of the product should be considered.

Bulking agents Bulking agents are intended to increase powder mass and improve cake structure at low drug concentrations. Since the concentration of antibodies is usually high, bulking agents are not necessary.

Stabilizers Stabilizers may be characterized as cryoprotectants, lyoprotectants, and reconstitution aids. Cryoprotectants are excipients that can preserve the native antibody structure during the freezing process. Cryoprotection may be explained by the mechanism of preferential hydration of the native antibody structure, which can be achieved by the preferential exclusion of the cryoprotectant from the protein surface in both the liquid and frozen state. Polyols, monosaccharides, disaccharides and hydrophilic polymers such as polyethyleneglycol have all been reported to act as cryoprotectants thereby preserving the native antibody structure during the freezing process.

Lyoprotection refers to the prevention of drying-induced conformational changes of the protein and is best achieved by disaccharides. It may be explained by the "water replacement mechanism," which states that dissacharides protect proteins during dehydration by hydrogen bonding to polar and charged groups on the surface of the native conformation.

Reconstitution aids may help rehydration of the lyophilized powder prior to administration (i.e. they reduce the tendency of the antibody to form aggregates during reconstitution). Low concentrations of nonionic surfactants such as polysorbate in either the powder or the diluent are usually effective in preventing aggregate formation during rehydration. Although the exact mechanism is still unknown, it may be assumed that the surfactant interferes with the intermolecular protein interactions and/or serves as a wetting agent that hastens the dissolution of the freeze-dried cake (Webb et al. 2002).

The proper choice of stabilizers requires several product characteristics to be considered, namely (1) elegant and mechanically strong cake structure, (2) fast reconstitution, and (3) long-term chemical and physical antibody stability. In view of this, the excipient(s) should allow for preferential hydration of the native antibody structure in the frozen state, and hydrogen bonding to polar and charged groups upon dehydration. In addition, the formulation should provide an amorphous glassy matrix with a high glass transition temperature in the dried state to achieve long-term product stability at ambient conditions. Generally, disaccharides are superior to polyols and/or hydrophilic polymers, since they may function as cryo- and lyoprotectants. Their crystallization tendency is usually low, which is an additional advantage over mannitol, for instance. However, one group of compounds, namely reducing sugars should be avoided, since they have the propensity to degrade proteins via Maillard reaction between sugar carbonyls and protein free amino acid groups.

Numerous reports provide evidence that trehalose and sucrose are first choice for stabilizing antibodies and fragments thereof during freeze-drying and storage in the dried solid state (Andya et al. 2003). The mechanism of stabilization by these sugars has been proposed to occur by acting as water substitute and producing a glassy matrix that restricts mobility. Moreover, a diluent effect may not be excluded. Usually sucrose and trehalose provide equivalent protection against chemical and physical degradation of freeze-dried antibodies upon storage. Concentrations that are 3- to 4-fold below the iso-osmotic concentration and equivalent to a 360 : 1 molar ratio of sugar to antibody are generally sufficient to ensure product stability even at elevated temperature of 40 °C (Cleland et al. 2003).

10.5.3 Crystalline Suspensions

Antibody therapies generally require frequent delivery of between 100 mg and 1 g of protein per dose to achieve clinical efficacy. These doses are typically administered through large volume i.v. infusions in a hospital setting. Delivery of these large doses in a small volume appropriate for subcutaneous injection is likely to improve therapeutic opportunities and patient compliance of antibody treatments. However, highly concentrated solutions often result in very high viscosity, which may cause manufacturing problems, poor overall stability and delivery problems as indicated in Section 10.5.1. Crystallization of full-length antibodies or antibody fragments is a novel approach that holds great promise for the development of high-dose, low-viscosity antibody suspensions (Yang et al. 2003).

Recently, a large-scale batch crystallization process has been developed which allows full-length monoclonal antibodies to be crystallized efficiently in high yields (>90%) (Shenoy et al. 2002). Small crystals with excellent chemical and physical stability upon storage at room temperature including full retention of biological activity *in vivo* can be produced. Crystals and crystal formulations of different size, shape, and morphology, including spherical nanocrystalline com-

posite particles with different dissolution properties, may be achieved by manipulation of the crystallization protocol (Yakovlevsky et al. 2005). In addition, the process can streamline the production of pharmaceutical antibody formulations by replacing some of the purification steps.

Once crystallized, the antibodies can be formulated into high-concentration suspensions that are biocompatible when injected subcutaneously. *In vivo* release rates may be controlled by varying the size and morphology of the crystals and/or the vehicle composition, thus allowing fast and carrier-free slow release dosage forms to be developed. PEG/ethanol mixtures have proven to be appropriate nonaqueous vehicles for providing low-viscosity formulations that maintain both crystallinity and integrity of various monoclonal antibodies such as rituximab, infliximab, and trastuzumab. Suspensions containing 200 mg mL^{-1} of crystalline trastuzumab in PEG/ethanol did not show any aggregation upon storage over a period of 20 weeks at 4 °C. Viscosity remained low and allowed injection of 1 mL suspension with a 26-gauge needle in less than 5 s. Efficacy in a preclinical mouse model of human breast cancer was clearly demonstrated. Histological analysis of the injection sites revealed rapid dissolution of the crystals after subcutaneous (s.c.) injection and high biocompatibility.

Crystalline infliximab suspensions injected subcutaneously in rats revealed slow release rates with an extended serum pharmacokinetic profile (i.e. a longer biological half-life compared with s.c. or i.v. injections of the commercially available solution). Furthermore, a higher area under the curve (AUC), indicating a higher bioavailability, was observed.

These examples clearly indicate that crystalline antibodies and suspensions thereof are an improved and versatile formulation approach for high-dose antibody delivery by the subcutaneous route. In addition, antibody crystals and stabilized crystal formulations may be advantageously encapsulated in a polymeric carrier to produce controlled-release microparticles.

10.5.4
Carrier-based Systems

In addition to crystalline suspensions, carrier-based delivery systems have gained increasing interest to provide parenteral controlled-release of whole antibodies or antibody fragments. One approach is the use of microparticles based on biodegradable polymers such as poly-D, L-lactide-co-glycolide (PLGA). PLGA polymers are available in a range of molecular weights and monomer ratios, thus providing a number of variables with which the antibody release rate can be adjusted. PLGA is legally approved for parenteral use, since it is nontoxic, nonimmunogenic and well tolerated after subcutaneous injection. A variety of microencapsulation techniques are available to produce PLGA microparticles (Benoit et al. 1996). They may be classified as phase separation or coacervation, emulsion solvent evaporation, and spray drying. The polymer is generally dissolved in an organic solvent, and the antibody drug may be encapsulated in either solid form or solution state.

When producing microencapsulated formulations of whole antibodies or antibody fragments, it is important that the chemical, physical, and biological properties of the antibody remain intact during encapsulation (Bilati et al. 2005). Since conditions such as exposure to water/organic solvent interfaces and homogenization may compromise the structural integrity of dissolved antibody molecules, formulation approaches based on PEGylated and/or solid crystalline antibodies are of particular interest. The solubility profile of PEG, including a certain shielding effect when attached to antibodies, opens up new opportunities for emulsion-based encapsulation techniques, which typically lead to adsorption and/or aggregation problems when applied to non-PEGylated antibodies or fragments. The use of stable nanocrystalline antibodies or antibody formulations is another option that may allow successful encapsulation with enhanced preservation of the native antibody structure during the encapsulation process (Yakovslevsky et al. 2005). Typically, the release rate from microparticles can be varied from days to months by adjusting the microencapsulation parameters, the polymer properties, the antibody loading, the crystal size, shape, and morphology, and the formulation used to prepare the nanocrystals. The potential of this challenging technology for controlled-release antibody formulations has to be verified in the future.

10.6 Formulation and Manufacturing of Local Delivery Systems

10.6.1 Inhalation Powders

Long-term stability of monoclonal antibodies is usually enhanced when stored in a dry solid rather than a liquid state. Contrary to parenteral delivery systems, dry powder formulations for local pulmonary delivery of antibody drugs require not only drug stability issues, but also aerosol performance (i.e. aerodynamic properties) to be addressed. Aerodynamic properties defining the fraction of aerosolized drug to be delivered to the lung are strongly affected by particle size and morphology. Since freeze-drying procedures usually generate cakes rather than powders, the process is not the method of choice when particles with good dispersibility and defined aerodynamic particle size distributions are required, such as for pulmonary delivery. Several drying techniques have been explored for their ability to produce fine inhalation powders. So far, spray drying is the most popular method, but supercritical fluid and spray freeze drying technologies have recently emerged as promising alternatives.

10.6.1.1 Spray Drying

Spray drying is a manufacturing process that typically yields powder particles small enough for aerosol delivery to the lower airways of the lung (1–7 μm). However, processes involved in spray drying impose potential stress to antibody

drugs, including exposure to high temperature, distribution at air–water interfaces, and dehydration. In the absence of sugar or polyol stabilizers, any of these stress parameters may induce structural perturbations to antibody molecules, thus compromising their biological activity or immunogenicity. In fact, a significant decrease in β-sheet content and a corresponding increase in turn and unordered content upon spray drying was monitored with several antibodies using FTIR or Raman spectroscopy (Sane et al. 2004). A correlation between the extent of structural perturbations immediately after spray drying and the rate of aggregation upon long-term storage of the formulation was reported. This clearly indicates that preservation of the native mAb structure during spray drying is essential for long-term product stability, as was already pointed out for freeze-dried products.

To protect full-length mAbs and antibody fragments during spray drying, different excipients such as polyols, sugars, hydrophilic polymers, surfactants, amino acids, and/or proteins are generally included in the final formulation. The stabilizing mechanism of surfactants is thought to be a competitive adsorption at the air–water interface, thereby retaining the native antibody structure. The effect of polyols and sugars may be explained by preferential hydration and/or water substitution by hydrogen bonding to the antibody molecules. The stabilizing effects are concentration dependent (i.e. a certain excipient to mAb mass ratio is required to preserve the native structure). However, any excessive excipient can lead to a decrease in the physical stability of the formulation, which in turn may affect aerosol performance. This has been demonstrated for spray-dried rhuMAbE25 stabilized with mannitol, trehalose, or lactose, respectively (Andya et al. 1999). Trehalose was found to increase powder cohesiveness in a concentration-dependent manner. Lactose exhibited acceptable powder performance, but protein glycation was observed during storage. Mannitol at a molar ratio of higher than 200:1 resulted in crystallization, thus compromising aerosol performance. In conclusion, a balance must be achieved between addition of enough stabilizer to improve protein stability without compromising aerosol performance.

10.6.1.2 **Spray Freeze-drying**

Spray freeze-drying, as opposed to spray drying, produces particles with light and porous characteristics, which offer more favorable aerodynamic properties and thus a better aerosol performance (Maa et al. 1999). The process is highly efficient in terms of product recovery (>95%). However, selection of appropriate formulation excipients is crucial for product quality, since crystallization can deteriorate aerodynamic powder properties. Application of supercritical fluid (SCF) technologies is especially attractive for reasons of mild process conditions, cost-effectiveness, feasibility of scaling up, possible sterilizing properties of supercritical carbon dioxide, and capability of producing powder particles with defined physicochemical properties. Several concepts have been described in the literature using a SCF (usually carbon dioxide) either as antisolvent or propellant during a low-temperature spray-drying process. Since limited data are available on

antibody stability during SCF processing, further research is required to identify stress parameters, and provide rationales for product development (Jovanovich et al. 2004).

10.6.2 Various Dosage Forms

As of today, limited data are available on the stability of antibodies or fragments in combination with oral and peroral delivery systems. Toothpaste, mouthwash, or chewing gum might be attractive formulations for local administration of antibodies in the mouth. To protect the antibody from pH- and/or enzyme-induced degradation in the upper gastrointestinal tract, controlled-release formulations based on gastroresistant polymers are a viable approach (Kälkert and Reich 2004). Delivery to the small intestine can be achieved using enteric-coating polymers such as Eudragit L or S that are insoluble at low pH, but soluble in the neutral environment of the small intestine. Direct compression of stable crystalline antibody powders with subsequent tablet coating, pellet formation, or microencapsulation based on stabilized antibody formulations are options to date. Colon targeting represents a greater technological challenge, as these dosage forms must pass through the whole upper gastrointestinal tract before delivering the antibody to the colon. Four different delivery principles based on pH-dependent dissolution, time-dependent erosion or dissolution, pressure-induced disintegration, or enzymatic degradation are available (Bauer 2001). Their clinical reliability for local antibody delivery to the colon has to be demonstrated.

10.7 Outlook

Site-specific drug delivery has been an ultimate goal of pharmaceutical product design. Antibodies and antibody fragments have the potential to realize this objective in various directions. To fully exploit their targeted effector and vector function, multidisciplinary collaborative efforts are key elements of future product development. Pharmaceutical research must focus on innovative formulation concepts for parenteral and local controlled delivery of tailor-made antibodies and antibody fragments. Major technological challenges are the design of high-dose, low-viscosity sustained-release systems, and antibody fragments with improved *in vitro* and *in vivo* stability. Moreover, optimization of biophysical properties of stealth immunoliposomes and antibody/drug conjugates are of major importance for further progress in tumor and brain targeting including gene delivery.

References

Andya, J.D., Maa, Y.-F., Costantino, H.R., Nguyen, P.-A., Dasovich, N., Sweeney, T.D., Hsu, C.C., Shire, S.J. (1999) The effect of formulation excipients on protein stability and aerosol performance of spray-dried powders of a recombinant humanized anti-IgE monoclonal antibody. *Pharm Res* 16: 350–358.

Andya, J.D., Hsu, C.C., Shire, S.J. (2003) Mechanisms of aggregate formation and carbohydrate excipient stabilization of lyophilized humanized monoclonal antibody formulations. *AAPS PharmSci* 5(2) Article 10.

Bauer, K.H. (2001) Colonic drug delivery: review of material trends. *Am Pharm Rev* 4: 8–16.

Benoit, J.P., Marchais, H., Rolland, H., Velde, V.V. (1996) Biodegradable microspheres: advances in production technology. In: S. Benita (ed.) *Microencapsulation: Methods and Industrial Applications*. New York: Marcel Dekker, pp. 35–72.

Bilati, U., Allemann, E., Doelker, E. (2005) Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles. *Eur J Pharm Biopharm* 59: 372–388.

Brannon-Peppas, L., Blanchette, J.O. (2004) Nanoparticle and targeted systems for cancer therapy. *Adv Drug Deliv Rev* 56: 1649–1659.

Calicati, P., Veronese, F.M. (2003) Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. *Adv Drug Deliv Rev* 55: 1261–1277.

Cao, Y., Lam, L (2004) Bispecific antibody conjugates in therapeutics. *Adv Drug Deliv Rev* 55: 171–197.

Carpenter, J.F., Pikal, M.J., Chang, B.S., Randolph, T.W. (1997) Rational design of stable lyophilized protein formulations: some practical advice. *Pharm Res* 14: 969–975.

Chang, L., Shephard, D., Sun, J., Quellette, D., Grant, K.L., Tang, X., Pikal, M.J. (2005a) Mechanism of protein stabilization by sugars during freeze-drying and storage: native structure preservation, specific interaction, and/or immobilization in a glassy matrix. *J Pharm Sci* 94: 1427–1444.

Chang, L., Shephard, D., Sun, J., Xiahu, T., Pikal, M.J. (2005b) Effect of sorbitol and residual moisture on the stability of lyophilized antibodies: implications for the mechanism of protein stabilization in the solid state. *J Pharm Sci* 94: 1445–1455.

Chapman, A.P. (2002) PEGylated antibodies and antibody fragments for improved therapy: a review. *Adv Drug Deliv Rev* 54: 531–545.

Chen, B., Bautista, R., Yu, K., Zapata, G.A., Mulkerrin, M.G., Chamow, S.M. (2003) Influence of histidine on the stability of physical properties of a fully human antibody in aqueous and solid forms. *Pharm Res* 20: 1952–1960.

Chi, E.Y., Krishnan, S., Randolph, T.W., Carpenter J.F. (2003) Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnativeprotein aggregation. *Pharm Res* 20: 1325–1336.

Cleland, J., Lam, X., Kendrik, B., Yang, J., Yang, T.-H., Overcashier, D., Brooks, D., Hsu, C., Carpenter, J.F. (2001) A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. *J Pharm Sci* 90: 310–321.

Greenwald, R.B., Choe, Y.H., McGuire, J., Conover C.D. (2004) Effective drug delivery by PEGylated drug conjugates. *Adv Drug Deliv Rev* 55: 217–250.

Gupta, S., Kaisheva, E. (2003) Development of a multidose formulation for a humanized monoclonal antibody using experimental design techniques. *AAPS PharmSci* 5(2) Article 8.

ICH Guideline Q1A (1993) *Stability Testing Guidelines: Stability Testing of New Drug Substances and Products, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use*. http://www.ich.org/cache/compo/363-272-1.html

ICH Guideline Q5C (1995) *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products, International Conference on Harmonization*

of Technical Requirements for Registration of Pharmaceuticals for Human Use. http://www.ich.org/cache/compo/363-272-1.html

Jovanovich, N., Bouchard, A., Hofland, G.W., Witkamp, G.-J., Crommelin, D.J.A., Jiskoot, W. (2004) Stabilization of protein dry powder formulations using supercritical fluid technology. *Pharm Res* 21: 1955–1969.

Kälkert, K., Reich, G. (2004) Spray dried gelatine/Eudragit microparticles as a gastroresistant release system for proteins. *Proc Int Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology*, 15–18 March, Nuremberg, pp. 269–270.

Kupper, M.B., Huhn, M., Spiegel, H., Ma, J. K.C., Barth, S., Fischer, R., Finnern, R. (2005) Generation of human antibody fragments against *Streptococcus mutans* using a phage display chain shuffling approach. *BMC Biotechnol* 5: 4–12.

Lam, X.M., Yang, J.Y., Cleland, J.L. (1997) Antioxidants for prevention of methionine oxidation in recombinant monoclonal antibody HER2. *J Pharm Sci* 86: 1250–1255.

Liu, J., Nguyen, M.D.H., Anya, J.D., Shire, S. J. (2005) Reversible self-association increases the viscosity of a concentrated monoclonal antibody in aqueous solution. *J Pharm Sci* 94: 1928–1940.

Maa, Y.-F., Nguyen, P.-A., Sweeney, T.D., Shire, S.J., Hsu, C.C. (1999) Protein inhalation powders: spray drying vs spray freeze drying. *Pharm Res* 16: 249–254.

McNeel, D.G., Eickhoff, J., Lee, F.T., King, D.M., Alberti, D., Thomas, J.P., Friedl, A., Kolesar, J., Marnocha, R., Volkman, J., Zhang, J., Hammershaimb, L., Zwiebel, J.A., Wildung, G. (2005) Phase I trial of a monoclonal antibody specific for αvβ3 integrin (MEDI-522) in patients with advanced malignancies, including an assessment of effect on tumor perfusion. *Clin Cancer Res* 11: 7851–7860.

Minton, A.P. (2005) Influence of macromolecular crowding upon the stability and state of association of proteins: predictions and observations. *J Pharm Sci* 94: 1668–1675.

Park, J.W., Hong, K., Kirpotin, D.B., Colbern, G., Shalaby, R., Baselga, J., Shao, Y., Nielson U.B., Marks J.D., Moore, D., Papahadjopoulos, D., Benz, C.C. (2002) Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery. *Clin Cancer Res* 8: 1172–1181.

Pavlon, A.K., Belsey M.J. (2005) The therapeutic antibodies market to 2008. *Eur J Pharm Biopharm* 59: 389–396.

Payne, G. (2003) Progress in immunoconjugate cancer therapeutics. *Cancer Cell* 3: 207–217.

Reithmeier, H., Winter, G. (2002) Development of an experimental setup for accelerated preformulation studies of liquid protein dosage forms. *Proc 4th World Meeting ADRITELF/APGI/APV*, Florence, 8/11 April, pp. 829–830.

Ross, J.S., Gray, K., Gray, G.S., Worland, P.J., Rolfe, M. (2003) Anticancer antibodies. *Am J Clin Pathol* 119: 472–485.

Sane, S.U., Wong, R., Hsu, C.C. (2004) Raman spectroscopic characterization of drying-induced structural changes in a therapeutic antibody: correlating structural changes with long term stability. *J Pharm Sci* 93: 1005–1018.

Sacre, S.M., Anreakos, E., Taylor, P., Feldmann, M., Foxwell, B.M. (2005) Summary of rheumatoid arthritis therapeutics in development. *Expert Rev Mol Med* 7: Issue 16.

Shenoy, B., Govardhan, C.P., Yang, M., Margolin, A.L. (2002) Crystals of whole antibodies and fragments thereof and methods for making and using them. International Patent WO 02/072636A2.

Schnyder, A., Huwyler, J. (2005) Drug transport to brain with targeted liposomes. *NeuroRx* 2: 99–107.

Wang, W. (1999) Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int J Pharm* 185: 129–188.

Webb, S.D., Cleland, J.L., Carpenter, J.F., Randolph, T.W. (2002) A new mechanism for decreasing aggregation of recombinant human interferon-γ by a surfactant: slowed dissolution of lyophilized formulations in a solution containing 0.03% polysorbate 20. *J Pharm Sci* 91: 543–558.

Weir, A.N.C., Nesbitt, A., Chapman, A.P., Popplewell, A.G., Antoniw, P., Lawson, A. D.G. (2001) Formatting antibody fragments to mediate specific therapeutic functions. *Biochem Soc Trans* 30: 512–516.

Wu, A.M., Senter, P.D. (2005) Arming antibodies: prospects and challenges for immunoconjugates. *Nat Biotechnol* 23: 1137–1146.

Yakovlevsky, K., Chamachkine, M., Khalaf, N., Govardhan, C.P., Jung, C.W. (2005) Spherical protein particels and methods of making and using them. European Patent EP 14925554.

Yang, M.X., Shenoy, B., Disttler, M. Patel, R., McGrath, M., Pechenov, S., Margolin, A.L. (2003) Crystalline monoclonal antibodies for subcutaneous delivery. *Appl Biol Sci* 100: 6934–6939.

11
Immunogenicity of Antibody Therapeutics

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11.1
Introduction

Since the first description of hybridoma technology in the 1970s (Kohler and Milstein 1975), a great deal of attention has been devoted to the development of monoclonal antibodies (mAbs) as therapeutic agents. However, it took 11 years before the first monoclonal antibody, OKT3, was approved for prevention of allograft rejection and another 7 years before the marketing authorization of Reopro to assist percutaneous coronary surgery was approved. There are many reasons for this slow development (Merluzzi et al. 2000), such as the difficulties of large-scale production, the high immunogenicity of the first generation of murine-derived monoclonal antibodies (Kuus-Reichel et al. 1994) and their lack of effector functions in humans, and the disappointing results in cancer trials, mainly caused by their bad penetration in cancer tissue (Reff and Heard 2001).

Several technical advances have been made over the years in both the development and the production of monoclonal antobodies. Recombinant DNA technology has made it possible to exchange the murine constant parts of the immunoglobulin chains with the human counterparts (chimeric mAbs) and later to graft murine complementarity determining regions (CDRs), which determine specificity, into a human immunoglobulin backbone, creating humanized mAbs. Today, transgenic animals, phage display technologies (Bradbury 1999) and other developments allow the production of completely human mAbs (Kellerman and Green 2002).

These technological advances have led to the introduction of an increasing number of therapeutics mAbs, some of which have provided major breakthroughs in the treatment of serious chronic diseases such as rheumatoid arthritis (Taylor 2003). Table 11.1 lists the monoclonals that have been allowed marketing authorization in the US and/or Europe to date. At least 400 others are in the pipeline and some of these will certainly reach the market in the near future.

However, the expectation that human mAbs would be devoid of immunogenicity proved to be naive. The scientists involved in the generation of completely

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Table 11.1 Monoclonal antibodies registered in the EU and/or the US.

Trade name	*Generic name*	*Type of mAb*	*Ig type*	*% antibodies*[a]
Humira	adalimumab	Human	IgG1	12
Remicade	infliximab	Chimeric	IgG1	24
Reopro	abciximab	Chimeric	Fab	6
Herceptin	trastuzumab	Humanized	IgG1	1
Mabthera	rituximab	Chimeric	IgG1	1
Xolair	omalizumab	Humanized	IgG1	0
Simulect	basiliximab	Chimeric	IgG1	0
Synagis	palivizumab	Humanized	IgG1	1
Campath	alemtuzumab	Humanized	IgG1	2
Zenapax	daclizumab	Humanized	IgG1	9

a Frequency of antibody induction based on package inserts.

human antibodies generated from transgenic mice claimed: "Fully human mAbs are anticipated to be nonimmunogenic and thus to allow repeated administration without human anti-human antibody response" (Yang et al. 2001), but although humanization has reduced the immunogenicity of mAbs, completely human mAbs have been shown to induce antibodies (Table 11.1) as has been predicted (Clark 2000). As we know from other biopharmaceuticals, proteins that are considered completely identical to an endogenous protein may induce antibodies, sometimes in the majority of patients. The study of the immunogenicity of these therapeutic proteins has shown the dependence on many other factors, besides structural factors, such as the degree of nonself (Schellekens 2002). Importantly, because the antibodies induced by mAbs may interfere with efficacy and may enhance immune-mediated side effects, the issue of immunogenicity should be considered for every new therapeutic mAb.

In this chapter we will discuss the methods available to assess the immunogenicity of mAbs, consider the immunological mechanisms responsible for the induction of antibodies, and the factors that influence these mechanisms. We will discuss the biological and clinical consequences of immunogenicity and the methods to predict and prevent it.

11.2
Assays for Antibodies Induced by Monoclonal Antibodies

When comparing the immunogenicity reported for different mAbs or even different trials with the same mAb, it is important to realize that the assays for measuring antibodies have not been standardized. Standard ELISA type immunoassays are not appropriate for measuring these antibodies because of the high level of crossreactivity between the therapeutic mAb and the antibodies it may

induce. The bridging assay has been advocated as the best assay (Buist et al. 1995; Pendley et al. 2003). In this assay the mAb is used to capture the antibodies present in the patient sera and the captured antibodies are detected by adding the labeled mAb as a probe (Thurmond et al. 1998). Such a bridging assay is independent of the type of antibodies to be detected. This enables the use of antisera induced in animals as positive control, although these sera will mainly contain antibodies directed to the constant part of the monoclonal antibodies, whereas human patients will mainly generate antibodies to the variable regions.

The bridging immune assay may miss a low-affinity IgM type of immune response because of the washing steps involved. Therefore, for the early immune response the use of surface plasmon resonance technology such as Biacore is advocated rather than the ELISA type of assay methodology (Ritter et al. 2001). On the other hand, the detection limit of ELISAs for high-affinity antibodies is generally lower than that for Biacore assays. In summary, the methods are complementary and should be used in parallel.

Both the bridging assay and the surface plasmon resonance technology determine the presence of binding antibodies and can be used as screening assays. In addition, it may be important to assay for the presence of neutralizing antibodies. These antibodies may interfere with the biological and clinical activity of the mAbs. Assays for neutralizing activity are based on the inhibition of a biological effect of the mAb *in vitro*. Because every mAb has its own specific biological effect, assays for neutralizing activity need to be designed for every individual mAb and are especially difficult to standardize because the basis is a bioassay.

Another aspect that needs to be considered in designing assays, sampling timing, and interpreting data is the relative long half-life (several weeks) of therapeutic mAbs, which may interfere with the detection of induced antibodies and therefore may lead to false negative results. Sampling sera up to 20 weeks after the patient has received the last injection avoids the interference of circulating mAbs. The presence of natural antibodies, receptors, and immune complexes may also interfere with assays and lead to either false positive or false negative results.

11.3 Mechanisms of Antibody Induction

As with other therapeutic proteins, there are two main mechanisms by which antibodies against mAbs are induced. If the mAbs are of foreign origin, like the first-generation mAbs derived from murine cells, the antibody response is comparable to a vaccination reaction. Often a single injection is sufficient to induce high levels of neutralizing antibodies which may persist for a considerable length of time. The other mechanism is based on breaking B-cell tolerance, which normally exists to self antigens, such as human immunoglobulins. To break B-cell tolerance prolonged exposure to proteins is necessary. In general it takes months

before patients produce antibodies which are mainly binding and disappear when treatment is stopped.

It is likely that in the case of mAbs the induction of antibodies occurs by both mechanisms. B-cell tolerance may explain why patients do not make antibodies to constant regions of mAbs of human origin. It is, however, unlikely that tolerance exists for the full repertoire of variable regions an individual may produce.

To induce a classical immune reaction, a degree of non-self is necessary. The trigger for this type of immunogenicity is the difference between the human and murine immunoglobulin structure. The triggers for breaking tolerance are essentially different. The production of autoantibodies may occur when the self antigens are exposed to the immune system in combination with a T-cell stimulus or danger signal such as bacterial endotoxins, microbial DNA rich in CpG motifs, or denatured proteins (Goodnow 2001). This mechanism explains the immunogenicity of biopharmaceuticals containing impurities. When tolerance is broken via this mechanism, the response is often weak with low levels of low-affinity antibodies.

To induce high levels of IgG, the self antigens should be presented to the immune system in a regular array form with a spacing of 50–100 Å, a supramolecular structure resembling a viral capsid (Chakerian et al. 2002). Apparently the immune system has evolved to react vigorously to these types of structures, which normally are only found on viruses and bacteria. The most important factor in the immunogenicity of biopharmaceuticals is the presence of aggregates. Aggregates present the self antigens in a repeating form, which is such a potent inducer of autoantibodies.

11.4 Factors Influencing the Immunogenicity

Many factors influence the immunogenicity of mAbs (Table 11.2). The degree of non-self has been considered the main factor contributing to the immunogenicity of mAbs. Indeed, the exchange of the murine constant regions with human counterparts has resulted in a substantial reduction the induction of antibodies (Table 11.3). It is less clear whether further humanization has resulted in an

Table 11.2 Factors influencing the immunogenicity of monoclonal antibodies.

Product-related	*Other*
Presence of foreign sequences	Dose schedule
Complete/incomplete antibody	Patient characteristics
Specificity of the mAb	Concomitant treatment
Formation of immune complexes	
Fc functions of the mAb	
Purity and formulation	

Table 11.3 Immunogenicity of monoclonal antibodies related to murine sequences.[a]

Antibody response	*Marked*	*Tolerable*	*Negligible*	n
Murine mAbs	84%	7%	9%	44
Chimeric mAbs	40%	27%	33%	15
Humanized mAbs	9%	36%	55%	22

a Marked, >15% of patients; tolerable 2–15% of patients; negligible. <2% of patients. Data from Hwang and Foote (2005).

additional decrease in immunogenicity (Hwang and Foote 2005). The DNA sequence homology between the V regions of different species is higher than between the C regions. This explains why the V regions of chimeric mAbs sometimes show a higher homology with the V regions in the human germline than those of humanized mAbs (Clark 2000). Fully human mAbs have also been reported to induce antibodies, pointing to other factors that are responsible for antibody induction.

Although more injections and higher doses are associated with a higher immune response, this is not necessarily true for all mAbs. Rechallenge with mAbs is associated with a higher antibody response than the first treatment. However, in some cases chronic treatment and higher doses are less immunogenic than episodic treatment and lower dose (Hanauer 2003). The induction of tolerance by continuous treatment and higher doses has been used to explain the reduced induction of antibodies. These data should, however, be interpreted with caution because under these treatment conditions the level of circulating mAbs is higher and more persistent; the presence of circulating mAbs during the time of blood sampling may mask the detection of induced antibodies. The few studies that have compared subcutaneous and intravenous routes of administration of mAbs showed little difference in immunogenicity (Livingston et al. 1995).

Although it has been suggested that smaller proteins are less likely to be immunogenic, $F(ab')_2$ fragments of murine antibodies have been reported to be at least as immunogenic as complete antibodies. The use of Fab fragments, however, was associated with a substantial reduction of the induction of antibodies.

The immune status of the patients influences the antibody response. Cancer patients and transplantation patients are important categories receiving mAb therapy. These patients are usually immunocompromised by the disease or by immunosuppressive treatments. It has been shown with other therapeutic proteins that immunocompromised patients are less likely to produce antibodies than patients with a normal immune status. Sometimes immunosuppressive drugs such as methotrexate are given to patients on mAb therapy with the purpose of inhibiting an antibody response. Other immunosuppressive agents that have been reported to block the antibody response are cyclosporin and 15-deoxyspergualin. However, the use of these agents sometimes leads to severe toxicity.

The type of ligand also influences the immunogenicity of mAbs. In general, cell-bound antigens as target lead to a higher level of antibody induction than soluble targets. An exception to this rule is mAbs directed to antigens on immune cells with the purpose of inducing immunosuppression; these mAbs also suppress an antibody response.

In contrast with many other biopharmaceuticals, mAbs by definition have T-cell activation properties and may themselves act as the second signal to initiate an immune raction or break immune tolerance. Fc functions such as macrophage activation and complement activation may boost the antibody response. Removal of *N*-linked glycosylation at the Fc part of the immunoglobulin may reduce Fc function and thereby lead to a diminished immunogenicity.

It is known from the use of therapeutic proteins other than mAbs that purity, stability, and formulations are the most important risk factors for inducing antibodies. The presence of aggregates, denatured proteins, and other impurities has been identified as the main factor influencing immunogenicity. However, in the case of mAbs these factors have hardly been investigated as most attention has been directed to the degree of non-self. But, with the advent of fully human mAbs, properties such as purity and stability will increase in importance.

11.5 Consequences of the Immunogenicity of Monoclonal Antibodies

In patients with anti-mAb antibodies an increased incidence of immune complex syndrome, allergic reactions, and infusion reactions has been reported (Baert et al. 2003). There also seems to be a dependency of the type of antibody reaction on these side effects. Patients with a slow but steadily increasing antibody titer are reported to show more infusion-like reactions than patients with a short temporary response (Ritter et al. 2001).

Monoclonal antibodies are mainly present in the circulation and therefore their pharmacokinetic behavior is highly sensitive to the presence of induced antibodies. These antibodies may either increase or diminish their half-life, depending on the affinity of the antibodies and properties of the mAbs.

The presence of antibodies raised by the mAbs may decrease their efficacy either by decreasing their half-life or by neutralizing their antigen-binding capacity (Baert et al. 2003). The more specific the immune response, the lesser the chance that the antibodies interfere with the efficacy of a new mAb treatment because of the lack of crossreactivity.

There are some suggestions that an immune reaction to mAbs may in certain conditions increase their efficacy (Koprowski et al. 1984; Wagner et al. 1990). A correlation has been reported between the level of the immune response to mAbs and the prognosis of the patient. This may be explained by an anti-idiotypic response increasing the immunogenic response to tumor antigens. Alternatively, the level of antibody response may only be a reflection of the general immune status of the patients.

11.6 Prediction of the Anti-mAb Response

As shown by comparing murine and chimeric/humanized mAbs, the level of non-self is a predictor of an immune response. This is the classical response to a foreign protein. However, completely human mAbs still are capable of eliciting antibodies based on breaking immune tolerance. It is likely that the quality of the preparation and its formulation are important factors, although this has not been studied in detail. Aggregated and solubilized immunoglobulins have been used in the past to break or induce immune tolerance, respectively, so aggregation is likely to be a major factor in the induction of antibodies. The role of protein modification (e.g. oxidation, deamidation, etc.) needs further investigation, as does the optimal formulation to avoid immunogenicity.

The monoclonal system may provide a unique opportunity to study the factors influencing immunogenicity because of the availability of a complete range of mAbs ranging from completely murine, murine/human hybrid to completely human. In addition, transgenic animals with the nearly complete immunoglobulin repertoire, which were developed for the production of human mAbs, are available. These animals also have an immune tolerance to mAbs that is comparable to the immune tolerance in patients. Comparing the immune response of a human mAb in these transgenic mice with the immune response in the non-transgenic strain will offer the possibility of discriminating between the factors important for the classical immune response and those which contribute to the breaking of immune tolerance.

Immune tolerant transgenic mice may also provide an important model for the study of the mechanisms responsible for the induction of antibodies and possible prevention and treatment by immune suppressive drugs.

Monkeys also have the potential to predict the immune response in patients as their response is mainly anti-idiotypic. However, other important factors may be missing in monkeys, such as disease state and concomitant therapy. Indeed, in one of the few studies in which the responses in monkeys and patients were compared there were major differences in incidence and type of response (Stephens et al. 1995).

In theory, *in vitro* T-cell stimulation tests and computational models are also available to predict immunogenicity. However T-cell proliferation assays have the drawback that all antibodies are capable of inducing some level of T-cell activation. The computational alogarithms which predict binding of antigens to HLA class II only give limited information on the interaction of the mAbs with the immune system, and also underdetected epitopes.

Competition antibody assays have also been used to predict immunogenicity of mAbs. Sera of patients who were positive for antibodies to murine mAbs were tested in a surface plasmon resonance-based competition assay to variants of humanized antibodies (Gonzales et al. 2002). Lesser reactivity of the sera was interpreted as a sign of reduced immunogenicity of these variants. These assays, however, show the antigenicity of these variants, which is not necessarily predic-

tive of their immunogenicity (El Kasmi et al. 2000). Although these assays help to define the immunogenic sites, they may miss new epitopes that may be present in the variants and they also ignore other factors important for immunogenicity such as aggregates and impurities. The same is true for studies in which the affinity of the grafted CDR was compared with its capacity to bind to patient sera in a competition radioimmunoassay to define the construct with the best clinical potential (Iwahashi et al. 1999).

11.7 Reduction of Immunogenicity of Monoclonal Antibodies

The immunogenicity of mAbs is associated with side effects and loss of efficacy and should be avoided. Various methods can be used to reduce the induction of antibodies, including:

- Replacing rodent sequences by human sequences
- Immunosuppressive treatment
- Altering Fc functions
- Reducing the size
- Attachment of polymers
- Improving product quality
- Optimizing formulation
- Inducing tolerance.

The main approach to reducing the immunogenicity of mAbs has been replacing the murine parts of the molecules by human-derived sequences as discussed earlier. The immunogenic response may be reduced by immunosuppressive treatment (Baert et al. 2003). This immunosuppressive effect seems to be dependent on treatment schedule (Hanauer 2003).

As with other therapeutic proteins, covalently linking polymers such as polyethylene glycol and low molecular weight dextran to mAbs reduces their immunogenicity (Fagnani et al. 1995; Trakas and Tzartos 2001; Chapman 2002). However, these modifications in general make the molecules less active, necessitating higher doses. This and the increased half-life of the proteins increases their exposure to the immune system, which may enhance the immunogenic potential.

Tolerance to mAbs has been induced by using soluble forms (Isaacs 2001). Tolerance induction to mAbs reacting with cell-associated targets may be restricted to the isotypic parts of the immunoglobulin. To achieve tolerance to the idiotypic parts of mAbs reacting with cell-bound protein, variants may be used which lack the affinity for cells. Tolerance may also be induced by pretreating with PEGylated mAbs.

Factors such as the presence of contaminants, impurities, and the effect of formulation, which have been shown to be important factors for the immunogenicity of other therapeutic proteins, have hardly been studied for mAbs. It can,

however, be assumed that these factors are also important for the induction of antibodies by mAbs. Improving the quality of mAb products and optimization of production and formulation may reduce the immunogenicity further.

11.8 Conclusion

As with other therapeutic drugs, it is safe to assume that all mAbs will induce an immune response, although the incidence may differ widely between individual mAb products. An immune response to mAbs is associated with an increase in toxicity and a decrease of efficacy. Although the reduction of non-human sequences has reduced the induction of antibodies, complete human mAbs are still immunogenic. The immunological mechanisms which lead to an antibody response to are not completely understood. More research is required to prevent immunogenicity completely.

References

Baert, F., Noman, M., Vermeire, S., van Assche, G., D'Haens, G., Carbonez, A., Rutgeerts, P. (2003) Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *N Engl J Med* 348: 601–608.

Bradbury, A. (1999) Display technologies expand their horizons. *Trends Biotechnol* 17: 137–138.

Buist, M.R., Kenemans, P., van Kamp, G.J., Haisma, H.J. (1995) Minor human antibody response to a mouse and chimeric monoclonal antibody after a single i.v. infusion in ovarian carcinoma patients: a comparison of five assays. *Cancer Immunol Immunother* 40: 24–30.

Chakerian, B., Lenz, P., Lowy, D.R., Schiller, J.T. (2002) Determinants of autoantibody induction by conjugated papillomavirus virus-like particles. *J Immunol* 169: 6120–6126.

Chapman, A.P. (2002) PEGylated antibodies and antibody fragments for improved therapy: a review. *Adv Drug Deliv Rev* 54: 531–545.

Clark, M. (2000) Antibody humanization: a case of the 'Emperor's new clothes'? *Immunol Today* 21: 397–402.

El Kasmi, K.C., Deroo, S., Theisen, D.M., Brons, N.H.C., Muller, C.P. (2000) Crossreactivity of mimotopes and peptide homologues of a sequential epitope with a monoclonal antibody does not predict cross reactive immunogenicity. *Vaccine* 18: 284–290.

Fagnani, R., Halpern, S., Hagan, M. (1995) Altered pharmacokinetic and tumour localization properties of Fab' fragments of a murine monoclonal anti-CEA antibody by covalent modification with low molecular weight dextran. *Nucl Med Commun* 16: 362–369.

Gonzales, N.R., Schuck, P., Schlom, J., Kashmiri, S.V.S. (2002) Surface plasmon resonance-based competition assay to assess the sera reactivity of variants of humanized antibodies. *J Immunol Methods* 268: 197–210.

Goodnow, C.C. (2001) Pathways for self-tolerance and the treatment of autoimmune diseases. *Lancet* 357: 2115–2121.

Hanauer, S.B. (2003) Immunogenicity of infliximab in Crohn's disease. *N Engl J Med* 348: 2155–2156.

Hwang, W.Y.K., Foote, J. (2005) Immunogenicity of engineered antibodies. *Methods* 36: 3–10.

Isaacs, J.D. (2001) From bench to bedside: discovering rules for antibody design, and

improving serotherapy with monoclonal antibodies. *Rheumatology* 40: 724–738.

Iwahashi, M., Milenic, D.E., Padlan, E.A., Bei, R., Schlom, J., Kashmiri, S.V.S. (1999) CDR substitutions of a humanized monoclonal antibody (CC49): contributions of individual CDRs to antigen binding and immunogenicity. *Mol Immunol* 36: 1079–1091.

Kellerman, S.A., Green, L.L. (2002) Antibody discovery: the use of transgenic mice to generate human monoclonal antibodies for therapeutics. *Curr Opin Biotechnol* 13: 593–597.

Kohler, G., Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.

Koprowski, H., Herlyn, D., Lubeck, M., DeFreitas, M., Sears, H.F. (1984) Human anti-idiotype antibodies in ancer patients: is the modulation of the immune response beneficial for the patient. *Proc Natl Acad Sci USA* 81: 216–219.

Kuus-Reichel, K., Grauer, L.S., Karavodin, L.M., Knott, C., Krusemeier, M., Kay, N.E. (1994) Will immunogenicity limit the use, efficacy and future development of therapeutic monoclonal antibodies? *Clin Diagn Lab Immunol* 1: 365–372.

Livingston, P.O., Adluri, S., Zhang, S., Chapman, P., Raychaudhuri, S., Merritt, J. A. (1995) Impact of immunological adjuvants and administration route on HAMA respones after immunication with murine monoclonal antibody MELIMMUNE-1 in melanoma patients. *Vac Res* 4: 87–94.

Merluzzi, S., Figini, M., Colombatti, A., Canevari, S., Pucillo, C. (2000) Humanized antibodies as potential drugs for therapeutic use. *Adv Clin Pathol* 4: 77–85.

Pendley, C., Schantz, A., Wagner, C. (2003) Immunogenicity of therpeutic monoclonal antibodies. *Curr Opin Mol Ther* 5: 172–179.

Reff, M.E., Heard, C. (2001) A review of modifications to recombinant antibodies: attempt to increase efficacy in oncology applications. *Oncol/Hematol* 40: 25–35.

Ritter, G., Cohen, L.S., Williams, C., Richards, E.C., Old, L.J., Welt, S. (2001) Serological analysis of human anti-human antibody responses in colon cancer patients treated with repeated doses of humanized monoclonal antibody A33. *Cancer Res* 61: 6851–6859.

Schellekens, H. (2002) Immunogenicity of therapeutic proteins: clinical implications and future prospects. *Clin Ther* 24: 1720–1740.

Stephens, S., Emtage, S., Vetterlein, O., Chaplin, L., Bebbington, C., Nesbitt, A., Sopwith, M., Athwall, D., Noval, C., Bodmer, M. (1995) Comprehensive pharmacokinetics of a humanized antibody and analysis of residual anti-idiotypic responses. *Immunology* 85: 668–674.

Taylor, P.C. (2003) Antibody therapy for rheumatoid arthritis. *Curr Opin Pharmacol* 3: 323–328.

Thurmond, L.M., Reese, M.J., Donaldson, R.J., Orban, B.S. (1998) A kinetic enzyme immunoassay for the quantitation of antibodies to a humanized monoclonal antibody in human serum. *J Pharm Biomed Anal* 16: 1317–1328.

Trakas, N., Tzartos, S.J. (2001) Conjugation of acetylcholine receptor-protecting Fab fragments with polyethylene glycol results in a prolonged half-life in the circulation and reduced immunogenicity. *J Neuroimmunol* 120: 42–49.

Wagner, V., Reinsberg, J., Oehr, P., Briele, B., Schmidt, S., Werner, A., Krebs, D., Biersack, H.J. (1990) Clinical course of patients with ovarian arcinoma after induction of anti-idiotypic antibodies against tumor-associated antigen. *Tumor Diagn Ther* 11: 1–4.

Yang, X., Jia, X., Corvalan, J.R.F., Wang, P., Davis, C.G. (2001) Development of ABX-EGF, a fully human anti-EGF receptor monoclonal antibody, for cancer therapy. *Crit Rev Oncol/Hematol* 38: 17–23.

12
Regulatory Considerations

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12.1
Introduction

In the 30 years since the publication of George Kohler and Cesar Milstein's paper describing hybridoma technology (Kohler and Milstein 1975), therapeutic monoclonal antibodies (mAbs) and the concept of mAbs as "magic bullets" have gone from initial disappointment to great success. The first therapeutic mAb, OKT3, was licensed in 1986, but it was not until the late 1990s that the potential of therapeutic mAbs began to be realized. Between the licensure of OKT3 and 1996 only one additional therapeutic mAb and five diagnostic mAbs were licensed. Since 1997, 17 mAbs (16 therapeutic and one diagnostic) and two Fc-fusion proteins have been approved by the US Food and Drug Administration (FDA; Table 12.1).

The early failure of most mAbs to progress to phase III clinical trials has been attributed to the insufficient characterization of the mAb and its *in vivo* performance, incomplete preclinical testing, and inadequately designed clinical trials (Stein 1997). In addition, many of the early clinical trials employed murine mAbs, which have a short half-life in humans, are inefficient at eliciting effector functions, and frequently induced human anti-mouse antibodies (HAMA) (Glennie and Johnson 2000). Overall, only 3% of therapeutic murine mAbs evaluated in clinical trials have been successful and ultimately approved (Reichert et al. 2005).

The major factor contributing to the more recent successes of therapeutic mAbs has been the ability to genetically engineer chimeric mAbs (murine or other non-human variable regions expressed with human constant regions) or humanized mAbs (murine or other non-human complementarity determining regions grafted onto human framework regions expressed with human constant regions). In direct contrast to their murine counterparts, chimeric and humanized mAbs

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Table 12.1 Approved monoclonal antibodies and Fc-fusion proteins.

Trade name	*USAN name*	*Use*	*Indication*	*Year approved*	*Type*
Orthoclone OKT3	muromomab	Therapeutic	Immunologic	1986	Murine
OncoScint	satumomab pendetide	Diagnostic	Oncologic	1991	Murine
ReoPro	abciximab	Therapeutic	Cardiac	1994	Chimeric
CEA-Scan	arcitumomab	Diagnostic	oncologic	1996	Murine
Myoscint	imciromab pentetate	Diagnostic	Cardiac	1996	Murine
Verluma	nofetumomab	Diagnostic	Oncologic	1996	Murine
Prostascint	capromab pendetide	Diagnostic	Oncologic	1996	Murine
Rituxan	rituximab	Therapeutic	Oncologic	1997	Chimeric
Zenapax	daclizumab	Therapeutic	Immunologic	1997	Humanized
Simulect	basiliximab	Therapeutic	Immunologic	1998	Chimeric
Synagis	pavilizumab	Therapeutic	Infectious disease	1998	Humanized
Remicade	infliximab	Therapeutic	Immunologic	1998	Chimeric
Herceptin	trastuzumab	Therapeutic	Oncologic	1998	Humanized
Enbrel	etaneracept	Therapeutic	Immunologic	1998	Fc Fusion Protein
Mylotarg	gemtuzumab ozogomicin	Therapeutic	Oncologic	2000	Humanized
Campath	alemtuzumab	Therapeutic	Oncologic	2001	Humanized
Zevalin	ibritumomab tiuxetan	Therapeutic	Oncologic	2002	Murine
Humira	adilimumab	Therapeutic	Immunologic	2002	Human
Amevive	alefacept	Therapeutic	Immunologic	2003	Fc fusion protein
Xolair	omalizumab	Therapeutic	Immunologic	2003	Humanized
Bexxar	tositumomab	Therapeutic	Oncologic	2003	Murine
Raptiva	efalizumab	Therapeutic	Immunologic	2003	Humanized
Erbitux	cetuximab	Therapeutic	Oncologic	2004	Chimeric
Avastin	bevacizumab	Therapeutic	Oncologic	2004	Humanized
NeutroSpec	fanolesomab	Diagnostic	Immunologic	2004	Murine
Tysabri	natalizumab	Therapeutic	Immunologic	2004	Humanized

are less immunogenic, exhibit longer half-lives, and efficiently promote effector functions in humans. Indeed, the approval rates for chimeric and humanized mAbs at present are 21% and 18%, respectively (Reichert et al. 2005).

The expression of fully human mAbs through hybridoma technology is generally inefficient but development of appropriate fusion partners continues (Karpas et al. 2001). The generation of fully human mAbs through the expression of human immunoglobulin genes in transgenic animals or by phage display librar-

ies however, has facilitated the development of human mAbs that share the advantages of chimeric and humanized mAbs and are also predicted to be even less immunogenic than chimeric and humanized mAbs. Such fully human mAbs, however, do not undergo selection on a human background so the potential exists to select mAbs with unusual structures that may be immunogenic or that cross-react with autoantigens. In spite of the effort to produce fully human mAbs, it is not expected that these technologies will provide a great advantage over chimeric mAbs in reducing immunogenicity (Clark 2000).

Several factors influence whether or not a mAb will be immunogenic in patients. These include; the patient population (immunosuppressed, autoimmune), intercurrent illnesses which may disrupt the distribution of the mAbs, the presence of pre-existing antibodies (rheumatoid factor may react with some IgGs), concomitant medications (chemotherapy or immunosuppressive drugs), increases in the dose and/or frequency of administration, and the route of administration. The subcutaneous and intramuscular routes of administration are generally found to be more immunogenic than the intravenous route.

Due to the multiple factors influencing immunogenicity, as well as inherent differences in the assays developed for the detection of HAMA, human antichimeric antibodies (HACA), and human anti-humanized or anti-human antibodies (HAHA) for each product, a direct comparison of the immunogenicity among products cannot be made. It can be seen from Table 12.2, however, that simply removing the Fc portion of a murine mAb reduces the incidence of HAMA to levels more consistently observed for chimeric and humanized mAbs.

To date only one human mAb generated by phage display has been approved. The majority of all mAbs entering clinical trials since 2001, however, have been generated by antibody phage display or in transgenic mice expressing human immunoglobulin genes (Reichert et al. 2005). It is anticipated that within the

Table 12.2 Immunogenicity of licensed monoclonal antibodies.[a]

Antibody type	*Total*	*% patients with HAMA, HACA, or HAHA*
Murine	8	Whole mAbs[b]: <3% to >80% (loss of effectiveness of OKT3 seen when titers were >1:1000) Fab or Fab′ fragments: <1% to 8%
Chimeric	5[c]	<1% to 13%
Humanized	8	<1% to 10%
Human	1	12%[d]

HAMA, human anti-mouse antibodies; HACA, human antichimeric antibodies; HAHA, human anti-humanized or anti-human antibodies.

a All immunogenicity data taken from package inserts.

b <3% of patients developed HAMA against Zevalin (at 90 days post treatment), which ablates B cells. All other murine mAbs induced HAMA in 55% to >80% of patients.

c Four are whole mAbs, one is a Fab.

d When used as a monotherapy, 12% patients made HAHA against Humira. When used in conjunction with methotrexate, <1% patients made HAHA.

next decade data will become available that demonstrate whether or not such fully human mAbs are indeed less immunogenic than chimeric or humanized mAbs.

The introduction of promising mAbs into the clinic is not only attributable to established biotechnology and pharmaceutical companies, but also to start-up companies, as well as academic researchers. This chapter is intended to assist small business and academic sponsors who have limited experience in preparing submissions for Investigational New Drug (IND) applications to the FDA. The primary focus will be on product and preclinical issues that should be addressed prior to the initiation of phase I clinical trials for both therapeutic and *in vivo* diagnostic mAbs. These product development issues should also be considered when a mAb is to be used with devices for enriching or purging specific cell populations or in conjunction with cell therapies. Issues arising relative to product or preclinical development as clinical trials progress and the necessity to provide additional information will also be addressed.

12.2 Regulatory Authority

The statutory authority for the regulation of biological products and drugs for human use in the USA are derived from the Public Health Service Act and the Food, Drug and Cosmetic Act, respectively (http://www.fda.gov/opacom/laws/lawtoc.htm). The implementing regulations can be found in Title 21 of the Code of Federal Regulations (CFR). The regulations for biological products are found in 21CFR Part 600. Other applicable regulations include 21CFR 210 and 21 CFR 211, which describe good manufacturing practices and 21CFR 312, which describes requirements for submission of an IND. Information and relevant forms for submitting an IND application can be found at http://www.fda.gov/cder/regulatory/applications/ind_page_1.htm. The statutory authority for the regulation of devices came under the Medical Device Amendments of the Food, Drug and Cosmetic Act with the implementing regulations for devices located in 21CFR Part 800. The website for the CFR is www.gpo.gov/nara/cfr/index.html.

The development program for an mAb (note that Fc-fusion proteins are grouped with mAbs) is dependent upon the intended use in humans. MAbs are developed as therapeutic or *in vivo* diagnostic agents and also as agents used in the manufacture of other products for *in vivo* use (ancillary mAbs). These ancillary mAbs can be used either alone or in conjunction with devices, such as for the *ex vivo* enrichment of specific cell populations for *in vivo* administration (e.g. hematopoietic stem cells) or for the *ex vivo* purging of unwanted cell types (e.g. tumor cells).

Most mAbs, including mAbs conjugated with toxins or radioisotopes, are regulated as biologics. As of October 1, 2003, the regulatory oversight of most mAbs was moved upon the transfer of the Division of Monoclonal Antibodies (DMA)

as well as the Office of Therapeutic Research and Review (OTRR) from the Center for Biologics Evaluation and Research (CBER) to the Center for Drug Evaluation and Research (CDER). Currently, the CMC portion of an application is regulated by the DMA in the Office of Biotechnology Products (OBP), whereas the pharmacology/toxicology and clinical portions are regulated based on clinical indication by the divisions in the Office of New Drugs (OND). The Office of Cellular, Tissue, and Gene Therapies (OCTGT), CBER has oversight of anti-idiotype (Id) mAbs and Id-KLH products used as vaccines as well as ancillary mAbs used in cell therapies but the DMA provides collaborative reviews for the development of these mAbs.

All therapeutic and *in vivo* diagnostic mAbs and mAbs used *ex vivo* with devices or as ancillary reagents in cell therapy protocols, should be characterized and manufactured under current Good Manufacturing Practices (cGMP), regardless of the FDA Center with regulatory oversight for that product. Although drug-conjugated mAbs such as Mylotarg, in which the mAb is used as a mode of localization or used to affect the biodistribution of the drug, are regulated as drugs, the mAb itself should be manufactured using guidelines for biologics. MAbs included as part of *in vitro* diagnostic kits (regulated either by CBER or the Center for Devices and Radiological Health (CDRH)) will not be discussed in this chapter.

In addition to the regulations, the FDA publishes guidance documents that reflect the FDA's current thinking on a particular topic. Guidance documents clarify requirements imposed by Congress or promulgated by the FDA by explaining how IND Sponsors and the Agency should comply with those statutory and regulatory requirements. They often provide specific detail that is not included in the relevant statutes and regulations. The recommendations in these documents are not legal requirements and are therefore not binding on either the Sponsor or the FDA. The Sponsor, however, is required to provide an alternative scientifically based proposal for those recommendations they choose not to follow. All guidance documents, including those cited in the references in this chapter, can be found on the CDER web site at http://www.fda.gov/cder/guidance/index.htm. Several guidance documents are developed through the International Conference on Harmonisation (ICH). The goal of the ICH is to harmonize the interpretation and application of regulatory requirements for pharmaceuticals among the United States, the European Union, and Japan. ICH documents are also posted on the CDER web site or can be found at http://www.ich.org.

The guidance entitled "Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use" (mAb PTC 97, www.fda.gov/cber/gdlns/ptc_mab.pdf) (Food and Drug Administration 1997a) is a comprehensive document, which describes and recommends steps that should be taken in the manufacture, characterization, quality control, and product testing of mAbs. This document also describes considerations for preclinical studies and the design of phase I and phase II clinical trials. Therapeutic mAbs including drug–mAb conjugates, mAbs for *in vivo* diagnostic use, and those for use *ex vivo* with

therapeutic devices or in cell therapy protocols, should be developed according to the guidance provided in this document.

12.3 Chemistry, Manufacturing, and Controls Considerations

This section contains a summary of quality control testing that should be performed at various stages of the manufacturing process. The mAb PTC 97 document should be carefully reviewed for specific details. This chapter, however, provides updates on some of these recommendations. In addition, the information that should be included in an IND for phase I studies can be found in the Guidance "Content and Format of Investigational New Drug Applications (INDs) for Phase 1 Studies of Drugs, Including Well-Characterized, Therapeutic Biotechnology-Derived Products" (Food and Drug Administration 1996a).

12.3.1 Cell Line Qualification

Most mAbs have been expressed as hybridoma proteins or recombinant proteins in rodent cell lines. Epstein–Barr virus (EBV)-transformed human or primate cell lines have also been used, but thorough studies for the detection of human pathogenic viruses and the demonstration of the removal EBV during purification of the product were necessary prior to use of these products in clinical trials. Because of these safety concerns, the use of EBV-infected primate or human cell substrates is not recommended. Products derived from such cell lines are not eligible for abbreviated safety testing allowed when products are intended for serious and life-threatening conditions (see Section 12.3.7). If mAbs derived from such cell lines show clinical potential, expression of the mAb as a recombinant protein in a non-primate, non-human cell line is a desirable alternative.

In general, for mammalian cell lines a Master Cell Bank (MCB) should be established and demonstrated to be free from bacterial, fungal, and mycoplasma contamination. The MCB should also be tested for the presence of adventitious and species-specific viruses. Murine hybridomas are considered to be inherently capable of producing infectious murine retrovirus and thus, it is not necessary to test these cell banks for the presence of endogenous retrovirus. All other cell substrates, however, including other rodent cells, should be tested for retrovirus. Authenticity testing should be performed to confirm the cell line species of origin, identity, and lack of cell line cross-contamination.

A Working Cell Bank (WCB) may be established to extend the lifetime of the MCB and requires less extensive testing than the MCB. The WCB should be free from bacterial, fungal, and mycoplasma contamination and tested for authenticity. It is not required to develop a WCB if the MCB is of sufficient size to last throughout product development. It is recommended, however, that a WCB be

established, qualified, and used for production by the time of a Biologics License Application (BLA) submission.

Antibody fragments (Fab, sFv, and sFv fusion proteins) are usually produced in bacteria, which do not require adventitious virus testing. An MCB should be established and demonstrated to be free of other microbial, fungal, and bacteriophage contamination.

Cell substrates from other species, yeast, insect or plant, or transgenic animals and plants, have been used infrequently, but it is anticipated that their use for mAb production will increase. In addition to relevant information obtained from the mAb PTC 97, specific guidance on cell substrates or alternative sources can be found in the documents entitled, ICH Q5D: "Guidance on Quality of Biotechnological/Biological Products: Derivation and Characterization of Cell Substrates Used for the Production of Biotechnological/Biological Products" (Food and Drug Administration 1998a), "Points to Consider in the Manufacture and Testing of Therapeutic Products for Human Use Derived from Transgenic Animals (1995)" (Food and Drug Administration 1995) and in the draft guidance entitled "Draft Guidance for Industry: Drugs, Biologics and Medical Devices Derived from Bioengineered Plants for Use in Humans and Animals (2002)" (Food and Drug Administration 2002a).

12.3.2 Quality Control Testing

Lot-to-lot safety testing should be performed at three stages of the manufacturing process: (1) on the unprocessed bulk drug (nonsterile, filtered, harvested tissue culture supernatant); (2) on the drug substance (bulk purified product), and (3) on the drug product (final formulated and filled product). The unprocessed bulk drug should be assessed for bioburden and shown to be free of mycoplasma and adventitious viruses. Three lots should be quantitated for endogenous retrovirus in order to establish a target level for removal of retrovirus during purification (see Section 12.3.6). In recent years, polymerase chain reaction (PCR) methods have been developed as alternatives to traditional methodologies for determining levels of endogenous retrovirus (Brorson et al. 2001, 2002) and assessing the presence of mycoplasma (Eldering et al. 2004) or adventitious virus by MAP testing (Bauer et al. 2004). PCR methods that have been properly validated may be acceptable alternatives to the traditional, more expensive and cumbersome assays. Dialogue with the FDA to discuss the acceptability of such assays is encouraged.

Acceptable limits for bioburden should be established for all stages of the purification process, but the drug product should be sterile. Sterility testing for licensed products is described in 21CFR 610.12. For sterility testing of mAbs during clinical development, procedures described in 21CFR 610.12 or in the US Pharmacopeia or European Pharmacopoeia are acceptable. *In vitro* adventitious virus testing should be performed routinely on all unprocessed bulk drug

production lots using multiple relevant cell lines, while *in vivo* testing is generally done once on the unprocessed bulk drug and repeated only when production methods change.

Other safety tests include assessing levels of endotoxin and polynucleotides. Testing for levels of residual host cell DNA is usually performed on the drug substance. Subsequent to the publication of the mAb PTC 97, the World Health Organization (WHO) Expert Committee on Biological Standardization revised its recommendation so that a maximum of 10 ng of residual DNA from continuous cell lines per dose of a purified product is acceptable (Griffiths 1987), rather than the 100 pg stated in the mAb PTC 97.

Endotoxin testing should be performed on the drug product and is typically performed on the drug substance as well. The endotoxin limit is defined as K/M, where $K = 5.0\,\mathrm{EU\,kg^{-1}}$ for parenteral drugs and M is the maximum human dose per kg of body weight administered in a single one-hour period. Thus, for a dose of $2\,\mathrm{mg\,kg^{-1}}$, the endotoxin limit would be $(5.0\,\mathrm{EU\,kg^{-1}})/(2\,\mathrm{mg\,kg^{-1}}) = 2.5\,\mathrm{EU\,mg^{-1}}$ (Food and Drug Administration 1987). Note that other routes of administration (e.g. intrathecal administration) have different acceptable limits of endotoxin.

CFR 610.13(b) requires that a rabbit pyrogen test be performed for commercial drug product. It is acceptable to assess levels of endotoxin rather than perform the rabbit pyrogen test during clinical development. When a BLA is submitted, the method for endotoxin detection should be validated against the rabbit pyrogen test. This study should demonstrate that at the maximum level of endotoxin per release specification, no positive result is observed in the rabbit pyrogen test (USP <151>). When this validation is successfully completed, it is acceptable to substitute endotoxin testing for the rabbit pyrogen test as a lot-release test for commercial product (21CFR 610.9).

21CFR 610.11 describes the General Safety test, which is used to detect extraneous toxic contaminants in biological products. Licensed mAbs as well as those under clinical development are exempted from the General Safety Test (21CFR 601.2(c)(1)).

In addition to the testing described above, routine analysis on the drug substance should include tests to establish biochemical purity, molecular integrity, identity, and potency. The drug product should be tested for protein concentration, potency, purity, identity, pH, and, when appropriate, moisture, preservative, and excipients.

Potency assays should be based on the proposed mechanism of action for the mAb. While ELISA tests or other binding assays are often employed as potency assays, unless the mAb works by blocking the binding of the antigen to its intended target, binding assays alone are not sufficient to establish potency. If the mAb is proposed to work through effector functions such as complement-dependent cytotoxicity or antibody-dependent cytotoxicity, by the induction of apoptosis, or other mechanisms, cellular-based assays that reflect these mechanisms should be developed. MAbs conjugated with drugs or toxins should employ a cytotoxicity assay to establish potency.

Tests to establish biochemical purity should include assays that demonstrate the reduction of process contaminants to levels below detection or, in some cases, to minimal acceptable levels. Such process contaminants include host cell proteins and DNA, materials that may be introduced during culture, such as bovine or human insulin or transferrin, bovine serum albumin, or immunoglobulin (from bovine serum), surfactants used to protect cells in agitated suspension cultures from shear and mechanical force, methotrexate or other agents used to maintain the antibody-expressing construct under selected pressure, inducing agents intended to maximize product expression, Protein A (or other proteins used in immunoaffinity columns), and solvents and detergents used in virus inactivation steps.

Assays to detect host cell proteins are usually developed for each product but commercial kits are now available. If commercial kits are used they should be demonstrated to be suitable for use for each product and its host cell substrate. Release specifications should be based on relevant product quality attributes. For early clinical development, release specifications are considered preliminary but should be quantitative when possible. Acceptance criteria may be broad unless constrained by safety considerations. Specifications such as "For information only" or "conforms to reference standard" are not usually acceptable as upper or lower limits of acceptability are not delineated or the reference standard itself, for a variety of reasons, may not always provide the same results. This is of particular importance for potency assays as potency should not vary significantly among lots of drug product as product development and clinical studies progress. "Conforms to reference standard" may be an acceptable release specification if the reference standard is fully characterized and the attribute for that assay is quantitated. For example, a reference standard may display a certain number of major and minor bands within a specific p*I* range on isoelectric focusing gels. Subsequent lots may "conform to reference standard" if they also have the specified number of bands within the specified p*I* range.

12.3.3
Transmissible Spongiform Encephalopathy (TSE)

TSEs are a concern for biotechnology products because bovine products (serum, transferrin, insulin, albumin) or human plasma-derived products (transferrin, albumin, IgG) may be used during manufacturing. Due to the mad-cow disease epidemic in Europe in the 1990s, the emergence of variant Creutzfeldt–Jakob disease (vCJD), and the inability to assess levels of prion protein in blood, cell substrates, raw materials, and unpurified bulk drug substance, the FDA issued a guidance in 2002 to reduce the possible risk of transmission of CJD and vCJD by human blood and blood products (Food and Drug Administration 2002b). This Guidance distinguishes the risks between CJD and vCJD and permits the use of donors for plasma derivatives from some affected countries because such products are highly processed materials. Although the use of plasma derivatives from donors who have resided in the UK, France, and on US military bases abroad may

be permitted, such products may be subject to an immediate recall if any donor subsequently develops vCJD. Therefore, it is strongly recommended that the use of such plasma derivatives be avoided. Of particular concern for mAb production are Protein A preparations used in affinity chromatography that have been purified over human IgG immunoaffinity columns. Protein A resins are now available that do not use human IgG in the purification process, which addreses this particular risk.

Bovine-derived products used during manufacture should be from countries known to be free of bovine spongiform encephalopathy (BSE). The United States Department of Agriculture (USDA) keeps a list of countries (see www.aphis.usda.gov/NCIE/country.html) with verified or suspected cases of BSE and animal products from these countries should not be used.

Because of the safety issues surrounding TSEs, the FDA has encouraged sponsors to adapt cell lines to serum-free, protein-free media and to use Protein A resins where the Protein A was not purified over human IgG. It is anticipated that in the future, validated assays to detect TSEs will be developed. Until that time, however, sponsors should provide the FDA with information regarding the source and country of origin for every animal- and human-derived component used in manufacture. This list should include the bovine and human plasma-derived components discussed above as well as amino acids used in the tissue culture medium, enzymes used to make protein hydrolyzates, cholesterol, Tween, and any other reagent used in the manufacturing process that may be animal- or human-derived. It is important to keep track of the lot numbers for each animal or human-derived raw material, should new developments regarding TSEs and the spread of vCJD arise.

12.3.4 Product Stability

Expiration dates are not established prior to submission of a BLA. Stability studies, however, are required during clinical development to ensure product quality for the duration of the clinical study. Therefore, stability testing protocols for both drug substance and drug product should be developed and initiated prior to the phase I clinical trials. The stability protocols should include tests for physicochemical integrity, potency, sterility, and other specific assays as appropriate. Samples of drug substance or drug product are usually tested frequently during the first year of the protocol and then on a 6-month basis through the second year. Stability studies extending longer than 2 years usually involve testing on a yearly basis. Accelerated stability testing (i.e. testing of samples stored at temperatures exceeding the recommended storage temperature) are often useful for identifying which tests are stability-indicating. Tests should be performed in parallel with a properly qualified and stored reference standard. Refer to the ICH documents Q5C: "International Conference on Harmonisation; Final Guideline on Stability Testing of Biotechnological/Biological Products" and Q1A (R): "Sta-

bility Testing of New Drugs and Products (Revised guidelines)" for more detailed recommendations (Food and Drug Administration 1996b, 2002c).

12.3.5 Reference Standard

A reference standard should be developed and appropriately qualified using defined physicochemical characteristics, specificity and potency attributes. It should be stored under appropriate conditions and tested periodically to document its integrity. The reference standard should be used for lot-to-lot comparisons performed for both drug substance and drug product release as well as for stability studies. New reference standards should be qualified when major manufacturing changes are made. Typically, the reference standard is qualified using a more comprehensive panel of biochemical, biophysical, and immunological assays than those used for release or stability testing. A thorough characterization of the lot that is used for a reference standard provides the basis for future comparability studies (see Section 12.3.8).

12.3.6 Virus Clearance and Inactivation Studies

Prior to the initiation of phase I clinical trials, virus clearance and inactivation studies that demonstrate an adequate level of removal or inactivation of a relevant model virus should be completed. For murine hybridomas and other rodent cell lines, the relevant model virus is murine leukemia virus (MuLV). For primate or human cell lines, the relevant model virus would be any viruses known to be present in that particular cell line (e.g. EBV). Studies on the clearance and/or inactivation of additional model viruses should be performed on material that will be manufactured using the process anticipated for licensure and should ideally be completed prior to the pivotal clinical trial. These studies may need to be repeated when major manufacturing changes are made. The ICH Q5A: "Guidance on Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin" (Food and Drug Administration 1998b) as well as the mAb PTC 97 documents describe the appropriate design, implementation, and interpretation of such studies.

Generic or modular virus clearance studies may be applied to subsequent mAbs manufactured at a given facility and are described in more detail in the mAb PTC 97. In general, a generic clearance study is one in which virus removal or inactivation has been demonstrated for several steps in the purification process of a model antibody. These data may then be applied to subsequent mAbs purified using the identical process, provided they are of the same species and class and are derived from the same cell substrate. A modular clearance study is one in which a single step in a purification process may differ from that of a model antibody. In such cases, only the unique module needs to undergo a virus clearance study, while

the values obtained from the other modules of the model antibody may be applied to the new mAb.

Alternatively, bracketed virus reduction/inactivation studies may be performed. If more than one mAb will be manufactured at the same facility with similar, but not identical purification schemes (e.g. differences in ionic strength or pH of an elution buffer), studies that bracket the range of differences may be performed. Subsequent mAbs for which the purification parameters fall within the tested range may use the virus clearance values obtained from the bracketed study (Brorson et al. 2003).

12.3.7
Abbreviated Product Safety Testing for Feasibility Trials in Serious or Immediately Life-Threatening Conditions

Feasibility clinical trials are pilot studies to provide an early characterization of safety and an initial proof of concept in specific patient populations. They are limited in scope and are generally conducted at a single clinical site with a small number of patients. An immediately life-threatening condition is defined in 21CFR 312.34 as a "stage of disease in which there is a reasonable likelihood that death will occur in a matter of months or in which premature death is likely without early treatment." For such phase I clinical trials, the full battery of product safety tests is not required. Sterility (bacteria and fungi) should be performed and it is strongly recommended that mycoplasma and endotoxin testing be performed. If the purification scheme contains two orthogonal robust virus removal/inactivation steps (virus removal/inactivation based on different mechanisms), neither adventitious virus testing nor virus clearance studies need be performed. If clinical trials progress beyond these phase I feasibility trials, the full battery of safety testing, as well as virus clearance studies for a relevant model virus, should be performed prior to initiating phase II trials. Abbreviated testing does not apply to human or primate cell substrates. Clinical reviewers determine whether the indication in the IND application meets the criteria for a serious or life-threatening condition.

12.3.8
Comparability

Changes in the manufacturing of an mAb are expected during product development and, depending on the nature of the change, may necessitate an assessment of the comparability of the product pre and post change. The purpose of the assessment is to ensure that the manufacturing changes have not affected the safety, identity, purity, or efficacy of the mAb (Chirino and Mire-Sluis 2004). Demonstration of the comparability of a product made using two different manufacturing schemes or at different manufacturing facilities becomes more important during phase III clinical trials or after product approval, than when changes are implemented earlier in the course of product development. In IND applica-

tions for phase I studies, however, the drug product being proposed for use in the clinical trial should be appropriately compared to the drug product used in the animal toxicology studies in order to extrapolate the preclinical safety data to the clinical scenario (Food and Drug Administration 1996a). As clinical trials progress and product development matures, it is expected that changes will be introduced to improve the manufacturing process and thus, plans to demonstrate comparability between the product generated by the old and new manufacturing schemes should be devised. Major changes should be in place at the start of phase III trials but a scale-up of the process or additional manufacturing changes are occasionally introduced during phase III trials. Comparability studies during phase III may also include an analysis of key process intermediates (including, but not limited to, cell culture metrics and process contaminants), as appropriate.

In addition to maintaining a current reference standard, samples from several lots manufactured by each production scheme or scale should be properly retained. An early and thorough characterization of the mAb, including physical, chemical, biological, and immunological characteristics, determines the attributes that a mAb should retain after manufacturing changes or scale-up are introduced. The need for additional preclinical or clinical testing when manufacturing changes occur is discussed in Section 12.4.6 below. Sponsors are strongly encouraged to consult with the FDA regarding plans for demonstrating product comparability.

12.4 Considerations for Preclinical Testing

The primary goals of preclinical safety evaluation are: to determine a safe starting dose and subsequent dose escalation schemes in humans; to identify target organs and potential toxicities; to establish safety parameters for clinical monitoring and to provide a risk assessment for the intended human population (Serabian and Pilaro 1999). A summary of the types of studies utilized in the preclinical assessment of biotechnology products, including mAbs, can be found in two specific documents, mAb PTC 97 and ICHS6: "Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals" (Food and Drug Administration 1997b). In general, the unique properties of mAbs that make them desirable therapeutic agents (i.e. high specificity and affinity for their targets) limit the types of preclinical studies that can be performed, resulting in the need for flexibility in preclinical development strategies. Hence, some conventional "small molecule" drug approaches may not be useful or appropriate.

When an IND is submitted to the FDA for initiation of a first-in-human study, the pharmacology/toxicology data for an mAb should include the following:

- Tissue cross-reactivity studies
- Data justifying relevant species
- Pharmacodynamic studies

- Pharmacokinetic assessment
- Toxicology studies.

A generalized description of the pharmacology and toxicology information needed to support a phase I study is provided in the "Guidance for Industry: Content and Format of Investigational New Drug Applications (INDs) for Phase 1 Studies of Drugs, Including Well Characterized, Therapeutic, Biotechnology-Derived Products" (Food and Drug Administration 1996a). This section of the chapter will address and expand upon the principles and concepts that relate specifically to pharmacology and toxicology studies performed with mAb products.

12.4.1 Tissue Cross-Reactivity

MAbs, although developed for a specific epitope/antigen, may cross-react with different, but similar, epitopes and/or bind to the intended epitope in unexpected tissues. Since the binding of pharmacologically active antibodies to nontarget tissues may have serious toxicological consequences, both the mAb PTC 97 and ICH S6 documents stress the importance of characterizing epitope/antigen distribution across a panel of human tissues, prior to the initiation of a phase I clinical trial. In general, tissue cross-reactivity studies help define the desired and undesired binding properties of the mAb, provide a greater understanding of the potential *in vivo* toxicities, reveal potential target tissues and organs and provide confidence in the selection of a suitable animal species for preclinical studies.

Tissue cross-reactivity is assessed by immunohistochemistry. For these studies, a panel of 37 human tissues from at least three donors should be examined to ensure an accurate representation of possible *in vivo* tissue binding (Food and Drug Administration 1997b). A list of the suggested human tissues is provided in the mAb PTC 97. The tissues should be cryopreserved, rather than paraffin-embedded, due to the possibility for "false negatives" that may result from inconsistent or poor antigen retrieval, which often occurs with paraffin-embedded tissue sections. The mAb to be tested (test article) should be the one designated for the clinic and can be either unconjugated and detected by a labeled secondary antibody or conjugated directly to detection molecules such as peroxidase, biotin, or fluorescein. Since direct labeling of an mAb may alter its binding affinity and tissue-staining pattern, the affinity of a conjugated test article should be comparable to that of the unconjugated mAb. At least two concentrations (a low and high concentration) of test article should be investigated. The projected clinical serum concentration may be considered, but the test article concentrations should be optimized for the assay to produce strong, specific binding, with limited background staining. Positive and negative control tissues and a species- and isotype-matched negative control antibody should be incorporated into the study design. In unique cases where adequate assay development is impossible, alternative approaches (e.g. use of a chimeric or murine mAb test article) may be considered;

however, it is advantageous to discuss these alternatives with the FDA prior to implementation.

12.4.2 Relevant Species

Since the specificity of an mAb, developed against a human substrate, often limits its immunoreactivity to orthologous substrates in test species, it is extremely important to identify pharmacologically relevant animal species appropriate for pharmacodynamic, pharmacokinetic, and toxicology studies. With regard to mAbs, a relevant species is one in which the mAb (1) is pharmacologically active due to its specific cross-species binding to the orthologous antigen/epitope and (2) demonstrates a tissue cross-reactivity pattern similar to human (Food and Drug Administration 1997b). Relative affinity of the mAb to the non-human epitope, compared with the human epitope, should be assessed as well and may be useful in the interpretation of the toxicology data. In determining appropriate species, it may be helpful to first screen several species for mAb binding by immunohistochemistry on a limited panel of tissues or by flow cytometry on cells expressing the orthologous antigens or for mAb activity by functional assays (e.g. enzyme induction, cell signaling, and physiological changes). A subsequent, comprehensive comparison of mAb tissue cross-reactivity between non-human and human tissues will confirm the relevancy of the species choice. All data supporting the choice of a relevant species should be included in the initial IND application. Two studies performed by Boon et al., one with a chimeric anti-CD40 mAb in cynomolgus monkeys (Boon et al. 2002a) and the other with a humanized anti-CD4 mAb in rhesus monkeys (Boon et al. 2002b), provide excellent examples of the contribution of tissue cross-reactivity studies in establishing a preclinical safety assessment program. In neither study were the tissue distributions of the mAb binding identical for the human and non-human primate; however, patterns of tissue and cell-type binding were similar enough to suggest that a toxicology study in the particular non-human primate would provide adequate safety data.

12.4.3 Pharmacodynamic and Pharmacokinetic Studies

Pharmacodynamic studies, particularly those contributing to understanding the mechanism of action of the mAb and those providing "proof of concept" (i.e. potential human efficacy), should be included in the initial IND submission. *In vitro* cell culture experiments and *in vivo* studies with animals displaying the disease are commonly utilized to demonstrate efficacy. If cross-species reactivity of the mAb is limited, xenograft or transgenic models expressing the human antigen of interest may be useful tools. Overall, these types of studies are important because they aid in the estimation of the effective dose, dosing regimens and most appropriate plasma concentrations.

Pharmacokinetic (PK) parameters are most often calculated from the administration of the mAb to healthy animals of a relevant species. However, use of an animal model that shares common pathophysiology/symptoms of the clinical disease may not only better reflect the pharmacodynamic properties and clinical outcome, but may also provide a more accurate PK profile. For example, if the mAb is directed towards an antigen that is overexpressed in tumor tissue, presence of the tumor will likely alter the biodistribution of the mAb, thereby decreasing its blood concentration. As the tumor burden diminishes, more antibody will be available systemically, ultimately affecting overall animal exposure to the mAb. Assessing the PK profile in nonrelevant animal species (those lacking a high-affinity binding epitope) is discouraged, because the major mechanisms of elimination of the mAb from circulation (i.e. binding to antigen and internalization) are low or absent. Thus, changes in clearance attributable to saturation of antigen binding would not be observed.

PK studies assess the absorption, distribution, and excretion of a mAb and may be performed in independent studies or incorporated into general toxicology studies. Unlike "small molecule" drugs, proteins, including mAbs, are not metabolized by hepatic cytochrome P450 mechanisms, but are catabolized into their individual amino acids that can be reused for protein synthesis and energy production. Thus, metabolism assessments used for "small molecule" drugs are not warranted. When mAbs are administered intravenously, they most often display a biphasic elimination profile, consisting of a rapid distribution phase and a long elimination phase in which antibody recycling and catabolism occur (Ghetie and Ward 2002). MAbs of various isotypes and subtypes have different elimination half-lives, following similar trends to that identified for endogenous antibodies. Endogenous IgG1, IgG2, and IgG4 have half-lives of approximately 23 days, whereas the half-lives of IgG3 and IgM average 7.5–9 days and 5 days, respectively (Trang 1992). In general, half-lives of mAbs, particularly as they become more "humanized", are shorter in animals than what is observed in humans; the projected exposure difference should be considered when extrapolating animal data to the human setting in the course of designing a clinical trial dosing regimen.

PK profiles of mAbs can be influenced by a variety of factors, including, but not limited to, affinity for specific and non-specific binding proteins, immunogenicity (see Section 12.4.5), posttranslational processing (see Section 12.4.6), concentration, antibody isotype, manipulation of the antibody (e.g. fragments, fusion proteins), formulation, and route of administration. It is therefore important to maintain consistency between the preclinical and clinical material and study designs.

12.4.4 Toxicology

All preclinical toxicology studies should be performed in relevant species, in order to prevent misleading safety interpretations. For example, Klingbeil and Hsu observed that administration of humanized mAb Hu1D10 to antigen-positive

monkeys resulted in severe acute adverse effects (e.g. respiratory suppression, increased heart rate, and urticaria) that in some cases required life-sustaining intervention, while no such adverse effects were observed in antigen-negative and control monkeys (Klingbeil and Hsu 1999). In this case, if pharmacological relevancy was ignored, the Hu1D10-related toxicities would have gone undetected and a "false negative" safety interpretation would have been established. Conversely, the use of nonrelevant animals may result in "false positive" safety conclusions, most often reflective of toxicities arising from product immunogenicity (see Section 12.4.5).

In many cases, the only appropriate animal species for a mAb is a non-human primate. The most common non-human primate used for preclinical toxicology studies is the cynomolgus monkey; additional non-human primates that may be used are rhesus and marmoset monkeys and chimpanzees. However, because chimpanzees are a protected species many limitations to conducting nonclinical studies with these animals exist, including (1) difficulty in obtaining protein-naive animals, (2) limited numbers of animals available, (3) limits to dose frequency and level, (4) limited numbers of animals per treatment group, and (5) inability to sacrifice animals at the end of the study to obtain histopathology data. Thus, sponsors confronted with the chimpanzee as the only relevant species often consider alternative approaches. These approaches include analogous mAbs, transgenic models, and animal models of disease (Food and Drug Administration 1997b).

An analogous mAb is one developed against the antigen of another species (e.g. recognizes the ortholog of the original human target). While this approach allows for conducting the necessary nonclinical studies, it is not without disadvantages. The manufacturing of the analogous mAb differs from that being developed clinically; as a result, the product may contain different impurity and contaminant profiles and exhibit disparate potency and/or pharmacology. Conducting appropriate studies to define the pharmacology of the analogous mAb, to the greatest extent possible, can reduce the impact of these disadvantages. The pharmacology of the analogous mAb should be compared with that of the product intended for human use. An example of this approach is described for the evaluation of reproductive and chronic toxicities of infliximab (chimeric mAb to human TNFα), in which an analogous mAb was used to target mouse TNFα (Treacy 2000). A second alternative approach is the use of a transgenic mouse model expressing the human antigen.

The information gained from these models is most useful when the interaction of the product with the humanized antigen has similar physiological consequences to those expected in humans. For example, chronic and reproductive toxicity studies for keliximab, a chimeric mAb specific for human and chimpanzee CD4, were performed in a human CD4 transgenic mouse model (Bugelski et al. 2000).

A third option is one using an animal model of disease. These models are used rarely in practice due to complications of the underlying disease interfering with the interpretation of the toxicological data. They may, however, be useful in

ascertaining whether a particular toxicity is dependent on the conditions inherent in the disease, such as the expression of a disease-associated receptor, or is an independent finding. Overall, these alternative approaches are not employed routinely, but are considered when the scientific need for more information arises.

Normally, safety assessments (toxicology and PK studies) are performed in two species, one rodent and one nonrodent (Food and Drug Adminstration 1997b, 1997c). However, due to the potential limitations presented by different relevant species, the majority of biological products are assessed in only one species. ICHS6 specifically states that this approach is acceptable "in certain justified cases (e.g., when only one relevant species can be identified or where the biological activity of the biopharmaceutical is well understood)." Two notable product exceptions are immunotoxins, mAbs that are fused to protein toxins such as diphtheria toxin, ricin, or pseudomonas toxin (produced as one protein), and immunoconjugates, mAbs conjugated through an organic chemical linker to "small molecule" drugs, radioisotopes, or toxins. Toxicology studies for these product types should be performed in two species when no relevant species is available and nonspecific factors play a predominant role in toxicity, such as when (1) the toxin, "small molecule" drug or radioisotope portion of the molecule results in activity and/or toxicity in non-mAb targeted tissues, and (2) immunoconjugates are unstable and degrade to the individual components, thereby negating the specificity provided by the antibody.

In cases where degradation is prominent, separate studies with the individual components may be necessary to identify target tissues and toxicities exerted by the different components. Additional special considerations for both the toxicology and PK studies exist for immunotoxins and immunoconjugates, but are beyond the scope of this chapter; a detailed discussion is found in mAb PTC 97.

Good preclinical study design is essential for identifying potential endpoints of toxicity to be monitored in clinical trials and for determining the human starting dose. In general, toxicity studies should be performed according to Good Laboratory Practices (GLP, part 58 of the Code of Federal Regulations), and both males and females should be included or justification provided if one sex is excluded. The duration of mAb exposure, and frequency and route of administration should mimic, as closely as possible, the proposed clinical use. ICHM3: "Nonclinical Safety Studies for the Conduct of Human Clinical Trials for Pharmaceuticals" outlines the minimum length of repeat dose toxicology studies and the timing of these studies in relation to clinic trial durations and phases (Food and Drug Administration 1997c). Various dose levels should be selected, including a no adverse effect level dose (known commonly as the NOAEL) and a toxic dose. If the mAb is expected to be relatively nontoxic, the highest dose should be (1) a scientifically reasonable multiple of the highest projected clinical dose, (2) a dose reflective of a pharmacodynamic marker (e.g. saturation of antigen) or (3) the maximum feasible dose in the animals. In addition to terminal sacrifice, usually performed within 1–3 days after the last dose, a treatment-free recovery period should be included in the study design to determine reversibility of effects and/or potential delayed toxic effects.

Although no guidance is provided by CDER on toxicology endpoints, it is expected that physical examination, body weight, food consumption, ophthalmologic evaluation, clinical pathology, gross pathology, organ weights, histopathology, and immunogenicity endpoints will be included in general toxicology studies. A good description of applicable endpoints can be found in *Principles and Methods of Toxicology* (Wilson and Hardisty 2001). Additionally, ICHS7A: "Safety Pharmacology Studies for Human Pharmaceuticals" (Food and Drug Administration 2001) describes a core battery of "safety pharmacology" tests designed to investigate potential toxicities on the cardiovascular, respiratory and central nervous systems. For highly targeted biologics, such as mAbs, these "safety pharmacology" endpoints can be incorporated into the general toxicology study.

In addition to the general toxicology studies, other specialized toxicology studies, such as reproductive toxicology and carcinogenicity, may be necessary depending upon the product, clinical indication, and intended patient population (Food and Drug Administration 1997b). Specifically, embryo–fetal developmental reproductive toxicology studies are conducted when the intended human population includes women of child-bearing potential. However, reproductive studies with mAbs that have only non-human primate as a relevant species pose many challenges, including obtaining sexually mature animals, low conception rate, high abortion rate, limited number of offspring, and immunogenicity. As a result, these studies often can be deferred until later stages of drug development, provided that the product does not produce an unacceptable reproductive risk and women of child-bearing potential are using suitable contraception. Guidance on reproductive study designs can be found in ICHS5A: "Detection of Toxicity to Reproduction for Medicinal Products" (Food and Drug Administration 1994). Two-year carcinogenicity studies are usually not conducted for mAb products because (1) mAbs are not expected to be translocated to the nucleus of an intact cell and interact with DNA or other chromosomal material to induce mutation and transform cells (i.e. genotoxicity); (2) species cross-reactivity may limit the usefulness of current rodent models; and (3) the long lifespan of non-human primates and the large number of animals required make the studies impractical. However, if the mechanism of action of the mAb suggests that it, directly or indirectly, might support or induce proliferation of transformed cells, tumor promotion studies may be required. These studies assess the ability of the product to stimulate the growth of cells already transformed, such as dormant tumors or micrometastases, and may consist of *in vitro* proliferation assays of normal and transformed cells and/or *in vivo* studies utilizing alternative approaches (e.g. transgenic mouse models). The need for and design of such studies should be discussed with the FDA.

12.4.5 Immunogenicity

Many biotechnology-derived pharmaceuticals, including mAbs, induce the formation of anti-product antibodies (anti-mAb) in animals and humans. An

immune response in animals, however, does not reliably predict a similar response in humans. This is true particularly with the design of more "humanized" or fully human antibodies, because animals may recognize these proteins as "foreign," whereas humans may not. Nevertheless, it is important to measure antibody formation in the toxicology study to aid in its interpretation. Specifically, anti-product antibodies may affect the overall animal exposure to the active mAb by altering its rate of clearance or by neutralizing its function by inhibiting target binding. In addition, anti-product antibody formation may result in immune complex disease (or serum sickness), a condition caused by deposition of anti-mAb:mAb complexes in the vasculature with subsequent activation of inflammation pathways. Conversely, these anti-product antibodies may not affect product exposure or activity; thus, the presence of anti-product antibodies in the absence of PK effects or other toxicities is not sufficient to lead to the termination of a study. Instead, antibody responses should be characterized with respect to titer, number of animals, and function (i.e. neutralizing or non-neutralizing) and correlated with any pharmacological and/or toxicological changes in the animals. An in-depth discussion of this topic was published previously (Bussiere 2003).

The ability to assess the preclinical and clinical immunogenicity of biotechnology products is dependent upon the quality of the assay that is developed. Poorly designed assays most often impede product development and may result in post-marketing commitment studies upon licensure. Specifically for mAb products, assays detecting anti-product antibodies are complicated by the fact that antibodies are usually the detection reagents, the product, and the target of the assay. Thus, the sole presence of the product (mAb) in blood samples can interfere with the assay. Therefore, it is critical to either (1) collect blood samples at time points where the levels of mAb are negligible or (2) demonstrate that the presence of the mAb does not interfere with the specificity and sensitivity of the assay. Mire-Sluis and colleagues provide detailed recommendations for optimizing such immunoassays (Mire-Sluis et al. 2004).

12.4.6 Comparability

Since changes in the product-manufacturing scheme occur frequently during clinical development, the product used in nonclinical studies is not required to be "identical" to that going into the clinic, but it does have to be "comparable." The mAb PTC 97, ICH Q5E: "Comparability of Biotechnological/Biological Products Subject to Changes in their Manufacturing Process" (Food and Drug Administration 2005) and the document entitled "FDA Guidance Concerning Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology-Derived Products" (Food and Drug Administration 2002d) provide guidance regarding the demonstration of comparability in this setting.

Comparability of biotechnology products is usually determined by analytical and functional assays. Preclinical studies may be necessary if (1) the product quality is impacted adversely; (2) biochemical alterations in the active moiety are

known to affect product exposure *in vivo* (e.g. glycosylation, charge); (3) it is not possible to determine if a potential biochemical change will be significant clinically because the analytical testing may be insufficiently sensitive, precise, or accurate; (4) the relationship of efficacy and/or toxicity to the product is not sufficiently established to determine the significance of the differences observed from analytical testing; or (5) a change in the formulation includes the addition of unknown excipients (Green 2002). Bridging PK studies are most often required; however, depending on the extent of the final product changes, more extensive toxicology studies may be required and necessitate discussion with the FDA.

12.5 Conclusions

The recent successes in approving mAbs for commercial use for oncologic, immunotherapeutic, and infectious disease indications encourage the further development of new mAbs directed against novel targets to treat or diagnose a broadening array of diseases. Furthermore, the approvals of the drug-conjugate Mylotarg and the radioimmunoconjugates Zevalin and Bexxar are likely to spur development of a variety of antibody conjugates. The sequencing of the human genome and mapping of human genetic variation (International HapMap Consortium 2005) provides many new targets for mAb therapy and it is anticipated that submission of INDs with mAbs recognizing novel targets will increase in the coming years. Regulators rely upon a flexible, case-by-case, science-based approach to safety evaluation needed to support clinical development and marketing authorization. Sponsors are encouraged to contact the appropriate review office at FDA for their mAb product to request a meeting before submitting an IND or IDE. Effective communication between the FDA and the sponsor is a crucial element of the pathway from drug discovery to the clinic. While the regulatory pathway is complex, an early understanding of the regulatory process and careful product and preclinical characterization will enhance the chances of success.

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References

Bauer, B.A., Besch-Williford, C.L., Riley, L.K. (2004) Comparison of the mouse antibody production (MAP) assay and polymerase chain reaction (PCR) assays for the detection of viral contaminants. *Biologicals* 32: 177–182.

Boon, L., Holland, B., Gordon, W., Liu, P., Shiau, F., Shanahan, W., Reimann, K.A.,

Fung, M. (2002a) Development of anti-CD4 MAb hu5A8 for treatment of HIV-1 infection: preclinical assessment in non-human primates. *Toxicology* 172: 191–203.

Boon, L., Laman, J.D., Ortiz-Buijsse, A., den Hartog, M.T., Hoffenberg, S., Liu, P., Shiau, F., de Boer, M. (2002b) Preclinical assessment of anti-CD40 Mab 5D12 in cynomolgus monkeys. *Toxicology* 174: 53–65.

Brorson, K., Swann, P.G., Lizzio, E., Maudru, T., Peden, K., Stein, K.E. (2001) Use of a quantitative product-enhanced reverse transcriptase assay to monitor retrovirus levels in mAb cell-culture and downstream processing. *Biotechnol Prog* 17: 188–196.

Brorson, K., Xu, Y., Swann, P.G., Hamilton, E., Mustafa, M., de Wit, C., Norling, L.A., Stein, K.E. (2002) Evaluation of a quantitative product-enhanced reverse transcriptase assay to monitor retrovirus in mAb cell-culture. *Biologicals* 30: 15–26.

Brorson, K., Krejci, S., Lee, K., Hamilton, E., Stein, K., Xu, Y. (2003) Bracketed generic inactivation of rodent retroviruses by low pH treatment for monoclonal antibodies and recombinant proteins. *Biotechnol Bioeng* 82: 321–329.

Bugelski, P.J., Herzyk, D.J., Rehm, S., Harmsen, A.G., Gore, E.V., Williams, D.M., Maleeff, B.E., Badger, A.M., Truneh, A., O'Brien, S.R., Macia, R.A., Wier, P.J., Morgan, D.G., Hart, T.K. (2000) Preclinical development of keliximab, a Primatized anti-CD4 monoclonal antibody, in human CD4 transgenic mice: characterization of the model and safety studies. *Hum Exp Toxicol* 19: 230–243.

Bussiere, J.L. (2003) Animal models as indicators of immunogenicity of therapeutic proteins in humans. *Dev Biol (Basel)* 112: 135–139.

Chirino, A.J., Mire-Sluis, A. (2004) Characterizing biological products and assessing comparability following manufacturing changes. *Nat Biotechnol* 22: 1383–1391.

Clark, M. (2000) Antibody humanization: a case of the 'Emperor's new clothes'? *Immunol Today* 21: 397–402.

Eldering, J.A., Felten, C., Veilleux, C.A., Potts, B.J. (2004) Development of a PCR method for mycoplasma testing of Chinese hamster ovary cell cultures used in the manufacture of recombinant therapeutic proteins. *Biologicals* 32: 183–193.

Food and Drug Administration (1987) *Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-Product Endotoxin Test For Human and Animal Parenteral Drugs, Biological Products and Medical Devices*. FDA.

Food and Drug Administration (1994) International Conference on Harmonisation: Guideline on detection of toxicity to reproduction for medicinal products. [Docket No. 93D-0140]. *Federal Register* 59: 48746–48752.

Food and Drug Administration (1995) Points to consider in the manufacture and testing of therapeutic products for human use derived from transgenic animals. [Docket No. 95D-0131]. *Federal Register* 60: 44036.

Food and Drug Administration (1996a) Guidance for industry; content and format of investigational new drug applications (INDs) for phase 1 studies of drugs, including well-characterized, therapeutic, biotechnology-derived products. [Docket No. 95D-0386]. *Federal Register* 61: 1939–1940.

Food and Drug Administration (1996b) International Conference on Harmonisation: Final guideline on stability testing of biotechnological/ biological products [Docket No. 93D-0139]. *Federal Register* 61: 36466–36469.

Food and Drug Administration (1997a) Points to consider in the manufacture and testing of monoclonal antibody products for human use. [Docket No. 94D-0259]. *Federal Register* 62: 9196–9197.

Food and Drug Administration (1997b) International Conference on Harmonisation: S6 Guidance on preclinical safety evaluation of biotechnology-derived products. [Docket No. 97D-0113]. *Federal Register* 62: 6515–61519.

Food and Drug Administration (1997c) International Conference on Harmonisation: Guidance on nonclinical safety studies for the conduct of human clinical trials for pharmaceuticals. [Docket No. 97D-0147]. *Federal Register* 62: 62922.

Food and Drug Administration (1998a) International Conference on Harmonisation: Guidance on quality of biotechnological/biological products Q5D: Derivation and characterization of cell substrates used for the production of biotechnological/biological products. [Docket No. 97D-0159]. *Federal Register* 63: 50244–50249.

Food and Drug Administration (1998b) International Conference on Harmonisation: Guidance on viral safety evaluation of biotechnology products derived from cell lines of human or animal origin. [Docket No. 96D-0058]. *Federal Register* 63: 51074–51084.

Food and Drug Administration (2001) International Conference on Harmonisation: Guidance on S7A safety pharmacology studies for human pharmaceuticals. [Docket No. D-1407]. *Federal Register* 66: 36791–36792.

Food and Drug Administration (2002a) Draft Guidance for Industry: Drugs, biologics, and medical devices derived from bioengineered plants for use in humans and animals. [Docket No. 02D-324]. *Federal Register* 67: 57828–57829.

Food and Drug Administration (2002b) Guidance for Industry: Revised preventative measures to reduce the possible risk of transmission of Creutzfeldt-Jakob disease (CJD) and variant Creutzfeldt-Jakob disease (vCJD) by blood and blood products. [Docket No. 97D-0318]. *Federal Register* 67: 2226–2227.

Food and Drug Administration (2002c) International Conference on Harmonisation: Draft revised guidance on Q1A(R) stability testing of new drug substances and products. [Docket No. 93D-0139]. *Federal Register* 65: 21446–21453.

Food and Drug Administration (2002d) Guidance concerning demonstration of comparability of human biological products. [Docket No. 96D-0132]. *Federal Register* 70: 37861–37862.

Food and Drug Administration (2005) International Conference on Harmonisation: Guidance on Q5 comparability of biotechnological/biological products subject to changes in their manufacturing process. [Docket No. 2004D-0118]. *Federal Register* 59: 48746–48752.

Ghetie, V., Ward, E.S. (2002) Transcytosis and catabolism of antibody. *Immunol Res* 25: 97–113.

Glennie, M.J., Johnson, P.W. (2000) Clinical trials of antibody therapy. *Immunol Today* 21: 403–410.

Green, M. (2002) Comparability studies for human therapeutics, preclinical and pharmacokinetic aspects. In *Comparability Studies for Human Plasma-Derived Therapeutics – Workshop*. http://www.fda.gov/cbr/minutes/plasma053002.htm.

Griffiths, E. (1987) WHO Expert Committee on Biological Standardization. Highlights of the Meeting of October 1996. *Biologicals* 25: 339–362.

Karpas, A., Dremucheva, A., Czepulkowski, B.H. (2001) A human myeloma cell line suitable for the generation of human monoclonal antibodies. *Proc Natl Acad Sci USA* 98: 1799–1804.

Klingbeil, C., Hsu, D.H. (1999) Pharmacology and safety assessment of humanized monoclonal antibodies for therapeutic use. *Toxicol Pathol* 27: 1–3.

Kohler, G., Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.

Mire-Sluis, A.R., Barrett, Y.C., Devanarayan, V., Koren, E., Liu, H., Maia, M., Parish, T., Scott, G., Shankar, G., Shores, E., Swanson, S.J., Taniguchi, G., Wierda, D., Zuckerman, L.A. (2004) Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. *J Immunol Methods* 289: 1–16.

Reichert, J.M., Rosensweig, C.J., Faden, L.B., Dewitz, M.C. (2005) Monoclonal antibody successes in the clinic. *Nat Biotechnol* 23: 1073–1078.

Serabian, M.A., Pilaro, A.M. (1999) Safety assessment of biotechnology-derived pharmaceuticals: ICH and beyond. *Toxicol Pathol* 27: 27–31.

Stein, K.E. (1997) Overcoming obstacles to monoclonal antibody product development and approval. *Trends Biotechnol* 15: 88–90.

International HapMap Consortium (2005) A haplotype map of the human genome. *Nature* 437: 1299–1320.

Trang, J.M. (1992) Pharmacokinetics and metabolism of therapeutic and diagnostic antibodies. In: Ferraiolo, B., Mohler, M.A., Gloff, C.A. (eds) *Protein Pharmacokinetics and Metabolism*. New York: Plenum Press, pp. 223–270.

Treacy, G. (2000) Using an analogous monoclonal antibody to evaluate the reproductive and chronic toxicity potential for a humanized anti-TNFalpha monoclonal antibody. *Hum Exp Toxicol* 19: 226–228.

Wilson, N., Hardisty, J.H. (2001) Short-term, subchronic and chronic toxicology studies. In: Hayes, W. (ed.) *Principles and Methods of Toxicology*. Philadelphia, PA: Taylor and Francis, pp. 917–957.

13
Intellectual Property Issues

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13.1
Introduction

Intellectual property (IP) is a core aspect of any commercialization effort in the pharmaceutical industry. While protecting the ownership of substances of therapeutic value has always been a central concern for the pharma companies, recent developments in IP laws, the steadily increasing number of patents on both methods and compounds, and not least a fair number of high-profile litigation cases have put the IP area into the spotlight. The court battles especially have given the field a somewhat mixed reputation. While everyone agrees that protecting one's inventions is necessary, there are also some concerns that extensive IP protection on every aspect of research may inhibit research and drug development in the long run. Before analyzing the specific intellectual property right (IPR) issues on therapeutic antibodies, it may therefore be helpful to summarize two central aspects of IP protection.

13.2
Why Intellectual Property Rights are Important

While trademarks and copyrights do play a role in product development and sales and marketing of a product, the most relevant aspect of the property law for technologies and products in the pharmaceutical industry pertains to patents. Patents are not at all a new invention. The patent system dates back to the seventeenth century, and terms such as "international priority dates" were introduced more than 100 years ago. A patent is a deal between an inventor and the society. If an inventor is willing to disclose his or her invention to the public in enough

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detail that anyone could copy it, then the inventor should be allowed for a certain time period to have the sole right of commercializing the invention. After this time period, however, all rights to the invention would belong to the public, and anyone would be free to use it. At its very core, a patent is one of the very few monopolies accepted in today's world.

The major importance of patents for the pharma industry is based on the huge costs of drug development and the associated risks. A company involved in R&D of drugs needs to cover the costs for their activities, both for work done on the compounds introduced into the market, as well as for the many projects that do not come to fruition. The time span given by the patent on a compound, where the drug developer is essentially free of competition, is the only time in which those investments can be recuperated. As soon as the patent expires, other companies that have not invested in the drug development are free to compete. This is the reason why patenting is central not only for large pharma companies, but also for universities and small companies, which research on new compounds or methods.

Most players in the field will not have the knowledge or the financial capacity to follow a drug development project from researching the lead to market introduction; most will need to involve other players sooner or later. A large company is not likely to join any project on a compound with unclear IP ownership, as this may mean that its investments might not bring any future monetary value.

The second crucial issue with patents is to understand that a patent is by its very nature a negative right. A patent and the monopoly associated with it does not allow its owner to do whatever is claimed in the patent. However, it gives the owner the right to stop others from doing what is claimed in the patent. To give an example, a patent might cover a specific antibody and its use as therapeutic agent against cancer. In this case, anyone who wants to market this antibody for this purpose will need a license from the owner of this patent. However, the owner of the patent might have used a method to find the antibody covered by IP from a third party. To produce that antibody, he or she might need a production method, which is patent protected, too. It may also be that the antigen recognized by the antibody is patent protected, together with all ligands binding to it for therapeutic purposes, including antibodies in general. In such a scenario, the owner of the patent on that specific antibody will need to negotiate three licenses to be entitled to commercialize the antibody – one each for the research tool, the production method, and the antigen. In each case a license might bear payment of significant royalty by the owner of the antibody. This phenomenon is known as "royalty stacking," as royalties are a very common condition in licenses. Given the large number of patents filed or granted, royalty stacking is a significant issue in the pharma field in general, and the recombinant antibody field, in particular.

A company having central patents on key technologies or compounds is in a strong position in a particular field of product development. Indeed, any company having a proprietary position beginning with the development tools to produc-

tion, which are covered largely by its own patents without the need of paying large amounts of license fee for any reach-through IPs, will have a larger share on potential revenues and would enjoy a dominant position in a specific drug development sector. Consequently, from the reverse perspective, entering a field in which several aspects are already covered by IP might be less attractive for a commercial organization, unless the licenses can be readily obtained and/or the potential revenue generation prospects are very high.

We have highlighted below the status of major IP positions in the recombinant antibody area and some of the interesting developments that have taken place in the field in recent years. We have also considered providing a roadmap for the newcomers in the field to facilitate solving some of the puzzles of the recombinant antibody IPs.

13.3
Recombinant Antibody Technologies

As incredible as it may sound from the perspective of current biotechnology commercialization, it is a fact that Georges Kohler and Cesar Milstein, inventors of mouse hybridoma, never attempted to patent their technology. Although many companies since established have utilized freely available mouse monoclonal antibody technology, only one completely mouse antibody is currently in the market, primarily because of the deleterious effect of mouse antibodies in humans, commonly known as the HAMA (human antibody mouse antibody) response. Of course, hybridoma technology has significantly improved during the past decades to generate hunanization technology, chimeric antibody technology, and completely human antibody technology. This has also led to the generation of patents covering every single aspect of such technologies and any improvements thereon, and production of antibodies generated by such technologies.

Because of this plethora of patents, many companies interested in entering the antibody field have been navigating the IP landscape with significant caution, while some of the IP holders in this field have been embroiled in lengthy legal battles to sort out their exact stake about the freedom of operation. Indeed, a great many elements involving discovery, engineering, and manufacturing of an antibody need to be considered in the case of production and marketing of a monoclonal antibody (mAb). Consequently, both dominant and reach-through IP issues need to be sorted out by an antibody developer and company financiers since these factors influence directly many facets of conducting a business in the mAb area, such as permissibility of development, freedom of operation as well as the royalty stacking issues. Most important IP issues in the mAb field deal with the following areas: (1) humanization of mouse monoclonals, (2) human antibody isolation by transgenic mouse system, (3) application of phage display in generating human antibodies and antibody fragments *in vitro*, and (4) production and manufacturing of humanized and/or human antibodies.

13.4
Antibody Humanization

Celltech Group plc (currently UCB SA) and Protein Design Labs (Currently PDL Biopharma) are the two companies that have the proprietary rights in the area of humanization of antibodies. Celltech's process is covered under the Adair patent (US 5,859,205), while Protein Design Labs has broad-ranging intellectual property coverage in the humanization process, covered under the Queens patents (US 5,585,089, US 5,693,761, US 5,693,762, US 6,180,370). PDL appears to have the dominant position in humanization technology, which it utilizes along with its in-house expertise to develop its own antibodies as well as to humanize promising murine antibodies of its partner companies. For a protracted period of about 6 months in 2003, Genentech and Protein Design Labs had developed significant disputes regarding PDL's antibody humanization patents and certain of Genentech's humanized antibodies. However, in late 2003, the two companies announced the resolution of such disputes and agreed that Genentech would exercise licenses under the patent licensing master agreement between the parties for various humanized products. Currently, there are seven marketed products that utilize PDL's humanization technology and more than 40 humanized antibodies in late stage clinical trials.

Companies currently holding patent licenses for technologies responsible for the discovery of various marketed antibodies are shown in Table 13.1.

13.5
Human Antibody Technology

Two primary technologies used for the discovery of recombinant human antibodies are transgenic mouse system and phage display method. Two leading companies that have strong intellectual patent position in the transgenic mouse technology area are Abgenix and Medarex. Abgenix's technology is known as XenoMouse technology, while Medarex's transgenic mouse system is called HuMAb-Mouse. Abgenix was launched as a wholly-owned subsidiary by Cell Genesys in 1996 by incorporating the XenoMouse technology as its primary technology platform. Medarex obtained its strong IP in its transgenic mouse technology by the acquisition of GenPharm in October of 1997. Although both companies were initially at bitter odds with each other, their cross-licensing agreement in March 1997 of their respective IP rights has proved to be a successful strategy for both companies since each has generated well over 50 collaborations and partnerships since conclusion of this agreement. Both technologies have successfully produced an exceedingly increased number of human antibodies, of which at least 33 are in the clinic with no apparent patient immune response to the products (*Nature Biotechnology*, September 2005).

Unlike the transgenic mouse system, there are several players in the *in vitro* human antibody discovery area who have certain proprietary rights applicable to

Table 13.1 Patented technologies applied to the FDA approved therapeutic monoclonal antibodies.

Product name/type	*Developer/marketer*	*Indication*	*Mechanism of action*	*Year approved*	*Patented technologies of organizations*
Orthoclone OKT3 Murine	Ortho Biotech	Acute kidney transplant rejection	Anti-CD3	1986	–
Zevalin Murine	IDEC Pharma (currently Biogen IDEC), Schering AG	Relapsed or refractory low-grade, follicular, or transformed B-cell non-Hodgkin's lymphoma (NHL)	Anti-CD20	2002	Corixa (GlaxoSmith Kline)
Bexxar Murine	Corixa, Glaxo Smith Kline	NHL, CLL	Anti-CD20	2003	University of Michigan
ReoPro Chimeric		Coronary intervention and angioplasty	GpIIa/IIIb antagonist	1994	Centocor, Malvern, PA
MAbThera/ Rituxan Chimeric	IDEC Pharma (Biogen IDEC), Genentech, Roche	CD20-positive non-Hodgkin's lymphoma	Anti-CD20	1997	Celltech
ReoPro Chimeric	Centocor (J & J), Eli Lilly	Refractory unstable angina	GpIIa/IIIb antagonist	1997	Celltech
Synagis Chimeric	MedImmune, Gaithersburg, MD/ Abbott, Chicago, IL	Respiratory syncytial virus disease	Anti-RSV F protein	1998	PDL, Celltech, Genentech, Centocor
Simulect Chimeric	Novartis Pharma	Kidney transplant rejection	Inhibits interleukin-2 activation of T cells	1998	Celltech
Remicade Chimeric	Centocor (J & J), Schering-Plough	Rheumatoid arthritis	Anti-TNF-α	1999	Celltech, Genentech
Erbitux Chimeric	ImClone Systems, Bristol Meyers Squibb, Merck KgaA	Colorectal cancer	Anti-EGFR	2004	Genentech

Table 13.1 *Continued*

Product name/type	*Developer/marketer*	*Indication*	*Mechanism of action*	*Year approved*	*Patented technologies of organizations*
Zenapax Humanized	PDL, Roche	Kidney transplant rejection	Inhibits interleukin-2 activation of T cells	1997	Celltech
Herceptin Humanized	Genentech, Roche	Metastatic breast cancer	Anti-Her-2/neu receptor	1998	PDL, Celltech
Remicade Humanized	Centocor	Crohn's disease	Anti-TNF-α	1998	
Mylotarg Humanized	Celltech, Wyeth	Chemotherapeutic mAb for the treatment of CD33-positive acute myeloid leukemia in patients 60 and older in first relapse	Anti-CD33	2000	PDL
Campath Humanized	Genzyme (Ilex Oncology), Berlex (Schering AG)	B-cell chronic lymphocytic leukemia (B-CLL)	Anti-CD52	2001	Cambridge University, BTG
Xolair Humanized	Genentech,Novartis Pharma, Tanox	Allergic asthma	Anti-IgE	2003	PDL
Raptiva Humanized	Genentech, XOMA	Psoriasis	Prevention of the activation of T cells and their migration to the site of inflammation	2003	PDL
Avastin Humanized	Genentech	Angiogenesis in colorectal cancer	Anti-VEGF	2004	PDL
Humira Human	Cambridge Antibody Technology, Abbott	Rheumatoid arthritis	Anti-TNF-α	2002	CAT, MRC, Scripps, Stratagene, Genentech

antibody discovery. Some of the forerunners are Cambridge Antibody Technologies (CAT), Morphosys, Dyax, Biosite, BioInvent, Affitech, and Xoma. However, all of these companies realized that they need other IPs from within their peer groups to be able to practice antibody discovery and development for commercialization purposes. Consequently, during the past several years significant cross-licensing of IPs within these group of companies have taken place.

CAT originally acquired certain aspects of its core technology platform from the Medical Research Council of the UK and was issued six patents in the US (Table 13.2) as well as one in Europe (EP0368684). In addition, CAT was recently issued several continuations in part and divisional patents. CAT's core technologies are covered by three main families of patents, commonly known as Winter II, Griffiths and McCafferty patents. Winter II patent covers antibody expression libraries, McCafferty patent is for phage display, and the Griffiths patent is for the isolation of human antibodies to human proteins by phage display.

The US Winter II patent (US 6,248,516) is entitled "Single domain ligands, receptors comprising said ligands, methods for their production, and use of said ligands and receptors," and was issued on June 19, 2001. It is generally directed to antibody variable domain expression libraries carrying a diversity of CDR3 sequences and methods of making such libraries. This patent is co-owned by the UK Medical Research Council, Scripps Research Institute, La Jolla, CA, USA and

Table 13.2 Key antibody phage display patents of Cambridge Antibody Technology.

Patent	*Description*	*Patent status United States*	*Europe*
Griffiths	Methods for obtaining of anti self antibodies from antibody phage display libraries (covers isolation of human antibodies against all human proteins except for those that generate natural antibodies in humans)	Granted in March, 1999 (US 5,885,793)	Pending
McCafferty	Covers phage display of antibody fragments	Granted in October, 1999 (US 5,969,108)	Granted in November, 1996 (EP 589 877)
Winter II	The use of antibody genes for constructing a library	Granted in June, 2001 (US 6,248,516)	Granted in April 1994 (EO-O-368-684)[a]
Huse/Lerner/Winter	The use of antibody genes for constructing a library	Granted in September 2001 (US 6,291,158; US 6,291,159; US 6,291,160 and US 6,291,161)	Pending

a Claim 32 of the Winter II patent was subsequently amended following opposition by Morphosys.

Stratagene Corporation. CAT obtained an exclusive commercialization rights to the IP. CAT obtained four US patents covering antibody expression libraries – the "Huse/Lerner/Winter" patents entitled: "Method for Producing Polymers Having a Preselected Activity" (US 6,291,158 and US 6,291,159) and "Method for Tapping the Immunological Repertoire" (US 6,291,160 and US 6,291,161). The patents are generally directed to nucleic acid libraries for expression of functional immunoglobulin variable domains (or portions thereof) and methods of making such libraries. All of these patents are co-owned by the Medical Research Council, The Scripps Research Institute and Stratagene. Following the settlement of interference proceedings in June 1999, CAT is the sole exploiter of the intellectual property rights arising under the patents, subject to certain rights reserved by the co-owners and their pre-existing licensees.

With its dominant IP position in antibody libraries and antibody phage display, CAT pursued vigorously for several years both execution of its IP rights as well as maintaining those rights through litigation in some cases, such as those with Morphosys and Crucell, and business negotiations with others, such as those with Xoma and Dyax.

Morphosys owns the HuCAL antibody library system comprising synthetically prepared genes. The company has since expanded its technology to include HuCAL-GOLD, Cys Display, HuCAL-EST etc. Table 13.3 lists Morphosys' most important patents.

Table 13.3 Key antibody phage display patents of Morphosys Technology.

Patent (primary authors)	*Description*	*Patent status*	
		United States	*Europe*
Pack, Lupas	Mini antibodies, kept together by heterodimerization	Granted in Sept 2001 (US 6,294,353)	Pending
Pack, Lupas	Mini antibodies, kept together by heterodimerization of complementary association domains	Granted in Feb 2004 (US 6,692,935)	Pending
Knappik et al.	HuCal libraries, construction of modular synthetic antibody libraries, methods for using them.	Granted in Oct 2001 (US 6,300,064)	Granted in June 2002 (EP 859841)
Knappik et al.	HuCal libraries, antibody libraries based on a consensus framework, methods for using the libraries	Granted in Feb 2004 (US 6,696,248) and Mar 2004 (US 6,706,484)	Pending
Frisch et al.	HuCal libraries, displaying ESTs	Granted in Nov 2003 (US 6,300,064)	Pending
Lohning	HuCal Gold libraries, CysDisplay	Granted in Jun 2004 (US 6,753,136)	Pending

Dyax owns the Ladner patent family (US 5,223,409, US 5,403,484, US 5,571,698, US 5,837,500), which is a dominant patent family for practicing basic phage display technology. Additionally, Dyax has been practicing its Fab antibody library, the basic technology that it acquired through the purchase of Target Quest NV, a Dutch company. Some of the most important patents of Dyax in the phage display area has been noted in Table 13.4.

Another leading phage display company is San Diego, CA-based Biosite, which obtained patent rights to US Patents US 5,427,908 and US 5,580,717 and European Patent EP 527839B1, through acquisition from Affymax Technologies N.V. in 1998. The patents are directed to phage display of Fab and multichain antibodies wherein one of the polypeptide chains comprises a heavy-chain variable domain or region and a light-chain variable domain or region. Additionally, Affimed, based in Heidelberg, Germany, cross-licensed its IgM display library against the Ladner IP estate of Dyax, as well as obtaining a license from CAT to carry out its antibody discovery activities.

The Swedish antibody company BioInvent utilizes a phage display library system called n-CoDeR for the fast and efficient discovery of human antibodies. It is utilized in the development of proprietary drug candidates in diseases areas as HIV, atherosclerosis, cancer, and osteoarthritis as well as in development partnerships with pharmaceutical and biotech companies. The n-CoDeR library

Table 13.4 Key antibody phage display patents of Dyax Technology.

Patent (primary author)	*Description*	*Patent status*	
		United States	*Europe*
Ladner	Displaying proteins other than scFv on the surface of viruses/phages	Granted in June 1993 (US 5,223,409)	Pending
Ladner	A virus, presenting a protein other than scFv fused to a coat protein of said virus on the surface of the virus	Granted in April, 1995 (US 5,403,484)	Pending
Ladner	Making display libraries of proteins other than scFv, and selecting a member with desired binding properties from that library	Granted in May, 1996 (US 5,571,698)	Pending
Ladner	Making display libraries of proteins other than by mutating a protein via randomization, and selecting a member with desired binding properties from that library	Granted in November 1998 (US 5,837,500)	Pending
Ladner	Making display libraries of antibodies, and selecting a member with desired binding properties from that library	Granted in Dec 2005 (US6,979,538)	Pending

is covered primarily by the EP1352959, EP0988378B1, US 6 989 250. Bioinvent has also finalized a cross-licensing agreement with Xoma, and obtained licenses from CAT, Micromet, and Dyax. BioInvent's more than 160 patents and pending applications cover its core technology to discover antibody candidates and various aspects thereof, as well as antibody product candidates in development and their use in therapy.

Another approach is followed by Domantis, which is located in both Cambridge, UK and Cambridge, MA, USA. The Company's Domain antibodies, about 13 kDa in size, correspond to either the variable heavy or the variable light region of a human antibody. Some features of this format allow for specific selection methods, which was developed by Domantis. Domain antibody libraries, their selection methods, and therapeutic formats with extended serum half-life are covered by WO05/035572, WO04/101790, WO04/081026, WO04/058821, WO04/003019, and WO03/002609 and their equivalents, amongst others. Furthermore, Domantis has licenses of Winter II, Huse/Lerner/Winter, McCafferty and Griffiths from the UK Medical Research Council. The relevance of those patents has been explained above in more detail.

Affitech, based in Oslo, Norway has the IP for a phagemid system of antibody display that contains full-length pIII protein. It has been granted the European rights (EP 547201) to its already approved US patent (US 5,849,500) and several divisionals (US 5,985,588, US 6,127,132, US 6,387,627, and US 6,730,483) on the use of phagemid display of antibodies and antibody fragments that it exclusively licensed from Deutsches Krebsforschungszentrum Stiftung des Offentlichen Rechts (German Cancer Research Center), Heidelberg, Germany. It has another European divisional application currently pending (EP 1065271), however, both the European patent and the divisional is being opposed by CAT at the European patent office. Recently, the original patent has been revoked in the first court hearing but the ruling is under consideration for an appeal. It should be emphasized here that since Norway is not covered by most of the dominant antibody display patents, Affitech's business strategy allows the company to practice all aspects of phage display-based antibody selection without any license from the primary IP holders, and to export non-infringing affinity-matured antibody products for the international market. Additional to that Affitech has recently entered into cross-licensing agreements with Dyax for Ladner IP and with XOMA for its antibody expression technology. Affitech's patent portfolio is summarized in Table 13.5.

CAT and Morphosys were entangled in litigation for several years over their respective IPs. However, since the ruling by the US District Court in March 2002 that Morphosys' HuCAL antibody library technology is distinct and independent from CAT's patent coverage by Griffith's IP, the two companies have ended their disputes after signing cross-licensing agreements. Additionally, CAT has also cross-licensed its phage display patents with (1) Dyax's Ladner phage display IP, (2) Xoma's antibody expression technology, (3) Micromet and Enzon Pharmaceutical's single chain antibody technology, and (4) Crucell's Mabstract phage display technology.

Table 13.5 Phage display library intellectual properties of Affitech.

Inventors:	Breitling, Little, Dübel, Braunagel and Klewinghaus
Priority date:	08 July 1991
Owner:	German Cancer Research Centre (DKFZ)
Licensee:	Affitech AS
License terms:	Exclusive with right to sublicense

Patent/application	*Priority date*	*Pat./appl. no.*	*Status*
European parental	8 July 1991	EP 0547201	Granted*
European divisional	8 July 1991	EP 1065271	Pending**
US parental	8 July 1991	US 5,849,500	Granted
US divisional 1	8 July 1991	US 5,985,588	Granted
US divisional 2 ***	8 July 1991	US 6,127,132	Granted
US divisional 3	8 July 1991	US 6,387,627	Granted
US divisional 4	8 July 1991	US 6,730,483	Granted

* Revoked, revocation under appeal
** Under opposition
*** Title: "Phagemid library for antibody screening"

13.6 Antibody Production

"Boss" patent of Celltech and old "Cabilly" of Genentech were the earliest patents for making monoclonal antibodies. Boss patent covered the production of engineered antibodies and antibody fragments together with vectors and host cells related to these processes. The old Cabilly patent (US 4,816,567) covered the production of altered and native immunoglobulins in recombinant cell culture. In 2001 Genentech obtained New Cabilly patent, the circumstances leading up to the issuance of this patent and the impact of the New Cabilly is detailed below. The other dominant player in the antibody production area is Xoma Corporation of Berkeley, CA, which holds several IPs involving expression of antibodies in bacterial systems.

13.6.1 Genesis of New Cabilly

Celltech has had a broad ranging intellectual property position in recombinant antibody production in various cell types under the Boss patent (US 4,816,397), which was valid until 2006. The Boss patent covered production of engineered antibodies but not murine antibodies produced from hybridomas and may not cover production of antibodies from transgenic mice. Consequently, companies producing chimeric and humanized antibodies and those produced by phage display were paying 1–3% royalty to Celltech. Celltech is also in possession of Bodner patent (US 5,219,996) for the production of antibody fragments in bacte-

ria. This patent runs up to 2008 and is of significance to Celltech for the development of its own product line as well as for generating royalties from other companies. Genentech, which owns the Cabilly patent (US 4,816,567, now known as the old Cabilly patent) for the production of altered and native immunoglobulins in recombinant cell culture, has had a cross-licensing deal with Celltech for payment of royalties to each other on sales of antibody products. Both the Boss and Cabilly patents were issued on the same date, March 28, 1989, even though Celltech's patent application was filed on March 25, 1983 while Genentech's was done on April 8, 1983. Prior to the grant of the old Cabilly patent, Genentech filed a continuation application, which was further amended in March 1990 and contained copied claims of the Boss patent. Genentech also applied to the US patent office for declaring an interference between Celltech's patent and Genentech's application. After a lengthy proceedings which lasted for 7 years, the Board of Interferences favored the Celltech patent. Genentech appealed the decision for a summary judgment motion in the US District Court in San Francisco and provided new evidence claiming to predate Celltech's original filing date. However, Genentech's appeal was denied, and a mediation process was ordered by the US District Court of the Northern District of California in San Francisco. This resulted in the following: (1) a settlement between the two companies was reached and Celltech did not challenge Genentech's priority of invention; (2) a US Patent No. US 6,331,415 ("New Cabilly patent") was granted to Genentech on December 18, 2001, and the US Patent and Trademark Office revoked the Boss patent of Celltech. This ruling resulted in the highly unusual situation that a patent essentially got a lifetime of 29 years granted, from 1989 to 2018. The reason is a quirk in the US law. In 1989 the lifetime of a patent was 17 years from the grant. However, before the mediation leading to Cabilly II was initiated the law changed, and the patent lifetime is now 20 years from filing; Cabilly II in its current form was technically filed during the mediation in 1998.

The newly issued patent has 36 claims for recombinant methods and vectors to produce immunoglobulin (Ig) molecules and functional Ig fragments in transformed host cells. The new Cabilly patent extends patent coverage on antibody manufacture until 2018, and Celltech will receive compensation from Genentech in terms of income from sales of products, which would otherwise have been covered by the Boss patent until its normal expiry date in 2006. Genentech also granted license of new Cabilly patent to Celltech for the production of its products.

The New Cabilly patent covers one of the principal processes used in the manufacturing of therapeutic and diagnostic antibody drugs, and could potentially block the production of antibody products by rival companies or increase their royalty burden.

Indeed, Gaithersburg, MD-based MedImmune has filed a lawsuit on April 11, 2003 in the US District court in Los Angeles charging that the settlement of Celltech and Genentech was illegal and anticompetitive under both Federal and State anti-trust laws and California's unfair competion law.The Plaintiff stated that Celltech and Genentech essentially received an exclusive and dominant posi-

tion until New Cabilly patent expires in 2018, essentially a 29-year monopoly. While the expiry of Boss and old Cabilly patents in 2006 would have allowed free access to the antibody manufacturing processes, various antibody manufacturers would have to consider now obtaining a license from Genentech. The case was resolved in late 2003 (MedImmune v. Genentech, Inc. et al., 2003 U.S. Dist. LEXIS 23443 (C.D. Cal. 2003)), amended Jan 14, 2004. Judge Pfaelzer of the District Court held that MedImmune is a licensee in good standing, and can therefore not bring forth a declaratory action for formal reasons, as there was no actual case of controversy.

But more importantly, MedImmune's alleged anti-trust and unfair competitions violations were rejected. Judge Pfaelzer held that the settlement between Genentech and Celltech was protected by the Noerr-Pennington doctrine, and granted a summary judgment for the defendants. The Noerr-Pennington doctrine permits collaboration between competitors to petition the government to take action that may restrain competition without incurring anti-trust liability by the act of collaborating. In the given case, the government action was the mediation between Genentech and Celltech with respect to Boss, Cabilly I and Cabilly II. The District Court found that the mediation of Judge Chesney was entitled to be protected under the Noerr-Pennington doctrine.

After an appeal, the Federal Circuit affirmed the District Court in a recent opinion (October 18, 2005). The Federal Circuit also dismissed the two further arguments of MedImmune: first, MedImmune had stated that permitting them to attack Cabilly served public interest, and second, according to MedImmune, if the Federal Circuit follows the District Court's argumentation that a licensee in good standing should not have the right to attack the rights the license is based upon (which it did), than the anti-trust and unfair competition counts should not be judged by the Federal Circuit but the United States Court of Appeals. The Federal Court did not follow MedImmune's argumentation in those cases.

In consequence, Cabilly II is still valid, and stronger than ever. There are some indications that the case may be brought to the Supreme Court. However, there are reasons to doubt that the Supreme Court will respond to the case.

13.6.2 Xoma Patents

Xoma Limited of Berkeley, California also holds a strong IP position in the expression of antibodies, but in bacterial systems. Xoma has received nine US patents to date relating to aspects of its bacterial cell expression system. The expression system includes an *araB* promoter, which allows controlled expression of the desired protein in bacterial host cells, and a *pelB* signal sequence, which allows protein secretion from the host cell. Xoma has also developed a genetically engineered *E. coli* host strain and an easy-to-use fermentation process that complements the *araB* and *pelB* technologies.

Out of the nine patents, six patents broadly cover key methods for the secretion of functional antibody molecules from bacteria, including antibody fragments

Table 13.6 List of Xoma's antibody expression intellectual properties.

Title	*Inventors*	*US Patents*
Modular Assembly of Antibody Genes, Antibodies Prepared Thereby and Use	Robinson, Liu, Horwitz, Wall, Better	5,618,920
		5,595,898
		5,576,195
		5,846,818
Novel Plasmid Vector with Pectate Lyase Signal Sequence	Lei, Wilcox	6,204,023
		5,698,435
		5,693,493
		5,698,417
		5,576,195
AraB Promoters and Method of Producing Polypeptides, Including Cecropins, by Microbiological Techniques	Lai, Lee, Lin, Ray, Wilcox	5,846,818

such as Fab and single-chain antibodies. On March 20, 2001, Xoma was issued its sixth patent in its antibody expression family of patents and the third patent in this family that broadly covers methods for the secretion of functional immunoglobulins from bacteria, including single-chain antibodies and antibody fragments (US 6,204,023, "Modular Assembly of Antibody Genes, Antibodies Prepared Thereby and Use"). Bacterial antibody expression is a key enabling technology for the discovery and selection, as well as the development and manufacture, of many recombinant antibody-based pharmaceuticals. Antibody discovery by phage display technology, for example, depends upon the expression of antibody domains in bacteria as properly folded, functional, secreted proteins, as described in Xoma's patent claims.

Xoma's other patents include one that relates to improved methods and genetic constructs for process control utilizing the *araB* promoter. Two patents relate to the technology for *pelB* signal sequence secretion. A number of foreign patents also have been granted to Xoma, which, along with pending applications, correspond to the issued US patents. Xoma's primary US-granted patents are listed in Table 13.6.

13.7 Litigations and Cross-licensing

From 1999, CAT was embroiled in patent opposition or infringement suits with Morphosys until December of 2002. The litigations between the companies were

fought in the US courts regarding CAT's Griffiths, McCafferty, Winter II, and Winter/Lerner/Huse patents. Additionally, MorphoSys also launched opposition at the European Patent Office against CAT's Winter II and McCafferty patents. However, since the ruling by the US District Court in March 2002 that Morphosys' HuCAL antibody library technology is distinct and independent from CAT's patent coverage by Griffith's IP, the two companies have ended their disputes after signing cross-licensing agreements.

There was, however, a substantial amount of payment from Morphosys to CAT, which included an annual payment of 1 million euros over the next 5 years. CAT will also receive other financial consideration from MorphoSys' activities related to its HuCAL GOLD libraries for a defined period of time in addition to milestone and royalty payments under the license for products developed using previous HuCAL libraries. Furthermore, CAT will receive an equity stake of 588 160 ordinary shares in MorphoSys under the license agreement. MorphoSys retains the option to buy out its obligations to CAT for a predefined fixed amount at any time during the duration of the agreement.

Both the ruling and the subsequent cross-licensing agreement should allow Morphosys to indemnify its several collaborators about the sole proprietorship of its HuCAL library and any potential patent infringement issues. Additionally, CAT has also cross-licensed its phage display patents with (1) Dyax's Ladner phage display IP, (2) Xoma's antibody expression technology, (3) Micromet and Enzon Pharmaceutical's single chain antibody technology and Crucell's Mabstract phage display technology.

CAT and Dyax expanded their licensing agreement of 1997 under which Dyax licensed its Ladner phage display patents to CAT in exchange for receiving a worldwide license for research and to develop therapeutic and diagnostic antibody products under all the antibody phage display patents controlled by CAT. In return, CAT will receive milestone and royalty payments on antibody products advanced into clinical trials by Dyax and Dyax's customers. CAT also gains the option to co-fund and co-develop with Dyax antibodies discovered by Dyax, as well as the right to share in Dyax's revenues from certain other applications of antibody phage display. In a further expansion of the agreement, in September 2003, Dyax realized from CAT, among other benefits, an increased number of options for licenses to develop therapeutic and diagnostic antibody products under CAT's patents for Dyax's own use and on behalf of its partners. CAT and Dyax have further agreed that, as a result of various co-licensing agreements, CAT shall not have to pay royalties to Dyax in respect of any antibody products CAT develops including Humira.

CAT also finalized a cross-licensing agreement with XOMA under which CAT and its collaborators receive license to use the Xoma antibody expression technology for developing products using CAT's phage-based antibody technology. In return, Xoma receives a license payment, and in addition receives the right to use CAT's phage antibody libraries for its target discovery and research programmes, with an option to develop antibodies into therapeutics. Xoma will pay license

payments to CAT if the company identifies and develops any therapeutic antibodies using CAT's libraries.

CAT and Crucell also formed a worldwide license agreement under which Crucell receives access to all CAT's antibody phage display technology patents, both pending and granted. This provided Crucell freedom under the CAT patents to fully exploit its proprietary MAbstract technology and other phage display technology. In return, CAT will receive an initial license fee from Crucell, and obtains an option to develop certain antibodies, opening the way for further collaboration between the companies. CAT will also receive milestone and royalty payments for any antibody products that Crucell or its partners develop that are derived from Crucell's MAbstract technology or other technology involving phage display.

13.8
Other Cross-licensing

Other notable cross-licensing agreements of recent years involved the antibody expression technology owned by XOMA. Among others, XOMA has cross-licensed with Morphosys, Dyax, BioInvent, and Affitech.

Indeed, such cross-licensing arrangements have provided a freedom-to-operate position for practicing human antibody discovery *in vitro* to several companies, akin to the transgenic mouse system of Abgenix and Medarex. Hopefully this will help to increase the interest level of many of the companies contemplating entering into the human antibody therapeutic area, since the constraints of entry to a greater extent has been minimized by the co-licensing process.

13.9
Litigation between CAT and Abbott

A recently resolved litigation case involved the distribution of royalties on Humira between CAT and Abbott. Humira is the first fully human antibody approved by the FDA for marketing on December 31, 2002. It is currently used for the treatment of early and late rheumatoid arthritis as well as psioratic arthritis. The antibody targets tumor necrosis factor alpha (TNF-α) and is in phase III trials for, amongst others, Crohn's disease and juvenile arthritis. Humira sales in 2005 were at $1.4 billion, and are expected to rise to $1.9 billion in 2006.

Humira was developed by CAT in collaboration with Knoll, which was later acquired by Abbott. The basic structure of the deal was that CAT was using their phage display technology to identify antibodies of clinical interest against targets provided by Knoll/Abbott. As a payment, CAT was to receive royalties at a little bit over 5%. However, under certain circumstances, the contract allowed for an offset of royalties due to CAT, if third-party rights were necessary to utilize CAT technology. Under that situation, the minimum royalty level to be received by

CAT was set at 2%. It should be mentioned that CAT was obliged under the initial contract to give a certain part of their royalty to the Medical Research Council (MRC), Scripps Institute, and Stratagene, where the CAT technology originated.

Abbott has disclosed that it is paying royalties on Humira to Serono and Peptech, and it is likely that royalties are also paid to Genentech. Consequently, Abbott reduced CAT's share of the royalties. CAT filed for legal proceedings, as they did not believe that these third-party rights are necessary to utilize CAT's technology and hence such a third-party royalty should not be deducted from the royalties paid to CAT. The case was brought to the High Court in London in November/December 2004. The Court ruled strongly in favor of CAT. The argumentation of Abbott was discarded in full. Abbott appealed, but before the proceedings at the Court of Appeal were initiated, an agreement between the parties was reached. This agreement was not only between CAT and Abbott but included also the licensors of CAT. The details were disclosed by CAT as follows:

- Abbott will pay CAT the sum of US$255 million, which CAT will pay to its licensors, the MRC, Scripps Institute and Stratagene, in lieu of their entitlement to royalties arising on sales of Humira from 1 January 2005 onwards.
- Abbott will also pay to CAT five annual payments of US$9.375 million commencing in January 2006, contingent on the continued sale of Humira. US$2 million from each of these payments will be payable to CAT's licensors.
- Abbott will pay CAT a reduced royalty of 2.688% from approximately 5.1% on sales of Humira from January 1, 2005. CAT will retain all of these royalties. CAT will also retain royalties received from Abbott in respect of sales of Humira up to December 31, 2004, net of approximately £1187.6 million of which will be paid to its licensors.
- CAT will refund to Abbott approximately £9.2 million for royalties paid from January 1, 2005 through June 30, 2005. Abbott will also pay CAT a reduced royalty of 4.75% on any future sales of ABT-874, from which CAT will pay a portion to the MRC and other licensors (according to CAT's 1997 agreement with the MRC).
- Abbott will capitalize and amortize the upfront payment, net of the refund, and annual payments to CAT through the term of the agreement. When this amortization is combined with the revised royalty rate of 2.688%, the blended effective royalty rate is reduced from the approximate 5.1% as previously instructed by the Court.

13.10 Importation of Data

For several years companies have wondered whether data or drugs generated by molecular screening methods outside of USA could be imported into the US

without infringing a US patent, and if such activities constitute a "Product made" under the Process Patent Amendments Act of 1988. This Act was passed by the US Congress to protect holders of US process patents in response to concerns that competitors can avoid infringement of method patents simply by carrying out the methods in other countries and importing the products into the USA. The amended patent statute included the section 35 U.S.C.271(g) which states that importing, using, selling, or offering for sale in the US a product that was made abroad by a process protected by a US patent would constitute infringement of that patent.

A verdict on August 22, 2003, by the US Court of Appeals for the Federal Circuit in the case of Bayer AG vs Housey Pharmaceuticals has clarified the scope of patent protection and ruled that drug discovery data are not "product" under the Act.

In this case Housey, the patent holder, sued Bayer, alleging that Bayer's importation of drug discovery data to the US violated the Act. However, Bayer contended that it was importing only information, and since 271(g) applies only to manufacturing processes in which a product is physically made, it could not infringe the Housey patent claims. Housey argued that 271(g) should be interpreted broadly to cover any patented method irrespective of whether it produces a physical product or not. The lower court disagreed with Housey and ruled that drug discovery data are not "product" covered by the Act. This ruling was subsequently upheld by the Federal Appeals Court, and by so doing determined that Bayer's importation of screening data was not an infringement of the Housey patents.

As a result of this decision many international companies would be able to carry out their drug discovery screening efforts outside of the US. Here it should be stated that the US Senate has been considering changing laws in order to close the possibility of importing data, which might trigger a new round of licenses and litigation.

13.11
The Single-Chain Antibody Technology

Single-chain antibodies (SCAs) are composed of the antigen-binding regions of antibodies on a single polypeptide chain. Micromet AG of Munich, Germany has recently consolidated the SCA patent estate, first by acquiring on June 29, 2001 from Curis, Inc. (Cambridge, MA, USA) all of its patent rights and patent applications directed to SCAs and SCA fusion proteins, and following to that by forming an exclusive marketing partnership with Enzon (Piscataway, NJ, USA) for its patents, patent applications, and technology pertaining to SCAs. Consequently, the comprehensive IP portfolio includes patents and patent applications claiming broad aspects of SCA technology. Table 13.7 lists the EU and US rights of the combined SCA IP portfolio of Micromet/Enzon.

Table 13.7 Single-chain antibody intellectual property portfolio of Micromet/Enzon.

Country	*Title*	*Patent number*
USA	Single Polypeptide Chain Binding Molecules	4,946,778
USA	Single Polypeptide Chain Binding Molecules	5,260,203
USA	Single Polypeptide Chain Binding Molecules	5,455,030
USA	Single Polypeptide Chain Binding Molecules	5,518,889
USA	Single Polypeptide Chain Binding Molecules	5,534,621
CANADA	Single Polypeptide Chain Binding Molecules	1,341,364
EP	Single Polypeptide Chain Binding Molecules	0281604
USA	Computer Based System And Method For Determining And Displaying Possible Chemical Structures For Converting Double or Multiple Chain Polypeptides to Single Chain Polypeptides	4,704,692
EP	Method For The Preparation of Binding Molecules	0349578
US	Multivalent Antigen Binding Proteins	5,869,620
US	Multivalent Antigen Binding Proteins	6,121,424
US	Multivalent Antigen Binding Proteins	6,027,725
US	Methods for Producing Multivalent Antigen Binding Proteins (As Amended)	6,025,165
US	Linker For Linked Fusion Polypeptides	5,856,456
US	Linker For Linked Fusion Polypeptides	5,990,275
US	Antigen-Binding Fusion ProteinsPolyalkylene	5,763,733
US	Antigen-Binding Fusion ProteinsPolyalkylene	5,767,260
US	Stabilized Monomeric Protein Compositions	5,656,730
US	Stabilized Monomeric Protein Compositions	5,917,021
US	Single-Chain Antigen-Binding Proteins Capable of Glycosylation, Production And Uses Thereof	6,323,322
US	Method For Targeted Delivery of Nucleic Acids	6,333,396
US	Nucleic Acid Molecules Encoding Single-Chain Antigen-Binding Proteins (As Amended)	6,103,889
US	Polypeptide linkers for production of biosynthetic proteins	5,482,858
US	Polypeptide linkers for production of biosynthetic proteins	5,258,498
CAN	Targeted Multifunctional Proteins	1,341,415
AU	Targeted Multifunctional Proteins	612 370
AU	Targeted Multifunctional Proteins	648 591
EP	Targeted Multifunctional Proteins	0318554
EP	Targeted Multifunctional Proteins	0623679
US	Biosynthetic Antibody Binding Sites	5,132,405
US	Biosynthetic Antibody Binding Sites	5,091,513
US	Biosynthetic Antibody Binding Sites	5,476,786
US	Genetically Engineered Antibody Analogues and Fusion Proteins Thereof	6,207,804
US	Biossynthetic Binding Protein for Immuno-targeting	5,534,254
US	Biossynthetic Binding Protein for Immuno-targeting	5,837,846
US	Biosynthetic Binding Protein for Immuno-targeting	5,753,204
US	Serine-rich Peptide Linkers	5,525,491
CAN	Serine-rich Peptide Linkers	2,100,671
AU	Serine-rich Peptide Linkers	664 030
EP	Serine-rich Peptide Linkers	0573551
US	Methods and Compositions for High Protein Production from Non-native DNA	5,658,763
US	Methods and Compositions for High Protein Production from Non-native DNA	5,631,158
US	Methods and Compositions for High Protein Production from Non-native DNA	5,733,782

Source: Micromet.

13.12
US Patent Issued on Polyclonal Antibody Libraries

In January 2002, it was announced that a US patent (US 6,335,163 B1) has been issued to Boston University, research partner of Symphogen, which holds a worldwide exclusive license to this newly issued patent and to the previously issued methods patent (US 5,789,208). The patent claims relate to the methods for the creation and use of libraries of proteins which comprise polyclonal antibodies to a common antigen or group of antigens, receptor proteins with related variable regions, or other immune related proteins with variable regions. The company believes that this patent gives it a unique position in the development of recombinant polyclonal antibodies.

Some of the important US patents relevant to the discovery, development, and production of recombinant antibodies, mentioned in the text and their assignee names are listed in Table 13.8.

Table 13.8 Selected list of relevant US patents.

Patent number	*Commonly used name (where known)*	*Assignee/company*
US 4,816,397	Boss patent	Celltech Group
US 4,816,567	Old Cabilly patent	Genentech Inc.
US 5,219,996	Bodner patent	Celltech Group
US 5,223,409	Ladner patent family member	Dyax Corporation
US 5,403,484	Ladner patent family member	Dyax Corporation
US 5,427,908	Not known	Biosite Inc.
US 5,571,698	Ladner patent family member	Dyax Corporation
US 5,580,717	Not known	Biosite Inc
US 5,585,089	Queen's patent family member	Protein Design Labs
US 5,595,721	Not known	Corixa Corporation
US 5,693,761	Queen's patent family member	Protein Design Labs
US 5,693,762	Queen's patent family member	Protein Design Labs
US 5,723,323	Kauffman patent family member	Applied Molecular Evolution, Inc.
US 5,763,192	Kauffman patent family member	Applied Molecular Evolution, Inc.
US 5,789,208	Not known	Symphogen
US 5,814,476	Kauffman patent family member	Applied Molecular Evolution, Inc.
US 5,817,483	Kauffman patent family member	Applied Molecular Evolution, Inc.
US 5,824,514	Kauffman patent family member	Applied Molecular Evolution, Inc.
US 5,837,500	Ladner patent family member	Dyax Corporation
US 5,849,500	Breitling patent family member	Affitech AS
US 5,859,205	Adair patent	Celltech Group
US 5,885,793	Griffiths patent	Cambridge Antibody Technology

Table 13.8 *Continued*

Patent number	*Commonly used name (where known)*	*Assignee/company*
US 5,967,862	Kauffman patent family member	Applied Molecular Evolution, Inc.
US 5,969,108	McCafferty patent	Cambridge Antibody Technology
US 5,985,588	Breitling patent family member	Affitech AS
US 6,015,542	Not known	Corixa Corporation
US 6,051,230	Not known	Peregrine Pharmaceutical
US 6,054,561	Not known	Chiron corporation
US 6,090,365	Not known	Corixa Corporation
US 6,127,132	Breitling patent family member	Affitech AS
US 6,180,370	Queen's patent family member	Protein Design Labs
US 6,204,023	Antibody Expression patent family member	Xoma Ltd.
US 6,248,516	Winter II patent	Cambridge Antibody Technology
US 6,287,537	Not known	Corixa Corporation
US 6,291,158	Huse/Lerner/Winter patent	Cambridge Antibody Technology
US 6,291,159	Huse/Lerner/Winter patent	Cambridge Antibody Technology
US 6,291,160	Huse/Lerner/Winter patent	Cambridge Antibody Technology
US 6,291,161	Huse/Lerner/Winter patent	Cambridge Antibody Technology
US 6,300,064	HuCAL patent family member	MorphoSys AG
US 6,331,415	New Cabilly patent	Genentech Inc.
US 6,335,163		Symphogen
US 6,387,629	Breitling patent family member	Affitech AS
US 6,451,312	Not known	Peregrine Pharmaceutical
US 6,692,935	Not known	MorphoSys AG
US 6,696,248	HuCAL patent family member	MorphoSys AG
US 6,730,483	Breitling patent family member	Affitech AS

13.13 Conclusion

In essence, a company venturing into the mAb therapeutic area must consider at the outset which discovery tool is the company's preferred system. Is it (1) humanization of mouse or rabbit antibodies, (2) human antibodies from transgenic mouse, or (3) *in vitro* antibody display technology? Following that comes the decision of mAb formating: should it be a full-length IgG or antibody fragments. This decision, to a large extent, is dependent upon the cellular functions, targeting, and efficacy of the antibody. This will also lead to the selection of manufacturing

Table 13.9 Essential technologies in recombinant antibody and primary intellectual property holders.

Technologies	*IP holders*
Antibody discovery	
Humanization	PDL
Transgenic mouse system	Abgenix
	Medarex
In vitro display technology	Affitech
	CAT
	Dyax
	Morphosys
Antibody production	
Mammalian	Genentech
Bacterial	Xoma
Plants	Various companies
Aquatic	Biolex
Yeast	GlycoFi
Antibody formats	
Single chain	Micromet/Enzon
Fab	Dyax
Domain antibodies	Domantis
Antibody modifications	
Glycosylation	Glycart (a Roche company)
	GlycoFi
	BioWa
Antibody variants	AME (an Eli Lilly company)
	Xencor

system since a full-length IgG must be made from an eukaryotic expression system because of the presence of oligosaccharides in the Fc region, while scFv and Fab formats could be produced from a prokaryotic system because of the lack of glycosylation moieties in such molecules. There are also plants, yeast, and aquatic organisms available for production that several companies are attempting in lieu of mammalian cell systems.

Although we have not considered here IP issues relating to the effect of modified glycosylation or antibody engineering for generating mAbs with superior efficacy or higher production yield or improved binding to the Fc receptor, several companies, such as Applied Molecular Evolution, Xencor, Glycart, GlycoFi, and BioWa in recent years have built important IP estates in such areas.

While the advice of a good patent attorney is imperative for an organization interested in entering or dealing with the labyrinth of mAb IPs, Table 13.9 depicting various technologies important for mAb discovery and production and the companies holding the most important IPs in respective areas should facilitate initial assessment of the recombinant mAb landscape and evaluation of the business perspectives of an mAb product.

Part III
Beyond IgG – Modified Antibodies

1
Immunoscintigraphy and Radioimmunotherapy

Jason L. J. Dearling and Alexandra Huhalov

1.1
Introduction

This chapter aims to provide an introduction to ways in which antibodies can be modified to optimize their role in the detection and therapy of solid tumors. These modifications include: alterations to the protein structure in order to improve antigen binding and pharmacokinetics; addition of a radionuclide to form a radioimmunoconjugate (RIC); and uses of the resultant RIC. The review is focused on solid tumors which continue to be a challenge to successful therapy. Antibody targeting of hematological cancers is discussed later in this volume (see Chapter 12; for review also see Goldenberg 2001).

Use of the antibody as a vehicle for radionuclide delivery means that the effector function is no longer solely attributed to the interaction of the antibody with the immune system but also the emissions of the radionuclide as it physically decays. The selection of the radionuclide will be influenced by the characteristics of the tumor, the antibody, and also whether the aim is to detect using medical imaging (radioimmunoscintigraphy, RIS) or destroy (radioimmunotherapy, RIT). RIS uses antibody-delivered radionuclides with emissions detectable outside the body combined with medical imaging techniques (principally single photon emission computed tomography (SPECT), or positron emission tomography (PET)) to detect solid tumors, either for diagnosis or to monitor therapy. RIT is based on similar basic principles but has different requirements. The two are closely related and often used in concert, as in prescouting dosimetry in which RIS is used to guide RIT. Localization of the labeled antibody to the tumor is a requirement for the success of RIS and RIT, though it is also a challenge for both techniques due to solid tumor structure. This chapter provides an overview of the progress that has been made in understanding the challenges of RIS and RIT and the optimal tools to improve clinical practise.

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1.2 Solid Tumors as Targets for Antibody-Based Therapeutics

For both RIS and RIT sufficient radionuclide must be delivered to the tumor but there are many obstacles on the way. Once the antibody has been labeled with an appropriate radionuclide the RIC is introduced into the blood, either as a bolus or infusion. It distributes throughout the body and is retained in the tumor. The vasculature of the tumor is the first obstacle for effective delivery of the RIC as it is inhibited by an inadequate tumor blood supply. A common characteristic of tumor growth is the uncontrolled growth rate of cancerous cells, a consequence of the loss of control of cell behavior. Tumor blood vessels do not keep pace with this increased rate of growth, leading to disorganized vasculature composed of poorly formed vessels with increased fenestrations and poor provision of nutrients to the tumor. Furthermore, tumor blood vessels exhibit a number of unusual structures, ending in blind ends, converging and merging, resulting in vascular arterial shunts, and structurally weak tumor blood vessels can collapse and shut down, temporarily or permanently (Jain 1988; Kallinowski 1996). Drug delivery is seriously affected by these factors. Conversely, the leaky nature of tumor blood vessels aid in tumor uptake, enabling the antibody to leave the vessel lumen and extravasate across the endothelium.

Antibody movement is principally driven by diffusion. Penetration into the tumor mass is limited by the relatively large size of whole antibodies (of the order of 150 kDa) (Jain 1999). Additionally, due to the absence or poor formation of the tumor lymphatic system, interstitial pressure, which is lower at the tumor periphery but elevated at its core, further reduces RIC penetration. The site and size of the tumor can also influence the use and success of RIT. Bulky tumors are less likely to respond to therapy, at least partially due to poor uptake. The size of the tumor is inversely related to the uptake of RIC at the tumor site (Hagan et al. 1986; Pedley et al. 1987; Behr et al. 1997a).

Micrometastases present an ideal target for RIT – they are accessible, a relatively homogeneous dose distribution is achievable throughout the tumor mass, and they might not be detected by current medical imaging techniques, excluding them from external beam radiotherapy (EBRT) (Vogel et al. 1996; Dearling et al. 2005).

1.3 Antigen

The type and pattern of antigen expression in tumor and normal tissue strongly influences the therapy regime and its outcome. The antigen is the feature of the tumor cell, typically a protein expressed at elevated levels on the cancer cell surface, that discriminates it from normal, noncancerous tissues. The antibody interacts with the antigen and is retained at the tumor site. Therefore the antigen must be expressed predominantly on tumor cells and at a level sufficiently high

to retain enough radionuclide for detection or therapy and to achieve a good differential between radionuclide at the tumor site and normal tissues. Antibody–antigen complexes formed in the blood are cleared via the liver, so elevated blood levels of shed antigen reduce the maximum amount of radionuclide that can accumulate in the tumor, as well as potentially leading to liver radiotoxicity. Expression of antigen at around 10^4–10^5 molecules per cell is desirable for successful targeting (Goldenberg et al. 1989; Sung et al. 1992). Expression of the antigen homogeneously throughout the tumor aids successful therapy, though the nuclides commonly used for RIT have long emission ranges, accounting for some degree of heterogeneity. Use of interferon has been reported to increase antigen expression, resulting in an increase in RIC tumor uptake (Meredith et al. 1996; Pallela et al. 2000). A cocktail of antibodies binding to different antigens might overcome heterogeneous expression throughout the tumor (Meredith et al. 1996).

From this initial discussion of the tumor as a target it becomes clear that the nature of solid tumor dictates some of the characteristics of successful RICs. Further requirements are made when we consider the clearance of proteins from the body and their use in RIS and RIT. The identification of these characteristics and how they may be achieved is the focus of the following section.

1.4 Antibodies as Vehicles for Radionuclide Delivery

The target of both RIS and RIT is the tumor but there are differences in the requirements of the RIC for optimal results. For RIS the molecule should localize rapidly at a high level to the tumor while clearing from the normal tissues in order to aid discrimination of the disease site. This occurs over a short time-frame, in the order of hours or days. For RIT the time-scale is different (therapy effectively taking place over around a week), and prolonged retention in the blood aids greater uptake in the tumor, resulting in a greater probability of tumor cell death. However, radiotoxicity to early responding, radiosensitive tissues, usually the bone marrow, limits administered dose. Antibody engineering has provided a number of ways in which RIS and RIT are being optimized (Table 1.1).

The development of antibody-based therapeutics was restrained by complex and laborious production and purification techniques until modern methods of genetic manipulation and protein engineering could be brought to bear on the task. With the development of these approaches antibodies could be engineered to have the characteristics of choice. One of the early modifications to the antibody structure was that of size, which can limit penetration into the tumor, but which also influences the rate of clearance of the RIC from the blood. Affinity and avidity of the antibody, properties that can greatly influence its pharmacokinetics and pharmacodynamics, have been optimized for improved tumor retention. Reducing the immunogenicity of the antibody has also been addressed. We will now

Table 1.1 Summary of optimization of protein constructs to act as radionuclide vehicles localizing to tumor deposits for detection (RIS) or therapy (RIT).

Modifiable property	
Size	Large size (e.g. whole IgG, 150 kDa) = longer circulation time, good tumor localization, increased normal tissue toxicity Small size (e.g. scFv, 27 kDa) = rapid renal clearance (<60 kDa) and poor tumor uptake
Affinity	Increased affinity improves tumor uptake, reaches a point where other factors become limiting
Avidity	Increased antigen-binding sites improve tumour uptake.
Immunogenicity	Patient immune response accelerates clearance leading to use of humanized or human-derived proteins

discuss these facets of antibody engineering for RIS and RIT of solid tumors in more detail.

1.4.1 Antibody Production

Historically material for antibody-based medical techniques was derived from immunized animals. This source carries several drawbacks, as a large number of animals were involved, producing a diverse range of polyclonal antibodies which required laborious purification. The development of hybridoma technology (Köhler and Milstein 1975) revolutionized the field of antibody research. In this technique, hybridization of myeloma cells with antibody-secreting cells from an immunized animal enables production of monoclonal antibodies raised against the same epitope, or determinant region on the antigen, for research and use. Mouse antibodies have been most commonly used in research, though rat antibodies have higher stability and their producer cells have more favorable yield (Clark et al. 1983).

Further developments in molecular biology have facilitated advances in antibody engineering. Large libraries of antibody sequences have been created, which allows for the selection of genes that encode for an antibody from a diverse repertoire. However, this requires that the protein, which binds to the antigen, and the gene encoding it, which gives the instructions for its structure, are associated. Display technologies in which the appropriate protein and its genetic code are physically linked have been developed to accomplish this. The display technologies employed include use of phage systems, display of the polypeptide on ribosomes, and cell surface systems using bacterial or yeast cells (McCafferty et al. 1990; Maynard and Georgiou 2000).

The field of RIS/RIT is not large, though growing, and consequently production must be efficient and cost-effective. Expression systems to produce these constructs in large quantities have contributed to the rapid developments in this field.

Almost any cell that can produce a protein can produce a usable antibody construct. Consequently both eukaryotic and prokaryotic cells have been used in their large-scale production. Traditionally, mammalian cell lines (e.g. recombinant CHO cell lines) have been employed for the expression of antibodies, but more recently insect cells and plants have been described (Yazaki et al. 2001a; de Graaf et al. 2002; Houdebine 2002; Stoger et al. 2002). Ideally, large amounts of product produced at low cost in a short time-frame are required, leading to the predominace of bacterial and yeast expression systems.

The IgG antibody class is the most widely used in this context. Its long residence time in the circulation is both beneficial and detrimental. While this allows good tumor localization, prolonged radionuclide exposure can result in radiotoxicity and increases immunogenicity. In order to overcome these issues properties including size, affinity, avidity, and immunogenicity have been engineered to improve tumor targeting.

1.4.2 Size

Reduction of the size of the antibody has implications for its circulation half-life, route of clearance from the blood, absolute tumor uptake and tumor penetration.

In order to reduce antibody size, enzymatic digestion can be used. Removal of the Fc region by papain gives two Fab regions, consisting of V_L-C_L and V_H-C_H1 chains linked by disulfide bonds (~55 kDa). Fab fragments exhibit rapid clearance and suboptimal tumor uptake due to their small size and monovalency (Buchegger et al. 1990, 1996; Casey et al. 1999, 2002; Behr et al. 2000). Digestion using pepsin gives an $F(ab)_2$ fragment of around 100 kDa. These fragments have better tumor penetration than whole IgG due to their smaller size. In a clinical comparison (Lane et al. 1994) $F(ab)_2$ fragments localized more rapidly than the parent IgG (8.2% injected activity per kg vs. 4.4% injected activity per kg for the parent IgG, both at 4.25 h, $P < 0.05$) (see also Buchegger et al. 1996). However, the circulation residence times of $F(ab)_2$ fragments are reduced, potentially reducing maximal tumor uptake. This may be a result of the removal of the Fc region. For IgG, the Fc region binds to the FcRn receptor in liver cells, leading to its recycling back into serum. This mechanism contributes to the maintenance of protein levels in the blood.

Protein engineering has employed recombinant technologies to reduce the size of constructs down to the smallest fragment recognizing antigen. Figure 1.1 shows a range of antibody formats. Longer circulation time enabling tumor uptake means that larger antibody molecules will generally have higher absolute localization in the tumor, which is beneficial for RIT, but poorer penetration into the tissue. Longer exposure of the bone marrow to the radionuclide will also result in radiotoxicity, limiting administered dose. While they penetrate more successfully into the tumor, smaller molecules have a shorter circulation time, clearing rapidly from the blood. Thus they achieve higher tumor-to-normal tissue ratios,

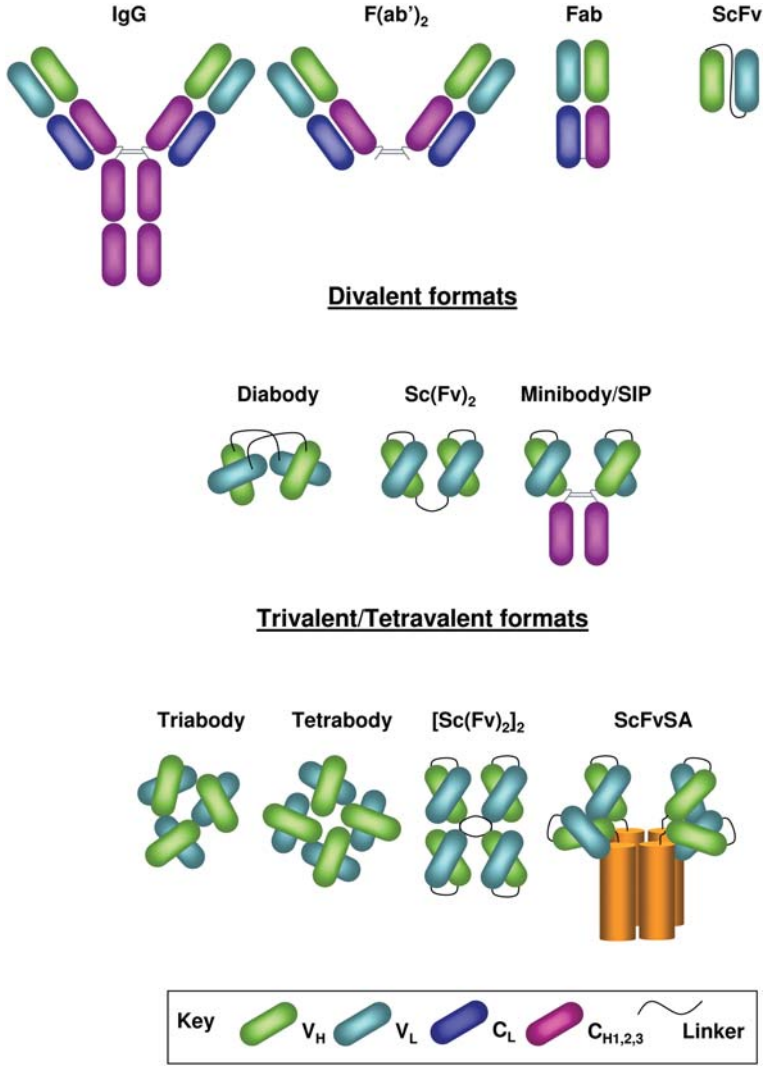

Fig. 1.1 Antibody construct formats. Developments made to optimize performance include varying size and avidity to modify blood clearance and improve tumor localization.

which is advantageous for discrimination of the tumor site from normal tissues, but at the cost of absolute uptake, challenging the sensitivity of RIS and the efficacy of RIT.

One of the smallest antigen-binding constructs used is the single-chain Fv (scFv). It also forms the building blocks of many antibody constructs. Consisting of a V_H and a V_L domain, the scFv has a M_w of ~27 kDa. The V_H and V_L domains, which otherwise dissociate and aggregate, bind either because disulfide bonds

have been engineered into the molecule, or because they are tethered by a flexible linker (Huston et al. 1988; Bird et al. 1988) which can be either chemically introduced or engineered into the polypeptide chain. scFvs clear rapidly from the blood, are monovalent and typically achieve low tumor accumulation, limiting their overall performance. While favorable tumor-to-normal tissue (T:N) ratios are obtained, tumor uptake is low (e.g. peaking at 3–5% injected dose per g at 1–4 h) in relation to whole antibodies. Proteins in the blood of <60 kDa are cleared by the kidney, and scFvs show high kidney uptake (Colcher et al. 1990; Milenic et al. 1991; Begent et al. 1996).

scFvs are retained in the glomerulum due to its negatively charged basement membrane. Tubular reabsorption and lysosomal degradation (Tsai et al. 2001) can lead to retention of the radionuclide, increasing radiotoxicity, particularly in the case of radiometals. This effect can be reduced by administration of L-lysine, reducing kidney accumulation of radionuclide (Behr et al. 1995, 1997b).

Increasing the size of the molecule by addition of polyethylene glycol (PEGylation) can decrease blood pool clearance rates, but fails to add otherwise to its performance (Pedley et al. 1994; Francis et al. 1996) and can interfere with antigen binding (Kubetzko et al. 2005). However multimeric antibodies based on scFvs have been produced that have proved advantageous: diabodies, triabodies, and tetrabodies have improved tumor-targeting capabilities. A similar use of scFvs has been investigated in their fusion to larger molecules, including enzymes, cytokines, and proteins with favorable blood maintenance such as human serum albumin (Michael et al. 1996; Bhatia et al. 2000; Cooke et al. 2002; Huhalov and Chester 2004) or into multivalent formats (see Section 1.4.4).

1.4.3 Affinity

The affinity of the antibody for its target antigen, or its affinity, is critical for tumor localization. The antibody/antigen-binding event involves the formation of around 20 noncovalent interactions, in the form of hydrogen bonds and salt bridges between amino acids on the topological surface of the antigen and the antibody-binding site. The kinetics are first order, and stronger or more numerous interactions move the equilibrium to the right, favoring formation of the antibody–antigen complex, reflecting the greater affinity of the two molecules. Often the affinity is quoted in the form of K_D, the dissociation constant, which is equal to k_{off}/k_{on}, where k_{on} is the association of the antibody onto the antigen and k_{off} is the dissociation of the complex.

Increases in affinity result from changes to the binding site of the antibody, specifically of the amino acids that form the noncovalent bonds between the two molecules. These changes might be randomly introduced into a library using error-prone polymerase chain reaction (PCR), complemenarity determining region (CDR) walking or chain shuffling (Barbas and Burton 1996; Lantto et al. 2002) while more directed changes require site-directed mutagenesis. Structural knowledge of the antibody, either directly from crystal structures or inferred

through comparison with similar molecules, will reveal those amino acids which are involved in binding and could be changed to increase the strength of binding.

Efforts have been made to continually increase the affinity of antibodies for their antigen, in the expectation that this will result in improved targeting. Preclinical *in vivo* studies have provided conflicting evidence of this. Certainly, high affinity is required, and increasing affinity does have a beneficial effect on antibody performance (Colcher et al. 1988; Schlom et al. 1992; Adams et al. 1998) although there are also reports of improvements having no effect (Behr et al. 1997a) on tumor targeting. It is probably the case that there is a threshold above which increases in affinity give no further advantage as other factors become limiting (Sung et al. 1992). Very high-affinity antibodies might pose problems. If the target antigen is not expressed solely on tumor cells then it might be retained in normal tissues, as was encountered in the case of anti-tenascin antibodies, the antigen also being expressed in the liver and spleen. Furthermore, very high-affinity antibodies might hinder tumor penetration of the antibody. Antibody binding to initially available antigen surrounding the blood vessel with very high affinity might not move on to penetrate the tumor. Antibody arriving at the tumor later will not be able to bind to antigen and might rediffuse back into the bloodstream. The "binding site barrier" (van Osdol et al. 1991; Saga et al. 1995) thus could decrease tumor uptake, though the effect is particularly significant when large amounts of protein are administered.

1.4.4 Avidity

The valency of binding of an antibody to its antigen is referred to as the avidity. Increasing the avidity of an antibody construct results in an overall increase in its binding, and its functional affinity (Karush 1970).

Autoradiographic study of radionuclide localiation in preclinical tumors and subsequent analysis of this data using mathematical models has emphasized the importance of valency in addition to affinity (Flynn et al. 2002). Multivalent molecules are retained longer than monovalent ones in radiosensitive (normoxic) regions of the tumor and are more likely to be successful vehicles for RIT.

Monovalent scFv and Fab antibody fragments have been engineered into multimeric constructs in order to improve their tumor retention. Chemical and genetic crosslinkages have been used. Incorporation of a free cysteine residue into the sequence results in crosslinking of two scFv molecules, and this has been shown to improve tumor uptake (Adams et al. 1993, 1995) as does linking two scFv molecules into one polyeptide (Goel et al. 2001a).

Another approach to increase avidity is to take advantage of the dimeric properties of the antibody chains. The V_H chain of one scFv will spontaneously associate with the V_L chain of another. The probability of this occurring can be increased by using a shorter polypeptide linker (e.g. shortening from 15 to 5 amino acids) to tether the V_H and V_L domain of the individual scFv, resulting in inter- rather than intramolecular association of V_H and V_L domains. These divalent formats,

also known as diabodies, possess improved pharmacokinetics compared with monovalent molecules (Nielsen et al. 2000). Divalent $scFv_2$ molecules have been demonstrated to have better tumor uptake than scFvs (tumor localization for the $scFv_2$ 3.57% injected dose per g, for scFv 1.25% injected dose per g, at 24 h) (Adams et al. 2006). Their use as RIS agents, when labeled with positron-emitting isotopes, has been investigated preclinically (Robinson et al. 2005). Clinical use of an ^{125}I-labeled diabody raised against the ED-B domain of fibronectin demonstrated tumor localization (Santimaria et al. 2003).

The tandem scFv, composed of two scFvs linked as a single polypeptide chain, again showed improved T:N ratios. When these molecules ($sc(Fv)_2$) spontaneously dimerized to form tetramers ($[sc(Fv)_2]_2$) absolute maximal tumor uptake was increased. Tumor uptake (% injected dose per g) for the tetravalent $[sc(Fv)_2]_2$ was 21.3 ± 1.3 compared with 9.8 ± 1.3 for the divalent $sc(Fv)_2$ and 17.3 ± 1.1 for the IgG (Goel et al. 2000). Similarly, in a comparison of the ^{177}Lu-labeled $[sc(Fv)_2]_2$ and its parental IgG the tumor localization at 8 h was 6.4% injected dose per g and 8.9% injected dose per g respectively (Chauhan et al. 2005). This was surprising in the light of the much more rapid blood clearance of the modified molecule. The $t_{1/2}\alpha$ was 4.40 min for the $[sc(Fv)_2]_2$, and 9.5 min for the whole IgG, and $t_{1/2}\beta$ were 375 min and 2193 min, respectively, conferring a significant advantage on the tetravalent form. Kidney accumulation was reduced with use of L-lysine (for further discussion of multivalent molecules see Todorovska et al. 2001; Wittel et al. 2004).

A different approach to forming multimers is to link antibody fragments to molecules that form homomultimers. For example, the minibody is an scFv linked to a human IgG1 C_H3 domain, the minibody molecule being bivalent because the C_H3 domains dimerize (Hu et al. 1996; Wu et al. 2000). The resultant molecule is similar in size to $F(ab')_2$ fragments (minibody 80 kDa versus $F(ab')_2$ 100 kDa). These molecules gave higher uptake than was achieved with diabody forms of the same scFv in the same xenograft system (Hu et al. 1996; Wu et al. 1996; Yazaki et al. 2001b). The maximal tumor uptake of the minibody at 6 h was 32.9 ± 11.18% injected dose per g while for the diabody it was 10.38 ± 0.81% injected dose per g at 1 h. Relevant for imaging purposes are the greatest tumor-to-blood ratios of 64.89:1 at 48 h for the minibody and 23.23:1 at 24 h for the diabody.

The Small ImmunoProtein (SIP) is a comparable molecule to the minibody, composed of the C_H4 region of a human Igε-S2, resulting in a dimeric scFv. This molecule improved on the performance of the diabody form, with maximal tumor uptake at 6 h of 6.14 ± 2.23% injected dose per g compared with 2.47 ± 0.65% injected dose per g, respectively (Borsi et al. 2002). Increasing the size of the $(scFv\text{-}C_H3)_2$ molecule further, from 80 to 105 kDa, to create the $(scFv\text{-}C_H2\text{-}C_H3)_2$ molecule, improved tumor targeting (5.7 ± 0.1% injected dose per g versus 12.2 ± 2.4% injected dose per g, respectively) while reducing kidney uptake (34.0 ± 4.0% injected dose per g versus 13.1 ± 1.5% injected dose per g) (Olafsen et al. 2005).

Other examples of multimers include p53 and streptavidin tetramerization. Tumor uptake of the p53 tetramers was low (4.32 ± 1.94% injected dose per g),

and was attributed to dissociation in serum (Willuda et al. 2001). Streptavidin has proved a more successful format (Dubel et al. 1995; Zhang et al. 2003) in terms of tumor targeting, and can also be used in combination with biotin in Pretargeted RIT (PRIT). In this technique a nonradiolabeled antibody is followed by a smaller molecule acting as the radionuclide vehicle.

1.4.5 Immunogenicity

As has been described, the origin of many antibodies under research and development is often rodent cells for genetic material and bacterial or yeast-based systems for production. Systemic administration of such products can lead to immune responses depending on the construct (for a discussion see Mirick et al. 2004). While human anti-mouse antibody (HAMA) responses are directed primarily toward the Fc regions, anti-idiotypic responses can also be encountered. The scale of the response can be increased by rapid introduction of large amounts of protein. The development of immune complexes will lead to faster clearance of RIC from the blood and complicate future treatments. Attempts to minimize or evade this detection and response altogether has led to a range of techniques. The protein may be disguised, or its origin may be changed, leading to chimeric, humanized, and fully human antibodies (Clark 2000). The majority of therapeutic antibodies used in clinical practice are humanized.

A chimeric antibody will have a mixture of sources, for example an human Fc region and the Fab fragment of a rodent antibody. However, it is still possible to elicit a response to the Fab region. Further "humanization" can be achieved through the transfer of the CDR loops, the regions of the rodent antibody bearing the amino acids that form the bonds with the antigen, onto a human antibody (Baca et al. 1997; Rader et al. 1998). In an analogous technique recombinant antibodies are "resurfaced" or "veneered" to provide fewer immunogenic sites (Pederson et al. 1994; Roguska et al. 1994) using structural information. Deimmunizaton, the removal of defined helper T cell epitopes, has also been reported. Fully human antibodies are the goal in this area. They can be produced using transgenic mice (Nagy et al. 2002; O'Connell et al. 2002), and human antibody fragments have been obtained using the phage display system in combination with fully human libaries (Knappik et al. 2000).

1.5 Radioisotope

1.5.1 Selection

Radionuclide choice is related to the aim of the procedure. Different isotopes are suited to imaging or therapy, depending on their properties (Table 1.2). The

Table 1.2 Radionuclides for radioimmunoscintigraphy and radioimmunotherapy.

Radionuclides		*Emission energy (MeV)*	*Physical $t_{1/2}$*
Imaging			
γ	^{99m}Tc	0.142	6.01 h
	^{111}In	0.173, 0.247	2.8 days
β^+	^{64}Cu	1.675	12.7 h
	^{72}As	1.17	1.1 days
	^{89}Zr	0.9	3.27 days
	^{124}I	1.53	4.18 days
Therapy			
Auger	^{111}In	0.86	2.8 days
	^{125}I	0.179	60.1 days
α	^{211}At	5.980	7.21 h
	^{212}Bi	6.051	1.01 h
	^{225}Ac	5.3–5.8	10 days
β^-	^{64}Cu	0.578	12.7 h
	^{67}Cu	0.58	2.58 days
	^{77}As	0.226	1.6 days
	^{90}Y	2.282	2.67 days
	^{131}I	0.606	8.04 days
	^{177}Lu	0.497	6.75 days
	^{186}Re	0.973	3.78 days

Radionuclides commonly used in RIS/RIT, or of potential utility, are shown. Some nuclides have both imaging and therapeutic emissions. Other elements, such as iodine, are represented in both the imaging and therapy sections, recommending them for use in predosing regimes.

emissions of the radionuclide will chiefly guide this selection, but the pharmacokinetics of the antibody construct will also have influence. The half-life of the imaging isotope should be in concordance with the *in vivo* behavior of the antibody. Sensitivity of the imaging procedure will be compromised if too few emissions are detected from the tumor site once the RIC has been allowed to clear from the blood pool and other normal tissues. An ideal range of half-life for the therapeutic isotope is around 1.5–3 times the time taken for the antibody to achieve maximal uptake in the tumor, and mathematical modeling has investigated the relative merits of varying half-lives (Flynn et al. 2002; Howell et al. 1998). This should lead to maximal absorbed dose and dose-rate to the tumor. For both RIS and RIT, administered dose is limited by radiotoxicity to the patient, though other factors, such as dose to medical workers and disposal of waste products should also be borne in mind. As well as procedural considerations

practical issues influence this selection, including production, ease of use and cost.

1.5.2 Emissions

Radionuclide emissions will determine use. The two main emissions used for imaging are gamma (γ) and positron (β^+, for positron emission tomography (PET) imaging). There are three main emissions used for therapy: Auger electrons, alpha particles (α) and beta particles (β^-). These therapy emissions vary in their biological effect. For example, Auger emissions are high-energy electrons which are very toxic but with a short path length (~0.1–5 μm), and are therefore more effective when internalized into the cell (Hofer 1996). Alpha particles are helium nuclei, again with short path lengths. Their lack of availability and short half-lives have limited their application, though they have proved useful in treatment of organ surface tumors when locally injected (so that systemic introduction does not hinder delivery) (McDevitt et al. 1998; Zalutsky and Vaidyanathan 2000) and vascular targeting (Akabani et al. 2002, 2003). Beta-emitting radionuclides are the most widely employed in this area, largely due to the predominance of ^{131}I. Beta emissions have a much longer range than either Auger or alpha articles, but are much less toxic. However their range is more suited to treatment of a small tumor deposit. The problem of heterogeneity of RIC distribution within the tumor, because of both delivery and antigen expression, has been discussed earlier. Use of a radionuclide with long emissions will smooth out this heterogeneity through the cross-fire effect; the radionuclide attached by the antibody to one cell will irradiate neighboring tumor cells whether they express the antigen or not. The emission ranges of commonly used radionuclides such as ^{131}I (0.83 mm) and ^{90}Y (5.2 mm) (Simpkin and Mackie 1990) make them potentially applicable to small deposits and larger bulky tumors, respectively (O'Donoghue, et al. 1995).

1.5.3 Antibody Radiolabeling

Modification of the antibody by addition of the radionuclide may be achieved through either direct or indirect labeling. Direct labeling, mainly through redox reactions, has been described in the use of ^{99m}Tc for RIS and isotopes of iodine for both imaging and therapy, depending on the radionuclide (Greenwood and Hunter 1963). The indirect method of labeling uses a bifunctional chelator to form a bridge between the protein and the radionuclide. The number of additions to the antibody will have an optimal point: too few will limit success of the procedure through insufficient information, while too many might interfere with antigen binding and limit tumor uptake.

1.5.4
Radiobiology

Radioisotope emissions are toxic because they alter biological molecules as they lose their energy. A highly energetic particle of large mass can cause direct damage to important biological structures such as DNA, or because the ionization of water leads to short-lived free radicals, including superoxide (O_2^-), and hyperoxide ($H_2O_2^-$) which are highly reactive and cause indirect damage in the cell. As oxygen is often involved in these reactions it is a potent radiosensitizer. Indirectly caused lesions, which are the main cause of damage by the β^- emitting radionuclides, are greatly reduced under oxygen-lacking (hypoxic) conditions in the tumor, which therefore reduce the success of irradiative therapies (see Thomlinson and Gray 1955; Tannock 1972).

The success of RIT is dependent on the absorbed dose to the tumor and the dose-rate. The dose-rate is important because cells can repair damage at a certain rate, so this must be exceeded in order to have a toxic effect. The time span of RIT is not fixed as with EBRT, but instead depends on the pharmacokinetics and biological half-life of the RIC and the physical half-life of the radioisotope. Once the RIC arrives at the tumor and the rate of cell kill exceeds the rate of proliferation (i.e. there is a net decrease in cell number), therapy has started. RIC uptake at the target site will continue to increase, reach a peak, and then decrease. Once the dose-rate required to overcome cell proliferation is again reached, typically around a week after injection, the therapy has effectively ended.

1.6
Improving Radioimmunoscintigraphy and Therapy

Methods investigated for improving RIS and RIT are generally applicable to both, with some exceptions. For RIS absolute uptake in the tumor is important in order to enable identification and relative tumor localization compared with normal tissues to allow discrimination and sensitivity. For RIT maximizing absolute uptake in the tumor is important, but minimal uptake in normal tissues is desirable in order to reduce radiotoxicity.

The chief methods of modifying RIC pharmacokinetics have already been discussed. Antibody construct size, affinity, avidity, and immunogenicity can all be optimized. Here we will consider ways in which the use of the RICs may be modified in order to improve performance, concentrating on clearing antibodies, dose fractionation and pretargeted RIT.

Use of a clearing agent is applicable to both RIS and RIT. Rapid clearance from the blood decreases radiotoxicity to normal tissues, but also reduces the amount of RIC that can localize to the tumor. A secondary antibody that reacts to the first increases the rate of clearance from the blood (Begent et al. 1982; Pedley et al. 1989). The advantages of this technique include that a greater amount of RIC

may be given, and therefore more will localize to the tumor, giving an increased maximal dose-rate and potentially higher overall absorbed dose to the tumor. However, the RIC is only cleared from the vascular space, and exposure of the liver and spleen to radionuclide is increased due to clearance.

Instead of giving a single injection of RIC the dose could be fractionated, that is split into a number of smaller injections over a period of time. This allows for the RIC to clear from normal tissues, allowing repair, but is retained in the tumor, which consequently receives a relatively constant low dose. Using this method a greater total amount of activity can be administered for a similar level of toxicity (Vriesendorp et al. 1993). The timing of the smaller administrations is crucial, and different investigators have reported for (Schlom et al. 1990; Buchsbaum et al. 1995; Goel et al. 2001b) and against fractionation (Pedley et al. 1993) using different regimes (for further discussion see DeNardo et al. 2002). Repeated therapy can reduce the tumor uptake of RIC due to vascular damage (Buchsbaum et al. 1999).

Separate administration of the antibody and radionuclide was first suggested in 1986 (Goodwin et al. 1986a,b). To take the use of the avidin/biotin system as an illustration, the antibody is labeled with avidin before being injected. An antibody construct with a long blood pool residence can be used, increasing tumor uptake. Following use of a clearing agent the radionuclide, attached to biotin, is administered. This is quickly cleared from normal tissues, minimizing toxicity, while delivering therapy to the tumor. Avidin/biotin-based systems have dominated this area, and some clinical applications of RIT use this technology. For example a streptavidin conjugate of the NR-LU-10 pancarcinoma antibody combined with ^{90}Y-DOTA-biotin (a single administration of 110 mCi m^{-2}) achieved a modest overall response rate, with four patients (out of 25) having freedom from progressive disease for 10–20 weeks (Knox et al. 2000).

While the avidin/biotin system has proved the principle it does have disadvantages, major problems being endogenous liver biotin and the immunogenicity of streptavidin. While other systems such as that based around oligonucleotides have been described, the affinity enhanced system (AES) is more relevant to our current survey. This centers upon a bispecific antibody with affinity for the tumor antigen and also for the radionuclide vehicle such as a hapten (Barbet et al. 1998; Schumacher et al. 2001) (for a review of PRIT see Gruaz-Guyon et al. 2005).

1.7 Summary

The ultimate goal of engineering antibodies for RIS and RIT is protein constructs that localize effectively to all tumor sites and clear from normal tissues. In this chapter we have discussed methods to achieve this goal through structural modification. The refinements made to the parent antibody structures have demon-

strated that use of antibody-based tumor imaging and therapy has much potential in cancer treatment.

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Abbreviations

AES	affinity enhanced system
CDR	complementarity determining region
CHO	Chinese hamster ovary fibroblast
DOTA	a molecule used as an indirect method of antibody radiometal labeling (1,4,7,10-tetraazacyclododecane – *N*,*N*′,*N*″,*N*‴ tetraacetic acid)
EBRT	external beam radiotherapy
Fab	fraction antigen binding, after papain digestion of monovalent Ig
Fc	fraction of IgG crystallizable – non-Fab part of IgG
IgG	immunoglobulin class G
K_D	dissociation constant, equal to k_{off}/k_{on}
kDa	kilodalton
M_w	molecular weight
PCR	polymerase chain reaction
PET	positron emission tomography
PRIT	pretargeted RIT
RIC	radioimmunoconjugate
RIS	radioimmunoscintigraphy
RIT	radioimmunotherapy
scFv	single-chain variable fragment
SPECT	single photon emission computed tomography
T:N	ratio of radionuclide localization between tumor and normal tissues
$t_{1/2}\alpha$, $t_{1/2}\beta$	mathematical terms (exponential half-lives) describing the biphasic rate of radiolabeled antibody blood clearance

References

Adams, G.P., McCartney, J.E., Tai, M.S. et al. (1993) Highly specific in vivo tumor targeting by monovalent and divalent forms of 741F8 anti-c-erbB-2 single-chain Fv. *Cancer Res* 53: 4026–4034.

Adams, G.P., McCartney, J.E., Wolf, E.J. et al. (1995) Enhanced tumor specificity of 741F8–1 (sFv')2: an anti-c-erbB-2 single-chain Fv dimer, mediated by stable radioiodine conjugation. *J Nucl Med* 36: 2276–2281.

Adams, G.P., Schier, R., Marshall, K. et al. (1998) Increased affinity leads to improved selective tumor delivery of single-chain Fv antibodies. *Cancer Res* 58: 485–490.

Adams, G.P., Tai, M-S., McCartney, J.E. et al. (2006) Avidity-mediated enhancement of *in vivo* tumor targeting by single-chain Fv dimers. *Clin Cancer Res* 12: 1599–1605.

Akabani, G., McLendon, R.E., Bigner, D.D., Zalutsky, M.R. (2002) Vascular targeted endoradiotherapy of tumors using alpha-particle-emitting compounds: Theoretical analysis. *Int J Radiat Oncol Biol Phys* 54: 1259–1275.

Akabani, G., Kennel, S.J., and Zalutsky, M. R. (2003) Microdosimetric analysis of particle-emitting targeted radiotherapeutics using histological images. *J Nucl Med* 44: 792–805.

Baca, M., Presta, L.G., O'Connor, S.J., Wells, J.A. (1997) Antibody humanization using monovalent phage display. *J Biol Chem* 272: 10678–10684.

Barbas, C.F. III, Burton, D.R. (1996) Selection and evolution of high-affinity human anti-viral antibodies. *Trends Biotechnol* 14: 230–234.

Barbet, J., Peltier, P., Bardet, S. et al. (1998) Radioimmunodetection of medullary thyroid carcinoma using indium-111 bivalent hapten and anti-CEA X anti-DTPA-indium bispecific antibody. *J Nucl Med* 39: 1172–1178.

Begent, R., Keep, P.A., Green, A.J. et al. (1982) Liposomally entrapped second antibody improves tumor imaging with radiolabelled (first) antitumor antibody. *Lancet* 2: 739–742.

Begent, R.H., Verhaar, M.J., Chester, K.A. et al. (1996) Clinical evidence of efficient tumor targeting based on single-chain Fv antibody selected from a combinatorial library. *Nat Med* 2: 979–984.

Behr, T.M., Sharkey, R.M., Juweid, M.E. et al. (1995) Reduction of the renal uptake of radiolabelled monoclonal antibody fragments by cationic amino acids and their derivatives. *Cancer Res* 55: 3825–3834.

Behr, T.M., Sharkey, R.M., Juweid, M.E. et al. (1997a) Variables influencing tumor dosimetry in radioimmunotherapy of CEA-expressing cancers with anti-CEA and anti-mucin monoclonal antibodies. *J Nucl Med* 38: 409–418.

Behr, T.M., Sharkey, R.M., Sgouros, G. et al. (1997b) Overcoming the nephrotoxicity of radiometal-labelled immunoconjugates: improved cancer therapy administered to a mouse model in relation to the internal radiation dosimetry. Cancer 80: 2591–2610.

Behr, T.M., Blumenthal, R.D., Memtsoudis, S. et al. (2000) Cure of metastatic human colonic cancer in mice with radiolabeled monoclonal antibody fragments. *Clin Cancer Res* 6: 4900–4907.

Bird, R.E., Hardman, K.D., Jacobson, J.W. et al. (1988) Single-chain antigen-binding proteins. *Science* 242: 423–426.

Bhatia, J., Sharma, S.K., Chester, K.A. et al. (2000) Catalytic activity of an in vivo tumor targeted anti-CEA scFv:: carboxypeptidase G2 fusion protein. *Int J Cancer* 85: 571–577.

Borsi, L., Balza, E., Bestagno, M. et al. (2002) Selective targeting of tumoral vasculature: comparison of different formats of an antibody (L19) to the ED-B domain of fibronectin. *Int J Cancer* 102: 75–85.

Buchegger, F., Pelegrin, A., Delaloye, B., Bischof-Delaloye, A., and Mach, J.P. (1990) Iodine-131-labelled MAb $F(ab')_2$ fragments are more efficient and less toxic than intact anti-CEA antibodies in radioimmunotherapy of large human colon carcinoma grafted in nude mice. *J Nucl Med* 31: 1035–1044.

Buchegger, F., Gillet, M., Doenz, F. et al. (1996) Biodistribution of anti-CEA $F(ab')_2$ fragments after intra-arterial and intravenous injection in patients with liver metastases due to colorectal carcinoma. *Nucl Med Commun* 17: 500–503.

Buchsbaum, D., Khazaelli, M.B., Liu, T. et al. (1995) Fractionated radioimmunotherapy of human colon carcinoma xenografts with ^{131}I-labeled monoclonal antibody CC49. *Cancer Res* 55: 5881s–5887s.

Buchsbaum, D.J., Khazaelli, M.B., Mayo, M.S., Roberson, P.L. (1999) Comparison of multiple bolus and continuous injections of ^{131}I-labeled CC49 for therapy in a colon cancer xenograft model. *Clin Cancer Res* 5: 3153s–3159s.

Casey, J.L., Pedley, R.B., King, D.J., Green, A.J., Yarranton, G.T., Begent, R.H.J. (1999) Dosimetric evaluation and radioimmunotherapy of anti-tumoour multivalent Fab' fragments. *Br J Cancer* 81: 972–980.

Casey, J.L., Napier, M.P., King, D.J. et al. (2002) Tumour targeting of humanised cross-linked divalent-Fab' antibody fragments: a clinical phase I/II study. *Br J Cancer* 86: 1401–1410.

Chauhan, S.C., Jain, M., Moore, E.D. et al. (2005) Pharmacokinetics and biodistribution of ^{177}Lu-labeled multivalent single-chain Fv construct of the pancarcinoma monoclonal antibody CC49. *Eur J Nucl Med Mol Imaging* 32: 264–273.

Clark, M. (2000) Antibody humanization: a case of the "Emperor's new clothes"? *Immunol Today* 21: 397–402.

Clark, M., Cobbold, S., Hale, G. et al. (1983) Advantages of rat monoclonal antibodies. *Immunol Today* 4: 100–101.

Colcher, D., Minelli, M.F., Roselli, M., Muraro, R., Simpson-Milenic, D., Schlom, J. (1988) Radioimmunolocalisation of human carcinoma xenografts with B72.3 second generation monoclonal antibodies. *Cancer Res* 48: 4597–4603.

Colcher, D., Bird, R., Roselli, M. et al. (1990) In vivo tumor targeting of a recombinant single-chain antigen-binding protein. *J Natl Cancer Inst* 82: 1191–1197.

Cooke, S.P., Pedley, R.B., Boden, R., Begent, R.H., and Chester, K.A. (2002) In vivo tumor delivery of a recombinant single chain Fv::tumor necrosis factor-alpha fusion protein. *Bioconjug Chem* 13: 7–15.

Dearling, J.L.J., Qureshi, U., Whiting, S., Boxer, G.M., Begent, R.H.J., and Pedley, R.B. (2005) Analysis of antibody distribution reveals higher uptake in peripheral intrahepatic deposits than central deposits. *Eur J Nucl Med Mol Imaging* 32: S83.

DeNardo, G.L., Schlom, J., Buchsbaum, D.J. et al. (2002) Rationales, evidence, and design considerations for fractionated radioimmunotherapy. *Cancer* 94: 1332–1348.

Dubel, S., Breitling, F., Kontermann, R. et al. (1995) Bifunctional and multimeric complexs of streptavidin fusd to single chain antibodies (scFv). *J Immunol Methods* 178: 201–209.

Flynn, A.A., Green, A.J., Pedley, R.B. et al. (2002) A model-based approach for the optimization of radioimmunotherapy through antibody design and radionuclide selection. *Cancer* 94: 1249–1257.

Francis, G.E., Delgado, C., Fisher, D., Malik, F., Agrawal, A.K. (1996) Polyethylene glycol modification: relevance of improved methodology to tumour targeting. *J Drug Target* 3: 321–340.

Goel, A., Colcher, D., Baranowska-Kortylewicz, J. et al. (2000) Genetically engineered tetravalent single-chain Fv of the pancarcinoma monoclonal antibody CC49: Improved biodistribution and potential for therapeutic application. *Cancer Res*, 60: 6964–6971.

Goel, A., Colcher, D., Baranowska-Kortylewicz, J. et al. (2001a) 99mTc-labeled divalent and tetravalent CC49 single-chain Fv's: novel imaging agents for rapid *in vivo* localisation of human colon carcinoma. *J Nucl Med* 42: 1519–1527.

Goel, A., Augustine, S., Baranowska-Kortyewicz, J. et al. (2001b) Single-dose versus fractionated radioimmunotherapy of human colon carcinoma xenografts using ^{131}I-labeled multivalent CC49 single-chain Fvs. *Clinical Cancer Res* 7: 175–184.

Goldenberg, A., Masui, H., Divgi, C., Kamrath, H., Pentlow, K., Mendelsohn, J. (1989) Imaging of human tumor xenografts with an indium-111-labelled anti-epidermal growth factor receptor monoclonal antibody. *J Natl Cancer Inst* 81: 1616–1625.

Goldenberg, D.M. (2001) The role of radiolabelled antibodies in the treatment of non-Hodgkin's lymphoma: the coming of age of radioimmunotherapy. *Crit Rev Oncol/Hematol* 39: 195–201.

Goodwin, D.A., Mears, C.F., McTigue, M., David, G.S. (1986a) Monoclonal antibody hapten radiopharmaceutical delivery. *Nucl Med Commun* 7: 569–580.

Goodwin, D.A., Mears, C.F., David, G.F., McTigue, M., McCall, M.J., Frincke, J.M., Stone, M.R. (1986b) Monoclonal antibodies as reversible equilibrium carriers of radiopharmaceuticals. *Int J Rad Appl Instrum B* 13: 383–391.

de Graaf, M., van der Meulen-Muileman, I.H., Pinedo, H.M., Haisma, H.J. (2002) Expression of scFvs and scFv fusion proteins in eukaryotic cells. *Methods Mol Biol* 178: 379–387.

Greenwood, F.C., Hunter, W.M. (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. *Biochem J* 89: 116–123.

Gruaz-Guyon, A., Raguin, O., Barbet, J. (2005) Recent advances in pretargeted radioimmunotherapy. *Curr Med Chem* 12: 319–338.

Hagan, P.L., Halpern, S.E., Dillman, R.O. et al. (1986) Tumor size: effect on monoclonal antibody uptake in tumor models. *J Nucl Med* 27: 422–427.

Hofer, K.G. (1996) Biophysical aspects of Auger processes. *Acta Oncol* 35: 789–796.

Houdebine, L.M. (2002) Antibody manufacture in transgenic animals and comparisons with other systems. *Curr Opin Biotechnol* 13: 625–629.

Howell, R.W., Goddu, S.M., and Rao, D.V. (1998) Proliferation and the advantage of longer- lived radionuclides in radioimmunotherapy. *Med Phys* 25: 37–42.

Hu, S., Shively, L., Raubitshek, A., Sherman, M. et al. (1996) Minibody: a novel engineered anti-carcinoebryonic antigen antibody fragment (single-chain Fv-CH3) which exhibits rapid high-level targeting of xenografts. *Cancer Res* 56: 3055–3061.

Huhalov, A., Chester, K.A. (2004) Engineered single chain antibody fragments for radioimmunotherapy. *Q J Nucl Med Mol Imaging* 48: 279–288.

Huston, J.S., Levinson, D., Mudgett-Hunter, M. et al. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc Nat Acad Sci USA* 85: 5879–5883.

Jain, R.K. (1988) Determinants of tumor blood flow: A review. *Cancer Res* 48: 2641–2658.

Jain, R.K. (1999) Transport of molecules, particles, and cells in solid tumors. *Ann Rev Biomed Eng* 1: 241–263.

Kallinowski, F. (1996) The role of tumor hypoxia for the development of future treatment concepts for locally advanced cancer. *Cancer J* 9: 37–40.

Karush, F. (1970) Affinity and the immune response. *Ann NY Acad Sci* 169: 56–64.

Knappik, A., Ge, L., Honegger, A. et al. (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomised with trinucleotides. *J Mol Biol* 296: 57–86.

Knox, S.J., Goris, M.L., Tempero, M. et al. (2000) Phase II trial of yttrium-90-DOTA-biotin pretargeted by NR-LU-10 antibody/streptavidin in patients with metastatic colon cancer. *Clin Cancer Res* 6: 406–414.

Köhler, G., Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.

Kubetzko, S., Sarkar, C.A., Pluckthun, A. (2005) Protein PEGylation decreases observed target association rates via a dual blocking mechanism. *Mol Pharmacol* 68: 1439–1454.

Lane, D.M., Eagle, K.F., Begent, R.H.J. et al. (1994) Radioimmunotherapy of metastatic colorectal tumours with iodine-131-labelled antibody to carcinoembryonic antigen: phase I/II study with comparative biodistribution of intact and $F(ab')_2$ antibodies. *Br J Cancer* 70: 521–525.

Lantto, J., Jirholt, P., Barrios, Y., Ohlin, M. (2002) Chain shuffling to modify properties of recombinant immunoglobulins. *Methods Mol Biol* 178: 303–316.

Maynard, J., Georgiou, G. (2000) Antibody engineering. *Annu Rev Biomed Eng* 2: 339–376.

McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J. (1990) Phage antibodies: Filamentous phage displaying antibody variable domains. *Nature* 348: 552–554.

McDevitt, M.R., Sgouros, G., Finn, R.D. et al. (1998) Radioimmunotherapy with alpha-emitting nuclides. *Eur J Nucl Med* 25: 1341–1351.

Meredith, R.F., Khazaeli, M.B., Plott, W.E. et al. (1996) Phase II study of dual ^{131}I-labelled monoclonal antibody therapy with interferon in patients with metastatic colorectal cancer. *Clin Cancer Res* 2: 1811–1818.

Michael, N.P., Chester, K.A., Melton, R.G. et al. (1996) In vitro and in vivo characterisation of a recombinant

carboxypeptidase G2::anti-CEA scFv fusion protein. *Immunotechnology* 2: 47–57.

Milenic, D.E., Yokota, T., Filpula, D.R. et al. (1991) Construction, binding properties, metabolism, and tumor targeting of a single-chain Fv derived from the pancarcinoma monoclonal antibody CC49. *Cancer Res* 51: 6363–6371.

Mirick, G.R., Bradt, B.M., Denardo, S.J., Denardo, G.L. (2004) A review of human anti-globulin antibody (HAGA, HAMA, HACA, HAHA) responses to monoclonal antibodies. Not four letter words. *Q J Nucl Med Mol Imaging* 48: 251–257.

Nagy, Z.A., Hubner, B., Lohning, C. et al. (2002) Fully human, HLA-DR-specific monoclonal antibodies efficiently induce programmed death of malignant lymphoid cells. *Nat Med* 8: 801–807.

Nielsen, U.B., Adams, G.P., Weiner, L.M., and Marks, J.D. (2000) Targeting of bivalent anti-ErbB2 diabody antibody fragments to tumor cells is independent of the intrinsic antibody affinity. *Cancer Res* 60: 6434–6440.

O'Connell, D., Becerril, B., Roy-Burman, A. et al. (2002) Phage versus phagemid libraries for generation of human monoclonal antibodies. *J Mol Biol* 321: 49–56.

O'Donoghue, J.A., Bardies, M., Wheldon, T. E. (1995) Relationships between tumor size and curability for uniformly targeted therapy with beta-emitting radionuclides. *J Nucl Med* 36: 1902–1909.

Olafsen, T., Kenanova, V.E., Sundaresan, G. et al. (2005) Optimizing radiolabelled engineered anti-p185^{HER2} antibody fragments for *in vivo* imaging. *Cancer Res* 65: 5907–5916.

Pallela, V.R., Rao, S.P., Thakur, M.L. (2000) Interferon-alpha-2b immunoconjugate for improving immunoscintigraphy and immunotherapy. *J Nucl Med* 41: 1108–1113.

Pederson, J.T., Henry, A.H., Searle, S.J. et al. (1994) Comparison of surface accessible residues in human and murine immunoglobulin Fv domains. Implication for humanization of murine antibodies. *J Mol Biol* 235: 959–973.

Pedley, R.B., Boden, J., Keep, P.A., Harwood, P.J., Green, A.J., and Begent, R.H.J. (1987) Relationship between tumour size and uptake of radiolabelled anti-CEA in a colon tumour xenograft. *Eur J Nucl Med* 13: 197–202.

Pedley, R.B., Dale, R., Boden, J.A., Begent, R.H.J., Keep, P.A., Green, A.J. (1989) The effect of second antibody clearance on the distribution and dosimetry of radiolabelled anti-CEA antibody in a human colonic tumor xenograft model. *Int J Cancer* 43: 713–718.

Pedley, R.B., Boden, J.A., Boden, R., Dale, R., Begent, R.H. (1993) Comparative radioimmunotherapy using intact or F(ab′)2 fragments of ^{131}I anti-CEA antibody in a colonic xenograft model. *Br J Cancer* 68: 69–73.

Pedley, R.B., Boden, J.A., Boden, R. et al. (1994) The potential for enhanced tumour localisation by poly(ethylene glycol) modification of anti-CEA antibody. *Br J Cancer* 70: 1126–1130.

Rader, C., Cheresh, D.A., Barbas, C.F. III. (1998) A phage display approach for rapid antibody humanization: designed combinatorial V gene libraries. *Proc Natl Acad Sci USA* 95 8910–8915.

Robinson, M.K., Doss, M., Shaller, C. et al. (2005) Quantitative immuno-positron emission tomography imaging of HER2-positive tumor xenografts with an iodine-124 labeled anti-HER2 diabody. *Cancer Res* 65: 1471–1478.

Roguska, M.A., Pederson, J.T., Keddy, C.A. et al. (1994) Humanization of murine monoclonal antibodies through variable domain resurfacing. *Proc Natl Acad Sci USA* 91: 969–973.

Saga, T., Neumann, R.D., Heya, T. et al. (1995) Targeting cancer micrometastases with monoclonal antibodies: A binding-site barrier. *Proc Natl Acad Sci USA* 92: 8999–9003.

Santimaria, M., Moscatelli, G., Viale, G.L. et al. (2003) Immunoscintigraphic detection of the ED-B domain of fibronectin, a marker of angiogenesis, in patients with cancer. *Clin Cancer Res* 9: 571–579.

Schlom, J., Molinolo, A., Simpson, J.F., Siler, K., Roselli, M., Hinkle, G., Houchens, D. P., Colcher, D. (1990) Advantage of dose fractionation in monoclonal antibody-directed radioimmunotherapy. *J Natl Cancer Inst* 82: 763–771.

Schlom, J., Eggensperger, D., Colcher, D. et al. (1992) Therapeutic advantage of high-affinity anticarcinoma radioimmunoconjugates. *Cancer Res* 52: 1067–1072.

Schumacher, J., Kaul, S., Klivenyi, G. et al. (2001) Immunoscintigraphy with positron emission tomography: gallium-68 chelate imaging of breast cancer pretargeted with bispecific anti-MUC1/anti-Ga chelate antibodies. *Cancer Res* 61: 3712–3717.

Simpkin, D.J., Mackie, T.R. (1990) EGS4 Monte Carlo determination of the beta dose kernel in water. *Med Phys* 17: 179–186.

Stoger, E., Sack, M., Fischer, R., Christou, P. (2002) Plantibodies: applications, advantages and bottlenecks. *Curr Opin Biotechnol* 13: 161–166.

Sung, C., Shockley, T.R., Morrison, P.F., Dvorak, H.F., Yarmush, M.L., Dedrick, R.L. (1992) Predicted and observed effects of antibody affinity and antigen density on monoclonal antibody uptake in solid tumors. *Cancer Res* 52: 377–384.

Tannock, I.F. (1972) Oxygen diffusion and the distribution of cellular radiosensitivity in tumours. *Br J Radiol* 45: 515–524.

Thomlinson, R.H., Gray, L.H. (1955) The histological structure of some human lung cancers and the possible implications for therapy. *Br J Cancer* 9: 539–549.

Todorovska, A., Roovers, R.C., Dolezal, O., Kortt, A.A., Hoogenboom, H.R., Hudson, P.J. (2001) Design and application of diabodies, triabodies and tetrabodies for cancer imaging. *J Immunol Methods* 248: 47–66.

Tsai, S.W., Li, L., Williams, L.E., Anderson, A.L., Raubitschek, A.A., Shively, J.E. (2001) Metabolism and renal clearance of ^{111}In-labeled DOTA-conjugated antibody fragments. *Bioconjug Chem* 12: 264–270.

van Osdol, W., Fujimori, K., Weinstein, J.N. (1991) An analysis of monoclonal antibody distribution in microscopic tumor nodules: Consequences of a "binding site barrier". *Cancer Res* 51: 4776–4784.

Vogel, C-A., Galmiche, M.C., Westermann, P. et al. (1996) Carcinoembryonic antigen expression, antibody localisation and immunophotodetection of human colon cancer liver metastases in nude mice: A model for radioimmunotherapy. *Int J Cancer* 67: 294–302.

Vriesendorp, H.M., Shao, Y., Blum, J.E., Quadri, S.M., and Williams, J.R. (1993) Fractionated intravenous administration of ^{90}Y-labeled B72.3 GYK-DTPA immunoconjugate in beagle dogs. *Nucl Med Biol* 20: 571–578.

Willuda, J., Kubetzko, S., Waibel, R. et al. (2001) Tumor targeting of mono-, di-, and tetravalent anti-p185(HER-2) mniantibodies multimerized by self-associating peptides. *J Biol Chem* 276: 14385–14392.

Wittel, U.A., Jain, M., Goel, A. et al. (2004) The *in vivo* characteristics of genetically engineered divalent and tetravalent single-chain antibody constructs. *Nucl Med Biol* 32: 157–164.

Wu, A.M., Chen, W., Raubitshek, A. et al. (1996) Tumor localisation of anti-CEA single-chain FVs: improved targeting by non-covalent dimers. *Immunotechnology* 2: 21–36.

Wu, A.M., Yazaki, P.J., Tsai, S. et al. (2000) High-resolution microPET imaging of carcinoembryonic antigen poitive xenografts by using a copper-64-labeled engineered antibody fragment. *Proc Natl Acad Sci USA* 97: 8495–8500.

Yazaki, P.J., Shively, L., Clark, C. et al. (2001a) Mammalian expression and hollow fiber bioreactor production of recombinant anti-CEA diabody and minibody for clinical applications. *J Immunol Methods* 253: 195–208.

Yazaki, P.J., Wu, A.M., Tsai, S.W. et al. (2001b) Tumor targeting of radiometal labeled anti-CEA recombinant T84.66 diabody and t84.66 minibody: comparison to radioiodinated fragments. *Bioconjug Chem* 12: 220–228.

Zalutsky, M.R., Vaidyanathan, G. (2000) Astatine-211-labelled radiotherapeutics: An emerging approach to targeted alpha-particle radiotherapy. *Curr Pharmac Des* 6: 1433–1455.

Zhang, M., Zhang, Z., Garmestani, K. et al. (2003) Pretarget radiotherapy with an anti-CD25 antibody-streptavidin fusion protein was effective in therapy of leukemia/lymphoma xenografts. *Proc Natl Acad Sci USA* 100: 1891–1895.

2
Bispecific Antibodies

Dafne Müller and Roland E. Kontermann

2.1
Introduction

Bispecific antibodies combine the antigen-binding sites of two antibodies within a single molecule. Thus they are able to bind two different epitopes simultaneously, either on the same or on different antigens. Besides applications for diagnostic purposes (e.g. recruiting detectable compounds), bispecific antibodies open up new avenues for therapeutic applications by redirecting potent effector systems to diseased areas or by increasing neutralizing or stimulating activities of antibodies (Fig. 2.1). They are thus able to improve efficacy and selectivity of natural effector functions and to expand effector functions to those not exerted by natural immunoglobulins (Fanger et al. 1992; Cao and Suresh 1998; van Spriel et al. 2000; Cao and Lam 2003).

In the last two decades a variety of different effector functions have been combined with bispecific antibodies, including the retargeting of effector molecules, effector cells, as well as carrier systems and viruses (Fig. 2.1). Thus, bispecific antibodies cover a wide range of applications from the fields of immunotherapy, chemotherapy, radiotherapy, and gene therapy (Cao and Suresh 1998). Developments for clinical applications of bispecific antibodies have mainly focused on the retargeting of different effector cells of the immune system (e.g. to tumor cells), although various other therapeutic strategies have been evaluated. The initial high expectations were not fulfilled, however, for several reasons, as reflected by the fact that as yet no bispecific antibody has been approved. The main problems have been the antibody formats (e.g. whole IgG molecules of murine origin), but also insufficient therapeutic effects. Developments in the field of antibody engineering have resulted in new approaches to improve the efficacy and safety of therapeutic antibodies. This had a substantial impact on the generation of novel bispecific antibody formats and led to a revival of interest in bispecific antibodies (Kufer et al. 2004).

Handbook of Therapeutic Antibodies. Edited by Stefan Dübel

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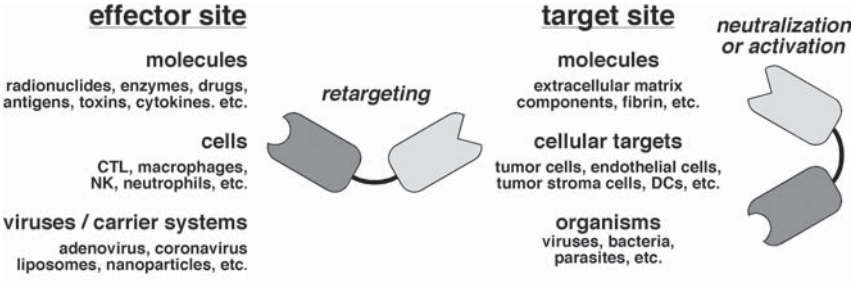

Fig. 2.1 Therapeutic applications of bispecific antibodies.

2.2 The Generation of Bispecific Antibodies

Bispecific antibodies are artificial molecules and are not produced by normal B cells. Various methods have been developed to generate them, including chemical conjugation of two antibodies or antibody fragments, fusion of two different antibody-secreting cells to a quadroma, and genetic approaches producing recombinant bispecific antibody molecules.

2.2.1 Somatic Hybridization

Early studies revealed that fusion of a B cell with a myeloma cell line results in a hybrid myeloma cell line (hybridoma) that is not subjected to allelic exclusion (Köhler and Milstein 1975). Subsequently, it was shown that fusion of two antibody-secreting hybridomas results in a quadroma, producing two different heavy chains and two different light chains within one cell, which randomly assemble to immunoglobulin molecules including bispecific molecules (Milstein and Cuello 1983). Besides two hybridomas secreting antibodies of known specificity, a hybridoma can be fused with splenic B cells (e.g. from immunized animals). In this case the resulting quadromas produce a large repertoire of bispecific antibodies from which those with the desired binding properties can be selected (Lloyd and Goldrosen 1991). Fusion of the two antibody-secreting cells can be achieved by standard methods (e.g. using polyethylene glycol (PEG) as fusogenic agent) (Link and Weiner 1993) or electrofusion (Cao et al. 1995; Kreutz et al. 1998). Quadromas with specificities for both antigens are then identified by immunological methods (e.g. by ELISA, flow cytometry, or immunoblotting). The enrichment of quadromas after fusion is facilitated by modified selection protocols that enrich for fused cells. The fusion of two parental cells resistant and sensitive to different chemicals (e.g. HAT/neomycin; HAT/ouabain) allows for a selection for double resistance (Staerz and Bevan 1986; De Lau et al. 1989; Link and Weiner 1993). The necessary resistant parental cells can be generated by growing cells in the presence of increasing concentrations of selection reagent or

by introducing resistance genes by genetic means (e.g. by retroviral gene transfer) (De Lau et al. 1989). Alternatively, selection methods based on fluorescence-activated cell sorting (FACS) using parental cells labeled with different fluorescent dyes have been established (Karawajew et al. 1987; Koolwijk et al. 1988; Kreutz et al. 1998).

Although methods to generate quadromas are well established, one of the major limitations of the hybrid hybridoma technology results from the production of a substantial number of nonfunctional molecules containing no or only one active binding site due to random heavy and light chain pairing (De Lau et al. 1991; Smith et al. 1992). On average, only 1 in 10 antibodies produced will be bispecific. Thus, elaborate purification steps are required to obtain homogeneous bispecific antibody preparations.

Interestingly, heterologous pairing of heavy chains with different isotypes or derived from different species can take place, while a preferential and species-restricted heavy–light chain pairing was reported (Corvalan and Smith 1987; Koolwijk et al. 1989; Link and Weiner 1993; Lindhofer et al. 1995). Heterologous heavy chain pairing can help to separate bispecific antibodies from parental or mismatched antibodies (e.g. by ionic exchange chromatography) (Link and Weiner 1993). Studies showed that bispecific antibodies derived from heterologous heavy chain pairings (e.g. mouse IgG1/IgG2a) are still able to bind to human Fc receptors and to recruit complement C1q (Koolwijk et al. 1989, 1991). However, an active Fc region within a bispecific antibody represents a third functional region, which might be undesirable for certain applications. Consequently, several groups have prepared bispecific $F(ab')_2$ molecules by proteolytic cleavage of bispecific antibodies derived from hybrid hybridomas (Fig. 2.2) (Warnaar et al. 1994; Tutt et al. 1995).

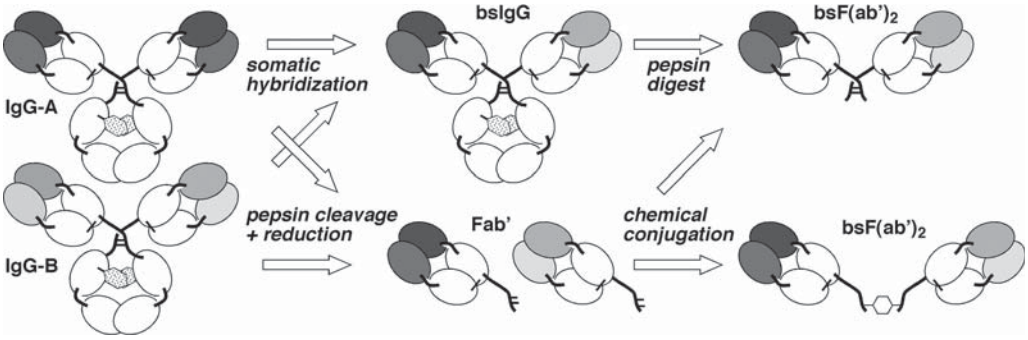

Fig. 2.2 Bispecific antibodies (bsIgG, $bsF(ab')_2$) generated by somatic hybridization or by chemical conjugation of two Fab′ fragments. bsIgG, bispecific IgG; $bsF(ab')_2$, bispecific $F(ab')_2$ fragment.

2.2.2 Chemical Conjugation

In 1961 Nisonoff and Rivers described for the first time bispecific antibodies which were generated by oxidation of Fab′ fragments derived from two polyclonal antibody preparations (Nisonoff and Rivers 1961). Although this oxidative reassociation strategy can also be applied to generate bispecific antibodies from monoclonal antibodies, it has the disadvantage of producing a mixture of monospecific and bispecific molecules (Paulus 1985). Improved methods employ homo- or heterobifunctional crosslinking reagents to conjugate two antibodies or antibody fragments such as Fab or Fab′ fragments (Fig. 2.2) (Graziano and Guptill 2004).

Heterobifunctional crosslinkers (e.g. SPDP (*N*-succinimidyl-3-(2-pyridyldithio) propionate)), introduce in a first reaction free thiol groups at amino groups which then can be used to form disulfide linkages with a second thiol-exposing protein. However, due to the presence of a large number of free amino groups in antibodies and antibody fragments this reaction causes random crosslinking and thus produces heterogeneous populations of crosslinked antibodies (Paulus 1985). This problem can be largely avoided using thiol-reactive homobifunctional reagents. Two such crosslinking reagents, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *o*-phenylenedimaleimide (*o*-PDM), have been primarily used for the generation of bispecific antibodies from Fab′ fragments exposing free thiol groups at the remaining hinge region (Brennan 1986; Glennie et al. 1987). These two reagents differ in the linkage produced between two proteins. While DTNB introduces a disulfide bond identical to that found in natural hinge regions, *o*-PDM generates more stable thioether bonds but a *o*-PDM moiety is left attached to the final product with the potential risk of immunogenicity (Fig. 2.3) (Graziano and Guptill 2004). Furthermore, conjugation of two Fab′ fragments with *o*-PDM requires the presence of an odd number of reactive thiol groups in the maleimidated Fab′ fragment (Fab′-A) due to the dual reactivity of *o*-PDM.

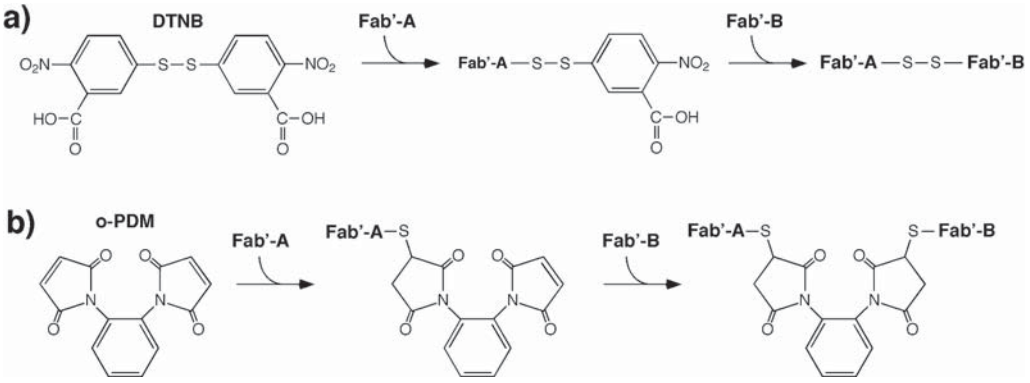

Fig. 2.3 Chemical crosslinking of two Fab′ fragments using DTNB (a) or *o*-PDM (b). DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); *o*-PDM, *o*-phenylenedimaleimide.

Several bispecific $F(ab')_2$ molecules that have entered clinical trials have been generated by the use of DTNB as crosslinking reagent (Keler et al. 1997; Russoniello et al. 1998). Production of such bispecific $F(ab')_2$ molecules is a multistep process starting from two monoclonal antibodies. In the first step Fab′ fragments are generated by pepsin digestion and reduction. The reduced first fragment is then incubated with the crosslinking reagent, the intermediate product purified by gel filtration and subsequently incubated with the reduced second fragment. The final product is again purified by gel filtration (Keler et al. 1997).

Problems associated with chemical crosslinking arise from the fact that the hinge regions contain varying numbers of cysteine residues depending on the antibody class and subclass (2–11 in the human IgG subclasses and 1–4 in the murine IgG subclasses). The presence of more than one thiol group may result in intrachain disulfide bond formation, which in the case of DTNB can be avoided by the use of dithiol complexing agents such as arsenite (Brennan et al. 1985). The conjugation of two Fab′ fragments derived from different subclasses or species possessing different numbers of free thiols may further leave free reactive thiols or lead to multimeric conjugates (Tutt et al. 1991; Graziano and Guptill 2004). These obstacles can be circumvented using genetically engineered Fab′ fragments possessing only one reactive thiol group at the hinge region. This approach also allows for the implementation of humanized or human Fab′ molecules leading to bispecific $F(ab')_2$ molecules with reduced immunogenicity (Shalaby et al. 1992).

2.2.3
Recombinant Bispecific Antibody Molecules

Initial clinical trials have revealed several limitations of bispecific IgG molecules derived from monoclonal antibodies (Segal et al. 1999; van Spriel et al. 2000). Besides production problems often leading to heterogeneous antibody preparations, therapeutic efficacy was limited by the induction of a neutralizing immune response against the non-human bispecific antibodies and severe Fc-mediated side effects such as cytokine-release syndrome, thrombocytopenia, and leukopenia.

Genetic engineering offers the possibility of generating novel bispecific antibodies with improved properties, especially for clinical applications. Using DNA from humanized or human antibodies (see Volume I of this book), recombinant antibodies can be generated that are fully or partially human and thus should have reduced immunogenicity compared with bispecific antibodies made from rodent monoclonal antibodies. In addition, various recombinant antibody formats are available which lack the Fc region of normal antibodies and therefore do not induce Fc-mediated side effects. Furthermore, generation of recombinant formats often results in a defined composition that facilitates production.

Over the past 15 years a plethora of different recombinant bispecific antibody molecules have been developed. These formats can be divided into (1) those based on variable domains of immunoglobulins only, (2) those which use constant immunoglobulin domains for heterodimerization, and (3) those which use non-

immunoglobulin sequences for heterodimerization (Kriangkum et al. 2001; Marvin and Zhu 2005; Plückthun and Pack 1997).

2.2.3.1 Small Recombinant Bispecific Antibody Formats Derived from the Variable Domain

Single-chain Fv (scFv) fragments are the prototype recombinant antibody molecules containing the complete antigen-binding site of an antibody. ScFv molecules are composed of the variable heavy and light chain domain interconnected by a short peptide sequence of approximately 15 amino acid residues. ScFv fragments can be readily obtained from hybridomas or from other sources, such as combinatorial antibody libraries (e.g. using phage display technology). In addition, they can be easily subjected to affinity maturation and humanization procedures (see Volume I).

Various small bispecific antibody formats can be generated using two scFv fragments with different antigen-binding activities (Kriangkum et al. 2001). The most commonly used formats that have been evaluated for therapeutic applications are bispecific tandem scFv molecules (taFv), bispecific diabodies (Db), and bispecific single-chain diabodies (scDb) as well as several derivatives thereof (Fig. 2.4). All three formats are composed of four variable domains (V_HA, V_LA, V_HB, V_LB) and possess a molecular weight of approximately 60 kDa. They represent the smallest bispecific antibody molecules derived from the entire antigen-binding sites of two antibodies.

Tandem scFv molecules (taFv) are easily generated by connecting two scFv molecules with an additional middle linker sequence (linker M) (Fig. 2.4). They therefore represent a single gene-encoded bispecific antibody format where each scFv unit forms a separate folding entity. Tandem scFv molecules can be expressed using various arrangements of the variable domains: V_HA-V_LA-linker M-V_HB-V_LB, V_LA-V_HA-linker M-V_HB-V_LB, V_LA-V_HA-linker M-V_LB-V_HB, or V_HA-V_LA-linker M-V_LB-V_HB. In all cases, the flanking linkers within the scFv units have a length

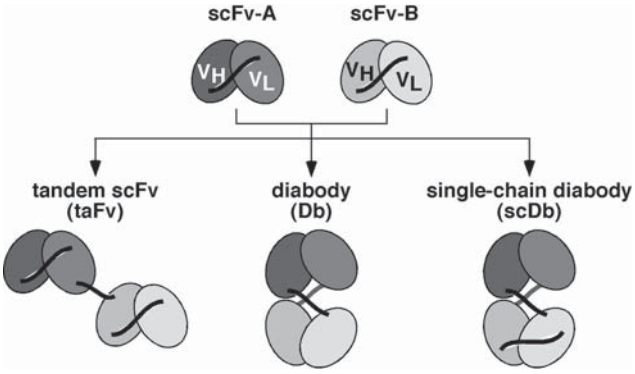

Fig. 2.4 Recombinant bispecific antibodies (tandem scFv, diabody, single-chain diabody) derived from two different scFv molecules (scFv-A, scFv-B).

of 15–20 amino acids to allow for an assembly of the variable heavy and light chain domain of each scFv into an active binding site. In contrast, the middle linkers can be of various length and composition. Examples include a very short Ala_3 linker (Brandaõ et al. 2003), a hydrophilic six-residue linker identified by a phage display approach (Korn et al. 2004a), glycine/serine-rich linkers (Kufer et al. 1997; McCall et al. 2001), linkers adopting a helical structure (Hayden et al. 1994), and linkers derived from various natural interconnecting sequences from immunoglobulins or immunoglobulin-like molecules (Grosse-Hovest et al. 2004; Ren-Heidenreich et al. 2004). Most of the described tandem scFv molecules have to be expressed in mammalian cells in order to obtain soluble protein, although several groups meanwhile have demonstrated soluble expression in bacteria (for an overview see: Kontermann 2005). In tandem scFv molecules the two antigen-binding sites are connected in a flexible manner, which might be advantageous for certain applications. However, until now the few direct comparisons (e.g. of tandem scFv and the more rigid single-chain diabody molecules (see below)), have not revealed functional differences in the efficacy of retargeting effector systems to target cells (Korn et al. 2004a).

Diabodies (Db) form when the linker sequence connecting the variable heavy and light chain domains of a scFv fragment is reduced to a length below 8–10 amino acid residues (Holliger et al. 1993). This reduction inhibits assembly of the V_H and V_L domains from one chain and promotes homodimerization of two V_H-V_L chains into a compact bivalent molecule containing two identical active binding sites. The diabody format can also be used to produce bispecific molecules by expressing two chains of the composition V_HA-V_LB and V_HB-V_LA (or V_LA-V_HB and V_LB-V_HA) within the same cell, which assemble into heterodimeric molecules containing two binding sites, one for each antigen (Fig. 2.4) (Holliger et al. 1993). Routinely, five residue linkers (e.g. with the sequence G_4S) are used to connect the variable domains, although other nonrepetitive linkers have been described (Völkel et al. 2001). These bispecific diabody molecules can be expressed in soluble form in bacteria or other systems such as *Pichia pastoris* or mammalian cells (for review see Kontermann 2005). However, the expression of two chains within one cell also leads to the assembly of homodimeric molecules. These molecules are functionally inactive as the V_H and V_L domains forming the antigen-binding sites are derived from two different antibodies. Attempts to improve the heterodimerization of the two chains include the introduction of interchain disulfide bonds (FitzGerald et al. 1997) and knobs-into-holes structures in the V_H-V_L interfaces (Zhu et al. 1997).

Single-chain diabodies (scDb) represent another approach to circumvent the problem of homodimerization observed for expression of bispecific diabodies. In this antibody format the two chains are connected by an additional middle linker (Fig. 2.4). Thus, all variable domains are present in a single polypeptide chain of the composition V_HA-V_LB-linker M-V_HB-V_LA or V_LA-V_HB-linker M-V_LB-V_HA, which assemble into monomeric molecules with a diabody-like structure containing two different antigen-binding sites (Brüsselbach et al. 1999). In the single-chain diabody configuration the two flanking linkers have a length of

approximately five residues, whereas the middle linker has the same length used for expression of scFv fragments (i.e. 15–20 residues) (Völkel et al. 2001). All single-chain diabodies analyzed so far could be expressed in bacteria in soluble form (for an overview see: Kontermann 2005). The single-chain diabody format was further modified to generate tetravalent bispecific molecules. This was achieved, for example, by reducing the middle linker to less than 12 amino acid residues, which led to homodimerization of two single-chain diabody chains into a molecule with a molecular weight of approximately 110 kDa (Kipriyanov et al. 1999; Völkel et al. 2001). Such a tetravalent bispecific tandem single-chain diabody directed against CD19 and CD3 mediated improved T-cell cytotoxicity *in vitro* in an autologous system and in combination with a costimulatory anti-CD28 antibody enhanced antitumor effects in an animal model (Cochlovius et al. 2000; Reusch et al. 2004).

For therapeutic applications the pharmacokinetic properties of these small bispecific antibodies are critical. Due to the small size of tandem scFv, diabodies, and single-chain diabodies, these molecules are rapidly cleared from the circulation (Kipriyanov et al. 1999; personal observations). Thus, several strategies are currently pursued to improve their pharmacokinetic properties. One approach is to increase the size of these molecules (e.g. by dimerization as in case of tandem single-chain diabodies, by fusion with other proteins or through PEGylation). Alternatively, direct *in vivo* expression of these molecules can result in high serum concentrations over a prolonged period of time with improved therapeutic efficacy (Blanco et al. 2003). This approach also obviates the need to purify the antibody molecules but requires a safe and efficacious gene transfer system (Kontermann et al. 2002; Sanz et al. 2004).

2.2.3.2 **Recombinant Bispecific Antibody Formats Containing Heterodimerization Domains**

A variety of other recombinant antibody formats have been developed that use heterodimerization domains for the assembly of bispecific molecules. One strategy applied the knobs-into-holes approach to the immunoglobulin C_H3 domain. Introduction of a knobs-into-holes structure into the C_H3 domain promotes heterodimerization of the immunoglobulin heavy chains or other fusion proteins containing these domains (Fig. 2.5a) (Carter 2001). These knobs-into-holes are generated by replacing in the first domain a small amino acid with a large amino acid and in the second domain at the adjacent position one or two large amino acids with small amino acids (Ridgeway et al. 1996; Atwell et al. 1997). However, although these mutations lead to heterodimerization of the heavy chains of whole IgG molecules, there is no preferential binding of the light chains. Initially, this problem was circumvented by using two parental antibodies possessing identical light chains (Merchant et al. 1998). More recently, single-chain Fv fragments were fused with knobs-into-holes Fc or C_H3 regions (Fig. 2.5a) generating bispecific scFv-Fc or scFv-C_H3 fusion proteins (minibodies) (Shahied et al. 2004; Xie et al. 2005).

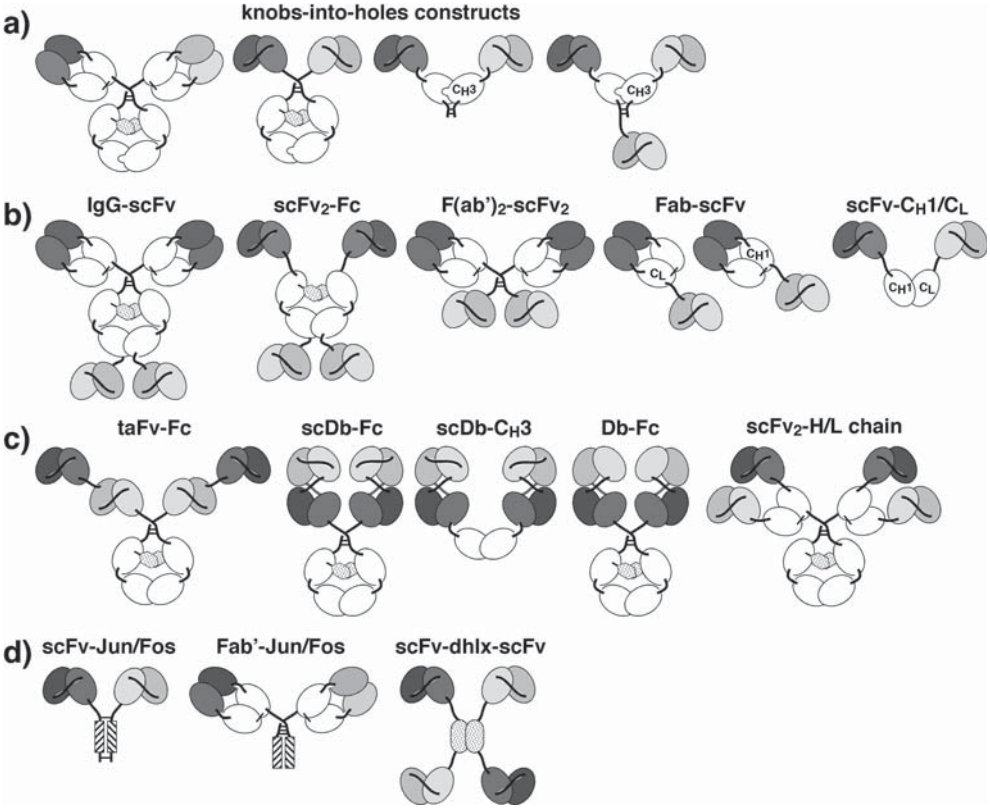

Fig. 2.5 Recombinant bispecific antibodies containing heterodimerization domains. (a) Knobs-into-holes strategies, (b) fusion of scFv fragments to constant immunoglobulin domains, (c) tetravalent and bispecific constructs, (d) fusion of scFv fragments to homo- or heterodimerizing peptides.

Alternatively, the interaction of the C_H1-C_L domains was employed for the generation of bispecific molecules fusing scFv fragments to the C_H1 and the C_L domain (Fig. 2.5b). This results in molecules with a structure similar to that of the above described scFv-C_H3 minibodies (Müller et al. 1998a). In another approach bispecific Fab-scFv fusion proteins were generated by fusing a scFv fragment either to the C-terminus of the C_H1 or the C_L domain of a Fab fragment (Schoonjans et al. 2000; Lu et al. 2002).

Various other bispecific but tetravalent antibody formats have also been developed that differ from the above described molecules in that they contain four antigen-binding sites per molecule (i.e. two for each antigen) (Fig. 2.5b,c). In one approach scFv fragments are fused either to the end of the C_H3 domain of an

intact antibody (IgG-scFv) or the hinge region ($F(ab')_2$-$scFv_2$ (Coloma and Morrison 1997). Alternatively, the above described small bispecific antibody molecules (tandem scFv, diabody, single-chain diabody, scFv-C_H1/C_L minibodies) were fused to the Fc region or C_H3 domain leading to IgG-like molecules with a molecular weight of 150–200 kDa (Connelly et al. 1998; Alt et al. 1999; Zuo et al. 2000; Lu et al. 2003, 2005; Schneider et al. 2005).

Finally, several approaches have utilized heterodimerizing peptides (e.g. jun-fos leucine zippers) for the generation of recombinant bispecific antibodies (Fig. 2.5d). Fusion of different scFv or Fab′ fragments to jun or fos peptides led to peptide-mediated heterodimerization. These dimers can be further stabilized by introducing flanking cysteine residues or using the natural hinge region (Kostelny et al. 1992; de Kruif and Logtenberg 1996). In addition, tetravalent and bispecific antibodies were constructed by linking two scFv fragments with a homodimerizing helix-loop-helix (dhlx) peptide (Müller et al. 1998b).

2.3 Bispecific Antibodies and Retargeting of Effector Cells

Antibodies are able to elicit therapeutic effects by recruiting effector cells of the immune system (e.g. natural killer (NK) cells, monocytes, macrophages, and granulocytes) to target cells, and inducing antibody-dependent cellular cytotoxicity (ADCC) and/or phagocytosis (Brekke and Sandlie 2003). Target cell-bound therapeutic antibodies, e.g. of the IgG1 isotype, are recognized by these effector cells through Fc receptors such as the high-affinity Fcγ receptor I (CD64) and the low-affinity Fcγ receptor III (CD16). However, the induction of ADCC is often limited since these antibodies also bind to non-activating Fc receptors (FcγRIIIb on polymorphonuclear leukocytes (PMNs)), inhibitory Fc receptors (FcγRIIb on monocytes/macrophages) or to Fc receptors on non-cytotoxic cells (FcγRII on platelets and B cells) (Peipp and Valerius 2002). In addition, therapeutic antibodies compete with serum IgGs for binding to the high-affinity Fcγ receptor I (CD64), leading to poor recruitment of monocytes and macrophages (Valerius et al. 1997). Finally, effector cells lacking Fc receptors, such as cytotoxic T lymphocytes, cannot be recruited with conventional antibodies. Developments in the field of bispecific antibodies for therapeutic applications have therefore been focused on the selective retargeting of potent effector cells of the immune system to tumor cells by binding with one arm to a tumor-associated antigen and with the other arm to a trigger molecule on the effector cell. Effector cells retargeted with bispecific antibodies include cytotoxic T lymphocytes, natural killer cells, monocytes and macrophages, as well as PMNs (e.g. neutrophils) by binding to trigger molecules expressed by these cells (Fig. 2.6) (Fanger and Guyre 1991; de Gast et al. 1997; van Spriel et al. 2000). Bispecific antibodies are thus able to elicit local target cell destruction by these effector cells and to circumvent limitations associated with conventional therapeutic antibodies, as described above (van de Winkel et al. 1997).

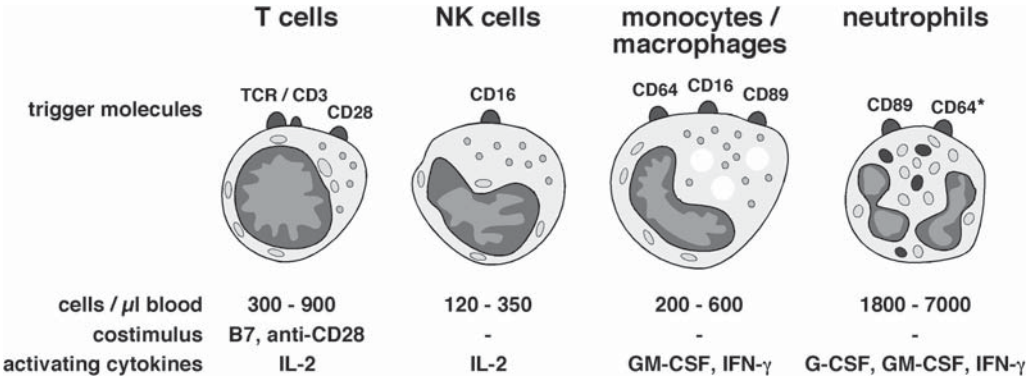

Fig. 2.6 Effector cells, trigger molecules and costimulating/activating molecules. *Upregulated after cytokine activation.

2.3.1 Retargeting of Cytotoxic T Lymphocytes

Cytotoxic T lymphocytes (CTLs) are considered to be the most potent killer cells of the immune system. Under physiological conditions, recognition and killing of a target cell is a highly controlled process involving antigen-specific binding of the T-cell receptor to major histocompatibility complexes (MHCs) on target cells. In order to be fully activated, CTLs need a second stimulus mainly provided by interaction of membrane-bound B7 molecules with CD28 on T lymphocytes during the process of T cell activation by antigen-presenting cells. CTLs can kill target cells by the perforin/granzyme pathways, leading to necrosis and apoptosis and by death receptor-mediated induction of apoptosis (e.g. through FasL/CD95) (Russell and Ley 2002).

Over the last two decades, a large number of bispecific antibodies have been developed for retargeting of CTLs to tumor cells, and more recently also to other cells such as cells of the tumor vasculature and tumor stroma (Molema et al. 2000; Wuest et al. 2001; Korn et al. 2004b; Lum and Davol 2005). Bispecific antibodies used for the retargeting of CTLs have the advantage of bypassing MHC-restricted target-cell recognition by CTLs, a process that is often inadequate due to downregulation or loss of MHC molecules on tumor cells (Bubenik 2003). Most bispecific antibodies developed for the retargeting of CTLs are directed against CD3, which is a multi-subunit complex associated with the T-cell receptor (TCR), but other trigger molecules such as CD2 or the TCR itself have also been evaluated (Fanger et al. 1992). Initially, bispecific antibodies for T-cell retargeting were generated by the hybrid hybridoma technology. Thus, these bispecific antibodies were whole IgG molecules or $F(ab')_2$ fragments of mouse or mouse/rat origin (Table 2.1). While potent tumor cell lysis was observed *in vitro* as well as

Table 2.1 Clinical trials with bispecific antibodies.

Antibody	*Specificity*	*Format*	*Costimulus*	*Indication*	*Status*
SHR-1	CD19 × CD3	IgG (rat/mouse)	–	Non-Hodgkin's lymphoma	Phase I
O6A (OKT3 × 6A4)	CD19 × CD3	IgG (mouse)	Anti-CD28	Non-Hodgkin's lymphoma	Phase I
OC/TR	FR × CD3	$F(ab')_2$ (mouse)	–	Ovarian cancer	Phase I/II
Bis-1	EGP2 × CD3	$F(ab')_2$ (mouse)	IL-2	Renal carcinoma	Phase I
OKT3 × Herceptin	HER2 × CD3	IgG (mouse/humanized)	IL-2, GM-CSF	Breast cancer	Phase I
Catumaxomab (removab)[a]	EpCAM × CD3	IgG (rat/mouse)	–	Malignant ascites, NSCLC, ovarian and breast cancer	Phase I–III
Ertumaxomab (rexomun)[a]	HER2/neu × CD3	IgG (rat/mouse)	–	Metastatic breast cancer	Phase II
MT103[b]	CD19 × CD3	Tandem scFv (mouse)	–	Non-Hodgkin's lymphoma	Phase I
rM28	HMW–MAA × CD28	Tandem scFv (mouse)	–	Melanoma	Phase I/II
HRS-3/A9	CD30 × CD16	IgG1 (mouse)	–	Hodgkin's lymphoma	Phase I/II
2B1	Her2/neu × CD16	IgG (mouse)	–	Lung, breast, ovarian, colon, kidney, prostate, pancreas, stomach cancer	Phase I
H22 × Ki-4	CD30 × CD64	$F(ab')_2$ (mouse/humanized)	–	Hodgkin's lymphoma	Phase I
MDX-220[c]	TAG72 × CD64	$F(ab')_2$	G-CSF	Prostate and colon cancer	Phase I/II
MDX-447[c]	EGFR × CD64	$F(ab')_2$ (humanized)	G-CSF	Renal cell carcinoma, head and neck, bladder, kidney, prostate cancer	Phase I/II
MDX-H210[c,d] (IDM-1, Osidem)	HER2/neu × CD64	$F(ab')_2$ (mouse/humanized)	G-CSF, GM-CSF, IFNγ	Renal cell carcinoma, breast, prostate, colon cancer	Phase III

a Fresenius; **b** micromet; **c** Medarex, **d** IDM.

in animal experiments, initial clinical trials faced several problems, despite reported clinical responses (Segal et al. 1999; van Spriel et al. 2000; Withoff et al. 2001). In most of these studies toxicity was observed due to release of inflammatory cytokines (cytokine storm), especially after parenteral administration of the antibodies, which might in part be caused by the presence of a Fc region. In addition, in all studies using bispecific IgG or $F(ab')_2$ molecules of mouse origin, the patients developed human anti-mouse antibody responses (HAMA). It was concluded that the ideal bispecific antibody for retargeting of CTLs, but also other effector cells, should (1) be highly selective for the target cell, (2) retarget relevant effector cells, (3) bind monovalently to the effector cells and activate them only upon binding to target cells, (4) lack a Fc region to avoid Fc-mediated side effects, (5) be human or humanized to avoid a neutralizing antibody response, and (6) be small enough to penetrate tumor tissues but large enough to circulate for a sufficient length of time (Segal et al. 1999).

In the course of progress of recombinant antibody technology, new bispecific antibody formats have been generated in order to address the problems of the HAMA response, Fc-mediated side effects and tumor penetration, but also production of bispecific antibodies with a defined composition. In parallel, much effort has gone into investigation of how to provide appropriate costimulatory signals, considered pivotal in the effective triggering of a cellular immune response. Several bispecific tandem scFv molecules, diabodies and single-chain diabodies with specificity for CD3 and tumor-associated antigens such as CD19, CD20, EpCAM, HER2/neu, and CEA have been reported to efficiently retarget T-cell cytotoxicity to tumor cells and to induce antitumoral activity *in vivo* (for review see Peipp and Valerius 2002; Kontermann 2005). In order to provide a costimulatory signal, anti-CD28 antibodies or recombinant antibody B7 fusion proteins were included in the therapeutic setting (Cochlovius et al. 2000; Holliger et al. 1999). The fusion of an extracellular region of B7 or parts of it to scFv fragments, bivalent diabodies or other antibody fragments directed against a tumor-associated antigen was shown to result in targeted delivery of the costimulatory signal to the target cell (Gerstmayer et al. 1997; Holliger et al. 1999; Biburger et al. 2005).

Interestingly, bispecific antibodies based on anti-CD3 antibody TR66 developed by Lanzavecchia and Scheidegger (1987) and expressed as tandem scFv molecules were shown to be potent T-cell activators even in the absence of a costimulatory signal (Löffler et al. 2000). These bispecific tandem scFv molecules were termed BiTEs (bispecific T cell engagers). *In vitro* studies revealed that efficient target cell lysis is induced by BiTEs at very low antibody concentrations (between 0.01 and 10 ng mL^{-1}) and low effector-to-target cell ratios (E : T = 1 : 5) (Wolf et al. 2005; Hoffmann et al. 2005). Thus, in addition to the fact that these bispecific antibodies are costimulation independent, they seem to be superior compared with other bispecific antibodies targeting CD3 in respect of the efficacy of tumor cell lysis and the amount of antibody required for T-cell retargeting (Wolf et al. 2005). Several BiTEs directed against different tumor-associated antigens, including CD19 and EpCAM, have been developed and evaluated *in vitro* and in animal

experiments (Dreier et al. 2003; Schlereth et al. 2005). Currently, one BiTE directed against CD19 and CD3 is in a clinical phase I trial for the treatment of non-Hodgkin's lymphoma (Table 2.1).

Other studies have shown that binding of certain monoclonal antibodies to CD28 can induce CTL-mediated cytotoxicity without the need for a first activation through the TCR complex (Tacke et al. 1997). These superagonistic antibodies bind to a particular epitope (aa 60–65) on CD28 different from the region that interacts with B7 (aa 99–104) (Lüdher et al. 2003). A non-superagonistic anti-CD28 antibody was recently used to construct a bispecific tandem scFv molecule directed against the melanoma-associated glycoprotein MAPG (HMW-MAA) (Grosse-Hovest et al. 2003). This antibody, r28M, was able to induce efficient tumor cell killing *in vitro* and *in vivo* in a CD3-independent way (Grosse-Hovest et al. 2005). Interestingly, although r28M activates T cells leading to T cell-mediated killing of tumor cells, a contribution to killing of a non-T cell population ($CD56^+$ NK cells) was also observed. It was found that activation of these non-T cells is induced indirectly by rM28 through cytokines secreted by the activated T cells in the presence of antigen-positive target cells (Grosse-Hovest et al. 2005). The observed potency of rM28 *in vivo* might also be due to the large proportion of dimeric molecules in the rM28 preparation (>50%) with a molecular weight of approximately 115 kDa leading to an extended serum half-life compared with monomeric tandem scFv molecules. To produce sufficient amounts for further studies, transgenic cows were generated secreting rM28 at high concentrations into the blood (Grosse-Hovest et al. 2004). Currently, rM28 is being tested in a phase I/II clinical trial for the intralesional treatment of metastatic melanoma and unresectable metastasis (Table 2.1).

Despite the observation that the presence of an Fc part in bispecific antibodies can cause increased toxicity, it was found that intact bispecific hybrid antibodies composed of mouse IgG2a and rat IgG2b and directed against CD3 and a tumor-associated antigen can elicit a strong antitumor response *in vitro* and *in vivo* (Ruf and Lindhofer 2001). It was postulated that these trifunctional bispecific antibodies are able to simultaneously activate T cells via binding to CD3 and accessory cells (NK cells, mononuclear blood cells) through interactions with their Fc region. These activated accessory cells can deliver necessary costimulatory signals to the T cells and can further increase the immune response through phagocytosis of tumor material, leading to long-lasting antitumor immunity (Zeidler et al. 2000, 2001). Two trifunctional bispecific antibodies, removab (catumaxomab) directed against EpCAM and CD3 and rexomun (ertumaxomab) directed against HER2 and CD3 are currently being tested in clinical trials for the treatment of various cancers including malignant ascites, ovarian cancer, and breast cancer (Table 2.1). Results from phase I studies demonstrated a good tolerability after repeated intravenous or intraperitoneal injections and clinical responses (Heiss et al. 2005). The occurrence and involvement of HAMA has not yet been addressed.

2.3.2 Retargeting of Fc Receptor-bearing Effector Cells

A second group of effector cells consists of those naturally recruited by binding to Fc receptors. These include NK cells, monocytes/macrophages and PMNs (e. g. neutrophils). Consequently, bispecific antibodies have been generated for the retargeting of these effector cells by binding to Fc receptors such as FcγRI (CD64), FcγRIII (CD16), and FcαR (CD89) (see Fig. 2.6). NK cell-mediated cytotoxicity is triggered by engagement of the low-affinity Fcγ receptor III (FcγRIIIA = CD16) that is constitutively expressed. Target cell destruction is achieved by mechanisms similar to those observed for CTLs. CD16 is also expressed by monocytes and macrophages, which, in addition, constitutively express the high-affinity FcγRI (CD64) and to some extend the FcαR (CD89). CD89 is the main Fc receptor on PMNs representing the largest effector cell population in the blood. These cells also express CD64 upon induction with interferon γ (IFNγ) and granulocyte colony-stimulating factor (G-CSF). In general, it was found that administration of growth factors (e.g. granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF) or cytokines (IFNγ and IL-2) can lead to further stimulation and proliferation of these effector cells (van Spriel et al. 2000).

Bispecific antibodies directed against Fc receptors are able to extend ADCC to cells normally not or only inefficiently recruited by conventional antibodies, such as PMNs or monocytes and macrophages. These cells are retargeted by binding to CD89 or CD64, respectively, and eliminate target cells directly through cytotoxicity or phagocytosis (Deo et al. 1998; Sundarapandiyan et al. 2001). In addition, since some Fc receptors such as CD64 are also present on antigen-presenting cells, bispecific antibodies can indirectly enhance antitumor immunity by increased antigen presentation (van Spriel et al. 2000).

Although preclinical studies with bispecific antibody-mediated retargeting of NK cells to tumor cells showed promising results *in vivo* (Hombach et al. 1993), first clinical trials were mainly characterized by toxicity (Weiner et al. 1995) and rather limited antitumor responses (Hartmann et al. 1997, 2001). This can be partly attributed to the bispecific antibody format used. Complete murine IgGs are thought to crosslink Fc receptors via the anti-CD16 antigen-binding site and the Fc region, inducing systemic leukocyte activation, characterized by extensive cytokine release. Another reason might be the specificity of most of the anti-CD16 antibodies used for construction, which do not differentiate between the activating (FcγRIIIA on NK cells) and non-activating (FcγRIIIB on PMNs) receptor isoforms (Hombach et al. 1993; Amoroso et al. 1999). Binding of the bispecific antibodies to FcγRIIIB as well as to shedded, soluble CD16 in human plasma might reduce their cytolytic activity unless a large molar excess of bispecific antibodies is applied. In addition, the establishment of the effective effector-to-target cell ratio at the tumor site is another critical point to be considered. Although under physiological conditions the expression pattern of CD64 predisposes it for monocyte/macrophage targeting, most bispecific antibody therapy

approaches include growth factor or cytokine treatment to induced CD64 upregulation on PMNs and to increase the effector cell population.

Diverse treatment schedules with IFNγ, G-CSF, or GM-CSF were investigated in clinical studies with MDX-H210 and MDX-447. These bispecific antibodies are partially or fully humanized $F(ab')_2$ molecules specific for CD16 × Her2/neu and CD16 × EGFR, respectively (Table 2.1). Although biological effects such as changes in circulating leukocyte subpopulation cell number and receptor expression, binding of the bispecific antibody to effector cells, enhanced ADCC/phagocytic capacity *in vitro*, cytokine release, and local infiltration of effector cells at the tumor site could be observed, clinical responses remained vague (Curnow 1997; Pullarkat et al. 1999; James et al. 2001; Lewis et al. 2001; Wallace et al. 2001; Repp et al. 2003). Now considerable interest focuses also on CD89 as trigger molecule, because it is constitutively expressed on neutrophils and the main trigger molecule to induce tumor cytolysis by these cells. Retargeting the cytotoxic potential of this large effector subpopulation to tumor cells normally not attacked by this effector mechanism seems feasible at least from *in vitro* studies (Deo et al. 1998).

Comparing a set of bispecific antibodies targeting CD20 or HER-2/neu and Fc receptors CD64, CD16, or CD89 in combination with G-CSF or GM-CSF revealed differences in the retargeting of effector cell cytotoxicity. This was dependent on Fc receptor expression, but also influenced by the growth factors and the tumor antigen involved (Stockmeyer et al. 2001).

Several recombinant bispecific antibodies, such as bispecific tandem scFv molecules directed against CD16 and various tumor-associated antigens (CD19, HER2/neu, HLA class II) have been recently developed for the retargeting of NK cells to tumor cells (McCall et al. 1999; Bruenke et al. 2004, 2005). Increasing binding of bispecific antibody molecules to the tumor-associated antigen, either by using high-affinity binding sites or by increasing the functional affinity with molecules containing two binding sites for the tumor-associated antigen, was shown to enhance antibody-mediated *in vitro* cytotoxicity (McCall et al. 2001; Xie et al. 2003; Shahied et al. 2004). In a further study the combination of bispecific diabodies directed against CD19 × CD3 and CD19 × CD16 demonstrated synergistic antitumor effects in a preclinical model of non-Hodgkin's lymphoma by retargeting different effector cell populations (Kipriyanov et al. 2002). Synergistic antitumor effects in a non-Hodgkin's lymphoma model were also observed by combined therapy with an anti-C19 × anti-CD16 bispecific diabody and the angiogenesis inhibitor thalidomide (Schlenzka et al. 2004). In summary, these findings underline the complexity of this still challenging approach, but also highlight potentials and perspectives of bispecific antibodies for retargeting effector cells to tumor cells.

2.4
Bispecific Antibodies and Retargeting of Effector Molecules

Besides retargeting of effector cells, bispecific antibodies can be applied to redirect effector molecules to other cells or structures associated with diseases. Thus, bispecific antibodies have been explored for the recruitment of a large number of different effector molecules, including radionuclides, drugs, toxins, enzymes, cytokines, complement components, and immunoglobulins (Cao and Lam 2003).

The use of bispecific antibodies circumvents chemical coupling of effector molecules. This might be especially advantageous in cases where chemical modifications may lead to inactivation of the effector molecules or the antibody. Furthermore, bispecific antibodies can be employed for the recruitment of natural effector molecules already present in the organism, such as components of the humoral immune system (Kontermann et al. 1997; Holliger et al. 1997). Importantly, the application of bispecific antibodies allows for an uncoupling of antibody-mediated targeting from delivery of effector molecules. This pretargeting strategy has extensively been studied for radioimmunotherapy (Gruaz-Guyon et al. 2005). In the first step, the bispecific antibody is injected, accumulates in the diseased tissue and unbound antibodies are allowed to clear from circulation and healthy tissues. In the second step, the effector molecule (e.g. a chelated radionuclide) is injected and is recovered by the second binding site of the bispecific antibody at the target site, while unbound effector molecules are rapidly eliminated (Fig. 2.7). Thus, side effects often seen with antibody conjugates can be reduced.

2.4.1
Bispecific Antibodies and Radioimmunotherapy

Radioimmunotherapy (RIT) is based on the selective antibody-mediated delivery of cell-damaging radionuclides into diseased tissues such as tumors. Several

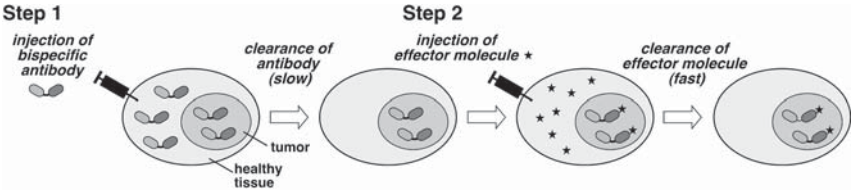

Fig. 2.7 Bispecific antibodies for pretargeting strategies. In the first step, the bispecific antibody is injected and allowed to accumulate in the tumor and to be cleared from circulation and healthy tissues. In the second step the small effector molecule is injected which is retained in the tumor by binding to the bispecific antibodies and is rapidly cleared from nontargeted tissue by renal excretion.

parameters influence efficacy of RIT, including tumor location, size, morphology, physiology and radiosensitivity, physical/chemical properties of the radionuclides and the nature of its radiation (low or high energy transfer), but also pharmacokinetic properties of the antibody (Goldenberg 2003). Several radionuclides are of clinical interest. Currently, mainly ^{131}I and ^{90}Y, both beta-emitters with a half-life of 193 and 64 h, respectively, are used for therapeutic applications, but various other beta-emitters with shorter half-lives (^{188}Rh) or alpha-emitters (^{211}At, ^{225}Ac) have also found increasing interest (Chang et al. 2002; Goldenberg 2003).

One problem associated with the application of antibody–radionuclide conjugates in therapy of solid tumors is an often observed toxicity in normal tissues due to an inappropriate tumor-to-normal tissue ratio. This limits total applicable dose and thus therapeutic efficacy. As described above, a pretargeting approach applying bispecific antibodies can uncouple the slow process of tumor targeting and antibody clearance from a rapid and selective delivery of the radionuclides.

In order to employ bispecific antibodies for RIT they have to bind with one arm to the radionuclide. This can be achieved using antibodies recognizing radionuclides complexed with a chelating agent such as DTPA (diethylenetriaminepentaacetic acid) or DOTA (1,4,7,10-tetra-azacyclododecane-N,N′,N″,N‴-tetraacetic acid) (Fig. 2.8a). This approach was further improved by generating bivalent molecules in respect to the chelating agent (i.e. containing two chelators (Fig. 2.8b)) (Le Doussal et al. 1990). The advantages of these molecules are increased functional affinity (affinity enhancement system) and binding of two radionuclides (e.g. ^{111}In) per molecule. In addition, the aromatic side chain of the tyrosine residue present in these molecules can be used for conjugation of other radionuclides such as ^{131}I (Morandeau et al. 2005). Alternatively, the chelating agent can be coupled to a hapten to which antibodies are available. HSG (histamine-succinyl-glycine) represents such a peptide hapten used for RIT (Fig. 2.8b). In its simplest form a chelating agent such as DOTA is conjugated to one HSG peptide. More advanced systems consist of two HSG peptides, leading again to increased functional affinity, and additional compounds for labeling with radionuclides. Several of these bivalent haptens have been developed which can be used to deliver various radionuclides (e.g. using the tyrosine side-chain of the central core or a chelating agent attached to these molecules) (Morandeau et al. 2005).

Various preclinical studies have shown that the use of the pretargeting approach in RIT results in reduced toxicity and allows for the administration of higher doses with enhanced antitumor effects (Sharkey et al. 2005a). Bispecific antibodies tested so far were directed against CEA, CD20, or the renal cell carcinoma marker G250 (Gautherot et al. 1997; Krannenborg et al. 1998; Sharkey et al. 2005b). Besides bispecific F(ab′)$_2$ molecules generated by chemical coupling, recombinant bispecific molecules (diabodies) have been developed for RIT (DeNardo et al. 2001). Currently a bispecific F(ab′)$_2$ molecule (hMN14 × m734) directed against carcinoembryonic antigen (CEA) and a ^{131}I-labeled di-DTPA molecule is tested in clinical trials for the treatment of patients with various CEA-positive tumors (Kraeber-Bodéré et al. 2003). This bispecific antibody fragment

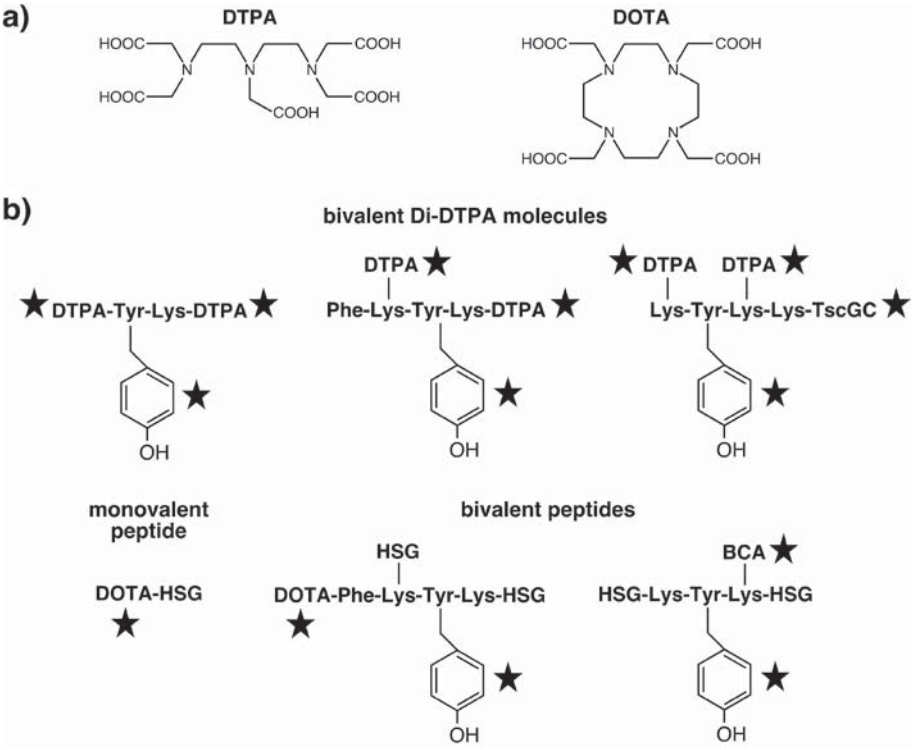

Fig. 2.8 (a) Structures of chelating agents DTPA and DOTA. (b) Examples of various mono- and bivalent haptens developed for pretargeting with bispecific antibodies. Stars indicate possible positions for labeling with radionuclides. DTPA, diethylenetriaminepentaacetic acid; DOTA, 1,4,7,10-tetra-azacyclododecane-N,N′,N″,N‴-tetraacetic acid; HSG, histamine-succinyl-glycine; BCA, bifunctional chelating agent; TscGC, thiosemicarbonylglyoxylcysteinyl.

is derived from a humanized anti-CEA antibody and a mouse anti-hapten antibody and was generated by chemical crosslinking with *o*-PDM. Thus far, the best results with this antibody have been obtained with a bispecific antibody dose of 40 mg m^{-2} given at 5-day intervals and treatment with doses up to 5.5 GBq in the absence of bone marrow involvement. Although further studies are inevitable to establish safety and efficacy, the encouraging results seen in clinical studies make this a promising approach to improve radioimmunotherapy with monoclonal antibodies.

2.4.2 Bispecific Antibodies and Targeting of Toxins and Drugs

Several proteins and small molecules (e.g. toxins and chemotherapeutic drugs) are known to be potent inhibitors of cell viability, growth, and proliferation.

Bispecific antibodies have been generated to target such therapeutically useful substances to target cells, with an emphasis on tumor therapy and autoimmune diseases (Embleton et al. 1991; Ferrini et al. 2001; Kuus-Reichel et al. 1995). The advantage is that chemical crosslinking, which might interfere with the activity of the therapeutic molecule and/or the antibody molecule, is not required. By this approach selectivity of the drug as well as target cell sensitivity can be increased (Ford et al. 2001). Toxins targeted with bispecific antibodies include ribosome-inactivating proteins such as saporin, gelonin, and ricin. In addition, several drugs (e.g. the anthracyclin doxorubicin, the antimetabolite drug methotrexate, and the vinca alkaloids vincristine, vindesine, and vinblastine) have been combined with bispecific antibodies for drug delivery (Cao and Lam 2003).

A study with a bispecific antibody generated by the hybrid-hybridoma technology and directed against CEA and doxorubicin (Dox) showed that the antibody can significantly reduce IC_{50} values for Dox with CEA-expressing tumor cell lines and improve inhibition of tumor growth in animal models (Ford et al. 2001). In another study, a remarkable antidotal activity was observed *in vivo* with a bispecific antibody targeting Dox to EGF receptor-expressing tumor cells, while antitumor effects were equal to those of the drug alone (Morelli et al. 1994). Of note, the analysis of a bispecific antibody directed against the IL-2 receptor and vincristine revealed additive and not synergistic effects of the antibody and the vinca alkaloid in the therapy of diabetic mice (Kuus-Reichel et al. 1995). This was attributed to an inactivation of the drug by the antibody, leading to an inaccessibility of the drug to the target cells. This finding indicates that antibodies have to be carefully selected in order to preserve the activity of the drug. Interestingly, synergistic toxic effects were described for the use of two bispecific antibodies, recognizing different nonblocking epitopes on saporin or gelonin redirected to human B cell lymphoma cell lines (Bonardi et al. 1993; Sforzini et al. 1995). Other studies showed that a combination of bispecific antibodies directed against a toxin and two different cell surface markers (e.g. CD22 and CD37 or CD7 and CD38) can also improve cytotoxicity in a synergistic manner (Flavell et al. 1992; French et al. 1995). These findings demonstrate that an increase in functional affinity, either for the drug or the target cell, can enhance therapeutic efficacy.

Currently, there are no data available on comparison of bispecific antibodies and antibody conjugates in a therapeutic preclinical or clinical setting. Initial studies of drug targeting with bispecific antibodies faced the same problems as observed for various other approaches (e.g. related to immunogenicity) (Bonardi et al. 1992). Further studies therefore have to be conducted to show if bispecific antibodies are superior to immunotoxins and antibody–drug conjugates in terms of production, safety, and efficacy.

2.5
Bispecific Antibodies as Agonists or Antagonists

Bispecific antibodies are able to bind to two different epitopes on the same or on different antigens. This dual binding can lead to an increase in func-

tional affinity and can improve the neutralizing or activating potential of antibodies.

Agonistic activities were demonstrated for a bispecific tandem scFv-Fc fusion protein directed against the T-cell antigen CD2. This antibody was shown to be a potent mitogen for T cells, displaying enhanced mitogenic properties compared with the combination of monoclonal antibodies (Connelly et al. 1998). In another study, a bispecific tandem scFv molecule directed against two epitopes on CTLA-4 was even able to convert CTLA-4 from an inhibitor to an activator of T cells. These T cell-activating activities could become clinically useful for boosting immunity (e.g. in vaccination or cancer immunotherapy) (Madrenas et al. 2004).

Bispecific antibodies are also potent antagonists by simultaneous binding to two epitopes on the same or different receptors. Thus, it was shown that a bispecific diabody directed against two epitopes on vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) efficiently blocked binding of VEGF to its receptor and inhibited VEGF-induced activation of the receptor and mitogenesis of endothelial cells, while neither of the parental scFv fragments showed any inhibitory activity (Lu et al. 1999). In further studies, a bispecific diabody as well as a tetravalent and bispecific diabody – Fc fusion protein (di-diabody) directed against VEGF receptor 2 and 3 blocked binding of VEGF and VEGF-C to their receptors and inhibited activation of both receptors (Lu et al. 2001, 2003; Jimenez et al. 2005). In a similar approach, IgG-like tetravalent and bispecific antibodies (diabody-Fc, $scFv_2$-H/L chain fusion proteins) were generated which bind simultaneously to the epidermal growth factor (EGF) receptor and the insulin-like growth factor (IGF) receptor (Lu et al. 2004). These antibodies blocked binding of EGF and IGF to their receptors and inhibited activation of several signal transduction proteins. In addition, due to the presence of an Fc region in these bispecific molecules they were able to mediate ADCC, which led to growth inhibition of human tumor xenografts *in vivo* (Lu et al. 2005). These studies underline that bispecific antibodies directed against cell surface receptors might be beneficial for therapeutic applications by simultaneously neutralizing two receptors and/or by improving Fc-mediated effector functions.

The approach of simultaneously targeting two essential receptors was also applied to phenotypic knockout by intrabodies. This was shown for a tetravalent and bispecific antibody molecule ($scFv_2$-Fc = intradiabody) directed against VEGFR2 and Tie-2, which was expressed in the endoplasmic reticulum (ER) of endothelial cells by attaching a KDEL retention signal to the C-terminus (Jendreyko et al. 2003). Compared with the ER-targeted parental scFv fragment, expression of the intradiabody resulted in a more efficient and longer lasting surface depletion of the two receptors and strong anti-angiogenic activity in *in vitro* endothelial cell tube formation assays. After adenoviral gene transfer of the bispecific antibody construct by subtumoral injection an efficient inhibition of tumor growth and tumor angiogenesis was observed (Jendreyko et al. 2005).

Further applications of neutralizing bispecific antibodies include the treatment of infectious diseases (e.g. viral infections), as shown with a tetravalent bispecific

antibody (tandem scFv-Fc fusion protein) directed against two surface antigens (S and pre-S2) of hepatitis B virus (Park et al. 2000).

2.6 Bispecific Antibodies and Somatic Gene Therapy

The *in vivo* transfer of DNA into living cells offers the possibility of curing monogenic hereditary diseases or expressing a therapeutic protein within the cell (e.g. a suicide protein for cancer therapy). Transfer is accomplished using nonviral carrier systems (liposomes, polymers) or viral vectors (e.g. adenovirus, adeno-associated virus, retroviruses) (El-Aneed 2004; Bartsch et al. 2005; Hendrie and Russell 2005). However, most systems lack specificity for the target cells, which limits efficacy and safety of gene transfer. Targeting to specific cell types and uptake into the cells can be achieved by incorporating ligands such as antibodies or peptides into the surface of the gene transfer vehicle (Walther and Stein 2000). Bispecific antibodies have been developed as adapter molecules to direct viral vectors to target cells, especially in cases where genetic fusion of antibody fragments with viral coat proteins was not successful (e.g. for adenoviral vectors) (Barnett et al. 2002; Everts and Curiel 2004). Bispecific antibodies have the advantage that binding of one arm to a viral coat protein can neutralize the wild-type tropism of the virus, as shown for adenovirus of the serotype 5 and adeno-associated virus (AAV) (Watkins et al. 1997; Bartlett et al. 1999). Thus, bispecific antibodies inhibit binding to natural receptors and allow for a retargeting to a specific receptor on the target cell (Fig. 2.9).

Both bispecific chemical conjugates ($F(ab')_2$) and recombinant bispecific antibodies (tandem scFv, single-chain diabodies) have been evaluated for retargeting

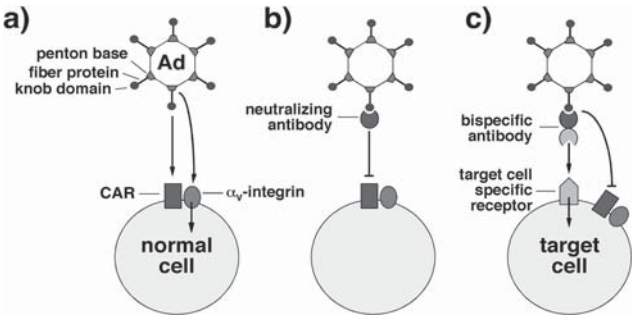

Fig. 2.9 Bispecific antibody-mediated retargeting of adenoviral vectors (serotype 5) to target cells. Wildtype adenovirus (Ad) binds with the fiber knob domain to the coxsackievirus and adenovirus receptor (CAR) on cells. Internalization is mediated by interaction of the penton base with α_v-integrins (a). Antibodies can inhibit adenoviral transduction by neutralizing the binding of the knob domain to CAR (b). Bispecific antibodies against the knob domain and a target cell-specific receptor redirect adenoviruses to new target cells and inhibit transduction of CAR-positive nontarget cells (c).

of recombinant viruses to target cells (Bartlett et al. 1999; Haisma et al. 1999; Kelly et al. 2000; Reynolds et al. 2000; Tillman et al. 2000; Grill et al. 2001; Heideman et al. 2001; Nettelbeck et al. 2001, 2004; Korn et al. 2004a; Würdinger et al. 2005). These approaches include retargeting to tumor cells (e.g. through binding to EGFR, EpCAM, HMW-MAA, TAG-72, and G250), endothelial cells (through binding to endoglin, αv integrins, ACE) and antigen-presenting cells (CD40 binding). In most cases increased and cell type-specific virus transduction was mediated by the antibodies *in vitro*, including transduction of primary tumor cells and spheroids (Grill et al. 2001; Nettelbeck et al. 2004). Importantly, these findings demonstrated that virus uptake is not abolished by the use of bispecific adapter molecules.

A few studies have already shown selective tumor targeting of adenoviral vectors *in vivo*, demonstrating the feasibility of this approach for therapeutic applications. In one study, a bispecific antibody directed against the adenoviral knob domain and angiotensin-converting enzyme (ACE) resulted in targeting of the adenoviruses to pulmonary capillary endothelium as shown by an increase in gene expression in the lung and reduced expression in nontarget organs, especially the liver (Reynolds et al. 2000). In a subsequent study, synergistic effects on selectivity were shown with a combination of transductional and transcriptional targeting using a bispecific antibody directed against ACE and the endothelial cell-specific VEGFR-1 promoter (Reynolds et al. 2001).

The applications of bispecific antibodies for adenoviral gene therapy focus mainly on tumor therapy mediating either the transfer of suicide genes (e.g. thymidine kinase in combination with ganciclovir as prodrug) or transduction by conditionally replicative adenoviruses (CRAds) that replicate only in tumor cells and destroy these cells through lysis (Kanerva and Hemminki 2005). In addition, bispecific antibodies have been developed to redirect other viruses such as coronaviruses (e.g. feline infectious peritonitis virus and felinized murine hepatitis virus), which can kill cancer cells through the formation of syncytia (Würdinger et al. 2005). Targeted adenoviruses have been also applied for vaccination strategies by gene transfer into antigen-presenting cells such as dendritic cells (DCs) (Tillman et al. 2000; de Gruijl et al. 2002). Due to low numbers of coxsackievirus and adenovirus receptor (CAR) on DCs, adenoviral transduction can be drastically improved with bispecific antibodies and can lead to efficient activation of DCs and antigen-specific stimulation of T cells.

2.7 Outlook

As has been extensively shown over the last two decades, bispecific antibodies can extend and improve therapeutic applications of monoclonal antibodies by combining target-specific binding with the recruitment of potent effector mechanisms. Although in recent years successful development and commercialization of monoclonal antibodies and antibody conjugates have come to fore, bispecific

antibodies seem to be experiencing a revival. Novel and improved bispecific antibodies generated for example by genetic engineering have already entered clinical trials. The near future will show if these bispecific antibodies will become new, clinically approved therapeutics for treating diseases.

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References

Alt, M., Müller, R., Kontermann, R.E. (1999) Novel tetravalent and bispecific IgG-like antibody molecules combining single-chain diabodies with the immunoglobulin γ1 Fc or C_H3 region. *FEBS Lett* 454: 90–94.

Atwell, S., Ridgway, J.B.B., Wells, J.A., Carter, P. (1997) Stable heterodimers from remodelling the domain interface of a homodimer using a phage display library. *J Mol Biol* 270: 26–35.

Amoroso, R.A., Alpaugh, R.K., Barth, M.W., McCall, A.M., Weiner, L.M. (1999) Production and characterization of mice transgenic for the A and B isoforms of human FcγRIII. *Cancer Immunol Immunother* 48: 443–455.

Barnett, B.G., Crews, C.J., Douglas, J.T. (2002) Targeted adenoviral vectors. *Biochim Biophys Acta* 1575: 1–14.

Bartlett, J.S., Kleinschmidt, J., Boucher, R. C., Samulski, R.J. (1999) Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab′γ)2 antibody. *Nat Biotechnol* 17: 181–186.

Bartsch, M., Weeke-Klimp, A.H., Meijer, D. K., Scherphof, G.L., Kamps, J.A. (2005) Cell-specific targeting of lipid-based carriers for ODN and DNA. *J Liposome Res* 15: 59–92.

Biburger, M., Weth, R., Wels, W.S. (2005) A novel bispecific tetravalent antibody fusion protein to target costimulatory activity for T-cell activation to tumor cells overexpressing ErbB2/HER2. *J Mol Biol* 346: 1299–1311.

Blanco, B., Holliger, P., Vile, R.G., Álvarez-Vallina, L. (2003) Induction of human T lymphocyte cytotoxicity and inhibition of tumor growth by tumor-specific d iabody-based molecules secreted from gene-modified bystander cells. *J Immunol* 171: 1070–1077.

Bonardi, M.A., Bell, A., Fench, R.R., Gromo, G., Hamblin, T., Modena, D., Tutt, A.L., Glennie, M.J. (1992) Initial experience in treating human lymphoma with a combination of bispecific antibody and saporin. *Int J Cancer Suppl* 7: 73–77.

Bonardi, M.A., French, R.R., Amlot, P., Gromo, G., Modena, D., Glennie, M.J. (1993) Delivery of saporin to human B-cell lymphoma using bispecific antibody: targeting via CD22 but not CD19, CD37, or immunoglobulin results in efficient killing. *Cancer Res* 53: 3015–3021.

Brandão, J.G., Scheper, R.J., Lougheed, S.M., Curiel, D.T., Tillman, B.W., Gerritsen, W. R., van den Eertwegh, A.J., Pinedo, H.M., Haisma, H.J., de Gruijl, T.D. (2003) CD40-targeted adenoviral gene transfer to dendritic cells through the use of a novel bispecific single-chain Fv antibody enhances cytotoxic T cell activation. *Vaccine* 21: 2268–2272.

Brekke, O.H., Sandlie, I. (2003) Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nat Rev Drug Discov* 22: 52–62.

Brennan, M. (1986) A chemical technique for the preparation of bispecific antibodies

from Fab fragments of mouse monoclonal IgG1. *BioTechniques* 4: 424–427.

Brennan, M., Davison, P.F., Paulus, H. (1985) Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G1 fragments. *Science* 229: 81–83.

Bruenke, J., Fischer, B., Barbin, K., Schreiter, K., Wachter, Y., Mahr, K., Titgemeyer, F., Niederweis, M., Peipp, M., Zunino, S., Repp, R., Valerius, T., and Fey, G.H. (2004) A recombinant bispecific single-chain Fv antibody against HLA classII and FcγRIII (CD16) triggers effective lysis of lymphoma cells. *Br J Haematol* 125: 167–179.

Bruenke, J., Barbin, K., Kunert, S., Lang, P., Pfeiffer, M., Stieglmaier, K., Niethammer, D., Stockmeyer, B., Peipp, M., Repp, R., Valerius, T., Fey, G.H. (2005) Effective lysis of lymphoma cells with a stabilised bispecific single-chain Fv antibody against CD19 and FcgammaRIII (CD16). *Br J Haematol* 130: 218–228.

Brüsselbach, S., Korn, T., Völkel, T., Müller, R., Kontermann, R.E. (1999) Enzyme recruitment and tumor cell killing in vitro by a secreted bispecific single-chain diabody. *Tumor Targeting* 4: 115–123.

Bubenik, J. (2003) Tumour MHC class I downregulation and immunotherapy. *Oncol Rep* 10: 2205–2208.

Cao, Y., Lam, L. (2003) Bispecific antibody conjugates in therapeutics. *Adv Drug Deliv Rev* 55: 171–197.

Cao, Y., Suresh, M.R. (1998) Bispecific antibodies as novel bioconjugates. *Bioconjug Chem* 9: 635–644.

Cao, Y., Vinayagamoorthy, T., Noujaim, A. A., Suresh, M.R. (1995) A rapid non-seletive method to generate quadromas by microelectrofusion. *J Immunol Methods* 187: 1–7.

Carter, P. (2001) Bispecific human IgG by design. *J Immunol Methods* 248: 7–15.

Chang, C.-H., Sharkey, R.M., Rossi, E.A., Karacay, H., McBridge, W., Hansen, H.J., Chatal, J.-F., Barbet, J., Goldenberg, D.M. (2002) Molecular advances in pretargeting radioimmunotherapy with bispecific antibodies. *Mol Cancer Ther* 1: 553–563.

Cochlovius, B., Kipriyanov, S.M., Stassar, M.J.J.G., Schuhmacher, J., Benner A., Moldenhauer, G., Little, M. (2000) Cure of Burkitt's lymphoma in severe combined immunodeficiency mice by T cells, tetravalent CD3 × CD19 tandem diabody, and CD28 costimulation. *Cancer Res* 60: 4336–4341.

Coloma, M.J., Morrison, S.L. (1997) Design and production of novel tetravalent bispecific antibodies. *Nat Biotechnol* 15: 159–163.

Connelly, R.J., Hayden, M.S., Scholler, J.K., Tsu, T.T., Dupont, B., Ledbetter, J.A., Kanner, S.B. (1998) Mitogenic properties of a bispecific single-chain Fv-Ig fusion generated from CD2-specific mAb to distinct epitopes. *Int Immunol* 10: 1863–1872.

Corvalan, J.R., Smith, W. (1987) Construction and characterisation of a hybrid-hybrid monoclonal antibody recognising both carcinoembryonic antigen (CEA) and vinca alkaloids. *Cancer Immunol Immunother* 24: 127–132.

Curnow, R.T. (1997) Clinical experience with CD64-directed immunotherapy. An overview. *Cancer Immunol Immunother* 45: 210–215.

de Gast, G.C., van de Winkel, J.G.J., Bast, B. E.J.E.G. (1997) Clinical perspectives of bispecific antibodies in cancer. *Cancer Immunol Immunother* 45: 121–123.

de Gruijl, T.D., Luykx-de Bakker, S.A., Tillman, B.W., van den Eertwegh, A.J., Buter, J., Lougheed, S.M., van der Bij, G.J., Safer, A.M., Haisma, H.J., Curiel, D.T., Scheper, R.J., Pinedo, H.M., Gerritsen, W. R. (2002) Prolonged maturation and enhanced transduction of dendritic cells migrated from human skin explants after in situ delivery of CD40-targeted adenoviral vectors. *J Immunol* 169: 5322–5331.

de Kruif, J., Logtenberg, T. (1996) Leucine zipper dimerized bivalent and bispecific scFv antibodies from a semisynthetic antibody phage display library. *J Biol Chem* 271: 7630–7634.

De Lau, W.B., van Loon, A.E., Heije, K., Valerio, D., Bast, B.J. (1989) Production of hybrid hybridomas based on HAT(s)-neomycin double mutants. *J Immunol Methods* 117: 1–8.

De Lau ,W.B., Keije, K., Neefjes, J.J., Oosterwegel, M., Rozemüller, E., Blast, B.J. (1991) Absence of preferential homologous

H/L chain association in hybrid hybridomas. *J Immunol* 146: 906–914.
DeNardo, D.G., Xiong, C.Y., Shi, X.B., DeNardo, G.L., DeNardo, S.J. (2001) Anti-HLA-DR/anti-DOTA diabody construction in a modular gene design platform: bispecific antibodies for pretargeted radioimmunotherapy. *Cancer Biother Radiopharm* 16: 525–535.
Deo, Y.M., Sundarapandiyan, K., Keler, T., Wallace, P.K., Graziano, R.F. (1998) Bispecific molecules directed to the Fc receptor for IgA (FcαRI, CD89) and tumor antigens efficiently promote cell-mediated cytotoxicity of tumor targets in whole blood. *J Immunol* 160: 1677–1686.
Dreier, T., Baeuerle, P.A., Fichtner, I., Grün, M., Schlereth, B., Lorenczewski, G., Kufer, P., Lutterbüse, R., Riethmüller, G., Gjorstrup, P., Bargou, R.C. (2003) T cell costimulus-independent and very efficacious inhibition of tumor growth in mice bearing subcutaneous or leukemic human B cell lymphoma xenografts by a CD19-/CD3- bispecific single-chain antibody construct. *J Immunol* 170: 4397–4402.
El-Aneed, A. (2004) An overview of current delivery systems in cancer gene therapy. *J Control Release* 94: 1–14.
Embleton, M.J., Charleston, A., Robins, R. A., Pimm, M.V., Baldwin, R.W. (1991) Recombinant ricin toxin A cytotoxicity against carcinoembryonic antigen expressing tumour cells mediated by a bispecific monoclonal antibody and its potentiation by ricin toxin chain. *Br J Cancer* 63: 670–674.
Everts, M., Curiel, D.T. (2004) Transductional targeting of adenoviral cancer gene therapy. *Curr Gene Ther* 4: 337–346.
Fanger, M.W., Guyre, P.M. (1991) Bispecific antibodies for targeted cellular cytotoxicity. *Trends Biotechnol* 9: 375–380.
Fanger, M.W., Morganelli, P.M., Guyre, P.M. (1992) Bispecific antibodies. *Crit Rev Immunol* 12: 101–124.
Ferrini, S., Sforzini, S., Canevari, S. (2001) Bispecific monoclonal antibodies for the targeting of type I ribosome-inactivating proteins against hematological malignancies. *Methods Mol Biol* 166: 177–192.
Flavell, D.J., Cooper, S., Morland, B., French, R., Flavell, S.U. (1992) Effectiveness of combinations of bispecific antibodies for delivering saporin to human acute T-cell lymphoblastic leuaemia cell lines via CD7 and CD38 as cellular target molecules. *Br J Cancer* 65: 545–551.
FitzGerald, K., Holliger, P., Winter, G. (1997) Improved tumour targeting by disulphide stabilized diabodies expressed in *Pichia pastoris*. *Protein Eng* 10: 1221–1225.
Ford, C.H., Osborne, P.A., Rego, B.G., Mathew, A. (2001) Bispecific antibody targeting of doxorubicin to carcinoembryonic antigen-expressing colon cancer cells lines in vitro and in vivo. *Int J Cancer* 92: 851–855.
French, R.R., Penney, C.A., Browning, A.C., Stirpe, F., Georg, A.J., Glennie, M.J. (1995) Delivery of the ribosome-inactivating protein, gelonin, to lymphoma cells via CD22 and CD38 using bispecific antibodies. *Br J Cancer* 71: 986–994.
Gautherot, E., Bouhou, J., Le Doussal, J.M., Manetti, C., Martin, M., Rouvier, E., Barbet, J. (1997) Therapy for colon carcinoma xenografts with bispecific antibody-targeted, iodine-131-labeled bivalent hapten. *Cancer* 80: 2618–2623.
Gerstmayer, B., Hoffmann, M., Altenschmidt, U., Wels, W. (1997) Costimulation of T-cell proliferation by a chimeric B7-antibody fusion protein. *Cancer Immunol Immunother* 45: 156–158.
Glennie, M.J., McBride, H.M., Worth, A.T., Stevenson, G.T. (1987) Preparation and performance of bispecific F(ab′ γ)2 antibody containing thioether-linked Fab′ γ fragments. *J Immunol* 139: 2367–2375.
Goldenberg, D.M. (2003) Advancing role of radiolabeled antibodies in the therapy of cancer. *Cancer Immunol Immunother* 52: 281–296.
Graziano, R.F., Guptill, P. (2004) Chemical production of bispecific antibodies. *Methods Mol Biol* 283: 71–85.
Grill, J., van Beusechem, V.W., van der Valk, P., Dirven, C.M.F., Leonhart, A., Pherai, D.S., Haisma, H.J., Pinedo, H.M., Curiel, D.T., Gerritsen, W.R. (2001) Combined targeting of adenoviruses to integrins and epidermal growth factor receptors increased gene transfer into primary

glioma cells and spheroids. *Clin Cancer Res* 7: 641–650.

Grosse-Hovest, L., Hartlapp, I., Marwan, W., Brem, G., Rammensee, H.-G., Jung, G. (2003) A recombinant bispecific single-chain antibody induces targeted, supraagonistic CD28-stimulation and tumor cell killing. *Eur J Immunol* 33: 1334–1340.

Grosse-Hovest, L., Müller, S., Minoia, R., Wolf, E., Zakhartchenko, V., Wenigerkind, H., Lassnig, C., Besenfelder, U., Müller, M., Lytton, S.D., Jung, G., Brem, G. (2004) Cloned transgenic farm animals produce a bispecific antibody for T cell-mediated tumor cell killing. *Proc Natl Acad Sci USA* 101: 6858–6863.

Grosse-Hovest, L., Wick, W., Minoia, R., Weller, M., Rammensee, H.-G., Brem, G., Jung, G. (2005) Supraagonistic, bispecific single-chain antibody purified from the serum of cloned, transgenic cows induces T-cell-mediated killing of glioblastoma cells *in vitro* and *in vivo*. *Int J Cancer* 117: 1060–1064.

Gruaz-Guyon, A., Raguin, O., Barbet, J. (2005) Recent advances in pretargeted radioimmunotherapy. *Curr Med Chem* 12: 3319–338.

Haisma, H.J., Pinedo, H.M., Rijswijk, A., der Meulen-Muileman, I., Sosnowski, B. A., Ying, W., van Beusechem, V.W., Tillman, B.W., Gerritsen, W.R., Curiel, D. T. (1999) Tumor-specific gene transfer via an adenoviral vector targeted to the pan-carcinoma antigen EpCAM. *Gene Ther* 6: 469–474.

Hartmann, F., Renner, C., Jung, W., Deisting, C., Juwana, M., Eichentopf, B., Kloft, M., Pfreundschuh, M. (1997) Treatment of refractory Hodgkin's disease with an anti-CD16/CD30 bispecific antibody. *Blood* 89 2042–2047.

Hartmann, F., Renner, C., Jung, W., da Costa, L., Tembrink, S., Held, G., Sek, A., König, J., Bauer, S., Kloft, M., Pfreundschuh, M. (2001) Anti-CD16/CD30 bispecific antibody treatment for Hodgkin's disease: role of infusion schedule and costimulation with cytokines. *Clin Cancer Res* 7: 1873–1881.

Hayden, M.S., Linsley, P.S., Gayle, M.A., Bajorath, J., Brady, W.A., Norris, N.A., Fell, H.P., Ledbetter, J.A., Gilliland, L.K. (1994) Single-chain mono- and bispecific antibody derivatives with novel biological properties and antitumour activity from a COS cell transient expression system. *Ther Immunol* 1: 3–15.

Heideman, D.A., Snijders, P.J., Craanen, M. E., Bloemena, E., Meijer, C.J., Meuwissen, S.G., van Beusechem, V.W., Pinedo, H.M., Curiel, D.T., Haisma, H.J., Gerritsen, W.R. (2001) Selective gene delivery toward gastric and esophageal adenocarcinoma cells via EpCAM-targeted adenoviral vectors. *Cancer Gene Ther* 8: 342–351.

Hendrie, P.C., Russell, D.W. (2005) Gene targeting with viral vectors. *Mol Ther* 12: 9–17.

Heiss, M.M., Strohlein, M.A., Jäger, M., Kimmig, R., Burges, A., Schoberth, A., Jauch, K.W., Schildberg, F.W., Lindhofer, H. (2005) Immunotherapy of malignant ascites with trifunctional antibodies. *Int J Cancer* 117: 435–443.

Hoffmann, P., Hofmeister, R., Brischwein, K., Brandl, C., Crommer, S., Bargou, R., Itin, c., Prang, N., Baeuerle, P.A. (2005) Serial killing of tumor cells by cytotoxic T cells redirected with a CD19-/CD3-bispecific single-chain antibody construct. *Int J Cancer* 115: 98–104.

Holliger, P., Prospero, T., Winter, G. (1993) "Diabodies": small bivalent and bispecific antibody fragments. *Proc Natl Acad Sci USA* 90: 6444–6448.

Holliger, P., Wing, M., Pound, J.D., Bohlen, H., Winter, G. (1997) Retargeting serum immunoglobulin with bispecific diabodies. *Nat Biotechnol* 15: 632–636.

Holliger P., Manze, O., Span, M., Hawkins, R., Fleischmann, B., Qianghua, L., Wolf, J., Diehl, V., Cochet, O., Winter, G., Bohlen, H. (1999) Carcinoembryonic antigen (CEA)-specific T-cell activation in colon carcinoma induced by anti-CD3xanti-CEA bispecific diabodies and B7 × anti-CEA bispecific fusion proteins. *Cancer Res* 59: 2909–2916.

Hombach, A., Jung, W., Pohl C., Renner C., Sahin U., Schmits R., Wolf, J., Kapp, U., Diehl, V., Pfreundschuh, M. (1993) A CD16/CD30 bispecific monoclonal antibody induces lysis of Hodgkin's cells by unstimulated natural killer cells in vitro and in vivo. *Int J Cancer* 55: 830–836.

James, N.D., Atherton, P.J., Jones, J., Howie, A.J., Tchekmedyian, S., Curnow, R.T. (2001) A phase II study of the bispecific antibody MDX-H210 (anti-HER2 × CD64) with GM-CSF in HER2+ advanced prostate cancer. *Br J Cancer* 85: 152–156.

Jendreyko, N., Popkov, M., Beerli, R.R., Chung, J., McGavern, D.B., Rader, C., Barbas, C.F. III (2003) Intradiabodies, bispecific, tetravalent antibodies for the simultaneous functional knockout of two cell surface receptors. *J Biol Chem* 278: 47812–4789.

Jendreyko, N., Popkov, M., Rader, C., Barbas, C.F. III (2005) Phenotypic knockout of VEGF-R2 and Tie-2 with an intradiabody reduces tumor growth and angiogenesis in vivo. *Proc Natl Acad Sci USA* 102: 8293–8298.

Jimenez, X., Lu, D., Brennan, L., Persaud, K., Liu, M., Miao, H., Witte, L., Zhu, Z.A. (2005) A recombinant, fully human, bispecific antibody neutralizes the biological activities mediated by both vascular endothelial growth factor receptors 2 and 3. *Mol Cancer Ther* 4: 427–434.

Kanerva, A., Hemminki, A. (2005) Adenoviruses for treatment of cancer. *Ann Med* 37: 33–43.

Karawajew, L., Micheel, B., Behrsing, O., Gaestel, M. (1987) Bispecific antibody-producing hybrid hybridomas selected by a fluorescence activated cell sorter. *J Immunol Methods* 92: 265–270.

Keler, T., Graziano, R.F., Mandal, A., Wallace, P.K., Fisher, J., Guyre, P.M., Fanger, M.W., Deo, Y.M. (1997) Bispecific antibody-dependent cellular cytotoxicity of HER2/neu-overexpressing tumor cells by Fcγ receptor type-I expressing effector cells. *Cancer Res* 57: 4008–4014.

Kelly, F.J., Miller, C.R., Buchsbaum, D.J., Gomez-Navarro, J., Barnes, M.N., Alvarez, R.D., Curiel, D.T. (2000) Selectivity of TAG-72-targeted adenovirus gene transfer to primary ovarian carcinoma cells versus autologous mesothelial cells in vitro. *Clin Cancer Res* 6: 4323–4333.

Kipriyanov, S.M., Moldenhauer, G., Schuhmacher, J., Cochlovius, B., Von der Lieth, C.W., Matys, E.R., Little, M. (1999) Bispecific tandem diabody for tumor therapy with improved antigen binding and pharmacokinetics. *J Mol Biol* 293: 41–56.

Kipriyanov, S.M., Cochlovius, B., Schäfer, H. J., Moldenhauer, G., Bähre, A., Le Gall, F., Knackmuss, S., Little, M. (2002) Synergistic antitumor effect of bispecific CD19 × CD3 and CD19 × CD16 diabodies in a preclinical model of non-Hodgkin's lymphoma. *J Immunol* 169: 137–144.

Köhler, G., Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.

Kontermann, R.E. (2005) Recombinant bispecific antibodies for cancer therapy. *Acta Pharmacol Sin* 26: 1–9.

Kontermann, R.E., Wing, M.G., Winter, G. (1997) Complement recruitment using bispecific diabodies. *Nat Biotechnol* 15: 629–631.

Kontermann, R.E., Korn, T., Jérôme, V. (2002) Recombinant adenoviruses for in vivo expression of antibody fragments. In: Welschof M, Kraus J (eds) *Recombinant Antibody Technology for Cancer Therapy: Reviews and Protocols*. Methods in Molecular Medicine. Totowa: Humana Press, pp. 421–433.

Koolwijk, P., Rozemüller, E., Stad, R.K., de Lau, W.B., Blast, B.J. (1988) Enrichment and selection of hybrid hybridomas by percoll density gradient centrifugation and flurescent-activated cell sorting. *Hybridoma* 7: 217–225.

Koolwijk, P., Spierenburg, G.T., Frasa, H., Boot, J.H., van de Winkel, J.H., Bat, B.J. (1989) Interaction between hybrid mouse monoclonal antibodies and the human high-affinity IgG FcR, huFcγRI, on U937. Involvement of only one of the mIgG heavy chains in receptor binding. *J Immunol* 143: 1656–1662.

Koolwijk, P., Boot, J.H., Griep, R., Bast, B.J. (1991) Binding of human complement subcomponent C1q to hybrid mouse monoclonal antibodies. *Mol Immunol* 28: 567–576.

Korn, T., Nettelbeck, D.M., Völkel, T., Müller, R., Kontermann, R.E. (2004a) Recombinant bispecific antibodies for the targeting of adenoviruses to CEA-expressing tumour cells: a comparative

analysis of bacterially expressed single-chain diabody and tandem scFv. *J Gene Med* 6: 642–651.

Korn, T., Muller, R., Kontermann, R.E. (2004b) Bispecific single-chain diabody-mediated killing of endoglin-positive endothelial cells by cytotoxic T lymphocytes. *J Immunother* 27: 99–106.

Kostelny, S.A., Cole, M.S., Tso, J.Y. (1992) Formation of a bispecific antibody by the use of leucine zippers. *J Immunol* 148: 1547–1553.

Kraeber-Bodéré, F., Faivre-Chauvet, A., Ferrer, L., Vuillez, F.-P., Brard, P.-Y., Rousseau, C., Resche, I., Devillers, A., Laffont, S., Bardiés, M., Chang, K., Sharkey, R.M., Goldenberg, D.M., Chatal, J.-F., Barbet, J. (2003) Pharmacokinetics and dosimetry studies for optimization of anti-carcinoembryonic antigen × anti-hapten bispecific antibody-mediated pretargeting of iodine-131-hapten in a phase I radioimmunotherapy trial. *Clin Cancer Res* 9: 3973s–3981s.

Krannenborg, M.H., Boerman, O.C., Oosterwijk-Wakka, J.C., de Weijert, M.C., Corstens, F.H., Oosterweijk, E. (1998) Two-step radio-immunotherapy of renal-cell carcinoma xenografts in nuce mice with anit-renal-cell-carcinoma × anti-DTPa bispecific monoclonal antibodies. *Int J Cancer* 75: 74–80.

Kreutz, F.T., Xu, D.Z., Suresh, M.R. (1998) A new method to generate quadromas by electrofusion and FACS sorting. *Hybridoma* 17: 267–273.

Kriangkum, J., Xu, B., Nagata, L.P., Fulton, R.E., Suresh, M.R. (2001) Bispecific and bifunctional single chain recombinant antibodies. *Biomol Eng* 18: 31–40.

Kufer, P., Mack, M., Gruber, R., Lutterbuse, R., Zettl, F., Riethmuller, G. (1997) Construction and biological activity of a recombinant bispecific single-chain antibody designed for therapy of minimal residual colorectal cancer. *Cancer Immunol Immunother* 45 193–197.

Kufer, P., Lutterbuse, R., Baeuerle, P.A. (2004) A revival of bispecific antibodies. *Trends Biotechnol* 22: 38–44.

Kuus-Reichel, K., Knott, C., Sam-Fong, P., Petrella, E., Corvalan, J.R. (1995) Therapy of streptozotocin induced diabetes with a bifunctional antibody that delivers vinca alkaloids to IL-2 receptor positive cells. *Autoimmunity* 22: 173–181.

Lanzavecchia, A., Scheidegger, D. (1987) The use of hybrid hybridomas to target human cytotoxic T lymphocytes. *Eur J Immunol* 17: 105–111.

Le Doussal, J.M., Martin, M., Gruaz-Guyon, A., Gautherot, E., Delaage, M., Barbet, J. (1990) In vitro and in vivo targeting of radiolabeled monovalent and bivalent haptens with dual specificity monoclonal antibody conjugates: enhanced divalent hapten affinity for cell-bound antibody conjugates. *J Nucl Med* 30: 1358–1366.

Lewis, L.D., Cole, B.F., Wallace, P.K., Fisher, J.L., Waugh, M., Guyre, P.M., Fanger M. W., Curnow, R.T., Kaufman, P.A., Ernstoff, M.S. (2001) Pharmacokinenic-pharmacodynamic relationship of the bispecific antibody MDX-H210 when administered in combination with interferon gamma: a multiple-dose phase-I study in patients with advanced cancer which overexpresses HER-2/neu. *J Immunol Methods* 248: 149–165.

Lindhofer, H., Mocika, R., Steipe, B., Thierfelder, S. (1995) Preferential species-restriced heavy/light chain pairing in rat/mouse quadromas. Implications for a single-step purification of bispecific antibodies. *J Immunol* 155: 219–225.

Link, B.K., Weiner, G.J. (1993) Production and characterization of a bispecific IgG capable of inducing T-cell-mediated lysis of malignant B cells. *Blood* 81: 3343–3349.

Lloyd, F. Jr., Goldrosen, M. (1991) The production of a bispecific anti-CEA, anti-hapten (4-amino-phthalate) hybrid-hybridoma. *J Natl Med Assoc* 83: 901–904.

Löffler, A., Kufer, P., Lutterbüse, R., Zettl, F., Daniel, P.T., Schwenkenbecher, J.M., Riethmüller, G., Dörken, B., Bargou, R.C. (2000) A recombinant bispecific single-chain antibody, CD19 × CD3: induces rapid and high lymphoma-directed cytotoxicity by unstimulated T lymphoctes. *Blood* 95: 2089–2103.

Lüdher, F., Huang, Y., Dennehy, K.M., Guntermann, C., Müller, I., Winkler, E., Kerkau, T., Ikemizu, S., Davis, S.J., Hanke, T., Hünig, T. (2003) Topological requirements and signaling properties of T cell-activating, anti-CD28 antibody superagonists. *J Exp Med* 197: 955–966.

Lu, D., Kotanides, H., Jimenez, X., Zhou, Q., Persaud, K., Bohlen, P., Witte, L., Zhu, Z. (1999) Acquired antagonistic activity of a bispecific diabody directed against two different epitopes on vascular endothelial growth factor receptor 2. *J Immunol Methods* 230: 159–171.

Lu, D., Jimenez, X., Zhang, H., Wu, Y., Bohlen, P., Witte, L., Zhu, Z. (2001) Complete inhibition of vascular endothelial growth factor (VEGF) activities with a bifunctional diabody directed against both VEGF kinase receptors, fms-like tyrosine kinase receptor and kinase insert domain-containing receptor. *Cancer Res* 61: 7002–7008.

Lu, D., Jimenez, X., Zhang, H., Bohlen, P., Witte, L., Zhu, Z. (2002) Fab-scFv fusion protein: an efficient approach to production of bispecific antibody fragments. *J Immunol Meth* 267: 213–226.

Lu, D., Jimenez, X., Zhang, H., Atkins, A., Brennan, L., Balderes, P., Bohlen, P., Witte, L., Zhu, Z. (2003) Di-diabody: a novel tetravalent bispecific antibody molecule by design. *J Immunol Methods* 279: 219–232.

Lu, D., Zhang, H., Ludwig, D., Persaud, A., Jimenez, X., Burtrum, D., Balderes, P., Liu, M., Bohlen, P., Witte, L., Zhu, Z. (2004) Simultaneous blockade of both the epidermal growth factor receptor and the insulin-like growth factor receptor signaling pathways in cancer cells with a fully human recombinant bispecific antibody. *J Biol Chem* 279: 2856–2865.

Lu, D., Zhang, H., Koo, H., Tonra, J., Balderes, P., Prewett, M., Corcoran, E., Mangalampalli, V., Bassi, R., Anselma, D., Patel, D., Kang, X., Ludwig, D.L., Hicklin, D.J., Bohlen, P., Witte, L., Zhu, Z. (2005) A fully human recombinant IgG-like bispecific antibody to both the epidermal growth factor receptor and the insulin-like growth factor receptor for enhanced antitumor activity. *J Biol Chem* 280: 19665–19672.

Lum, L.G., Davol, P.A. (2005) Retargeting T cells and immune effector cells with bispecific antibodies. *Cancer Chemother Biol Response Modif* 22: 273–291.

Madrenas, J., Chau, L.A., Teft, W.A., Wu, P. W., Jussif, J., Kasaian, M,. Carreno, B.M., Ling, V. (2004) Conversion of CTLA-4 from inhibitor to activator of T cells with a bispecific tandem single-chain Fv ligand. *J Immunol* 172: 5948–5956.

Marvin, J.S., Zhu, Z. (2005) Recombinant approaches to IgG-like bispecific antibodies. *Acta Pharmacol Sin* 26: 649–658.

McCall, A.M., Adams, G.P., Amoroso, A.R., Nielsen, U.B., Zhang, L., Horak, E., Simmons, H., Schier, R., Marks, J.D., Weiner, L.M. (1999) Isolation and characterization of an anti-CD16 single-chain Fv fragment and construction of an anti-HER2/neu/anti-CD16 bispecific scFv that triggers CD16-dependent tumor cytolysis. *Mol Immunol* 36: 433–445.

McCall, A.M., Shahied, L., Amoroso, A.R., Horak, E.M., Simmons, H.H., Nielson, U., Adams, G.P., Schier, R., Marks, J.D., Weiner, L.M. (2001) Increasing the affinity for tumor antigen enhances bispecific antibody cytotoxicity. *J Immunol* 166: 6112–6117.

Merchant, A.M., Zhu, Z., Yuan, J.Q., Goddard, A., Adams, C.W., Presta, L.G., Carter, P. (1998) An efficient route to human bispecific IgG. *Nat Biotechnol* 16: 677–681.

Milstein, C., Cuello, A.C. (1983) Hybrid hybridomas and their use in immunohistochemistry. *Nature* 305: 537–540.

Molema, G., Kroesen, B.J., Helfrich, W., Meijer, D.K.F., de Leij, L.F.M.H. (2000) The use of bispecific antibodies in tumor cell and tumor vasculature directed immunotherapy. *J Control Release* 64: 229–239.

Morandeau, L., Benoist, E., Loussouarn, A., Quadi, A., Lesaec, P., Mougin, M., Faivre-Chauvet, A., Le Boterff, J., Chatal, J.F., Barbet, J., Gestin, J.F. (2005) Synthesis of new bivalent peptides for applications in the affinity enhancement system. *Bioconjug Chem* 16: 184–193.

Morelli, d., Sardini, A., Villa, E., Menard, S., Colnaghi, M.I., Balsari, A. (1994) Modulation of drug-induced cytotoxicity by a bispecific monoclonal antibody that recognizes the epidermal growth factor receptor and doxorubicin. *Cancer Immunol Immunother* 38: 171–177.

Müller, K.M., Arndt, K.M., Strittmatter, W., Plückthun, A. (1998a) The first constant

domain (C_H1 and C_L) of an antibody used as heterodimerization domain for bispecific miniantibodies. *FEBS Lett* 422: 259–264.

Müller, K.M., Arndt, K.M., Plückthun, A. (1998b) A dimeric bispecific miniantibody combines two specificities with avidity. *FEBS Lett* 432: 45–49.

Nettelbeck, D.M., Miller, D.W., Jérôme, V., Zuzarte, M., Watkins, S.J., Hawkins, R.E., Müller, R., Kontermann, R.E. (2001) Targeting of adenovirus to endothelial cells by a bispecific single-chain diabody directed against the adenovirus fiber knob domain and human endoglin (CD105). *Mol Ther* 3: 882–891.

Nettelbeck, D.M., Rivera, A.A., Kupsch, J., Dieckmann, D., Douglas, J.T., Kontermann, R.E., Alemany, R., Curiel, D.T. (2004) Retargeting of adenoviral infection to melanoma: combining genetic ablation of native tropism with a recombinant bispecific single-chain diabody (scDb) adapter that binds to fiber knob and HMWMAA. *Int J Cancer* 108: 136–145.

Nisonoff, A., Rivers, M.M. (1961) Recombination of a mixture of univalent an tibody fragments of different specificity. *Arch Biochem Biophys* 93: 460–462.

Park, S.S., Ryu, C.J., Kang, Y.J., Kashmiri, S. V., Hong, H.J. (2000) Generation and characterization of a novel tetravalent bispecific antibody that binds to hepatitis B virus surface antigens. *Mol Immunol* 37: 1123–1130.

Paulus, H. (1985) Preparation and biomedical applications of bispecific antibodies. *Behring Inst Mitt* 78: 118–132.

Peipp, M., Valerius, T. (2002) Bispecific antibodies targeting cancer cells. *Biochem Soc Trans* 30: 507–511.

Plückthun, A., Pack, P. (1997) New protein engineering approaches to multivalent and bispecific antibody fragments. *Immunotechnology* 3: 83–105.

Pullarkat, V., Deo, Y., Link, J., Spears, L., Marty, V., Curnow, R., Groshen, S., Gee, C., Weber, J.S. (1999) A phase I study of a HER2/neu bispecific antibody with granulocyte-colony-stimulating factor in patients with metastatic breast cancer that overexpresses HER2/neu. *Cancer Immunol Immunother* 48: 9–21.

Ren-Heidenreich, L., Davol, P.A., Kouttab, N.M., Elfenbein, G.J., Lum, L.G. (2004) Redirected T-cell cytotoxicity to epithelial cell adhesion molecule-overexpressing adenocarcinomas by a novel recombinant antibody, E3Bi, in vitro and in an animal model. *Cancer* 100: 1095–1103.

Repp, R., van Ojik, H.H., Valerius, T., Groenewegen, G., Wieland, G., Oetzel, C., Stockmeyer, B., Becker, W., Eisenhut, M., Steininger, H., Deo, Y.M., Blijham, G.H., Kalden, J.R., van de Winkel, J.G.J., Gramatzki, M. (2003) Phase I clinical trial of the bispecific antibody MDX-H210 (anti-FcγRIxanti-HER-2/neu) in combination with Filgrastim (G-CSF) for treatment of advanced breast cancer. *Br J Cancer* 89: 2234–2243.

Reusch, U., Le Gall, F., Hensel, M., Moldenhauer, G., Ho, A.D., Little, M., Kipriyanov, S.M. (2004) Effect of tetravalent bispecific CD19 × CD3 recombinant antibody construct and CD28 costimulation on lysis of malignant B cells from patients with chronic lymphocytic leukaemia by autologous T cells. *Int J Cancer* 112: 509.

Reynolds, P.N., Zinn, K.R., Gavrilyuk, V.D., Balyasnikova, I.V., Rogers, B.E., Buchsbaum, D.J., Wang, M.H., Miletich, D.J., Grizzle, W.E., Douglas, J.T., Danilov, S.M., Curiel, D.T. (2000) A targetable, injectable adenoviral vector for selective gene delivery to pulmonary endothelium in vivo. *Mol Ther* 2: 562–578.

Reynolds, P.N., Nicklin, S.A., Kaliberova, L., Boatman, B.G., Grizzle, W.E., Balyasnikova, I.V., Baker, A.H., Danilov, S.M., Curiel, D.T. (2001) Combined transductional and transcriptional targeting improves the specificity of transgene expression in vivo. *Nat Biotechnol* 19: 838–842.

Ridgeway, J.B., Presta, L.G., Carter, P. (1996) "Knobs-into-holes" engineering of antibody CH3 domains for heavy chain heterodimerization. *Protein Eng* 9: 617–621.

Ruf, P., Lindhofer, H. (2001) Induction of a long-lasting antitumor immunity by a trifunctional bispecific antibody. *Blood* 98: 2526–2534.

Russell, J.H., Ley, T.J. (2002) Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol* 20: 323–370.

Russoniello, C., Soomasundaram, C., Schlom, J., Deo, Y.M., Keler, T. (1998) Characterization of a novel bispecific antibody that mediates Fcγ receptor type-I-dependent killing of tumor-ass
ociated glycoprotein-72-expressing tumor cells. *Clin Cancer Res* 4: 2237–2243.

Sanz, L., Blanco, B., Àlvarez-Vallina, L. (2004) Antibodies and gene therapy: teaching old "magic bullets" new tricks. *Trends Immunol* 25: 85–91.

Schlenzka, J., Moehler, T.M., Kipriyanov, S. M., Kornacker, M., Benner, A., Bahre, A., Stassar, M.J., Schafer, H.J., Little, M., Goldschmidt, H., Cochlovius, B. (2004) Combined effect of recombinant CD19 × CD16 diabody and thalidomide in a preclinical model of human B cell lymphoma. *Anticancer Drugs* 15: 915–919.

Schlereth, B., Fichtner, I., Lorenczewski, G., Kleindienst, P., Brischwein, K., da Silva, A., Kufer, P., Lutterbüse, R., Juhnhahn, I., Kasimir-Bauer, S., Wimberger, P., Kimmig, R., Baeuerle, P.A. (2005) Eradication of tumors from a human colon cancer cell line and from ovarian cancer metastases in immunodeficient mice by a single-chain Ep-CAM-/CD3-bispecific antibody construct. *Cancer Res* 65: 2882–2889.

Schneider, M.A., Bruhl, H., Wechselberger, A., Cihak, J., Stangassinger, M., Schlondorff, D., Mack, M. (2005) In vitro and in vivo properties of a dimeric bispecific single-chain antibody IgG-fusion protein for depletion of CCR2(+) target cells in mice. *Eur J Immunol* 35: 987–995.

Schoonjans, R., Willems, A., Schoonooghe, S., Fiers, W., Grooten, J., Mertens, N. (2000) Fab chains as an efficient heterodimerization scaffold for the production of recombinant bispecific and trispecific antibody derivatives. *J Immunol* 165: 7050–7057.

Segal, D.M., Weiner, G.J., Weiner, L.M. (1999) Bispecific antibodies in cancer therapy. *Curr Opin Immunol* 11: 558–562.

Sforzini, S., Bolognesi, A., Meazza, R., Marciano, S., Casalini, P., Durkop, H., Tazzari, P.L., Stein, H., Stirpe, F., Ferrini, S. (1995) Differential sensitivity of CD30+ neoplastic cells to gelonin delivered by anti-CD30/anti-gelonin bispecific antibodies. *Br J Haematol* 90: 572–577.

Shahied, L.S., Tang, Y., Alpaugh, R.K., Somer, R., Greenspon, D., Weiner, L.M. (2004) Bispecific minibodies targeting (HER2/neu and CD16 exhbit improved tumor lysis when placed in a divalent tumor antigen binding format. *J Biol Chem* 279: 53907–53914.

Shalaby, M.R., Shepard, H.M., Presta, L., Rodrgues, M.L., Beverley, P.C.L., Feldmann, M., Carter, P. (1992) Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protoncogene. *J Exp Med* 175: 217–225.

Sharkey, R.M., Karracay, H., Cardillo, T.M., Chang, C.H., McBride, W.J., Rossi, E.A., Horak, I.D., Goldenberg, D.M. (2005a) Improving the delivery of radionuclides for imaging and therapy of cancer using pretargeting methods. *Clin Cancer Res* 11: 7109s–7121s.

Sharkey, R.M., Karracay, H., Chang, C.H., McBride, W.J., Horak, I.D., Goldenberg, D. M. (2005b) Improved therapy of non-Hodgkin's lymphoma xenografts using radionuclides pretargeted with a new anti-CD20 bispecific antibody. *Leukemia* 19: 1064–1069.

Smith, W., Jarrett, A.L., Beattie, R.E., Corvalan, J.R. (1992) Immunoglobulins secreted by a hybrid-hybridoma: analysis of chain assemblies. *Hybridoma* 11: 87–98.

Staerz, U.D., Bevan, M.J. (1986) Hybrid hybridoma producing a bispecific monoclonal antibody that can focus effector T-cell activity. *Proc Natl Acad Sci USA* 83: 1453–1457.

Stockmeyer, B., Elsässer, D., Dechant, M., Repp, R., Gramatzki, M., Glennie, M.J., van de Winkel, J.G.J., Valerius, T. (2001) Mechanisms of G-CSF- or GM-CSF-stimulated tumor cell killing by Fc receptor-directed bispecific antibodies. *J Immunol Methods* 248: 103–111.

Sundarapandiyan, K., Keler, T., Behnke, D., Engert, A., Barth, S., Matthey, B., Deo, Y. M., Graziano, R.F. (2001) Bispecific antibody-mediated destruction of Hodgkin's lymphoma cells. *J Immunol Methods* 248: 113–123.

Tacke, M., Hanke, G., Hanke, T., Hunig, T. (1997) CD28-mediated induction of proliferation in resting T cells in vitro and in vivo without engagement of the T cell receptor: evidence for functionally distinct forms of CD28. *Eur J Immunol* 27: 239–247.

Tillman, B.W., Hayes, T.L., de Gruijl, T.D., Douglas, J.T., Curiel, D.T. (2000) Adenoviral vectors targeted to CD40 enhance the efficacy of dendritic cell-based vaccination against human papillomavirus 16-induced tumor cells in a murine model. *Cancer Res* 60: 5456–5463.

Tutt, A., Greenman, J., Stevenson, G.T., Glennie, M.J. (1991) Bispecific F(ab′γ)3 antibody derivatives for redirecting unprimed cytotoxic T cells. *Eur J Immunol* 21: 1351–1358.

Tutt, A.L., Reid, R., Wilkins, B.S., Glennie, M.J. (1995) Activation and preferential expansion of rat cytotoxic (CD8) T cells in vitro and in vivo with a bispecific (anti-TRC α/β × anti-CD2) F(ab′)2 antibody. *J Immunol* 155: 2960–2971.

Valerius, T., Würflein, D., Stockmeyer, B., Repp, R., Kalden, J.R., Gramatzki, M. (1997) Activated neutrophils as effector cells for bispecific antibodies. *Cancer Immunol Immunother* 45: 142–145.

van de Winkel, J.G.J., Bast, B., de Gast, G.C. (1997) Immunotherapeutic potential of bispecific antibodies. *Immunol Today* 12: 562–564.

van Spriel, A.B., van Ojik, H.H., van de Winkel, J.G.J. (2000) Immunotherapeutic perspective for bispecific antibodies. *Immunol Today* 21: 391–396.

Völkel, T., Korn, T., Bach, M., Müller, R., Kontermann, R.E. (2001) Optimized linker sequences for the expression of monomeridc and dimeric bispecific single-chain diabodies. *Protein Eng* 14: 815–823.

Wallace, P.K., Kaufman, P.A., Lewis, L.D., Keler, T., Givan, A.L., Fisher, J.L., Waugh, M.G., Wahner, A.E., Guyre, P.M., Fanger, M.W., Ernstoff, M.S. (2001) Bispecific antibody-targeted phagocytosis of HER-2/neu expressing tumor cells by myeloid cells activated in vivo. *J Immunol Methods* 248: 167–182.

Walther, W., Stein, U. (2000) Viral vectors for gene transfer: a review of theiruse in the treatment of human diseases. *Drugs* 60: 249–271.

Warnaar, S.O., de Paus, V., Lardenoije, R., Machielse, B.N., de Graaf, J., Bregonje, M., van Haarlem, H. (1994) Purification of bispecific $F(ab')_2$ from murine trinoma OC/TR with specificity for CD3 and ovarian cancer. *Hybridoma* 13: 519–526.

Watkins, S.J., Mesyanzhinov, V.V., Kurochkina, L.P., Hawkins, R.E. (1997) The "adenobody" approach to viral targeting: specific and enhanced adenoviral gene delivery. *Gene Ther* 4: 1004–1012.

Weiner, L.M., Clark, J.I., Davey, M., Li, W.S., Garcia de Palazzo, I., Ring, D.B., Alpaugh, R.K. (1995) Phase I trial of 2B1: a bispecific monoclonal antibody targeting c-erbB-2 and FcγRIII. *Cancer Res* 55: 4586–4593.

Withoff, S., Helfrich, W., de Leij, L.F.M.H., Molema, G. (2001) Bi-sepcific antibody therapy for the treatment of cancer. *Curr Opin Mol Ther* 3: 53–62.

Wolf, E., Hofmeister, R., Kufer, P., Schlereth, B., Baeuerle, P.A. (2005) BiTEs: bispecific antibody constructs with unique anti-tumor activity. *Drug Discov Today* 10: 1237–1244.

Würdinger, T., Verheije, M.H., Raaben, M., Bosch, B.J., de Haan, C.A., van Beusechem, V.W., Rottier, P.J., Gerritsen, W.R. (2005) Targeting non-human coronaviruses to human cancer cells using a bispecific single-chain antibody. *Gene Ther* 12: 1394–1404.

Wuest, T., Moosmayer, D., Pfizenmaier, K. (2001) Construction of a bispecific single chain antibody for recruitment of cytotoxic T cells to the tumour stroma associated antigen fibroblast activation protein. *J Biotechnol* 92: 159–168.

Zeidler, R., Mysliwietz, J., Csanady, M., Walz, A., Ziegler, I., Schmitt, B., Wollenberg, B., Lindhofer, H. (2000) The Fc-region of a new class of intact bispecific antibody mediates activation of accessory cells and NK cells and induces direct phagocytosis of tumour cells. *Br J Cancer* 83: 261–266.

Zeidler, R., Mayer, A., Gires, O., Schmitt, B., Mack, B.M., Lindhofer, H., Wollenberg, B., Walz, A. (2001) TNFα contributes to the antitumor activity of a bspecific,

trifunctional antibody. *AntiCancer Res* 21: 3499–3503.

Xie, Z., Shi, M., Feng, J., Yu, M., Sun, Y., Shen, B., Guo, N. (2003) A trivalent anti-erbB2/anti-CD16 bispecific antibody retargeting NK cells against human breast cancer cells. *Biochem Biophys Res Commun* 311: 307–312.

Xie, Z., Guo, N., Yu, M., Hu, M., Shen, B. (2005) A new format of bispecific antibody: highly efficient heterodimerization, expression and tumor cell lysis. *J Immunol Methods* 296: 95–101.

Zhu, Z., Presta, L.G., Zapata, G., Carter, P. (1997) Remodeling domain interfaces to enhance heterodimer formation. *Protein Sci* 6: 781–788.

Zuo, Z., Jimenez, X., Witte, L., Zhu Z. (2000) An efficient route to the production of an IgG-like bispecific antibody. *Protein Eng* 13: 361–367.

3
Immunotoxins and Beyond: Targeted RNases

Susanna M. Rybak and Dianne L. Newton

3.1
Introduction

Antibodies linked to cytotoxic compounds were designed to kill diseased cells while sparing normal ones. The benefits of this "smart drug" approach has great appeal and attempts at therapy with antibodies conjugated to drugs began in the 1950s (Mathe et al. 1958). A major milestone in the evolution of this therapeutic strategy was the development of murine monoclonal antibodies (Köhler and Milstein 1975). The increased specificity further encouraged targeting cancer-associated antigens expressed at high levels on tumor cells and at low levels on non-essential or easily renewable normal cells. Chemicals, radioisotopes, and toxins have been linked to antibodies to produce reagents that progressed from experimental studies to clinical trials (Milenic 2002; Wu and Senter 2005). Initially, standard chemotherapeutic drugs chemically linked to antibodies were widely explored as targeted cancer therapeutics (Blattler 1996). A major problem with those drug conjugates was the low molar cytotoxicity of the drugs, resulting in little improvement in efficacy or nonspecific toxicity over the unconjugated drug. This led to the coupling of chemical toxins that were several fold more potent than the chemotherapeutic drugs (reviewed in Payne 2003; Lambert 2005). The humanized anti-CD33 antibody calicheamicin conjugate (Mylotarg) is of this novel drug type, and has been approved for the treatment of CD33-positive acute myeloid leukemia (Hamann et al. 2002). In addition to giving proof of concept of the power of armed antibody targeting, the approval of Mylotarg demonstrates that the process of linking an antibody to another molecule to create a new drug is not too complex to be commercialized. Mylotarg is also the result of improvements in antibodies, such as humanization, that occurred through developments in technologies that solved some of the problems associated with murine monoclonal antibodies.

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3.1.1 Targeted Drug Architechture

The progression from murine to human monoclonal antibodies as well as the engineering of novel antibody forms and fragments are coupled to the recent rapid advances in antibody targeted therapeutics (reviewed in Carter 2001; Hoogenboom 2005). Recombinant DNA technology afforded the opportunity to design fusion proteins for specific applications by altering the features of the antibody or enzyme domains. For instance, the nature of the antibody used, IgG, $F(ab')_2$, Fab, or scFv, will depend on the application intended. Natural antibodies as well as $F(ab')_2$ fragments are bivalent and usually bind polyvalent cellular antigens with higher affinities than the monovalent Fab or single-chain Fv (scFv) fragments (Crothers and Metzger 1972). Generally it is recognized that high-affinity antibodies are preferable, yet valency and affinity have to be balanced against factors such as size which affect tumor penetration and plasma clearance rates. Comparative studies show that small antibody fragments clear from the blood faster (Colcher et al. 1990; Milenic et al. 1991; King et al. 1994). The disadvantage is that more fusion enzyme might have to be administered to attain adequate tumor uptake since rapid clearance decreases the latter parameter. Yet, due to the smaller size scFvs exhibit better tumor penetration and are more evenly distributed throughout the tumor compared with intact IgGs (Yokota et al. 1992).

Therefore, for many applications, only the antigen-binding domains of the antibody are required. In scFv analogs the two variable domains are coupled by peptide linkers (Bird et al. 1988; Huston et al. 1988) (reviewed in Huston et al. 1996, 1991). The linker needs to be of sufficient length to bridge the distance between the C-terminus of the first V domain and the N-terminus of the second V domain. Several studies have examined the characteristics of scFvs that are affected by the length and composition of the peptide linker (Whitlow et al. 1993; Desplancq et al. 1994; Alfthan et al. 1995; Kortt et al. 1997). Overall, the nature of the linker can affect affinity, dimerization, and aggregation of the scFv. Indeed, the length of the linker can be adjusted to prevent the V_H and V_L domains on the same chain from pairing with each other (Holliger et al. 1993). This can be exploited to create bispecific-binding proteins. Linkers should not interfere with the association of the two domains. Glycine provides flexibility to the $(GGGGS)_3$ peptide linker originally used by Huston et al. (1988). It is also devoid of charged and hydrophobic residues that might interact with the V domain surfaces and interfere either with the binding of these domains to each other or with the binding of the scFv to the antigen (Huston et al. 1991). Recently, the introduction of an interchain disulfide bond has been used to link V_H-V_L domains after genetically modifying each domain to introduce opposing cysteine residues (Glockshuber et al. 1990; Brinkmann et al. 1993; Rodrigues et al. 1995). The advantages of disulfide-linked Fvs (dsFv) compared with scFvs include enhanced serum stability, decreased tendency to aggregate, increased production, similar or increased antigen binding and improved antitumor activity in animals

(reviewed in Reiter and Pastan 1996). Disadvantages include the requirement of additional protein engineering and experiments to investigate possible effects of the introduced disulfide bond on the affinity of the Fv. Finally, in designing the Fv binding unit, the choice of the V region order may be important, that is, V_H-linker-V_L or V_L-linker-V_H. Both the affinity and secretion level of the protein can be influenced by the V domain order (reviewed in Huston et al. 1993).

In some of the studies described in this chapter chimeric or humanized antibodies are being used in the antibody domain in an attempt to reduce immunogenicity. Chimeric antibodies are built by incorporating entire murine variable regions within human constant regions while in humanized antibodies the only murine sequences are in the CDRs (complementarity determining regions), (reviewed in Jolliffe 1993).

All of this fundamental research contributed to the evolution of immunotoxin and targeted RNase architecture described in Sections 3.2 and 3.3.

3.1.2 Targeted Drug Strategies

Different strategies have been adopted for ultimately delivering an effector function to the target antigen. In direct targeting the effector moiety itself is the drug and is transported to the target by the antibody. Most approved and experimental antibody drugs directly target cancer cells and this review will focus on the application of direct antibody targeting to cancer therapy. However, other pathologies such as thrombolysis, dissolving clots formed inside blood vessels (Bode et al. 1985), viral and autoimmune diseases are amenable to direct antibody-targeted therapies as well. References to those studies can be found in Bolognesi and Polito (2004). Indirect targeting is another approach to increase the availability of the drug to its target (Bagshawe et al. 1988; Senter et al. 1988). The antibody–enzyme combination (drug activator) is bound to the tumor cell surface. When cleared from the circulation a pro-drug is administered that will be activated by the antibody enzyme complex. An advantage of the indirect approach for cancer (prodrug therapy) is that the antibody–enzyme does not have to translocate into the cell, which is an inefficient process. Thus, noninternalizing antigens that may be overexpressed on tumor cells become available for cancer therapy. Also, the activated drug can diffuse to nearby antigen-negative tumor cells. This helps to circumvent the problem of antigen heterogeneity encountered in direct targeting strategies. Excellent reviews by Bagshawe et al. (2004) and Wa (2004) bring prodrug therapy up to date, and a comparison between indirect and direct antibody targeting is provided by Rybak and Newton (1999a).

The following sections of this chapter examine advances in antibody-targeted proteins. Understanding that effectors had to be extremely potent led to conjugation of antibodies to very toxic plant and bacterial proteins (Pastan et al. 1986; Vitetta et al. 1987). The resulting hybrid proteins were named "immunotoxins." Immunotoxins cause potent cell killing *in vitro* and impressive results in murine models of cancer. In human patients both first-generation chemical conjugates

(Rybak and Youle 1991) and more recent derivations (Frankel et al. 2000) cause toxic side effects and immune responses that limit achievable therapeutic regimens. Yet, some successful clinical results spur continued refinement of these molecules as well as new approaches that are different from, but build on, the broad platform of immunotoxin technologies. For example, antibody-targeted ribonucleases (Section 3.3) may be a natural solution to managing the immunogenicity and toxicity of antibody-targeted proteins.

3.2 Immunotoxins

3.2.1 Diphtheria Toxin-based Immunotoxins

Toxins from plants and bacteria have evolved to kill cells (Fitzgerald 1996). Thus they have evolved structurally to withstand proteolytic degradation and susceptibility to intracellular inhibitors. They target elements in a cell that lead to cell death by inhibiting protein synthesis. The use of extremely toxic proteins isolated from plants and bacteria coupled to monoclonal antibodies to generate drugs that specifically kill target cells was inspired, in part, by the theory that the catalytic activity of extremely toxic poisons would be potent enough to kill tumor cells in spite of low levels of tumor uptake of antibody conjugates in humans. Section 3.2 focuses on the evolution of immunotoxins containing the three most studied toxins.

Diphtheria toxin (DT) is one of the bacterial toxins extensively studied and used in the construction of immunotoxins. It is secreted from *Corynebacterium diphtheria* as a single polypeptide chain consisting of three domains: the N-terminal catalytic domain, a translocation domain, and a C-terminal sequence that mediates binding to the cell surface. Originally the entire DT toxin was coupled to antibodies (Moolten et al. 1975). These immunotoxins were very potent but demonstrated a high level of nonspecific toxicity due to binding to nontarget cells. Understanding the DT structure led to substitution of the native cell binding domain with antibodies and other cell binding molecules. For example, the enzymatically active A chain of diphtheria toxin was coupled to the alternate receptor-binding domain of placental lactogen (Chang et al. 1977) but this did not make a functional immunotoxin. Studies that followed showed that the A chain of DT lacked efficient membrane protein translocation function to form a potent immunotoxin (Colombatti et al. 1986). This led to genetic manipulation of the DT molecule to create deletion mutants such as DAB_{389} that deleted portions of the DT-binding domain (Williams et al. 1990) and site-specific DT mutants such as CRM107 (Greenfield et al. 1987) and CRM9 (Hu and Holmes 1987) that changed critical amino acid residues necessary for native toxin binding. Both of the improved toxin variants retained critical B chain sequences necessary

for translocation to the cytosol and have been used to make successful immunotoxins.

Human-transferrin (Tf) chemically conjugated to a receptor-binding mutant of DT, CRM107 (Johnson et al. 1989) has progressed to phase III clinical trials for patients with brain cancer. Phase I clinical trial results demonstrated that Tf-CRM107 (delivered via a high-flow convection method utilizing stereotactically placed catheters) produced tumor response in patients with malignant brain tumors refractory to conventional therapy without severe neurological or systemic toxicity (Laske et al. 1997). In a phase II study, Tf-CRM107 treatment resulted in complete and partial tumor responses without severe toxicity in 35% of the evaluable patients (Weaver and Laske 2003). An important consideration with immunotoxin treatment is delivery of the drug. The success, in part, of Tf-CRM107 was the introduction of an intratumoral infusion under positive pressure. Direct administration to tumors increases the drug concentration and decreases systemic toxicity. Also, more treatment cycles can be given because the high concentration of the drug in the compartment overcomes possible inhibition by induced anti-drug antibodies.

Unfortunately the majority of solid tumors are not amenable to direct intratumoral administration and require systemic infusion. In this regard a cytokine DT fusion protein has received approval from the US Food and Drug Administration for the treatment of relapsed or refractory cutaneous T-cell lymphoma. DAB_{389}IL-2 (ONTAK, Ligand Pharmaceuticals) administered, as an intravenous infusion, is specifically targeted to cells expressing high-affinity IL-2 receptors. ONTAK is noted for its importance in establishing the clinical (Eklund and Kuzel 2005) and commercial relevance of immunotoxins. The experience gained by administering a targeted toxin to human patients is generating more confidence in their use. This, in turn, encourages the development of other toxin conjugates and fusion proteins. Although these two DT immunotoxins have made the most progress to date, many other ligands have been chosen for preclinical and clinical development of DT conjugates. A detailed analysis can be found in Frankel et al. (2002).

Anti-CD3 DT immunotoxins are extremely potent because crosslinking the T-cell receptor leads to rapid endocytosis (Youle et al. 1986). They have advanced from chemical conjugates with DT and DTA (Colombatti et al. 1986) to sophisticated fusion proteins (Table 3.1). A chemical conjugate (anti-CD3-CRM9) of an anti-rhesus CD3 antibody and CRM9, a DT-binding site mutant, significantly reduced lymph node T cells in monkeys (Neville et al. 1996). This encouraged the expression and purification of a recombinant anti-CD3 DT-scFv fusion protein using the scFv made from the anti-human CD3 antibody UCHT1 (Thompson et al. 1995). However, the binding affinity of the monovalent fusion protein was significantly decreased compared with the parental UCHT1 antibody. Problems with N-terminal heterogeneity were reported for a similar anti-human CD3 DT single-chain fusion protein, DT389-scFv (Hexham et al. 2001). Additional problems with toxicity, yield, and purity of anti-CD3 scFv fusion proteins spurred the

Table 3.1 Molecular evolution of immunotoxins.

Immunotoxin	*Target*	*Status*	*References*
Mab-DT[a]	SV40 antigens	Experimental	Moolten et al. 1975
UCHT1-DT	CD3	Experimental	Colombatti et al. 1986
UCHT1-DTA[a]	CD3	Experimental	Colombatti et al. 1986
Anti-CD3-CRM9[b]	CD3	Primate study	Neville et al. 1996
DT390-scFv(UCHT1)[c]	CD3	Experimental	Thompson et al. 1995
DT390-bisFv(G4S) [d]	CD3	Experimental	Thompson et al. 2001
Bic3[d]	CD3	Experimental	Vallera et al. 2005
Tf-CRM107[b]	Tf receptor[b]	Phase III	Weaver and Laske 2003
DAB_{389}IL2, ONTAK[c]	IL-2 receptor	FDA, approved	Eklund and Kuzel 2005
PE-anti-TAC[a]	CD25	Phase I, halted	Pastan 2003
Anti-Tac(Fv)-PE38[c]	CD25	Phase I	Kreitman et al. 2000
IL4(38–37)-PE38KDEL[c]	IL-4 receptor	Experimental, Phase I	Garland et al. 2005
B3-PE38, LMB1	LeY antigen	Phase I	Pai et al. 1996
BR96scFv-PE40 SGN-10[c]	LeY	Phase I	Posey et al. 2002
RFB4(ds)-PE38, BL22[e]	CD22	Phase I	Kreitman et al. 2005
HA22[e]	CD22	Experimental	Ho et al. 2004
HA22 R490A[e]	CD22	Experimental	Bang et al. 2005
UCHT-1-R[a]	CD3	GVHD, *ex vivo*	Filipovich et al. 1987
Anti-CD25-RTA	CD25	GVHD, *ex vivo*	Solomon et al. 2005
Anti-CD5-RTA[a]	CD5	GVHD, phase I	Hertler et al. 1989
Anti-B4-Blocked ricin	CD19	Phase I, phase II	O'Toole et al. 1998
RFB4-dgA[a]	CD22	Phase I	Amlot et al. 1993
IgG-HD37-dgA + IgG-RFB4-dgA	CD19 + CD22	Phase I	Messmann et al. 2000
IgG-RFB4-SMPT-dgA	CD22	Phase I	Sausville et al. 1995
RFB4-rRTA	CD22	Experimental	Smallshaw et al. 2003

a DT, entire diphtheria toxin molecule; DTA, A chain of DT; R, entire ricin; RTA, A chain of ricin; dgA, deglycosylated ricin A chain; PE, entire *Pseudomonas* toxin molecule.
b CRM9, CRM107, binding site mutants of DT; Tf, transferrin.
c DT390, DT389, DT deletion mutants; PE38, PE40; PE deletion mutants; PE38KDEL, PE38 with endoplasmic reticulum retention signal; scFv, single-chain antibody.
d Bivalent DT fusion proteins.
e BL22, disulfide stabilized single-chain fusion protein; HA22, improved scFv in BL22; HA22R490A, improved PE38 in HA22.

development of novel solutions. A bivalent anti-CD3 single-chain DT was constructed with linkers that reduced aggregation and was expressed with new refolding methods to improve yield (Vallera et al. 2005). The novel bivalent fusion protein was effective in a murine model of established human T-cell leukemia without toxicity to the mice seen with the monovalent fusion protein. Another study combined two anti-CD3 single-chain antibodies to create a single-chain bivalent fusion protein (Thompson et al. 2001). Aggregation and yield was markedly reduced by optimizing culture conditions in *Pichia pastoris* (Woo et al. 2006). These new anti-T-cell immunotoxins could prove useful for the treatment of

malignant and autoimmune T-cell disorders. Moreover, they illustrate that imaginative solutions can be found to practical problems as antibody-targeted immunotoxins evolve from chemical conjugates to more complex recombinant antibody toxin fusion proteins.

3.2.2 Pseudomonas Exotoxin-based Immunotoxins

Pseudomonas exotoxin A (PE) secreted by the pathogen *Pseudomonas aeruginosa* is another bacterial toxin under extensive evaluation for immunotoxin use. It has a molecular mass of 60 kDa and the same enzymatic activity as DT (Collier 1988). Both catalyze the ADP-ribosylation of elongation factor 2, thus inhibiting protein synthesis and killing the cell. Immunotoxins built with PE also changed as the different domains of the toxin were understood. An early clinical trial of an anti-CD25 (anti-TAC) PE chemical conjugate had to be halted due to severe liver toxicity caused by nonspecific cell binding of the holotoxin (Pastan 2003). Thus truncated PE molecules (PE38, PE40) were engineered to delete the native toxin cell binding activities while retaining the translocation and enzymatic functions. Notably, a chemical conjugate of modified PE (PE38) linked to the anti-Lewis Y (Le^Y) antibody B3 (LMB-1) was the first immunotoxin to show objective tumor responses in patients with solid tumors (Pai et al. 1996). LMB-1 was supplanted by recombinant immunotoxins in which the Fv portion of the antibody was expressed as an scFv directed against CD25 genetically fused to a truncated toxin (Chaudhary et al. 1989). A slightly smaller molecule (Anti-Tac (Fv)-PE38) was administered to patients with hematologic malignancies in a phase I clinical trial (Kreitman et al. 2000).

Although remissions occurred in some patients, new variations continue to be sought for PE immunotoxins targeting blood cancers. One example is IL-4(38–37)-PE38KDEL that combines targeting the IL-4 receptor with PE38 fused to KDEL, to facilitate transport to the endoplasmic reticulum (Kay et al. 2005). The IL-4-PE immunotoxin did not show any objective responses in patients with advanced solid tumors, possibly due to high levels of neutralizing human antitoxin antibodies (Garland et al. 2005). However, treating patients with more accessible tumor in the vasculature may be more successful since some responses were seen with intratumoral administration.

Single-chain PE fusion proteins have also been designed to try to improve on the activity shown in solid cancers by LMB-1. The anti-Le^Y BR96 scFv-PE40 fusion protein (SGN-10, Seattle Genetics) was evaluated in a phase I trial in patients with advanced solid carcinomas (Posey et al. 2002). Even the smaller scFv-PE fusion elicited an antitoxin antibody response in humans and modest vascular leak syndrome was manifested.

Further iterations in the design of PE single-chain fusion proteins have increased the stability of the scFv, allowing for more efficient delivery of the recombinant immunotoxins (detailed in FitzGerald et al. 2004; Pastan 2003). One of the most exciting of the PE immunotoxins is BL22. BL22 is a fusion protein

comprising the cloned variable domains of murine anti-CD22 antibody RFB4, joined by an engineered disulfide bond and fused to PE38. The results of a phase I clinical trial are most impressive for patients with hairy cell leukemia (HCL) (Kreitman et al. 2005). There were 19 complete remissions and six partial responses in patients with HCL. Hairy cells express significantly higher levels of the CD22 receptor compared with CD22-positive tumor cells that were among the other the B-cell malignancies treated. Improved versions of BL22 designed to effectively target malignant cells that express fewer CD22-positive sites are currently being designed using hot spot mutagenesis and phage display (Salvatore et al. 2002). HA22 is a mutant of BL22 with mutations in heavy-chain CDR3 and it was more cytotoxic than BL22 (Ho et al. 2004). HA22 (R490A) is an even more improved version of HA22. It was fused to PE38 that has a mutation located in the catalytic domain (III) of the immunotoxin HA22. This resulted in increased cytotoxic and antitumor activity but without increased toxicity to mice (Bang et al. 2005).

In light of the promising results with PE, numerous studies are reporting new treatment possibilities for pancreatic cancer (Bruell et al. 2005) and mesothelioma (Li et al. 2004). Additionally, Proxinium (MacDonald and Glover 2005) a single-chain anti-Ep-CAM antibody PE38 fusion protein (Viventia Biotech) targets an epithelial cell adhesion molecule. It is being administered intratumorally in a phase I clinical trial for the treatment of squamous cell carcinoma of the head and neck. Like the transferrin-DT chemical conjugate described in Section 3.2.1 (Tf-CRM107), Proxinium is generally well tolerated without the systemic toxicities seen for this type of toxin with intravenous infusions. A detailed compilation of PE clinical trial results can be found in the reviews by Frankel et al. (2000, 2003).

3.2.3
Plant-based Immunotoxins

Ribosome-inactivating proteins (RIPs) from plants inactivate protein synthesis and kill cells by cleaving a single *N*-glycosidic bond of the 28S RNA of ribosomes. They have a rich history in medicinal and immunotoxin use (Olsnes 2004; Stirpe 2004). One class of RIPs are single-chain (A chain) enzymatically active proteins of about 30 kDa. A second class of these toxins consists of an A chain covalently linked to a B chain. The B chain binds the toxic A chain to the cell surface and aids in the translocation of the toxin into the cell. Although many plant toxins are suitable as immunotoxins, the toxin ricin produced by the castor bean plant (*Ricinus communis*) has been used most extensively (Thrush et al. 1996; Vallera 1988).

Similar to results with the bacterial toxins (Sections 3.2.1 and 3.2.2), a high level of nonspecific toxicity results from use of the intact toxin due to cell binding sites on the toxin. For this reason intact ricin conjugates were used *ex vivo* to deplete T cells to prevent graft-versus-host disease (GVHD) and to circumvent problems of graft failure/rejection in patients needing bone marrow transplanta-

tion. The goal was to remove specific undesirable cell types from the bone marrow before infusion into the patient without damaging the hematopoietic stem cells. Both the CD3 and CD5 antigens were targeted in such a clinical trial in 1987 using a cocktail of three ricin conjugates (Filipovich et al. 1987). While *ex vivo* T-cell depletion of the graft can prevent development of GVHD it can also lead to a delay in immune reconstitution as well as an increase in opportunistic infections and recurrence of the cancer. An approach that enables a selective depletion of the donor T cells that causes GVHD while preserving anticancer and antimicrobial functions would be optimal. Recently, a study demonstrated that an anti-CD25 ricin A chain immunotoxin, which reacts with a cell surface activation antigen (IL-2 alpha subunit), might more selectively deplete T cells (Solomon et al. 2005).

Most ricin immunotoxin clinical trials have explored using the A chain of ricin. As described in Sections 3.2.1 and 3.2.2, bacterial toxins were found to need portions of the B chain to aid in translocation. Separating the A and B chains of ricin decreases the nonspecific binding but also the translocation activity of the B chain across the cell membrane. Therefore, the internalization capacity of the antigen is critical for A chain toxins. Blocked ricin is an altered ricin derivative that has its nonspecific binding eliminated by chemically blocking the galactose-binding domains of the B chain (Lambert et al. 1991). Anti-B4-blocked ricin (Anti-B4-bR) is an immunotoxin composed of the murine anti-B4 monoclonal antibody and "blocked ricin." The anti-B4 antibody is directed against the CD19 antigen expressed on more than 95% of normal and neoplastic B cells. Anti-B4-bR is extremely potent due to preservation of the translocation function. Early clinical trials looked promising (Rybak and Youle 1991; Grossbard et al. 1992). Toxicities were manageable and objective responses were obtained. Clinical results with blocked ricin were reviewed in O'Toole et al. (1998) and compared with results using ricin A chain and PE in (Gottstein et al. 1994). To date they are still being tested in phase I and II clinical trials. Serious complications from vascular leak syndrome resulted in the death of a patient in a phase II trial of a blocked ricin in patients with small cell lung cancer (Fidias et al. 2002). Nearly all the patients developed human antibodies against both components of the immunotoxin.

Recently a long-term follow-up of results in patients with chronic lymphocytic leukemia (CLL) treated with anti-B4-blocked ricin was published (Tsimberidou et al. 2003). No patients achieved an objective response. Although found to have an acceptable safety profile in some patients, it was immunogenic even in patients who had previously received immunosuppressive chemotherapy.

Early clinical trials with ricin immunotoxins were done in patients with non-Hodgkin's lymphoma, chronic B- and T-cell lymphocytic leukemia, breast cancer, colon cancer, and melanoma (reviewed in Rybak and Youle 1991). Blood-borne malignancies such as B- and T-cell cancers are thought to be more amenable to immunotoxin therapy because the tumor cells are accessible to the treatment. The CD5 antigen is widespread on cancer cells and was used as a target antigen with ricin A chain (Hertler et al. 1989). It does not mediate potent entry of the

A chain, perhaps explaining disappointing results. Early clinical trials illuminated facets of ricin immunotoxin therapy in humans related to toxicity and immunogenicity (Hertler 1988). Modifications to reduce toxicities included deglycosylated A chain (dgA) constructs to reduce hepatic uptake and new linkers (SMPT) to increase plasma stability. Even so, toxicity not predicted from animal studies persisted, notably vascular leak syndrome (VLS) (Sausville et al. 1995). Although associated with most immunotoxin clinical trials, VLS is particularly a problem for ricin-containing immunotoxins. VLS is characterized by weight gain, edema, serum albumin decrease, and pulmonary edema (Amlot et al. 1993; Sausville et al. 1995; Messmann et al. 2000). Changes in the ricin molecule were made in an attempt to address VLS problems. Recombinant ricin A chains were engineered with mutations in amino acid sequences common to proteins known to cause vascular leak. One mutant was comparable to unmodified ricin A chain except that it did not cause VLS in mice at the same dose and, when conjugated to RFB4 (RFB4-rRTA), was more effective in xenografted immunodeficient mice (Smallshaw et al. 2003). If mice develop VLS due to ricin these results may be predictive of an improved safety profile for ricin-based immunotoxins.

Stability has been another problem with ricin A chain conjugates. Patients were treated with a 1:1 mixture of anti-CD22 RFB4-dgA and anti-CD19 HD37 dgA (Combotox) (Messmann et al. 2000). Patients with 50 or more circulating tumor cells per mm^3 in the peripheral blood tolerated all doses without major toxicities. However those patients with less than 50 circulating tumor cells per mm^3 in the peripheral blood experienced unpredictable toxicity that included two deaths related to the immunotoxin. It was thought that biochemical heterogeneity and/or aggregation of the HD37-dgA preparation may have played a role in the clinical toxicities encountered.

Although ricin is the major plant toxin developed as an immunotoxin, it should be noted that pokeweed antiviral protein, gelonin, momordin, and saporin are among other plant toxins most commonly used to construct immunotoxins. Numerous preclinical (Bolognesi and Polito 2004) and clinical studies have been described and have compared these toxins (Frankel et al. 2000). New candidate plant toxins continue to be isolated and characterized as potent immunotoxins (Bolognesi et al. 2000). Though all these toxins exhibit some difference in levels of toxicity to animals and cell-killing potency, none of them are in clinical trials as natural product drugs; none of them are homologs of human proteins.

The evolution of antibodies, toxins, and consequently immunotoxins described in Section 3.2 is due to advances in recombinant DNA technology and antibody engineering. Representative examples of the changes in immunotoxin design and structure are listed in Table 3.1. Immunotoxins have advanced from heterogeneous chemical conjugates using the whole toxin, to using only catalytic A chains, to sophisticated mutations of the bacterial toxins to decrease nonspecific binding. This was coupled with linkage to increasingly sophisticated recombinant antibody designs. Improvements in ricin immunotoxins relied more on chemical methods to solve problems, with the exception of mutated ricin A chain to reduce VLS. Though the science is impressive there have been few major breakthroughs

in clinical use of immunotoxins more than 30 years after their introduction in the 1970s. The most promising areas appear to be in compartmentalized treatment such as Tf-CRM107 administered directly to brain tumors, *ex vivo* T-cell depletion, blood-borne malignancies, and treatment of minimal residual disease in solid tumors where the need for tumor penetration is obviated. Prophylactic tactics are being sought to try to manage the immunogenicity and toxicity. Finally combination trials with other therapeutics may yield better results in the future.

3.3 Targeted RNases

3.3.1 Background

To avoid the problems of immunogenicity and toxicity alluded to in Section 3.2, members of the pancreatic RNase A family have been proposed as possible alternatives to plant and bacterial toxins in the construction of immunotoxins (Rybak and Newton 1999b). Since these small extracellular proteins normally reside in the plasma and tissues of humans, they and their homologs could be expected to cause fewer problems when reinfused into human patients and, in fact, they have been safely administered to humans (Aleksandrowicz 1958; Mikulski et al. 2002). Furthermore, they were well tolerated immunologically in humans (Glukhov et al. 1976; Mikulski et al. 2002).

Moreover, numerous reports link RNases and antitumor activity. In 1955 bovine pancreatic RNase A injected into tumor-bearing mice was reported to impede tumor growth (Ledoux 1955a,b). Thus investigations into the clinical use of RNase A were stimulated and it was used in human clinical trials for the treatment of leukemia. Patients with chronic myelocytic leukemia were given daily subcutaneous injections of 0.5–1 mg of the bovine enzyme and were reported to have a decrease in spleen size and show general improvement (Aleksandrowicz 1958). A dimeric member of this protein superfamily, bovine seminal RNase (BS-RNase), was shown to possess antitumor activity *in vitro* and *in vivo* (Matousek 1973; Laccetti et al. 1994; Soucek et al. 1996; Pouckova et al. 1998). Though antitumor properties have been long associated with diverse members of the pancreatic RNase family, the potency, tumor specificity, and reproducibility in various tumor systems did not encourage serious clinical development in the light of other advances in chemotherapy.

In the early 1990s it was shown that by covalently linking the RNase protein to antibodies or fusing the RNase gene to genes encoding cell-binding ligands, human and mammalian RNases could become potent and specific cytotoxic agents directed towards tumor cells (Rybak et al. 1991, 1992). Subsequently, other studies have confirmed these observations, providing a substantial body of experimental and preclinical studies in support of targeted RNases. Both chemical

conjugates and recombinant fusion proteins consisting of various targeting domains linked to RNase proteins or fused to RNase genes have been made (Table 3.2).

3.3.2 Targeted Human and Mammalian RNases

Initially the transferrin (Tf) receptor was targeted with bovine pancreatic RNase (Rybak et al. 1991, 1993; Newton et al. 1992). The Tf receptor is an integral membrane glycoprotein that binds and internalizes Tf-iron complexes into cells. It is widely expressed on both tumor and normal cells, but the number of Tf receptors is coupled to growth rate (Trowbridge and Omary 1981). Most tumor cells proliferate more rapidly than normal cells and express more Tf receptors, thus this antigen was shown to have potential for tumor targeting (Trowbridge and Domingo 1981; Trowbridge 1988). Effector proteins linked to Tf are rapidly internalized by receptor-mediated endocytosis, an important criteria for generation of potent selective cell-killing agents (Taetle et al. 1986).

A complication of targeting the Tf receptor is that anti-Tf antibody drug conjugates cross the blood–brain barrier (Friden et al. 1991) and Tf receptors are present on the luminal side of capillary endothelial cells (Jefferies et al. 1984). Thus systemic administration of an antibody linked to a toxin could target brain endothelium unless the endothelial cells were not sensitive to killing by the toxic protein. However, as described in Section 3.2.1, interstitial infusion of a Tf-DT conjugate (Tf-CRM107) in patients with brain tumors was shown to be possible without serious toxicities, presumably because the conjugate did not interact with Tf receptors inside capillaries (Laske 1995). Tf-CRM107 has advanced to phase III trials, showing that the Tf receptor can be targeted successfully. In that regard, an anti-Tf receptor antibody RNase conjugate was directly compared with the same antibody conjugated to ricin. The RNase conjugate was 1000-fold less cytotoxic *in vitro* than the same antibody conjugated to ricin A chain but equally effective *in vivo* in a solid flank model of brain cancer (Newton et al. 1992). Thus, cell culture cytotoxicity assays may not be reflective of true *in vivo* antitumor efficacy for RNase-based targeted compounds. Transferrin was also conjugated to two human RNases (Suzuki et al. 1999).

RNase fusion proteins have been constructed with growth factors like epidermal growth factor (EGF) (Jinno et al. 1996, 2002; Psarras et al. 1998; Suwa et al. 1999; Yoon et al. 1999), fibroblast growth factor (FGF) (Futami et al. 1999; Hayashida et al. 2005; Tanaka et al. 1998), cytokines such as IL-2 (Psarras et al. 2000), peptide hormones (Gho and Chae 1999), and ligands (Huhn et al. 2001) as the targeting agent (Table 3.2). Some of these fusion proteins are fully humanized since both domains are built from human proteins. Herceptin (trastuzumab), a humanized antibody against the receptor tyrosine kinase ErbB2, has been approved for therapy of metastatic breast cancer, but efforts to increase its efficacy are being sought (Carter 2001). A fully human antitumor RNase targeting the

Table 3.2 Targeted human and mammalian RNases.

Name	*RNase*	*Ligand*	*Structure*	*Cell surface target*	*Biological target*	*Cytotoxicity in vitro IC_{50}[a] (nmol L^{-1})*	*Refs*
LHRH-RNase	Bovine	Human	Hormone fusion	LHRH receptor	Carcinoma	500–700	Gho and Chae 1999
Tf-RNase[b]	Bovine	Human	Ligand conjugate	Tf receptor	Leukemia	40	Rybak et al. 1991
Anti-TFR-RNase[b]	Bovine	Murine	Mab conjugate	Tf receptor	Leukemia	20	Newton et al. 1992
H17-BSRNase	Bovine	Murine	scFv fusion[b]	hPLAP[c]	Carcinoma	4–400	Deonarain and Epenetos 1998
hpRNase scFv	Human	Chimeric	scFv fusion	Tf receptor	Carcinoma	5–90	Zewe et al. 1997
hpRNase-IL2	Human	Human	Cytokine fusion	IL2 receptor	T-lymphocytes	20	Psarras et al. 2000
RNase-FGF	Human	Human	GF fusion[b]	FGR receptor	Endothelium	100	Tanaka et al. 1998[e]
RNase-EGF	Human	Human	GF fusion	EGF receptor	Carcinoma	300–600	Jinno et al. 1996[e]
ECP-EGF	Human	Human	GF-fusion	EGF receptor	Carcinoma	150	Jinno et al. 2002
Tf-hRNase[d]	Human	Human	Ligand conjugate	Tf receptor	Glioma	2[d]	Suzuki et al. 1999
hERB-hRNase	Human	Human	scFv Fusion	ErbB-2 receptor	Carcinoma	10–60	Lorenzo et al. 2004
EDNscFv	Human	Chimeric	scFv fusion	Tf receptor	Carcinoma	1–20	Newton et al. 1994
Tf-EDN[d]	Human	Human	Ligand conjugate	Tf receptor	Glioma	5[d]	Suzuki et al. 1999
CH2Ang	Human	Chimeric	Fab fusion	Tf receptor	Carcinoma	0.05	Rybak et al. 1992
AngscFv	Human	Chimeric	scFv fusion	Tf receptor	Carcinoma	4–200	Newton et al. 1996
Ang-EGF	Human	Human	GF fusion	EGF receptor	Carcinoma	10	Yoon et al. 1999
Ang-CD30	Human	Human	Ligand fusion	CD30	Lymphoma	0.24	Huhn et al. 2001
scFv-ANG	Human	Humanized	scFv fusion	CD22	Lymphoma	60	Krauss et al. 2005
Dimeric scFvANG	Human	Humanized	scFv fusion	CD22	Lymphoma	74	Arndt et al. 2005

a Concentration required to inhibit protein synthesis 50%.
b Tf, transferrin; TFR, transferrin receptor; scFv, single-chain antibody; GF, growth factor.
c Tumor-associated isoform of human placental alkaline phosphatase.
d Assayed in the presence of retinoic acid to enhance cytotoxicity; resistant to intracellular RNase inhibitor.
e Multiple studies listed in Section 3.2.

ErbB-2 receptor induced a dramatic reduction in tumor volume in mice bearing an ErbB-2-positive tumor (Lorenzo et al. 2004).

Bovine seminal RNase (BSRNase) was targeted against the tumor-associated human hPLAP that is an isoform of placental alkaline phosphatase present on solid carcinomas such as ovarian and testicular as well as on some bladder and head and neck cancers (Epenetos et al. 1984). The scFv (Savage et al. 1993) was constructed from the H17E2 antibody (Travers and Bodmer 1984) against hPLAP. The scFv was found to localize to human xenografts in a murine model of human cancer more rapidly than the IgG form (Deonarain and Epenetos 1998). H17-BSRNase exhibited a wide range of cytotoxicity against tumor cell lines (Table 3.2).

3.3.3
RNase Fusion Protein Architecture

Genetically engineered RNase fusion proteins have been constructed with several different architectures (Fig. 3.1). In one variation the 5′ region of the human angiogenin ribonuclease gene (ANG) was fused to the 3′ region of the C_H2 domain (Rybak et al. 1992) of a chimeric anti-human Tf receptor antibody, E6 (Hoogenboom et al. 1990). This fusion protein (CH2ANG) displayed activity at picomolar concentrations. This construct was 4 logs more toxic to antigen-expressing cells than were chemical RNase conjugates (Table 3.2). The major problem with CH2ANG was that myeloma cell expression yielded only 1–5 ng mL^{-1}, which made purification difficult. To increase yield, single-chain Fv antibody fusions were constructed with three different human RNases: eosinophil-derived neurotoxin (EDN) (Newton et al. 1994), human pancreatic RNase (hpRNase) (Zewe et al. 1997), or ANG (Newton et al. 1996b; Krauss et al. 2005). Stable active RNase-scFvs were expressed as insoluble protein in inclusion bodies (Newton et al. 1996b) and later in transient mammalian cell expression systems (Krauss et al. 2005) or as proteins secreted from *E. coli* (Arndt et al. 2005). Another RNase single-chain fusion protein, H17-BSRNase (Deonarain and Epenetos 1994, 1995) was designed using BS-RNase to allow RNase dimerization since this RNase itself dimerizes by virtue of two intersubunit disulfide bonds and an exchanged N-terminus (D'Alessio et al. 1991). H17-BSRNase was cytotoxic to target cells despite being directly attached to the V_L domain of the antibody without an intervening spacer peptide. Altogether the scFv antibody fusion proteins shown in Fig. 3.1 express activity in the nanomolar range irrespective of the RNase, orientation, or linkers used. The extremely potent activity of CH2ANG may be due to possible bivalency of the Fab fragment or may reflect its homogeneous composition resulting in a more perfectly formed fusion protein with full retention of enzymatic and binding activities.

Humanized and human antibodies are being used in antibody-mediated therapeutics to reduce the immune response. Although allergic reactions can occur upon retreatment of patients that have developed antibodies against the reagent, the major effect of the induced antibodies appears to be on the half-life of the

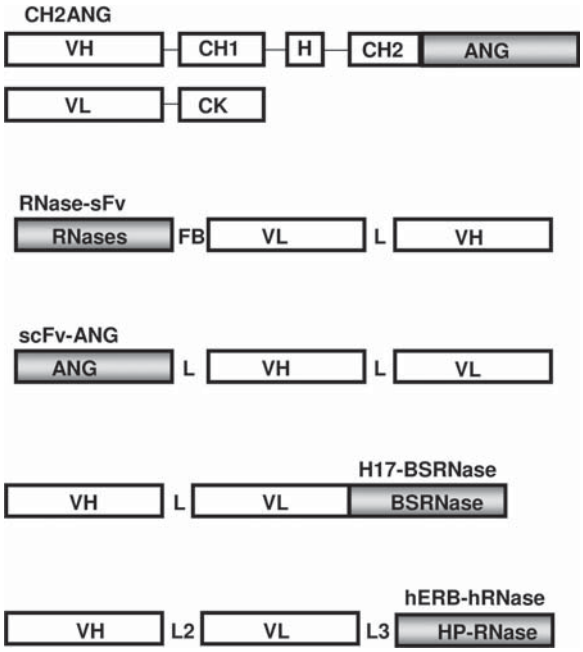

Fig. 3.1 Configurations of recombinant antibody RNase fusion proteins. CH2ANG, the gene for ANG was fused to the 3′ end of a chimeric antihuman TfR receptor (E6) antibody (Hoogenboom et al. 1990). It is shown as a Fab enzyme but the possibility of dimerization to a $F(ab')_2$ enzyme existed because of the hinge region and the C_H2 domain (Rybak et al. 1992). Three human RNases – ANG, EDN, and hpRNase – were expressed as fusion proteins with a chimeric scFv derived from E6 in the configuration shown for RNase scFv. The RNases were separated from the V_L by the FB peptide (residues 48–60 of staphylococcal protein A (AKKLNDAQAPKSD)). The V_L and V_H domains were joined by the flexible linker, L, $(GGGGS)_3$ originally described by Huston et al. (1988). This configuration was found to be optimal for RNase scFvs expressed as insoluble protein from inclusion bodies (Newton et al. 1996a,b). scFv ANG, targeted by a humanized single-chain antibody with specificity to the CD22 antigen, was produced from transiently transfected mammalian Chinese hamster ovary cells (Krauss et al. 2005). H17-BSRNase, the gene for bovine seminal Rnase, was fused to a murine scFv directed against tumor-associated hPLAP (Deonarain and Epenetos 1998). hERB-hRNase is directed to the ErbB-2 receptor. L2, the 15-residue junction peptide $SS(G_4S)_2GGS$; L3, the peptide AAASGGPEGGS connecting the scFv and the RNase (Lorenzo et al. 2004).

protein (shortened), which impedes targeting and tumor uptake (reviewed in Rybak et al. 1991; Khazaeli et al. 1994). Thus far it appears that the immune response is attenuated in patients that receive humanized antibodies (Carter 2001; Khazaeli et al. 1994; Stephens et al. 1995), emphasizing the importance of attaching less immunogenic effectors such as RNases to these antibodies. The studies described in Section 3.3 show that the concept of using targeted human and mammalian RNases has been well established in experimental studies but

not been developed in rigorous preclinical studies. The most advanced targeted RNase is described in detail in Section 3.4.

3.4 Targeted Onconase

3.4.1 Background

Studies with human and mammalian RNases have converged with those of a new direction in cancer research. Sequencing an anticancer protein from frog eggs showed that it belonged to the RNase A superfamily (Ardelt et al. 1991). This new RNase, named Onconase (ranpirnase, Alfacell Corporation), was originally isolated from *R. pipiens* oocytes by following cytotoxic activity against cancer cells *in vitro* (Darzynkiewicz et al. 1988; Mikulski et al. 1990a,b, 1992a,b; Lee et al. 2000) and *in vivo* (Mikulski et al. 1990a,b). Phase I and phase I/II clinical trials of Onconase as a single therapeutic agent in patients with a variety of solid tumors have been completed (Mikulski et al. 1993, 2002; Vogelzang et al. 2001) and have progressed to phase III clinical trials in the United States and Europe for the treatment of malignant mesothelioma (Vogelzang et al. 2000). Although Onconase is an amphibian protein, there have been few problems associated with repeated administration in humans (Mikulski et al. 2002). Recently it was demonstrated that Onconase could selectively enhance activation-induced (e.g. PHA- or mixed lymphocyte reaction-induced) apoptosis of peripheral blood lymphocytes at nanomolar concentrations (Halicka et al. 2002). These results could partly explain an apparent lack of significant adverse immunological reactions observed in Onconase-treated patients.

Since Onconase is currently in clinical trials, understanding the mechanism underlying its antitumor properties is important. Unlike intracellular pancreatic-type ribonucleases that hydrolyze cellular RNAs as a general metabolic function, Onconase targets a specific intracellular RNA that causes changes in intracellular signaling. Onconase binds to the surface of a tumor cell through an as yet unidentified receptor(s) (Wu et al. 1993). Binding is saturable, correlates with cytotoxicity and routing to the cytosol occurs via the Golgi apparatus (Wu et al. 1995). The potent cell killing activity of Onconase is due to damage to tRNA (Lin et al. 1994; Iordanov et al. 2000; Saxena et al. 2002) that causes a physiologically relevant death signal in mammalian cells (Iordanov et al. 2000). The caspase cascade is activated, resulting in apoptosis (Iordanov et al. 2000; Grabarek et al. 2002). However, unlike DNA damage and apoptosis (Benhattar et al. 1996), Onconase-induced apoptosis through tRNA damage is not affected by the absence of a functional p53 protein (Iordanov et al. 2000), an advantage to targeting RNA with Onconase.

Another postulated mechanism of Onconase is that it acts as an intracellular catalyst for the generation of interfering RNAs (RNAi) that could also trigger

apoptosis depending upon the microenvironment of the cell (Ardelt et al. 2002). Although apoptosis seems to be a general result of Onconase treatment in all tumor cells tested thus far, specific mechanisms may differ in varying cell types. For example treatment of Jurkat leukemia cells by Onconase corresponds to altered nucleocytoplasmic distribution and reduced expression of the transcription factor NF-κB (Tasi et al. 2004).

Onconase was submitted to the Cancer Drug Discovery and Development Program of the National Cancer Institute (Monks et al. 1991; Grever et al. 1992). The patterns of cell sensitivity of Onconase to all the other agents in the NCI screen database were computed using COMPARE software (Paull et al. 1989). Interestingly, a significant correlation coefficient was found with bleomycin (Abraham et al. 2003), a chemotherapeutic agent now thought to act, in part, by cleaving tRNA. Since it acts on RNA, not DNA, the efficacy of Onconase can be increased in combination therapy with standard DNA damaging chemotherapeutic agents (Lee et al. 2003; Mikulski et al. 1990a,b, 1992a,b; Vasandani et al. 1999) even in the presence of the mdr1 form of multidrug resistance (Rybak et al. 1996). Increased sensitivity to drugs may be due, in part, to the ability of Onconase to decrease tumor interstitial fluid pressure that is an impediment to drug delivery (Lee et al. 2000). Further information on the structure and therapeutic potential of Onconase is found in a recent review (Saxena et al. 2003).

3.4.2
Onconase Conjugates

Attachment of Onconase to targeting ligands markedly improves its specificity and potency (Rybak and Newton 1999b). Chemical conjugates of Onconase to date are listed in Table 3.3. Analogous to Tf-linked toxins (Raso and Basala 1984) and other RNases (Table 3.2) Onconase linked to an anti-Tf receptor antibody was markedly more cytotoxic to human K562 erythroleukemia cells than unconjugated Onconase (IC_{50} 130 versus 5000 nmol L^{-1}, respectively (Table 3.3).

Onconase was conjugated to MRK16, an anti-P-glycoprotein (Pgp) monoclonal antibody (Newton et al. 1996a). A multidrug-resistant phenotype is caused by expression of the Pgp membrane protein encoded by the MDR1 gene (*ABCB1*) (reviewed in Endicott and Ling 1989; Fork and Hait 1990; Ambudkar et al. 1999). The interaction of MRK16-Onconase conjugates with vincristine (VCR) against parental and multidrug-resistant (MDR), Pgp-expressing, human colon carcinoma cells was investigated *in vitro* and *in vivo*. Both reducible disulfide and nonreducible thioether-linked MRK16-Onconase conjugates were more cytotoxic on the Pgp-expressing $HT29^{mdr1}$ cells than on the Pgp-negative parental ($HT29^{par}$) cells (IC_{50s}, disulfide conjugate 20 nmol L^{-1} versus 130 nmol L^{-1}; thioether conjugate, 100 nmol L^{-1} versus 300 nmol L^{-1}, $HT\text{-}29^{mdr1}$ and $HT\text{-}29^{par}$ respectively) (Table 3.3). As expected, MRK16-Onconase conjugates with the reducible disulfide bond were more potent, presumably since the Onconase could more easily separate from the antibody and enter the cytosol. Immunofluorescent studies demonstrated that the enhanced toxicity of Onconase conjugates was also related to

Table 3.3 *In vitro and in vivo* efficacy of Onconase and Onconase chemical conjugates.

Onconase or conjugate	*Cell Line*[a]	*IC_{50} (nmol L^{-1})*[b]	*% ILS*[b]	*Refs*
Onconase	K562	5000	ND	Rybak et al. 1993
	HT-29^{par}	6000	0	Newton et al. 1996a
	HT-29^{mdr1}	6000	0	Newton et al. 1996a
	Daudi	1200	0	Newton et al. 2001
Anti-TfR-Onconase[c]	K562	130	ND	Rybak et al. 1993
MRK-16-Onconase[d] (DS)[e]	HT-29^{par}	130	ND	Newton et al. 1996a
MRK-16-Onconase[d] (Thio)[f]	HT-29^{par}	300	ND	Newton et al. 1996a
MRK-16-Onconase[d] (DS)[e]	HT-29^{mdr1}	20	93	Newton et al. 1996a
MRK-16-Onconase[d] (Thio)[f]	HT-29^{mdr1}	100	75	Newton et al. 1996a
LL2-Onconase	Daudi	0.02	44	Newton et al. 2001
RFB4-Onconase[g]	Daudi	0.02	153	
LL2-EDN[g]	Daudi	>100	ND	
LL2-hRNase[g]	Daudi	>100	ND	

a The cell lines are as follows: K562, human erythroleukemia; Daudi, human lymphoma; HT-29^{par}, human colon carcinoma; HT-29^{mdr1}, human multidrug-resistant colon carcinoma.
b IC_{50}, concentration of RNase or conjugate required to inhibit protein synthesis by 50%; ILS, increase in lifespan.
c Anti-TfR, 5E9 antibody against the human transferrin receptor.
d MRK-16, an anti-P-glycoprotein (Pgp) monoclonal antibody
e DS, disulfide-linked chemical conjugate.
f Thio, thioether-linked chemical conjugate.
g D.L.N., unpublished data.

enhanced internalization of Onconase by the antibody (Newton et al. 1996b). The MRK16 antibody reacts with a portion of the Pgp that functions as an ATP-dependent drug efflux pump not thought to internalize (Hamada and Tsuruo 1986). Thus enhanced internalization by MRK16 most likely reflects the ability of the antibody to concentrate Onconase at the cell surface, where it is able to self-internalize as previously reported (Wu et al. 1993). The implications are that Onconase may be effectively targeted with non-internalizing antibodies. These results were surprising because plant and bacterial toxins must be linked to ligands that efficiently internalize them to the cytosol such as the rapidly internalized 5E9 antibody (Johnson 1991).

Moreover, MRK16-Onconase conjugates sensitized VCR-resistant human colon cancer cells to VCR and this correlated with increased levels of VCR in these cells (Newton et al. 1996a,b). This resulted in an increase in the median survival times of athymic nude mice given transplants of VCR-resistant tumor cells by 40 days, demonstrating that reversal of drug resistance was not an *in vitro* phenomenon (Newton et al. 1996a,b). These results suggest that MRK16-Onconase conjugates could exhibit a dual role: (1) they could directly kill drug-resistant cells; (2) they could decrease resistance to a chemotherapeutic drug such as VCR.

CD22, a B lymphocyte-restricted member of the immunoglobulin superfamily, is a member of the sialoadhesin family of adhesion molecules that include sialo-

adhesin and myelin-associated glycoprotein (Kelm et al. 1994). Sialoadhesin and CD22 mediate cellular interactions by recognizing specific cell surface sialylated glycoconjugates (Nath et al. 1995). Binding of CD22 to glycoconjugates on neighboring cells alters signaling through the membrane immunoglobulin of B cells by binding cytosolic proteins (Doody et al. 1996). CD22 is an attractive molecular target because of its restricted expression; it is not exposed on embryonic stem or pre-B cells, nor is it normally shed from the surface of antigen-bearing cells (Li et al. 1989). Moreover, it is highly expressed on B cells in non-Hodgkin's lymphoma. A murine anti-CD22 monoclonal antibody (LL2, originally designated EPB-2 (Pawlak-Byczkowska et al. 1989)) was developed for imaging and treatment of non-Hodgkin's lymphoma. LL2 has a highly restricted specificity; it does not cross-react with peripheral blood cells, including the blood's normal B cells, yet is reactive with virtually all cases of non-Hodgkin's lymphoma (Stein et al. 1993).

RFB4, another murine IgG1 antibody against CD22, originally characterized by Campana et al. (1985), was shown to exhibit B-cell specificity that would be favorable for constructing immunotoxins (Li et al. 1989) since it rapidly internalizes (Shih et al. 1994; Shan and Press 1995). Onconase has been conjugated to both LL2 (Newton et al. 2001) and RFB4 (D.L.N., unpublished results). Covalently linking Onconase to these anti-CD22 antibodies increased its cytotoxicity thousands of times (Onconase vs. anti-CD22 Onconase conjugates, IC_{50}s 1200 and 0.02 nmol L^{-1}, respectively) (Table 3.3). Surprisingly, LL2 conjugated to two different human RNases that were successfully conjugated to other antibodies (Table 3.2) did not kill human Daudi cells. To date, all antibodies active with human RNases were also active when conjugated to Onconase. These results imply that human RNases may have a more restricted use than Onconase in targeted therapies.

Anti-CD22 antibodies have been developed to target B-cell lymphomas with plant and bacterial toxins such as derivatives of PE (Kreitman et al. 1993; Mansfield et al. 1996, 1997), deglycoslyated ricin A chain (dgA) (Ghetie et al. 1991, 1992; van Horssen et al. 1996, 1999), and ribosomal inactivating proteins (Flavell et al. 1997; Bolognesi et al. 1998). Some of these anti-CD22 immunotoxins are listed and compared with anti-CD22 Onconase conjugates in Table 3.4. The *in vitro* potency and specificity of anti-CD22 Onconase on Daudi cells is comparable with anti-CD22 immunotoxin conjugates that also kill lymphoma cells in the picomolar range.

Toxins evolved to kill cells and evade host defense mechanisms. Harnessing that extreme toxicity to specifically kill pathological cells is the goal in the immunotoxin field. Though many changes to the toxin molecule were made to try to decrease the nonspecific effects of toxins, severe nonspecific toxicities continue to hamper the clinical effectiveness of these compounds. For instance, all immunotoxins used to date cause VLS to some degree in humans, but the most severe cases have been observed in those patients treated with immunotoxins containing ricin A chain (Frankel et al. 2000). In contrast, VLS has not been associated with Onconase in the clinic (Mikulski et al. 1993, 2002). Moreover, unlike RFB4-ricin

Table 3.4 Comparison of anti-CD22 targeted agents.

Targeted agent	In vitro *potency* IC_{50} *(nmol* L^{-1}*)*[a]	In vivo *schedule*	In vivo *toxicity* LD_{50} *(mg/kg)*[a]	*Refs*
LL2-PE38KDEL[b]	0.01–0.03	ip[c] QD × 4	6	Kreitman et al. 1993
LL2-onconase	0.02	ip QD × 5	350	Newton et al. 2001
RFB4-PE35	0.005	iv QD[c] × 4	5	Mansfield et al. 1996
RFB4(dsFv)PE38	0.005	iv QOD[c] × 3	5	Mansfield et al. 1997
RFB4-dgA[b]	0.001	ip bolus	14	Ghetie et al. 1991
RFB4-onconase[d]	0.02	ip QD × 5	550	
OM124-saporin	0.005	Q3D × 3	0.5	Bolognesi et al. 1998
OM124-momordin	0.009	Q3D × 3	1.75	Bolognesi et al. 1998

a IC_{50}, 50% inhibitory concentration; LD_{50}, 50% lethal dose.
b PE, *Pseudomonas* toxin; dgA, deglycosylated ricin A chain.
c ip, intraperitoneally; iv, intravenously; QD, every day; QOD, every other day; Q3D, every third day.
d D.L.N., unpublished data

A chain, the Onconase conjugate does not damage endothelial cells in culture (D.L.N., unpublished results) when tested in the *in vitro* assay described to be predictive for VLS (Soler-Rodriguez et al. 1993). Onconase is not a toxin. It has been administered to humans and was predicted to cause less toxicity when conjugated to antibodies. As shown in Table 3.4, both anti-CD22 Onconase conjugates are markedly less lethal to mice than immunotoxins. In fact, RFB4(dsFv)PE38, the construct that has caused complete remissions in some hairy cell leukemia patients (Kreitman et al. 2005) is more than 10 times as lethal to mice than RFB4-Onconase.

Anti-CD22 Onconase conjugates are also effective in murine models of cancer. Treatment schedules were designed to assess tumor prevention as well as treatment of minimal and more advanced cancer with both LL2- (Newton et al. 2001) and RFB4-Onconase conjugates (D.L.N., unpublished results). In all of the experiments the conjugate was always effective in significantly increasing the survival of tumor-bearing mice (the increase in lifespan ranged from 40 to 200% over that of mock-treated mice). Again this potency and specificity is comparable to anti-CD22 immunotoxins made with plant and bacterial toxins.

Perhaps as important, considering the lethal toxicities associated with ricin A chain immunotoxin aggregation (Messmann et al. 2000), formulation studies show that both LL2- (Newton et al. 2001) and RFB4-Onconase are very stable. No aggregation either visibly or by HPLC analysis was noted even after the thawing of RFB4-onconase samples stored at –20°C or –70°C (D.L.N., unpublished results). Taken together, targeted Onconase looks promising even though each version has been a first-generation chemical conjugate composed of a heterogeneous mix of molecules. The DNA for Onconase has been cloned, paving the way for the creation of more sophisticated immunofusions as described in Sections

3.2.1, 3.2.2, 3.3.2, and 3.3.3. Yet, because of possible production problems due to misfolded recombinant proteins and poor yield, a new derivative of Onconase has been made to allow site-specific chemical conjugation. (K. Shogen, personal communication). Future generations of targeted Onconase will draw on a variety of possible solutions to enable the best drug for each application.

3.5 Outlook

Immunotoxins have entered the arsenal of anticancer drugs. Problems in clinical use have been identified and attempts to improve the therapeutic index of immunotoxins are being developed. It is widely anticipated that they have the potential to be effective anticancer agents. Many different laboratories have now shown that targeted RNases effectively kill cancer cells in preclinical studies and because they are not toxins naturally display a large therapeutic index. One of these, Onconase, is currently in phase III clinical trials for mesothelioma. Its specificity and potency, particularly against non-Hodgkin's lymphoma, is improved by antibody targeting. Comparisons to classical immunotoxins show that targeted Onconase is effective in preclinical models, causes less nonspecific toxicity in mice and has favorable formulation properties. Anti-CD22 Onconase (RN321) is being developed by the National Cancer Institute in collaboration with Alfacell, Corp., Bloomfield, NJ, USA and is expected to enter clinical trials shortly.

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Abbreviations

RNase	ribonuclease
scFv	single-chain antibody
dsFv	disulfide linked single-chain antibody
DT	diphtheria toxin
PE	*Pseudomonas* exotoxin A
Le^Y	Lewis Y antigen

RIPs	ribosome inactivating proteins
dgA	deglycosylated ricin A chain
Tf	transferrin
EGF	epidermal growth factor
FGF	fibroblast growth factor
ILS	increased lifespan
i.p.	intraperitoneally
i.v.	intravenously
VLS	vascular leak syndrome

References

Abraham, A.T., Lin, J.J., Newton, D.L., Rybak, S., Hecht, S.M. (2003) RNA cleavage and inhibition of protein synthesis by bleomycin. *Chem Biol* 10: 45–52.

Aleksandrowicz, J. (1958) Intracutaneous ribonuclease in chronic myelocytic leukemia. *Lancet* 2: 420.

Alfthan, K., Takkinen, K., Sizmann, D., Soderlund, H., Teeri, T.T. (1995) Properties of a single-chain antibody containing different linker peptides. *Protein Eng* 8: 725–731.

Ambudkar, S.V., Dey, S., Hrycyna, C.A., Ramachandra, M., Pastan, I., Gottesman, M.M. (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. *Annu Rev Pharmacol Toxicol* 39: 361–398.

Amlot, P.L., Stone, M.J., Cunningham, D., Fay, J., Newman, J., Collins, R., May, R., McCarthy, M., Richardson, J., Ghetie, V., Ramilo, O., Thorpe, P.E., Uhr, J.W., Vitetta, E.S. (1993) A phase I study of an anti-CD22-deglycosylated ricin A chain immunotoxin in the treatment of B-cell lymphomas resistant to conventional therapy. *Blood*. 82: 2624–2633.

Ardelt, W., Mikulski, S.M., Shogen, K. (1991) Amino acid sequence of an anti-tumor protein from *Rana pipieno* ocytes and early embryos. *J Biol Chem* 266: 245–251.

Ardelt, B., Ardelt, W., Darzynkiewicz, Z. (2002) Cytotoxic ribonucleases and RNA interferencde (RNAi). *Cell Cycle* 2: 22–24.

Arndt, M.A., Krauss, J., Vu, B.K., Newton, D.L., Rybak, S.M. (2005) A dimeric angiogenin immunofusion protein mediates selective toxicity towards CD22+ tumor cells. *J Immunother* 28: 245–251.

Bagshawe, K.D., Springer, C.J., Searle, F., Antoniw, P., Sharma, S.K., Melton, R.G., Sherwood, R.F. (1988) A cytoxic agent can be generated selectively at cancer sites. *Br J Cancer* 58: 700–703.

Bagshawe, K.D., Sharma, S.K., Begent, R.H. (2004) Antibody-directed enzyme prodrug therapy (ADEPT) for cancer. *Expert Opin Biol Ther* 4: 1777–1789.

Bang, S., Nagata, S., Onda, M., Kreitman, R. J., Pastan, I. (2005) HA22 (R490A) is a recombinant immunotoxin with increased antitumor activity without an increase in animal toxicity. *Clin Cancer Res* 11: 1545–1550.

Benhattar, J., Cerottini, J.P., Saraga, E., Metthez, G., Givel, J.C. (1996) p53 mutations as a possible predictor of response to chemotherapy in metastatic colorectal carcinomas. *Int J Cancer* 69: 190–192.

Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S.M., Lee, T., Pope, S.H., Riordan, G.S., Whitlow, M. (1988) Single-chain antigen- binding proteins. *Science* 242: 423–426.

Blattler, W.A., Chari, R.V.J., Lambert, J.M. (1996) Immunoconjugates. In: *Cancer Therapeutics: Experimental and Clinical Agents*. Teicher, B. (ed.), Humana Press Inc., Totowa, NJ, 371–394.

Bode, C., Matsueda, G.R., Hui, K.Y., Haber, E. (1985) Antibody-directed urokinase: a

specific fibrinolytic agent. *Science* 229: 765–767.

Bolognesi, A., Polito, L. (2004) Immunotoxins and other conjugates: Preclinical Studies. *Mini-Rev Med Chem* 4: 563–583.

Bolognesi, A., Tazzari, P., Olivieri, F., Polito, L., Lemoli, R., Terenzi, A., Pasqualucci, L., Falini, B., Stirpe, F. (1998) Evaluation of immunotoxins containing single-chain ribosome-inactivating proteins and an anti-CD22 monoclonal antibody (OM124): in vitro and in vivo studies. *Br J Haematol* 101: 179–188.

Bolognesi, A., Polito, L., Tazzari, P.L., Lemoli, R.M., Lubelli, C., Fogli, M., Boon, L., deBoer, M., Stirpe, F. (2000) In vitro anti-tumour activity of anti-CD80 and anti-CD86 immunotoxins containing type 1 ribosome-inactivating proteins. *Br J Haematol* 110: 351–361.

Brinkmann, U., Reiter, Y., Jung, S.H., Lee, B., Pastan, I. (1993) A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Proc Natl Acad Sci USA* 90: 7538–7542.

Bruell, D., Bruns, C.J., Yezhelyev, M., Huhn, M., Muller, J., Ischenko, I., Fischer, R., Finnern, R., Jauch, K.W., Barth, S. (2005) Recombinant anti-EGFR immunotoxin 425(scFv)-ETA demonstrates anti-tumor activity against disseminated human pancreatic cancer in nude mice. *Int J Mol Med* 15: 305–313.

Campana, D., Janossy, G., Bofill, M., Trejdosiewicz, L.K., Ma, D., Hoffbrand, A.V., Mason, D.Y., Lebacq, A.M., Forster, H.K. (1985) Human B cell development. I. Phenotypic differences of B lymphocytes in the bone marrow and peripheral lymphoid tissue. *J Immunol* 134: 1524–1530.

Carter, P. (2001) Improving the efficacy of antibody-based cancer therapies. *Nat Rev Cancer* 1: 118–129.

Chang, T.M., Dazord, A., Neville, D.M. Jr. (1977) Artificial hybrid protein containing a toxic protein fragment and a cell membrane receptor-binding moiety in a disulfide conjugate. II. Biochemical and biologic properties of diphtheria toxin fragment A-S-S-human placental lactogen. *J Biol Chem* 252: 1515–1522.

Chaudhary, V.K., Queen, C., Junghans, R.P., Waldmann, T.A., FitzGerald, D.J., Pastan, I. (1989) A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin. *Nature* 339: 394–397.

Colcher, D., Bird, R., Roselli, M., Hardman, K.D., Johnson, S., Pope, S., Dodd, S.W., Pantoliano, M.W., Milenic, D.E., Schlom, J. (1990) In vivo tumor targeting of a recombinant single-chain antigen-binding protein. *J Natl Cancer Inst* 82: 1191–1197.

Collier, R.J. (1988) Activity relationships in diptheria toxin and pseudomonas aeruginosa exotoxin A. In: *Immunotoxins*, Frankel, A.E., (ed.), Kulwer Academic Publishers, Boston/Dorcrecht/Lancaster. 25–35.

Colombatti, M., Greenfield, L., Youle, R. (1986) Cloned fragment of diphtheria toxin linked to T cell-specific antibody identifies regions of B chain active in cell entry. *J Biol Chem* 261: 3030–3035.

Crothers, D.M., Metzger, H. (1972) The influence of polyvalency on the binding properties of antibodies. *Immunochemistry* 9: 341–357.

D'Alessio, G., Donato, A.D., Parente, A., Piccoli, R. (1991) Seminal RNase: a unique member of the ribonuclease superfamily. *Trends Biochem Sci* 16: 104–106.

Darzynkiewicz, Z., Carter, S.P., Mikulski, S. M., Ardelt, W.J., Shogen, K. (1988) Cytostatic and cytotoxic effects of Pannon (P-30 protein) a novel anti-cancer agent. *Cell Tissue Kinet* 21: 169–182.

Deonarain, M.P., Epenetos, A.A. (1994) Targeting enzymes for cancer therapy:old enzymes in new roles. *Br J Cancer* 70: 786–794.

Deonarain, M.P., Epenetos, A.A. (1995) Construction, refolding and cytotoxicity of a single chain Fv-seminal ribonuclease fusion protein expressed in *Escherichia coli*. *Tumor Targeting* 1: 177–182.

Deonarain, M.P., Epenetos, A.A. (1998) Design, characterization and antitumor cytotoxicity of a panel of recombinant, mammalian ribonuclease-based immunotoxins. *Br J Cancer* 77: 537–546.

Desplancq, D., King, D.J., Lawson, A.D.G., Mountain, A. (1994) Multimerization behaviour of single chain Fv variants for

the tumour-binding antibody B72.3. *Protein Eng* 7: 1027–1033.
Doody, G.M., Dempsey, P.W., Fearon, D.T. (1996) Activation of B lymphocytes: integrating signals from CD19: CD22 and FcgRIIb1. *Curr Opin Immunol* 8: 378–382.
Eklund, J.W., Kuzel, T.M. (2005) Denileukin diftitox: a concise clinical review. *Expert Rev Anticancer Ther* 5: 33–38.
Endicott, J.A., Ling, V. (1989) The biochemistry of multidrug resistance. *Annu Rev Biochem* 58: 137–171.
Epenetos, A.A., Travers, P., Gatter, K.C., Oliver, R.D., Mason, D.Y., Bodmer, W.F. (1984) An immunohistological study of testicular germ cell tumours using two different monoclonal antibodies against placental alkaline phosphatase. *Br J Cancer* 49: 11–15.
Fidias, P., Grossbard, M., Lynch Jr., T.J. (2002) A phase II study of the immunotoxin N901-blocked ricin in small-cell lung cancer. *Clin Lung Cancer* 3: 219–222.
Filipovich, A., Vallera, D., Youle, R., Haake, R., Blazar, B., Arthur, D., Neville, D., Ramsay, N., McGlave, P., Kersey, J. (1987) Graft-versus-host-disease prevention in allogeneic bone marrow transplantation from histocompatible siblings. *Transplantation* 44: 62–69.
FitzGerald, D. (1996) Why toxins. *Semin Cancer Biol* 7: 87–95.
FitzGerald, D.J., Kreitman, R., Wilson, W., Squires, D., Pastan, I. (2004) Recombinant immunotoxins for treating cancer. *Int J Med Microbiol* 293: 577–582.
Flavell, D.J., Noss, A., Pulford, D.A.F., Ling, N., Flavell, S.U. (1997) Systemic therapy with 3BIT, a triple combination cocktail of anti-CD19: -CD22: and CD38-saporin immunotoxins, is curative of human B-cell lymphoma in severe combined immunodeficient mice. *Cancer Res* 57: 4824–4829.
Fork, J.M., Hait, W.N. (1990) Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmcol Rev* 42: 155–192.
Frankel, A.E., Kreitman, R.J., Sausville, E.A. (2000) Targeted toxins. *Clin Cancer Res* 6: 326–334.
Frankel, A.E., Powell, B.L., Lilly, M.B. (2002) Diphtheria toxin conjugate therapy of cancer. *Cancer Chemother Biol Response Modif* 20: 301–303.
Frankel, A.E., Neville, D.M., Bugge, T.A., Kreitman, R.J., Leppla, S.H. (2003) Immunotoxin therapy of hematologic malignancies. *Semin Oncol* 30: 545–547.
Friden, P.M., Walus, L.R., Musso, G.F., Taylor, M.A., Malfroy, B., Starzyk, R.M. (1991) Anti-transferrin receptor antibody and antibody-drug conjugates cross the blood-brain barrier. *Proc Natl Acad Sci USA* 88: 4771–4775.
Futami, J., Seno, M., Ueda, M., Tada, H., Yamada, H. (1999) Inhibition of cell growth by a fused protein of human ribonuclease 1 and human basic fibroblast growth factor. *Protein Eng* 12: 1013–1019.
Garland, L., Gitliz, B., Ebbinghaus, S., Pan, H., deHaan, H., Puri, R.K., VonHoff, D., Figlin, R. (2005) Phase 1 trial of intravenous IL-4 pseudomonas exotoxin protein (NBI-3001) in patients with advanced solid tumors that express the IL-4 receptor. *J Immunother* 28: 376–381.
Ghetie, M.A., Richardson, J., Tucker, T., Jones, D., Uhr, J.W., Vitetta, E.S. (1991) Antitumor activity of Fab′ and IgG-anti-CD22 immunotoxins in disseminated human B lymphoma grown in mice with severe combined immunodeficiency disease effect on tumor cells in extranodal sites. *Cancer Res* 51: 5876–5880.
Ghetie, M.A., Tucker, K., Richardson, J., Uhr, J.W., Vitetta, E.S. (1992) The anti-tumor activity of an anti-CD22 immunotoxin in SCID mice with disseminated Daudi lymphoma is enhanced by either an anti-CD19 antibody or an anti-CD19 immunotoxin. *Blood* 84: 702–707.
Gho, Y.S., Chae, C.B. (1999) Proliferation of LHRH-receptor positive human prostate and breast tumor cells. *Mol Cells* 9: 31–36.
Glockshuber, R., Malia, M., Pfitzinger, I., Pluckthun, A. (1990) A comparison of strategies to stabilize immunoglobulin Fv-fragments. *Biochemistry* 29: 1362–1367.
Glukhov, B.N., Jerusalimsky, A.P., Canter, V. M., Salganik, R.I. (1976) Ribonuclease treatment of tick-borne encephalitis. *Arch Neurol* 33: 598–603.

Gottstein, C., Winkler, U., Bohlen, H., Diehl, V., Engert, A. (1994) Immunotoxins: is there a clinical value? *Ann Oncol* 5: 97–103.

Grabarek, J., Ardelt, B., Du, L., Darzynkiewicz, Z. (2002) Activation of caspases and serine proteases during apoptosis induced by Onconase (Ranpirnase). *Exp Cell Res* 278: 61–71.

Greenfield, L., Johnson, V., Youle, R. (1987) Mutations in diphtheria toxin separate binding from entry and amplify immunotoxin selectivity. *Science* 238: 536–539.

Grever, M.R., Schepartz, S.A., Chabner, B.A. (1992) The National Cancer Institute: Cancer drug discovery and development program. *Semin Oncol* 19: 622–638.

Grossbard, M.L., Freedman, A.S., Ritz, J., Coral, F., Goldmacher, V.S., Eliseo, L., spector, N., Dear, K., Lambert, J.M., Blattner W.A. (1992) Serotherapy of B-cell neoplasms with anti-B4-blocked ricin: a phase I trial of daily bolus infusion. *Blood* 79: 576–585.

Halicka, D.H., Pozarowski, P., Ita, M., Ardelt, W., Mikulski, S.M., Shogen, K., Darzynkiewicz, Z. (2002) Enhancement of activation-induced apoptosis of lymphocytes by the cytotoxic ribonuclease Onconase (ranpirnase). *Int J Oncol* 21: 1245–1250.

Hamada, H., Tsuruo, T. (1986) Functional role for the 170 to 180-kDa glycoprotein specific to drug resistant tumor cells as revealed by monoclonal antibodies. *Proc Natl Acad Sci USA* 83: 7785–7789.

Hamann, P.R., Hinman, L.M., Hollander, I., Beyer, C.F., Lindh, D., Holcomb, R., Hallett, W., Tsou, H.R., Upeslacis, J., Shochat, D., Mountain, A., Flowers, D.A., Bernstein, I. (2002) Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. *Bioconjug Chem* 13: 47–58.

Hayashida, T., Ueda, M., Aiura, K., Tada, H., Onizuka, M., Yamada, M.S.H., Kitajima, M. (2005) Anti-angiogenic effect of an insertional fusion protein of human basic fibroblast growth factor and ribonuclease-1. *Protein Eng Des Sel* 18: 321–327.

Hertler, A.A., Schlossman, D.M., Borowicz, M.J., Poplack, D.G., Frankel, A.E. (1988) A phase I study of T101-ricin A chain immunotoxins in refractory chronic lymphocytic leukemia. *J Biol Res Mod* 7: 97–113.

Hertler, A.A., Schlossman, D.M., Borowicz, M.J., Laurent, G., Jansen, F.K., Schmidt, C., Frankel, A.E. (1989) An anti-CD5 immunotoxin for chronic lymphocytic leukemia: Enhancement of cytotoxicity with human serum albumin –monensin. *Int J Cancer* 43: 215–219.

Hexham, J.M., King, V., Dudas, D., Graff, P., Mahnke, M., Wang, Y.K., Goetschy, J.F., Plattner, D., Zurini, M., Bitsch, F., Lake, P., Digan, M.E. (2001) Optimization of the anti-(human CD3) immunotoxin DT389-scFv (UCHT1) N-terminal sequence to yield a homogeneous protein. *Biotechnol Appl Biochem* 34: 183–187.

Ho, M., Kreitman, R.J., Onda, M., Pastan, I. (2004) In vitro antibody evolution targeting germline hot spots to increase activity of an anti-CD22 immunotoxin. *J Biol Chem* 280: 607–617.

Holliger, P., Prospero, T., Winter, G. (1993) "Diabodies": Small bivalent and bispecific antibody fragments. *Proc Natl Acad Sci USA* 90: 6444–6448.

Hoogenboom, H.R. (2005) Selecting and screening antibody libraries. *Nat Biotechnol* 23: 1105–1116.

Hoogenboom, H.R., Raus, J.C.M., Volckaert, G. (1990) Cloning and expression of a chimeric antibody directed against the human transferrin receptor. *J Immunol* 144: 3211–3217.

Hu, V.W., Holmes, R.K. (1987) Single mutation in the A domain of diphtheria toxin results in a protein with altered membrane insertion behaviou. *Biochem Biophys Res Commun* 902: 24–30.

Huhn, M., Sasse, S., Tur, M.K., Matthey, B., Schinkothe, T., Rybak, S.M., Barth, S., Engert, A. (2001) Human angiogenin fused to human CD30 ligand (Ang-CD30L) exhibits specific cytotoxicity against CD30-positive lymphoma. *Cancer Res* 61: 8737–8742.

Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.S., Novotny, J., Margolies, M.N., Ridge, R.J., Bruccoleri, R.E., Haber, E., Crea, R., Oppermann, H. (1988) Protein engineering of antibody binding sites: Recovery of specific activity in an anti-

digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc Natl Acad Sci USA* 85: 5879–5883.

Huston, J.S., Mudgett-Hunter, M., Tai, M.S., McCartney, J., Warren, F., Haber, E., Oppermann, H. (1991) Protein engineering of single-chain Fv analogs and fusion proteins. *Methods Enzymol* 203: 46–88.

Huston, J.S., McCartney, J., Tai, M.S., Mottola-Hartshorn, C., Jin, D., Warren, F., Keck, P., Oppermann, H. (1993) Medical applications of single-chain antibodies. *Int Rev Immunol* 10: 195–217.

Huston, J.S., Margolies, M.N., Haber, E. (1996) Antibody binding sites. *Adv Protein Chem* 49: 329–450.

Iordanov, M.S., Ryabinina, O.P., Wong, J., Dinh, T.-H., Newton, D.L., Rybak, S.M., Magun, B.E. (2000) Molecular determinants of programmed cell death induced by the cytotoxic ribonuclease Onconase: Evidence for cytotoxic mechanisms different from inhibition of protein synthesis. *Cancer Res* 60: 1983–1994.

Jefferies, W.A., Brandon, M.R., Hunt, S.V., Williams, A.F., Gatter, K.C., Mason, D.Y. (1984) Transferrin receptors on endothelium of brain capillaries. *Nature* 312: 162–163.

Jinno, H., Ueda, M., Ozawa, S., Ikeda, T., Enomoto, K., Psarras, K., Kitajima, M., Yamada, H., Seno, M. (1996) Epidermal growth factor receptor-dependent cytotoxicity for human squamous carcinoma cell lines of a conjugate composed of human EGF and RNase 1. *Life Sci* 58: 1901–1908.

Jinno, H., Ueda, M., Ozawa, S., Ikeda, T., Kitajima, M., Maeda, T., Seno, M. (2002) The cytotoxicity of a conjugate composed of human epidermal growth factor and eosinophil cationic protein. *Anticancer Res* 22: 4141–4145.

Johnson, V.G., Wrobel, C., Wilson, D., Zovickian, J., Greenfield, L., Oldfield, E.H., Youle, R. (1989) Improved tumor-specific immunotoxins in the treatment of CNS and leptmeningeal neoplasia. *J Neurosurg* 70: 240–248.

Johnson, V.G., Youle, R.J. (1991) Intracellular routing and membrane translocation of diphtheria toxin and ricin. In: *Intracellular trafficking of proteins*, Steer, C.J., Hanover, J.A. (eds.), Cambridge University Press, Cambridge. 183–225.

Jolliffe, L.K. (1993) Humanized antibodies: enhancing therapeutic utility through antibody engineering. *Int Rev Immunol* 10: 241–250.

Kay, N.E., Bone, N.D., Lee, Y.K., Jelinek, D. F., Leland, P., Battle, T.E., Frank, D.A., Puri, R.K. (2005) A recombinant IL-4-*Pseudomonas* exotoxin inhibits protein sythesis and overcomes resistance in human CLL B cells. *Leuk Res* 9: 1009–1018.

Kelm, S., Pelz, A., Schauer, R., Filbin, M.T., Tang, S., deBellard, M.-E., Schnaar, R.L., Mahoney, J.A., Hartnell, A., Bradfield, P., Crocker, P.R. (1994) Sialoadhesin, myelin-associate glycoprotein and CD22 define a new family of sialic acid-dependent adhesion molecules of the immunoglobulin superfamily. *Curr Biol* 4: 965–972.

Khazaeli, M.B., Conry, R.M., LoBuglio, A.F. (1994) Human immune response to monoclonal antibodies. *J Immunother* 15: 42–52.

King, D.J., Turner, A., Farnsworth, A.P.H., Adair, J.R., Owens, R.J., Pedley, R.B., Baldock, D., Proudfoot, K.A., Lawson, A.D.G., Beeley, N.R.A., Millar, K., Millican, T.A., Boyce, B.A., Antoniw, P., Mountain, A., Begent, R.H.J., Shochat, D., Yarranton, G.T. (1994) Improved tumor targeting with chemically cross-linked recombinant antibody fragments. *Cancer Res* 54: 6176–6185.

Köhler, G., Milstein, C. (1975) Continous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.

Kortt, A.A., Lah, M., Oddie, G.W., Gruen, C. L., Burns, J.E., Pearce, L.A., Atwell, J.L., McCoy, A.J., Howlett, G.J., Metzger, D.W., Webster, R.G., Hudson, P.J. (1997) Single-chain Fv fragments of anti-neuraminidase antibody NC10 containing five- and ten-residue linkers form dimers and with zero-residue linker a trimer. *Protein Eng* 10: 423–433.

Krauss, J., Arndt, M.A.E., Vu, B.K., Newton, D.L., Rybak, S.M. (2005) Targeting malignant B-cell lymphoma with a humanized anti-CD22 scFv-angiogenin

immunoenzyme. *Br J Haematol* 128: 602–609.

Kreitman, R.J., Hansen, H.J., Jones, A.L., FitzGerald, D.J.P., Goldenberg, D.M., Pastan, I. (1993) *Pseudomonas* Exotoxin-based immunotoxins containing the antibody LL2 or LL2-Fab′ induce regression of subcutaneous human B-cell lymphoma in mice. *Cancer Res* 53: 819–825.

Kreitman, R.J., Wilson, W.H., White, J.D., Stetler-Stevenson, M., Jaffe, E.S., Giardina, S., Waldmann, T.A., Pastan, I. (2000) Phase I trial of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) in patients with hematologic malignancies. *J Clin Oncol* 18: 1622–1636.

Kreitman, R.J., Squires, D.R., Stetler-Stevenson, M., Noel, P., FitzGerald, D.J.P., Wilson, W.H., Pastan, I. (2005) Phase I trial of recombinant immunotoxin RFB4(dsFv)-PAE38 (BL22) in patients with B-cell malignancies. *J Clin Oncol* 23: 6719–6729.

Laccetti, P., Spalletti-Cernia, D., Portella, G., DeCorato, P., D'Alessio, G., Vecchio, G. (1994) Seminal RNase inhibits tumor growth and reduces the metastatic potential of Lewis lung carcinoma. *Cancer Res* 54: 4253–4256.

Lambert, J.M. (2005) Drug conjugated monoclonal antibodies for the treatment of cancer. *Curr Opin Pharmacol* 5: 543–549.

Lambert, J.M., McIntyre, G., Gauthier, M.N., Zullo, D., Rao, V., Steeves, R.M., Goldmacher, V.S., Blattler, W.A. (1991) The galactdose-binding sites of the cytotoxic lectin ricin can be chemically blocked in high yield with reactive ligands prepared by chyemical modificatgion of glycopeptides containing triantennaly N-linked oligosaccharides. *Biochemistry* 30: 3234–3247.

Laske, D., Oldfield, E., Youle, R. (1995) Immunotoxins for brain tumor therapy. In: *Proc Fourth Int Symp Immunotoxins*., Myrtle Beach.

Laske, D.W., Youle, R.J., Oldfield, E.H. (1997) Tumor regression with regional distribution of the targeted toxin TF-CRM107 in patients with malignant brain tumors. *Nat Med* 3: 1362–1368.

Ledoux, L. (1955a) Action of ribonuclease on certain ascites tumours. *Nature* 175: 258–259.

Ledoux, L. (1955b) Action of ribonuclease on two solid tumors *in vivo*. *Nature* 176: 36–37.

Lee, I., Lee, Y.H., Mikulski, S.M., Lee, J., Covone, K., Shogen, K. (2000) Tumoricidal effects of onconase on various tumors. *J Surg Oncol* 73: 164–171.

Lee, I., Lee, Y.H., Mikulski, S.M., Shogen, K. (2003) Effect of onconase +/− tamoxifen on ASPC-1 human pancreatic tumors in nude mice. *Adv Exp Med Biol* 530: 187–196.

Li, J.L., Shen, G.L., Ghetie, M.A., May, R.D., Till, M., Ghetie, V., Urh, J.W., Janossy, G., Thorpe, P.E., Amlot, P., Vitetta, E.S. (1989) The epitope specificity and tissue reactivity of four murine monoclonal anti-CD22 antibodies. *Cell Immunol* 118: 85–99.

Li, Q., Verschraegen, C.F., Mendoza, J., Hassan, R. (2004) Cytotoxic activity of the recombinant anti-mesothelin immunotoxin, SS1(dsFv)PE38: towards tumor cell oines extablished from asccites of patients with peritoneal mesothelioma. *Anticancer Res* 24: 1327–1336.

Lin, J.J., Newton, D.L., Mikulski, S.M., Kung, H.F., Youle, R.J., Rybak, S.M. (1994) Characterization of the mechanism of cellular and cell free protein synthesis inhibition by an anti-tumor ribonuclease. *Biochem Biophys Res Commun* 204: 156–162.

Lorenzo, C.D., Arciello, A., Cozzolino, R., Palmer, D.B., Laccedtti, P., Piccoli, R., D'Alessio, G. (2004) A fully human antitumor immunoRNase selective for ErbB-2-positive carcinomas. *Cancer Res* 64: 4870–4874.

MacDonald, G.C., Glover, N. (2005) Effective tumor targeting: Strategies for the delivery of armed antibodies. *Curr Opin Drug Dis Dev* 8: 177–183.

Mansfield, E., Pastan, I., FitzGerald, D.J. (1996) Characterization of RFB4-*Pseudomonas* exotoxin A immunotoxins tareted to CD22 on B-cell malignancies. *Bioconjugate Chem* 7: 557–563.

Mansfield, E., Amlot, P., Pastan, I., FitzGerald, D. (1997) Recombinant RFB4 immunotoxins exhibit potent cytotoxic activity for CD22-bearing cells and tumors. *Blood* 1997: 2020–2026.

Mathe, G., Loc, T., Bernard, J. (1958) Effet sur la leucemie 1210 de la souris d'un

combinasion par diazotation d'A methopterine et de -globulines de hamsters portteurs de cette leucemie par heterogreffe. *CR Acad Sci* 246: 1626–1628.

Matousek, J. (1973) The effect of bovine seminal ribonuclease (AS RNase) on cells of Crocker tumour in mice. *Experientia* 29: 858–859.

Messmann, R.A., Vitetta, E.S., Headlee, D., Senderowicz, A.M., Figg, W.D., Schindler, J., Michiel, D.F., Creekmore, S., Steinberg, S.M., Kohler, D., Jaffe, E.S., Stetler-Stevenson, M., Chen, H., Ghetie, V., Sausville, E.A. (2000) A phase 1 study of combination therapy with immunotoxins IgG-HD37-deglycosylated Ricin A chain (dgA) and IgG-RFB4-dgA (combotox) in patiens with refractory CD19 (+), CD22 (−) B cell lymphoma. *Clin Cancer Res* 6: 1302–1313.

Mikulski, S.M., Ardelt, W., Shogen, K., Bernstein, E.H., Menduke, H. (1990a) Striking increase of survival of mice bearing M109 Madison Carcinoma treated with a novel protein from amphibian embryos. *J Natl Cancer Inst* 82: 151–153.

Mikulski, S.M., Viera, A., Ardelt, W., Menduke, H., Shogen, K. (1990b) Tamoxifen and trifluoroperazine (Stelazine) potentiate cytostatic/cytotoxic effects of P-30 protein, a novel protein possessing anti-tumor activity. *Cell Tissue Kinet* 23: 237–246.

Mikulski, S.M., Viera, A., Darzynkiewicz, A., Shogen, K. (1992a) Synergism between a novel amphibian oocyte ribonuclease and lovastatin in inducing cytostatic and cytotoxic effects in human lung and pancreatic carcinoma cell lines. *Br J Cancer* 66: 304–310.

Mikulski, S.M., Viera, A., Shogen, K. (1992b) In vitro synergism between a novel amphibian oocytic ribonuclease (Onconase) and tamoxifen, lovastatin and cisplatin in human OVCAR-3 ovarian carcinoma cell line. *Int J Oncol* 1: 779–785.

Mikulski, S.M., Grossman, A.M., Carter, P.W., Shogen, K., Costanzi, J.J. (1993) Phase 1 human clinical trial of ONCONASE (P-30 protein) administered intravenously on a weekly schedule in cancer patients with solid tumors. *Int J Oncol* 3: 57–64.

Mikulski, S., Costanzi, J., Vogelzang, N., McCachres, S., Taub, R., Chun, H., Mittelman, A., Panella, T., Puccio, C., Fine, R., Shogen, K. (2002) Phase II trial of a single weekly intravenous dose of ranpirnase in patients with unresectable malignant mesothelioma. *J Clin Oncol* 20: 274–281.

Milenic, D.E. (2002) Monoclonal antibody-based therapy strategies: Providing options for the cancer patient. *Curr Pharm Des* 8: 1749–1764.

Milenic, D.E., Yokota, T., Filpula, D.R., Finkelman, M.A.J., Dodd, S.W., Wood, J.F., Whitlow, M., Snoy, P., Schlom, J. (1991) Construction, binding properties, metabolism, and tumor targeting of a single-chain Fv derived from pancarcinoma monoclonal antibody CC49. *Cancer Res* 51: 6363–6371.

Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., Gray-Goodrich, M., Campbell, H., Mayo, J., Boyd, M. (1991) Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst* 83: 757–766.

Moolten, F.L., Capparell, N.J., Zajdel, S.H., Cooperband, S.R. (1975) Antitumor effects of antibody-diphtheria toxin conjugates. II. Immunotherapy with conjugates directed against tumor antigens induced by simian virus 40. *J Natl Cancer Inst* 55: 473–477.

Nath, D., vanderMerwe, P.A., Kelm, S., Bradfield, P., Crocker, P.R. (1995) The amino-terminal immunoglobulin-like domain of sialoadhesin contains the sialic acid binding site. *J Biol Chem* 270: 26184–26191.

Neville, D.M., Scharff, J., Hu, H.Z., Rigaut, K., Shiloach, J., Slingerland, W., Jonker, M. (1996) A new reagent for the induction of T-cell depletion, anti-CD3-CRM9. *J Immunother Emphasis Tumor Immunol* 19: 85–92.

Newton, D.L., Ilercil, O., Laske, D.W., Oldfield, E., Rybak, S.M., Youle, R.J. (1992) Cytotoxic ribonuclease chimeras: Targeted tumoricidal activity *in vitro* and *in vivo*. *J Biol Chem* 267: 19572–19578.

Newton, D.L., Nicholls, P.J., Rybak, S.M., Youle, R.J. (1994) Expression and characterization of recombinant human

eosinophil-derived neurotoxin and eosinophil-derived neurotoxin-anti-transferrin receptor sFv. *J Biol Chem* 269: 26739–26745.

Newton, D.L., Pearson, J.W., Xue, Y., Smith, M.R., Fogler, W.E., Mikulski, S.M., Alvord, W.G., Kung, H.F., Longo, D.L., Rybak, S. M. (1996a) Anti-tumor ribonuclease combined with or conjugated to monoclonal antibody MRK16: overcomes multidrug resistance to vincristine in vitro and in vivo. *Int J Oncol* 8: 1095–1104.

Newton, D.L., Xue, Y., Olson, K.A., Fett, J. W., Rybak, S.M. (1996b) Angiogenin single-chain immunofusions: Influence of peptide linkers and spacers between fusion protein domains. *Biochemistry* 35: 545–553.

Newton, D.L., Hansen, H.J., Mikulski, S.M., Goldenberg, D.M., Rybak, S.M. (2001) Potent and specific antitumor activity of an anti-CD22-targeted cytotoxic ribonuclease: potential for the treatment of non-Hodgkin lymphoma. *Blood* 97: 528–535.

O'Toole, J.E., Esseltine, D., Lynch, T.J., Lambert, J.M., Grossbard, M.L. (1998) Clinical trials with blocked ricin immunotoxins. *Curr Top Microbiol Immunol* 234: 35–56.

Olsnes, S. (2004) The history of ricin, abrin and related toxins. *Toxicon* 44: 361–370.

Pai, L.H., Wittes, R., Setser, A., Willingham, M.C., Pastan, I. (1996) Treatment of advanced solid tumors with immunotoxin LMB-1: an antibody linked to *Pseudomonas* exotoxin. *Nat Med* 2: 350–353.

Pastan, I. (2003) Immunotoxins containing *Pseudomonas* exotoxin A: a short history. *Cancer Immunol Immunother* 52: 328–341.

Pastan, I., Willingham, M., FitzGerald, D. (1986) Immunotoxins. *Cell* 47: 641–648.

Paull, K.D., Shoemaker, R.H., Hodes, L. et al. (1989) Display and analysis of patterns of differential actitivy of drugs against human tumor cell lines: development of a mean graph and COMPARE algorithm. *J Natl Cancer Inst* 81: 1088–1092.

Pawlak-Byczkowska, E.J., Hansen, H.J., Dion, A.S., Goldenberg, D.M. (1989) Two new monoclonal antibodies, EPB-1 and EPB-2 reactive with human lymphoma. *Cancer Res* 49: 4568–4577.

Payne, G. (2003) Progress in immunoconjugate cancer therapeutics. *Cancer Cell* 3: 207–212.

Posey, J.A., Khazaelu, M.B., Bookman, M.A., Nowrouzi, A., Grizzle, W.E., thornton, J., Carey, D.E., Lorenz, J.M., Sing, A.P., Siegall, C.B., LoBuglio, A.F., Saleh, M.N. (2002) A phase I trial of the single-chain immunotoxin SGN-10 (BR96 sFv-PE40) in patients with advanced solid tumors. *Clin Cancer Res* 10: 3092–3099.

Pouckova, P., Soucek, J., Jelinek, J., Zadinova, M., Hlouskova, D., Polivkova, J., Navratil, L., Cinatl, J., Matousek, J. (1998) Antitumor action of bovine seminal ribonuclease. Cytostatic effect on human melanoma and mouse seminoma. *Neoplasma* 45: 30–34.

Psarras, K., Ueda, M., Yamamura, T., Ozawa, S., Kitajima, M., Aiso, S., Komatsu, S., Seno, M. (1998) Human pancreatic RNase1-human epidermal growth factor fusion: an entirely human immunotoxin analog with cytotoxic properties against squamous cell carcinomas. *Protein Eng* 11: 1285–1292.

Psarras, K., Ueda, M., Tanabe, M., Kitajima, M., Aiso, A., Komatsu, S., Seno, M. (2000) Targeting activated lymphocytes with an entirely human immunotoxin analogue: human pancreatic RNase1-human IL-2 fusion. *Cytokine* 12: 786–790.

Raso, V., Basala, M. (1984) A highly cytotoxic human transferrin-ricin A chain conjugate used to select receptor-modified cells. *J Biol Chem* 259: 1143–1149.

Reiter, Y., Pastan, I. (1996) Antibody engineering of recombinant Fv immunotoxins for improved targeting of cancer: Disulfide-stabilized Fv immunotoxins. *Clin Cancer Res* 2: 245–252.

Rodrigues, M.L., Presta, L.G., Kotts, C.E., Wirth, C., Mordenti, J., Osaka, G., Wong, W.L.T., Nuijens, A., Blackburn, B., Carter, P. (1995) Development of a humanized disulfide-stabilized anti-p185 HER2 Fv-β-lactamase fusion protein for activation of a cephalosporin doxorubicin prodrug. *Cancer Res* 55: 63–70.

Rybak, S.M., Newton, D.L. (1999) Natural and engineered cytotoxic ribonucleases: Therapeutic potential. *Exp Cell Res* 253: 325–335.

Rybak, S.M., Newton, D.L. (1999a) Immunoezymes. In: Antibody Fusion Proteins. eds. Chamow, S.M., Ashkenazi,

A., John Wiley & Sons, New York, N.Y.: 53–110.
Rybak, S.M., Youle, R.J. (1991) Clinical use of immunotoxins: monoclonal antibodies conjugated to protein toxins. *Immunol Allergy Clin N Am* 11: 359–380.
Rybak, S.M., Saxena, S.K., Ackerman, E.J., Youle, R.J. (1991) Cytotoxic potential of ribonuclease and ribonuclease hybrid proteins. *J Biol Chem* 266: 21202–21207.
Rybak, S.M., Hoogenboom, H.R., Meade, H.M., Raus, J.C., Schwartz, D., Youle, R.J. (1992) Humanization of immuntoxins. *Proc Natl Acad Sci USA* 89: 3165–3169.
Rybak, S.M., Newton, D.L., Mikulski, S.M., Viera, A., Youle, R.J. (1993) Cytotoxic Onconase and ribonuclease A chimeras: Comparison and *in vitro* characterization. *Drug Del* 1: 3–10.
Rybak, S.M., Pearson, J.W., Fogler, W.F., Volker, K., Spence, S.E., Newton, D.L., Mikulski, S.M., Ardelt, W., Riggs, C.W., Kung, H.F., Longo, D.L. (1996) Enhancement of vincristine cytotoxicity in drug-resistant cells by simultaneous treatment with Onconase, an antitumor ribonuclease. *J Natl Cancer Inst* 88: 747–753.
Salvatore, G., Beers, R., Kreitman, R.J., Pastan, I. (2002) Improved cytotoxic activity dtowards cell lines and fresh leukemia cells of a mutant anti-CD22 immunotoxin obtained by antibody phage display. *Clin Cancer Res* 8: 942–944.
Sausville, E.A., Headlee, D., Stetler-Stevenson, M., Jaffe, E.S., Solomon, D., Figg, W.D., Herdt, J., Kopp, W.C., Rager, H., Steinberg, S.M., Gethie, V., Shindler, J., Uhr, J., Wittes, R.E., Vitetta, E.S. (1995) Continuous infusion of the anti-CD22 immunotoxin IgG-RFB4-SMPT-dgA in patients with B-cell lymphoma: a phase I study. *Blood* 85: 3457–3465.
Savage, P., Rowlinson-Busza, G., Verhoeyen, M., Spooner, R.A., So, A., Windust, J., Davis, P.J., Epenetos, A.A. (1993) Construction, characterisation and kinetics of a single chain antibody recognising the tumour-associated antigen placental alkaline phosphatase. *Br J Cancer* 68: 738–742.
Saxena, S., Sirdeshmukh, R., Ardelt, W., Mikulski, S.M., Shogen, k., Youle, R.J. (2002) Entry into cells and selective degradation of tRNAs by a cytotoxic member of the RNase A family. *J Biol Chem* 277: 15142–15146.
Saxena, S.K., Shogen, K., Ardelt, W. (2003) Onconase and its therapeutic potential. *Lab Med* 34: 380–387.
Senter, P.D., Saulnier, M.G., Schreiber, G.J., Hirschberg, D.L., Brown, J.P., Hellstrom, I., Hellstrom, K.E. (1988) Anti-tumor effects of antibody-alkaline phosphatase conjugates in combination with etoposide phosphate. *Proc Natl Acad Sci USA* 85: 4842–4846.
Shan, D., Press, O.W. (1995) Constitutive endocytosis and degradation of CD22 by human B cells. *J Immunol* 154: 4466–4475.
Shih, L.B., Lu, H.H.A., Xuan, H., Goldenberg, D.M. (1994) Internalization and intracellular processing of an anti-B-cell lymphoma monoclonal antibody, LL2. *Int J Cancer* 56: 538–545.
Smallshaw, J.E., Ghetie, V., Rizo, J., Fulmer, J.R., Trahan, L.L., Ghetie, M.A., Vitetta, E.S. (2003) Genetic engineering of an immunotoxin to eliminate pulmonary vascular leak. *Nat Biotechnol* 21: 387–391.
Soler-Rodriguez, A.M., Ghetie, M.-A., Oppenheimer-Marks, N., Uhr, J.W., Vitetta, E.S. (1993) Ricin A-chain and ricin A-chain immunotoxins rqpidly damage human endothelial cells: Implications for vascular leak syndrome. *Exp Cell Res* 206: 227–234.
Solomon, S.R., Mielke, S., Savani, B.N., Montero, A., Wisch, L., Childs, R., Hensel, N., Schindler, J., Ghetie, V., Leitman, S.F., Mai, T., Carter, C.S., Kurlander, R., Read, E.J., Vitetta, E.S., Barrett, A.J. (2005) Selective depletionn of allorective donor lymphocytes-a novel method to reduce the severity of graft-versus-host disease in older patients undergoing matched sibling donor stem cell transplantation. *Blood* 106: 1123–1129.
Soucek, J., Pouckova, P., Matousek, J., Stockbauer, P., Dostal, J., Zadinova, M. (1996) Antitumor action of bovine seminal ribonuclease. *Neoplasma* 43: 335–340.
Stein, R., Belisle, E., Hansen, H.J., Goldenberg, D.M. (1993) Epitope specificity of the anti-B-cell lymphoma monoclonal antibody, LL2. *Cancer Immunol Immunother* 37: 293–298.

Stephens, S., Emtage, S., Vetterlein, O., Chaplin, L., Bebbington, C., Nesbitt, A., Sopwith, M., Athwal, D., Novak, C., Bodmer, M. (1995) Comprehensive pharmacokinetics of a humanized antibody and analysis of residual anti-idiotypic responses. *Immunology* 85: 668–674.

Stirpe, F. (2004) Ribosome-inactivating proteins. *Toxicon* 44: 371–383.

Suwa, T., Ueda, M., Jinno, H., Ozawa, S., Kitagawa, Y., Ando, N., Kitajima, M. (1999) Epidermal growth factor receptor-dependent cytotoxic effect of anti-EGFR antibody-ribonuclease conjugate on human cancer cells. *Anticancer Res* 19: 4161–4165.

Suzuki, M., Saxena, S.K., Boix, E., Prill, R.J., Vasandani, V.M., Ladner, J.E., Sung, C., Youle, R.J. (1999) Engineering receptor-mediated cytotoxicity into human ribonucleases by steric blockade of inhibitor interaction. *Nat Biotechnol* 17: 265–270.

Taetle, R., Castagnola, J., Mendelsohn, J. (1986) Mechanisms of growth inhibition by anti-transferrin receptor monoclonal antibodies. *Cancer Res* 46: 1759–1763.

Tanaka, M., Ozawa, S., Ando, N., Kitagawa, Y., Otani, Y., Seno, M., Futami, J., Ueda, M., Kitajima, M. (1998) Inhibitory effects of recombinant human RNase-FGF fused protein on angiogenesis and tumor growth. *Proc Am Assoc Cancer Res* 40: 68.

Tasi, S.Y., Ardelt, B., Hsieh, T., Darzynkiewicz, Z., Shogen, K., Wu, J.M. (2004) Treatment of Jurkat acute T-lymphocytic leukemia cells by Onconase is accompanied by an altered nucleocytoplasmic distribution and reduced expression of transcription factor NF-kB. *Int J Oncol* 25: 1745–1752.

Thompson, J., Hu, H., Scharff, J., Neville, D. M. Jr. (1995) An anti-CD3 single-chain immunotoxin with a truncated diphtheria toxin avoids inhibition by pre-existing antibodies in human blood. *J Biol Chem* 270: 28037–28041.

Thompson, J., Stavrou, S., Weetall, M., Hexham, J.M., Digan, M.E., Wang, Z., Woo, J.H., Yu, Y., A Mathias, Liu, Y., Ma, S., Gordienko, I., Lake, P., Neville, D.M. Jr. (2001) Improved binding of a bivalent single-chain immuntoxin results in increased efficacy for in vivo T-cell depletion. *Protein Eng* 14: 1035–1041.

Thrush, G.R., lark, L.R., Clinchy, B.C., Vitetta, E.S. (1996) Immunotoxins: an update. *Annu Rev Immunol* 14: 4971.

Travers, P., Bodmer, W. (1984) Preparation and characterization of monoclonal antibodies against placental alkaline phosphatase and other human trophoblast-associated determinants. *Int J Cancer* 33: 633–641.

Trowbridge, I.S. (1988) Transferrin receptor as a potential therapeutic agent. *Prog Allergy* 45: 121–146.

Trowbridge, I.S., Domingo, D.L. (1981) Anti-transferrin receptor monoclonal antibody and toxin-antibody conjugates affect growth of human tumor cells. *Nature* 294: 171–173.

Trowbridge, I.S., Omary, M.B. (1981) Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. *Proc Natl Acad Sci USA* 78: 3039–3043.

Tsimberidou, A.M., Giles, F.J., Kantarjian, H.M., Keating, M.J., O'Brien, S.M. (2003) Anti-B4 blocked ricin post chemotherapy in patients with chronic lymphocytic leukemia-long term follow-up of a monoclonal antibody-based approach to residual disease. *Leukemia Lymphoma* 44: 1719–1725.

Vallera, D.A., Myers, D.E. (1988) Immunotoxins containing ricin. In: *Immunotoxins*, Frankel, A.E. (ed.), Kluwer Academic Publishers, Boston, Dordrecht, Lancaster. 141–159.

Vallera, D.A., Todhunter, D., Kuroki, D.W., Shu, Y., Sicheneder, A., Panoskaltix-Mortari, A., Vallera, V.D., Chen, H. (2005) Molecular modification of a recombinant, bivalent anti-human CD3 immunotoxin (Bic3) results in reduced in vivo toxicity in mice. *Leuk Res* 29: 331–341.

van Horssen, P., Preijers, F., Voosterhout, Y., Witte, T.D. (1996) Highly potent CD22-recombinant ricin A results in complete cure of disseminated malignant B-cell xenografts in SCID mice but fails to cure solid xenografts in nude mice. *Int J Cancer* 68: 378–383.

van Horssen, P.J., van Oosterhout, Y.V.J.M., Evers, S., Backus, H.H.J., van Oijen, M. G.C.T., Bongaerts, R., deWitte, T. (1999) Influence of cytotoxicity enhancers in combination with human serum on the

activity of CD22-recombinant ricin A against B cell lines, chronic and acute lymphocytic leukemia cells. *Leukemia* 13: 241–249.

Vasandani, V.M., Castelli, J.C., Hott, J.S., Saxena, S., Mikulski, S.M., Youle, R.J. (1999) Interferon enhances the activity of the anticancer ribonuclease, Onconase. *J Interferon Cytok Res* 19: 447–454.

Vitetta, E.S., Fulton, R.J., May, R.D., Till, M., Uhr, J.W. (1987) Redesigning nature's poisons to create anti-tumor reagents. *Science* 238: 1098–1104.

Vogelzang, N., Taub, R., Shin, D., Costanzi, J., Pass, H., Gutheil, J., Georgiadis, M., McAndrew, P., Kelly, K., Chun, H., Mittelman, A., McCachren, S., Shogen, K., Mikulski, S. (2000) Phase III randomized trial of Ranpirnase (Onc) vs doxorubicin (DOX) in patients with unresectable malignant mesothelioma: analysis of survival. *Proc Am Soc Clin Oncol* 19: 577a.

Vogelzang, N., Aklilu, M., Stadler, W.M., Dumas, M., Mikulski, S. (2001) A phase II trial of weekly intravenous ranpirnase (Onconase), a novel ribonuclease in patients with metastatic kidney cancer. *Investig New Drugs* 19: 255–260.

Wa, D. (2004) Tumor-activated prodrugs – a new approach to cancer therapy. *Cancer Invest* 22: 604–619.

Weaver, M., Laske, D.W. (2003) Transferrin receptor ligand-targeted toxin conjugate (TF-CRM107) for therapy of malignant gliomas. *J Neurooncol* 65: 3–13.

Whitlow, M., Bell, B.A., Feng, S.L., Filpula, D., Hardman, K.D., Hubert, S.L., Rollence, M.L., Wood, J.F., Schott, M.E., Milenic, D.E., Yokota, T., Schlom, J. (1993) An improved linker for single-chain FV with reduced aggregation and enhanced proteolytic stability. *Protein Eng* 6: 989–995.

Williams, D.P., Snider, C.E., Strom, T.B., Murphy, J.R. (1990) Structure/function analysis of interleukin-2-toxin (DAB486-IL-2. Fragment B sequences required for the delivery of fragment A to the cytosol of target cells. *J Biol Chem* 265: 11885–11889.

Woo, J.H., Liu, Y.Y., Neville, D.M. Jr. (2006) Minimization of aggregation of secreted bivalent anti-human T cell immunotoxin in *Pichia pastoris* bioreactor culture by optimizing culture conditions for protein secretion. *J Biotechnol* 121: 75–85.

Wu, A.M., Senter, P.D. (2005) Arming antibodies: Prospects and challenges for immunoconjugates. *Nat Biotechnol* 23: 1137–1146.

Wu, Y.N., Mikulski, S.M., Ardelt, W., Rybak, S.M., Youle, R.J. (1993) Cytotoxic ribonuclease: A study of the mechanism of Onconase cytotoxicity. *J Biol Chem* 268: 10686–10693.

Wu, Y.N., Saxena, S.K., Ardelt, W., Gadina, M., Mikulski, S.M., DeLorenzo, C., D'Alessio, G., Youle, R.J. (1995) A study of the intracellular routing of cytotoxic ribonucleases. *J Biol Chem* 270: 17476–17481.

Yokota, T., Milenic, D.E., Whitlow, M., Schlom, J. (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res* 52: 3402–3408.

Yoon, J.M., Han, S.H., Kown, O.B., Kim, S. H., Park, M.H., Kim, B.K. (1999) Cloning and cytotoxicity of fusion proteins of EGF and angiogenin. *Life Sci* 64: 1435–1445.

Youle, R.J., Uckun, F.M., Vallera, D.A., Colombatti, M. (1986) Immunotoxins show rapid entry of diphtheria toxin but not ricin via the T3 antigen. *J Immunol* 136: 93–98.

Zewe, M., Rybak, S.M., Dubel, S., Coy, J.F., Welschof, M., Newton, D.L., Little, M. (1997) Cloning and cytotoxicity of a human pancreatic RNase immunofusion. *Immunotechnology* 3: 127–136.

Part IV
Emerging Concepts

4
Automation of Selection and Engineering

Zoltán Konthur

4.1
Introduction

The field of recombinant antibody technology first arrived with the development of two major milestones in molecular biology: the development of murine hybridoma technology (Köhler and Milstein 1975) and the discovery and ease of use of the polymerase chain reaction (PCR) to multiply DNA *in vitro* by Mullis and coworkers (Saiki et al. 1985; Mullis et al. 1986). Together these technologies allowed, in combination with antibody sequence information, the construction of many different recombinant antibody fragments with different specificities and a wide range of applications, revolutionizing diagnostic and therapeutic applications in medicine (Little et al. 2000). For therapeutic applications, however, the initial use of murine antibodies was greatly hampered due to difficulties in obtaining murine monoclonal antibodies for cross-species conserved antigens, a lack of biological function of murine effector domains in humans, and the immune intolerance provoked by mouse antibody sequences and glycosylation patterns during treatment – the so-called human anti-mouse antibody (HAMA) response (see Vol. I, Chap. 1 for more details). The generation of fully human antibodies was therefore desirable and technologies were developed, which meanwhile have become the standard for therapeutic antibodies (Weiner 2006).

4.1.1
Emergence of Antibody Phage Display

To overcome these limitations, in the early 1990s, the merger of recombinant antibody technology with bacteriophage surface display (Smith 1985) – yet another milestone development – resulted in a completely different strategy for the development of specific antibodies. For the first time, combinatorial human antibody libraries were generated (McCafferty et al. 1990; Barbas et al. 1991; Breitling et al. 1991; Clackson et al. 1991; Hoogenboom et al. 1991) allowing the isolation and production of functional antibody fragments based solely on the binding reaction

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of the antibody, hence uncoupling the generation of antibodies from an immune response (Winter and Milstein 1991). The strategy for generating combinatorial phage display libraries as well as the general enrichment process called biopanning is covered in depth in Vol. I, Chap. 3.

4.1.2 Phage Display and Automation

In the last 15 years, phage display has become the most frequently and most successfully employed *in vitro* selection system for the generation of human antibodies geared towards therapeutic applications. Hence, phage display is exploited commercially by a number of companies (Table 4.1) with many antibodies being in clinical trials. Despite this technology's high-throughput potential being regarded as low (Li 2000), the growing interest and demand for therapeutic antibodies has initiated increased streamlining processes of all aspects of phage display and many technological improvements have been achieved within the last 5 years.

In this chapter I describe some thoughts and considerations during setting up a (semi-)automated selection pipeline for the generation, screening, and downstream evaluation of monoclonal antibodies. While the major focus of the chapter is on using phage display for the *in vitro* selection of antibodies rather than conventional immunization and hybridoma-based monoclonal antibody techniques,

Table 4.1 Non-exclusive collection of companies using phage display for generating human antibodies.

Company name	*Antibody format for screening*	*Homepage*
Affitech	scFv	http://www.affitech.com
Antibodies by Design (Div. of Morphosys)	scFv	http://www.antibodiesbydesign.com
BioInvent	scFv	http://www.bioinvent.com
Cambridge Antibody Technology	scFv	http://www.cambridgeantibody.com
Crucell	scFv	http://www.crucell.com
Domantis	single domain	http://www.domantis.com
Dyax	Fab	http://www.dyax.com
Genentech	scFv	http://www.gene.com
MorphoSys	scFv, Fab	http://www.morphosys.com
Symphogen	scFv	http://www.symphogen.com
Wyeth Pharmaceuticals[a]	scFv	http://www.wyeth.com
Xerion Pharmaceuticals[a]	scFv	http://www.xerion-pharma.com
Xoma[a]	scFv, Fab	http://www.xoma.com

a Companies licensing antibody libraries from others in this list.

many of the considerations in respect to screening applications – especially for downstream evaluation – will apply to both.

4.2 General Considerations for the Automation of Antibody Generation

At the beginning of a project in which a pipeline for the generation and screening of antibodies is to be established, there are many technologies and automation possibilities to investigate. One of the major decisions to take, however, is to consider whether the pipeline is going to follow the unit-automation design strategy or should rather be a fully automated system. Unit automation refers to systems in which human intervention is necessary and the individual steps in a process pipeline are partially automated independent of each other (Menke 2002), for example with individual workstations that pipette PCR reactions together. In unit automation, individual processes rather than complex assays are automated and many commercially available workstations and individual automation solutions are available "off the shelf" (Table 4.2).

Fully automated systems refer to pipelines in which all steps of the process or an assay are covered without human intervention, for example, where robotic arms and conveyer belt-like systems are used to move plates from integrated and automated equipment under the control of a scheduling software monitoring and synchronizing all processes (Cohen and Trinka 2002). The benefits and drawbacks of these automatic systems are clear. They can work 24 hours a day, through 7 days a week, and no mistakes due to human handling errors can happen. However, they are very expensive, take a much longer time to develop, and allow little variation once installed without the need for a complete redesign.

In principle, almost everything can be automated; it is primarily a matter of financial resources that limits the extent of automation. In practice, the degree of laboratory automation is dependent upon the scope and timeline of the project pursued (Hamilton 2002).

Before the start of the design process a number of decisions have to be considered and a "project inventory" has to be compiled, dealing non-exclusively with the following, rather generalized question catalogue:

- What is the scientific aim of the project?
- What is the time-scale of the project?
- What type of throughput is aimed for?
- What is the starting point?
- Are targets available or easily accessible?
- What types of applications are aimed for?
- How many individual steps and assays are needed?
- Does one need to have a fully automated pipeline or does one automate purely for the sake of automation?
- Does the pipeline justify the costs?

Table 4.2 A collection of manufacturers providing automation technology and equipment.

Company name	*Equipment*	*Homepage*
Abgene	Microplate handling and labeling	www.abgene.com
Affymetrix Inc.	Microarray and detection equipment	www.affymetrix.com
Agilent Technologies Inc.	Bioanalyzers, microfluidic devices	www.agilent.com
Applied Biosystems	Microplate automation, liquid handling, detection, workstations, LIMS	www.appliedbiosystems.com
The Automation Partnership Ltd.	Sample storage	www.autoprt.co.uk
Beckman Coulter	Liquid handling, detection systems, workstations, laboratory automation	www.beckman-coulter.com
BIAcore	Labor-free detection systems	www.biacore.com
Bioveris Corporated	Microplate detection systems, magnetic bead-based technology	www.bioveris.com
Brooks Automation Inc.	Laboratory automation, sample handling	www.automationonline.com
Caliper Life Sciences	Workstations, liquid handling, screening systems	www.caliperls.com
Corning Incorporated	Label-free microplate detection	www.corning.com
CyBio AG	Liquid handling, detection, software and system integration	www.cybio-ag.com
Dmetrix, Inc.	Microscopy imaging instrumentation	www.dmetrix.net
GE Healthcare	Chromatography, microplate detection systems	www.gehealthcare.com
Genetix Ltd	Arraying technology	www.genetx.com
Genomic Solutions Inc.	Liquid handling, arraying technology	www.genomicsolutions.com
GeSiM mbH	Noncontact arraying	www.gesim.de
Luminex	Bead-based assay system	www.luminexcorp.com
Molecular Devices	Microplate readers, fluorometric imaging, microarray analysis, liquid handling, cellular imaging	www.moleculardevices.com
PerkinElmer Life and Analytical Sciences, Inc.	Plate readers and imagers, LIMS, workstations, laboratory automation	www.perkinelmer.com
QIAGEN GmbH	Liquid handling, workstations	www.qiagen.com
Scienion AG	Noncontact arraying	www.scienion.com
SSI Robotics	Labeling, storage systems, microplate handling, scheduling software	www.ssirobotics.com
Tecan	Liquid handling, microarray and microplate detection	www.tecan.com
Thermo Electron Corporation	Microtiter plate equipment, washers, liquid handling, laboratory robotics, workstations, LIMS, magnetic bead automation	www.thermo.com
Titertek	Microplate handling and reading, liquid handling, integrated systems	www.titertek.com
Tomtec	Microplate washer and sealers, liquid handling, workstations	www.tomtec.com
Zinsser Analytic	Liquid handling, workstations	www.zinsser-analytic.com

LIMS, Laboratory Information Management System.

- What is the overall cost per individual selection or individual antibody?
- Is it possible to partially use high-throughput robotic technology already installed for other purposes?
- Survey of accessible equipment and resources.

4.3 Development of an Antibody Generation Pipeline

The remainder of this chapter deals mainly with the approach we have taken at the Max Planck Institute for Molecular Genetics within the "Antibody Factory" (http://www.antibody-factory.de), a German National Genome Research Network NGFN initiative (http://www.ngfn.de). Our major goal in this project is to develop a streamlined process, which will eventually allow antibody selections against up to 500 target molecules per year, preferably with multiple antibody libraries and multiple selections for each target.

The process follows the unit-automation design strategy, where all procedures involved are handled as individual modules, finally being placed in a "virtual conveyer belt" type pipeline (Fig. 4.1). This approach allows individual modules of the system to be modified easily as well as allowing the pipline to be completely changed or extra modules to be added at later stages.

4.3.1 Selection Targets

Depending on the type of project pursued, there are several different stages at which the antibody generation pipeline can be entered. While for many therapeutic applications the number of targets is fairly limited and target accessibility, production, and cost are not issues, in projects aiming at the establishment of large antibody resources for proteomic applications it is quite the opposite.

4.3.1.1 Target Availability

Target availability in suitable format, amounts, and the production costs for generating the targets is pivotal. Therefore, large-scale projects often rely primarily on earlier established clone resources, for example for structural genomics (Bussow et al. 2004). Such clone collections are also available at the German Resource Center for Genomic Research in Berlin, (http://www.rzpd.de), or from companies such as Invitrogen (http://www.invitrogen.com). The collections range from full-length ORF (open-reading frame) Gateway or Creator clones, through N-terminally tagged cDNA expressions clones representing a smaller proportion of full-length ORFs and mainly partial (C-terminal) gene constructs, to non-expressing full-length clones such as the clones from the I.M.A.G.E. consortium (http://image.dbnl.gov) which in turn can be used as a template for subcloning or PCR amplification of the whole protein or parts thereof, such as

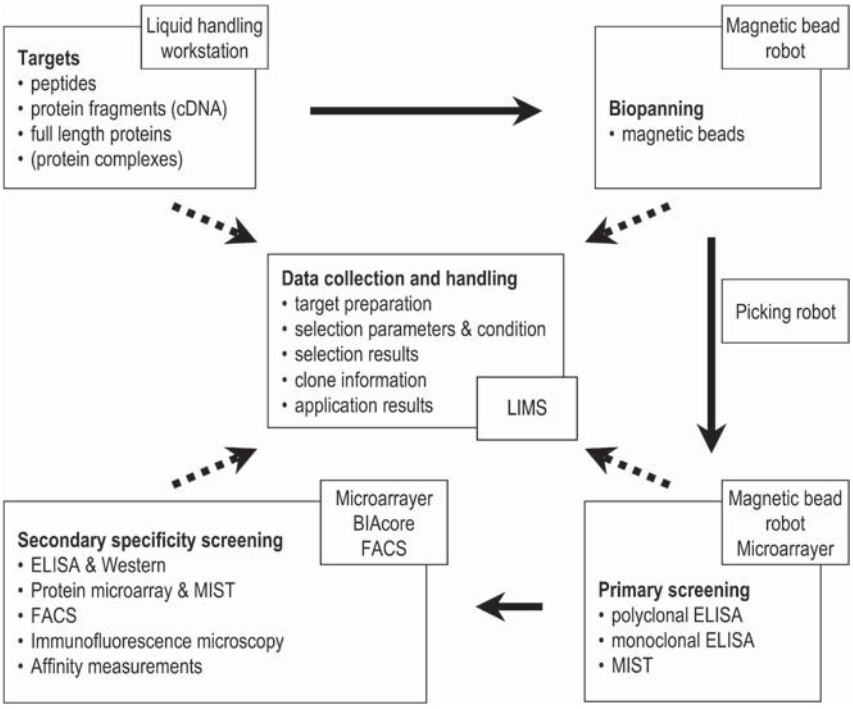

Fig. 4.1 Schematic representation of the antibody selection pipeline established at the Max Planck Institute for Molecular Genetics, Berlin. Unit automation preserves a highly open architecture allowing the individual modules and the pipeline to be easily modified and extended with novel applications. *MIST, Multiple Spotting Technique.

specific domains of interest. In our laboratory, we are mainly using an arrayed human fetal brain cDNA expression library that was established in-house as a clone resource for protein expression (Bussow et al. 2000, 2004). In addition, targets of special interest are also cloned or shuttled into suitable expression vectors from Gateway Entry clones by recombination.

4.3.1.2 Expression Systems

Currently, the main expression system in use for low-cost protein generation of full-length or partial protein constructs is to use *Escherichia coli* as the expression host. However, target proteins can also be obtained in alternative expression systems, ranging from *in vitro* coupled transcription–translation systems (Kigawa et al. 2004), over *Saccharomyces cerevisiae* (Holz and Lang 2004) or *Pichia pastoris* (Boettner et al. 2002) to various cell culture formats, e.g. insect cells (Albala et al. 2000). In addition, peptides derived from the target proteins can be used for the generation of antibodies, similar to immunization strategies (see other chapter in the book).

Since the selection system we use in our pipeline can handle up to 96 targets at a time, we are currently using only proteins expressed in *E. coli* BL21 strains with helper plasmids providing rare tRNA codons for enhanced expression of human proteins. The proteins or fragments thereof are cloned in vectors that possess N-terminal affinity tags, such as the His6, biotin (AVI tag), or GST tag usable for protein purification and immobilization. For our purposes, the biotin tag is of major importance as it allows the directed immobilization of the selection target onto our preferred selection matrix, streptavidin-coated magnetic beads (see Section 4.3.2). The tag consists of a 14-amino-acid sequence representing an optimized recognition site for the biotin holoenzyme synthetase BirA, which catalyzes the transfer of biotin to a specific lysine residue in this sequence (Schatz 1993; Beckett et al. 1999). Hence, our vectors have an N-terminal His6 and biotin tag, for efficient purification and immobilization. Since the BirA enzyme is *E. coli* specific, this tag cannot be used to produce biotinylated proteins in eukaryotic expressions systems, unless the host is modified in order to also express the biotin ligase (Mechold et al. 2005; van Werven and Timmers 2006).

4.3.1.3 High-Throughput Target Protein Expression and Purification

Several high-throughput strategies concerning the expression of human proteins in *E. coli* have been reported. All have to rely on the arrayed organization of the protein expression clones, preferably in 96- or 384-well format. While the 384-well format is usually used for the storage of glycerol stocks of individual clone collections, the actual target protein expression is taking place in 96-well plates, ranging from normal over large capacity (0.5 mL per well) to deep-well microtiter plates with working volumes around 1 mL per well. Büssow and coworkers at the Protein Structure Factory (http://www.proteinstrukturfabrik.de) developed an expression protocol using a rich medium for culturing the cells up to an OD_{600} of around 1.5, before protein expression is induced by adding IPTG (isopropyl-beta-D-thiogalactopyranoside; final conc. 1 mmol L^{-1}) for 4 h (Bussow et al. 2000, 2004). In our laboratory, we have adapted the protocol to 200 µL culture volumes in 0.5 mL large-capacity microplates, obtaining similar quantities of recombinant protein by incubating the cells at 1200 rpm in microtiter plate incubators (iEMS, Thermo). High-throughput protein purification methods have been set up for parallel handling of up to 96 clones at a time using liquid handling robots, for instance a Zinsser Speedy (Scheich et al. 2003) or a Qiagen 8000 (Kersten et al. 2003; Lueking et al. 2003). Many more liquid-handling systems and workstations are available on the market that can do the same thing (Table 4.2).

Purification of the proteins is based on affinity chromatography using Ni-NTA agarose beads (Qiagen), Talon resin (BD Biosciences) or glutathione agarose (Sigma). In many cases, however, randomly chosen expression constructs originating from cDNA expression libraries will mainly produce insoluble protein (~60%) captured in inclusion bodies within the cytoplasm (Bussow et al. 2000, 2004). Hence, methods to systematically analyze expression constructs for solubility have been developed and partially unit automated. This is a very important issue, especially in the light of crystallography and *in vitro* antibody selection

techniques. For instance, Stenvall and colleagues simply monitor the loss of protein concentration due to precipitation after dilution of the purified protein in presence or absence of a denaturing agent (urea) by measuring the protein concentration in the soluble fraction after centrifugation (Stenvall et al. 2005). Alternatively, methods exploit the property of green fluorescent protein (GFP) and its fusion proteins to fluoresce only in a properly folded state using a cell-free expression system (Coleman et al. 2004), or a flow cytometry-based approach is used, in which the whole cell fluores-cence is proportional to the amount of soluble protein in the *E. coli* cytoplasm (Hedhammar et al. 2005).

Currently, we are working on a two-step purification method, combining the benefits of metal ion-affinity chromatography with the selectivity of the streptavidin–biotin interaction. In the first purification step the recombinant protein is separated from the sole biotinylated *E. coli* protein BCCP (biotin carboxyl carrier protein), which does not bind to the chromatography support. In the second step, only the biotinylated recombinant human proteins are directly bound to the selection support matrix, separating the target protein from impurities as a result of unspecific binding of natural proteins to metal ions of the chromatography material.

4.3.2 Automating the Selection Procedure

The biopanning process in phage display runs over 3–5 rounds of selection and can be seen as an affinity-driven process during which binding molecules are continuously enriched out of an initially large pool of non-binders on an immobilized target until they finally represent the majority of all clones.

While variations in the selection process in itself are only relatively small, there are various strategies for target presentation. Proteins can be attached randomly or in a directed fashion to microtiter plate surfaces (Krebs et al. 2001), to magnetic beads (Walter et al. 2001), or to immunopins in the 96-well format (Lou et al. 2001). However, not all approaches are amenable for parallel selection and automation. For instance, selection efficacy on immunopins is limited by the rather small surface available for antigen presentation and lack of automation capability. Hence, I focus on approaches proven in respect of automation by published results and available equipment.

4.3.2.1 Panning in 96-well Microtiter Plates

Plastic 96-well microtiter plates were developed in 1952 in Hungary and have been used for countless immunological assays ever since the introduction of the enzyme-linked immunosorbent assay (ELISA) in the mid-1970s (Hamilton 2002). As a direct consequence, they have quickly become an indispensable tool in every routine diagnostic laboratory and hence, automated instrumentation for handling this format is widely available (Table 4.2).

For selection purposes, there are two ways of immobilizing the target molecules. The proteins are simply adsorbed to the polystyrene surface of the microtiter plate by applying 100 µL of antigen (10–100 µg mL^{-1}) in a bicarbonate buffer (pH 8) or phosphate-buffered saline (pH 7.4) and incubated overnight at 4°C. Alternatively, the target proteins are immobilized in a directed fashion using, for example, streptavidin-coated or antibody-coated plates directed against a tag sequence. The disadvantage of directed immobilization using antibodies is the much lower amount of bound target proteins, which can be as little as 5–10% compared with adsorption to plastic or directed immobilization of biotinylated proteins to streptavidin-coated surfaces.

For automation purposes, the general phage display biopanning can easily be adapted to the use of 96-well plates, since this process strongly resembles ELISA protocols with the difference that bound phage are amplified and not detected. Krebs and colleagues have successfully developed an automated protocol in which they use an ELISA washer for all washing cycles during the selection rounds and by simply modifying the phage amplification protocol during the individual biopanning cycles (Krebs et al. 2001). Surely, additional modifications to the selection process as well as a fully automated selection process utilizing a string of individual liquid handling robots, ELISA washers, incubators, and spectrophotometers under the control of a scheduling software will be seen in the near future.

4.3.2.2 Panning Using Magnetic Beads

As an alternative to microtiter plates, antigens can be coated onto magnetic particles. In our laboratory we use streptavidin-coated magnetic beads as the selection support and immobilize biotinylated target proteins (Walter et al. 2001). This has several advantages, such as directed immobilization, a uniform and dispersed presentation of the target, as well as a largely increased surface area in comparison to microtiter plates. Additionally, the use of magnetic beads easily allows specialized biopanning protocols, such as counterselections, to be performed. For example, adding a non-biotinylated isoform of the target protein can gear the selection towards antibodies recognizing only a certain desired conformation.

For unit automation of the biopanning procedure we use a pin-based magnetic particle processor (Kingfisher, Thermo), which enables the handling of 96 magnetic pins, corresponding to the positions of a 96-well microtiter plate. The processor has several positions for accommodating microtiter plates filled with individual buffers for washing and incubation and the individual steps of the biopanning procedure are performed by transferring the magnetic particles between wells by capture to and release from rod-shaped magnets covered with plastic caps (Rhyner et al. 2003). Controlling of the movements is software driven and parameters such as time, position, frequency, and strength of shaking movements can be adjusted, allowing reproducible control of each step of the phage display selection protocol for up to 96 parallel selections. Moving the beads from

solution to solution instead of changing the solutions with liquid-handling robots is, in our eyes, an additional advantage of using magnetic bead selection over microtiter plate-based schemes. It results in reduced background binding, since liquid remaining in the system from dead volume in the container can be avoided and problems arising from leaky liquid-handling systems are eliminated (Konthur and Walter 2002).

In any case, we can conclude at this stage that automating the biopanning process on its own can largely increase the throughput of targets against which antibodies are selected, but it also shifts the bottleneck of the overall selection pipeline further towards the isolation and evaluation of monospecific binders.

4.3.3 Primary Screening

The screening of individual antibody selections and rounds thereof is the biggest task to handle and the methods applied can vary widely according to the downstream application of the antibodies. However, it is generally accepted that at this stage the numbers of individual clones and screens can dramatically expand in the range of 10^2–10^4 per selection target.

Within the antibody development process, the primary screening procedure is the first step in the determination of target binding. In our laboratory, we use this term to refer to screening the individual selection rounds (polyclonal pools) for binding to the target protein and when screening sets of monoclonals – that is single bacterial expression clones – for each target for the first time.

4.3.3.1 Screening Polyclonal Antibody Pools

To ensure the highest possible success rate, we decided to perform polyclonal screening in an assay that is as close as possible to the selection process. Since the selection is carried out using magnetic bead-bound antigens, we perform an ELISA with phage from the individual selection rounds for evaluating specific enrichment in the same assay format. As a negative control we generally use only blocked selection support material, that is streptavidin-coated magnetic beads (Invitrogen, Dynabeads M-280) blocked with 2% skimmed milk powder. The ELISA is carried out according to general protocols with 30- to 60-minute incubation times for the primary (phage antibody) and secondary reagents (anti-M13-HRP, GE Healthcare) in an automated fashion using the magnetic particle processor. As in the selection procedure, during the ELISA the magnetic beads are moved from microtiter plate to microtiter plate prefilled with all necessary solutions. After addition of the beads to the substrate (2′,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt, Sigma), a first reading is taken after 30 min incubation at room temperature with a conventional spectrophotometer at OD_{405} and the values are exported and evaluated in Microsoft Excel (Fig. 4.2a).

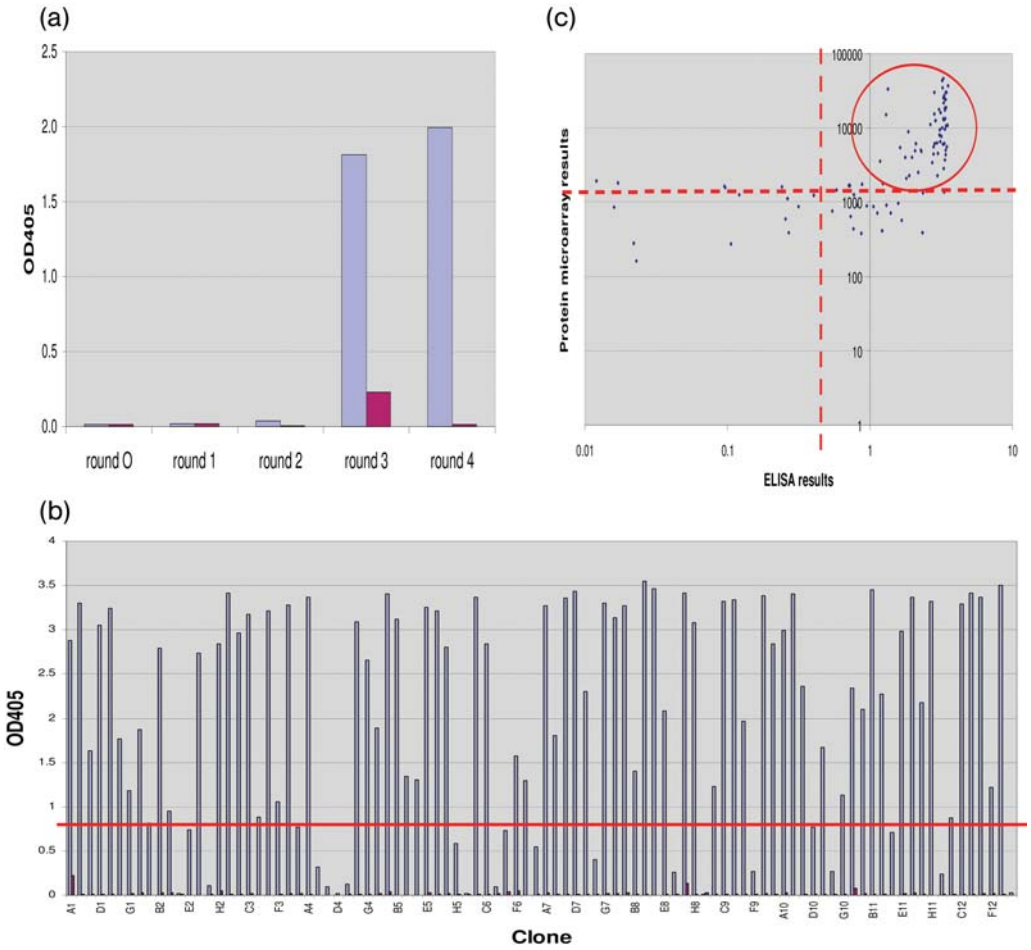

Fig. 4.2 Primary screening of an antibody phage display selection against a soluble human recombinant protein as a target. (a) An example of polyclonal enrichment of antibody fragments. (b) Conventional ELISA with 94 monoclonal soluble scFv fragments. blue: specific signal, red: background. (c) Correlation between the ELISA and the microarray binding assay MIST. Red dotted lines represent 50 times signal-to-background ratio; red circle represents clones positive in both assays.

Once a polyclonal enrichment for a specific selection target is successfully confirmed, we decide how many monoclonal entities we will further analyze from which selection rounds.

4.3.3.2 Generating Sets of Monoclonal Entities

To obtain single colonies for picking, we infect *E. coli* growing in the log-phase (OD_{600} ~0.4–0.6) with bacteriophage pools of the corresponding rounds and plate the infected cells onto 22 × 22 cm square dishes containing selective medium.

After overnight growth, individual colonies on the agar plates are visualized with a CCD camera mounted next to the picking gadget of a colony picker (Qbot, Genetix) and individual colonies are mapped and evaluated according to size and roundness with special image analysis software. Finally, up to 3000 selected colonies per hour are picked into bar-coded 384-well or 96-well plates prefilled with selective medium to generate master plates. After overnight incubation at 37°C without shaking, the master plates are replicated prior storage at −80°C to obtain two working copies each for further use. From the working copies, one is chosen for replicating into expression plates to obtain recombinant antibody fragments for characterization.

4.3.3.3 Primary Screening of Monoclonal Entities Without Changing Format

Depending on the phage display vector used, soluble antibody fragments can be obtained directly without subcloning simply by switching to a different *E. coli* host, for example HB2151. The underlying basis for this is the presence of an amber stop codon between the recombinant antibody fragment and the phage coat protein, which is read through only in amber-suppressor *E. coli* strains, such as TG1 and XL1-blue.

Conventional screening of specifically binding monoclonal entities involves the use of ELISA either in 384-well or 96-well plates and has been shown to be fully amenable to automation, reaching a throughput of 20.000 data points per day (Hallborn and Carlsson 2002). Another possibility to evaluate monoclonal binders at primary screening level is to make colony arrays consisting of around 20.000 *E. coli* clones, all harboring recombinant antibody molecules, which become accessible for analysis with directly labeled selection targets after lysis of the cells (de Wildt et al. 2000). However, this approach seems outdated ever since the introduction of protein microarray technology with multiplexing potential (Hultschig et al. 2006).

Currently there are two protein array methods used for the analysis of monoclonal antibody entities, which allow multiplexing. Sawyer and colleagues have set up a method to rapidly characterize primary cell fusions for the expression of mouse monoclonal antibodies obtained after a multiplexed immunization strategy (De Masi et al. 2005). For characterization, cell culture supernatants of cell fusions after only 12 days are spotted onto a glass microarray, which has previously been completely coated with 5 µg of an antigen used in the immunization process. Using a set of two different fluorescent-labeled secondary antibodies towards IgM and pan IgG of mouse, it was not only possible to find binding antibody molecules, but additionally to isotype them in a single multiplexing experiment.

In our pipeline, we are currently relying on classical ELISA as a primary screening tool to identify specific antibody fragments. However, we have developed a "Multiple Spotting Technique" (MIST), which allows the multiplexed analysis of hundreds of antibody fragments against a given set of target proteins on a single protein array (Angenendt et al. 2004). The technique is based on the simple but effective concept of addressing single positions on a chip multiple times

(Angenendt et al. 2003). After spotting multiple fields of antigens on the slide and blocking the remaining surface, a set of antibodies can be spotted onto the different antigens to not only find binders to each antigen but also to eliminate cross-specific binders at a very early stage of screening. Once we have thoroughly evaluated this technique and determined the maximum working capacity, we consider using MIST as the only primary screening tool in our antibody generation pipeline.

4.3.3.4 Primary Screening of Monoclonal Entities with Changing Format

In some laboratories the individual screening with monoclonal antibody fragments can only be conducted after the antibody fragments have been subcloned into different vectors (Krebs et al. 2001; Jostock et al. 2004). For example, McCafferty and coworkers subcloned the obtained scFv into an alkaline phosphatase fusion vector, which not only allowed the direct detection of the recombinant antibody without secondary detection reagents, but also increased the avidity of the antibody molecules, since alkaline phosphatase is a homodimer (Han et al. 2004).

Others are on the way to completely omit the screening of monoclonal entities produced in *E. coli* and primary screening of individual binders is directly performed in the desired application format. This can be especially useful when directly aiming at therapeutic application of the antibodies. For this purpose, the antibody heavy and light chain fragments are subcloned into specially designed acceptor vectors in batches before individual colonies are isolated and finally transfected to obtain fully human IgG1 antibodies (Jostock et al. 2004).

4.3.4 Secondary Screening of Monoclonal Entities

Re-evaluating identified binders by independent methods is referred to as secondary screening and normally results in additional information on the antibody, such as specificity, epitope binding, kinetic data, and the range of applications for which the antibody can be used.

4.3.4.1 Microtiter Plate and Bead-based Secondary Screening Assays

Next to ELISA, other microtiter plate-based assays are open to high-throughput screening such as the fluorometric microvolume assay technology (FMAT) (Hallborn and Carlsson 2002). Also common in use are homogeneous electrochemiluminescence assays on a magnetic bead basis (Schweitzer and Abriola 2002). Recently, a specially developed protocol for assessing all stages of monoclonal antibody production, including isotyping has been released (http://www.bioveris.com).

4.3.4.2 Cell-based and Tissue-based Secondary Screening

Additional information about antibodies is often desired, for instance when the antibodies are to target cell surface markers that are potential therapeutic targets

in cancer. These assays frequently include fluorescence-activated cell sorting (FACS), but also complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) and have been are covered in Chapter 1. Depending on the project pursued, the compatibility of the generated antibody with immunohistochemistry is of vital importance and multiple automated solutions for scoring data are available, which allow the investigation of protein expression patterns on a large scale using tissue microarrays (Warford et al. 2004; Uhlen and Ponten 2005).

4.3.4.3 **Protein Microarray-based Secondary Screening Assays**

Protein macro- and microarrays can be used at multiple steps within the pipeline. For example, we use protein microarrays containing a set of selection-relevant and -irrelevant recombinant human proteins for the monitoring of selection rounds with respect to increasing specificity. Templin and coworkers have used protein microarrays for the evaluation of specificity and affinity determination of individual phage display-derived Fab fragments (Poetz et al. 2005). Similarly, we have also used protein microarrays for the evaluation of oligoclonal mixtures and monoclonal antibody fragments and have also compared some of our antibody fragments with commercially available monoclonal antibodies in respect of specificity and sensitivity. Currently, we are also in the process of further advancing the use of protein microarray applications by combining the described features of the multiple spotting technique (Angenendt et al. 2003, 2004) with cell-free expression systems (Angenendt et al. 2006), which will allow the parallel testing of hundreds of single antibody-expressing clones at a time on a single chip in a multiplex assay format. In addition, the target protein can be represented by short oligopeptides (15-meres) directly synthesized onto a membrane support in a microarray format applying the SPOT synthesis and can be used for the mapping of the targeted epitope, provided it is linear (Frank 2002).

4.3.4.4 **Secondary Screening Assays Determining Affinity**

The most prominent technology for the determination of antibody affinities is the use of surface plasmon resonance (SPR). While classical BIAcore instrumentation is regarded as the gold standard, the product portfolio has been expanded recently by the Flexchip, a microarray-based SPR apparatus that allows the determination of up to 400 antibody affinity constants of in a parallel fashion (Wassaf et al. 2006). However, other alternative techniques have also been established, for example the Multiplexed Competitive Antibody Binning assay (MCAB), a bead-based assay applying the Luminex technology that allows fast affinity ranking of the antibodies under investigation (Jia et al. 2004). Another heterogeneous method to study antibody–antigen interactions is the Kinetic Exclusion Assay, a flow fluorimeter immunoassay based on the rapid separation and quantification of free and complexed antigen–antibody pairs monitored via a fluorescein-labeled secondary antibody in solution (Blake and Blake 2004).

4.3.5 Data Management

The selection of antibodies using phage display technology involves a great variety of methods and many individual experiments, which all need to be kept on record to guarantee good experimental practice and maintain high quality. Therefore, we have started to build a Laboratory Information Management System (LIMS) to store all antibody generation relevant data. The database is a Java-based web application built on the open source relational data base management system (RDBMS) PostgreSQL, Version 8.1 (http://www.postgresql.org). It covers areas as broad as "transcripts", "antibody_targets", "annotation", "expression_clones", "clones", "experiments", "experimentator" and "administrative data". In addition, we have linked our system to external databases to retrieve as much information to each target under way as possible with all our own data, for instance in which vector the target is expressed and whether it is a full-length product of a gene, or a mutant with amino acid substitution. The outline of the database was greatly inspired by the LIMS systems used for large-scale structural genomics projects, such as the Hex1 protein database of the Protein Structure Factory (http://www.proteinstructurfabrik.de) (Bussow et al. 2004, 2005).

4.4 Conclusion

Phage display has become a well-established and extremely powerful tool for the development of antibodies and, inevitably, plays a major role in research towards diagnostics and therapy.

During recent years, our general understanding of the phage display selection process as being difficult to automate has changed dramatically and many companies and organizations have started to develop (semi-)automated selection protocols similar to the one described in this chapter, or have successfully established downstream evaluation pipelines for the screening of thousands of monoclonal entities per day. With the increasing speed of novel technology developments in laboratory design and management, it is undoubtedly only a matter of time until fully automated selection systems with minimal user intervention are established, further raising the throughput of antibody development by phage display.

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References

Albala, J.S., Franke, K., McConnell, I.R., Pak, K.L., Folta, P.A., Rubinfeld, B., Davies, A.H., Lennon, G.G., Clark, R. (2000). From genes to proteins: high-throughput expression and purification of the human proteome. *J Cell Biochem* 80: 187–191.

Angenendt, P., Glokler, J., Konthur, Z., Lehrach, H., Cahill, D.J. (2003). 3D protein microarrays: Performing multiplex immunoassays on a single chip. *Anal Chem* 75: 4368–4372.

Angenendt, P., Wilde, J., Kijanka, G., Baars, S., Cahill, D.J., Kreutzberger, J., Lehrach, H., Konthur, Z., Glokler, J. (2004). Seeing better through a MIST: Evaluation of monoclonal recombinant antibody fragments on microarrays. *Anal Chem* 76: 2916–2921.

Angenendt, P., Kreutzberger, J., Glokler, J., Hoheisel, J.D. (2006). Generation of high-density protein microarrays by cell-free in situ expression of unpurified PCR products. *Mol Cell Proteomics* 5: 1658–1666.

Barbas, C.F., III, Kang, A.S., Lerner, R.A., Benkovic, S.J., (1991). Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc Natl Acad Sci USA* 88: 7978–7982.

Beckett, D., Kovaleva, E., Schatz, P.J. (1999). A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. *Protein Sci* 8: 921–929.

Blake, R.C., II and Blake, D.A. (2004). Kinetic exclusion assays to study high-affinity binding interactions in homogeneous solutions. *Methods Mol Biol* 248: 417–430.

Boettner, M., Prinz, B., Holz, C., Stahl, U., Lang, C. (2002). High-throughput screening for expression of heterologous proteins in the yeast *Pichia pastoris*. *J Biotechnol* 99: 51–62.

Breitling, F., Dubel, S., Seehaus, T., Klewinghaus, I., Little, M. (1991). A surface expression vector for antibody screening. *Gene* 104: 147–153.

Bussow, K., Nordhoff, E., Lubbert, C., Lehrach, H., Walter, G. (2000). A human cDNA library for high-throughput protein expression screening. *Genomics* 65: 1–8.

Bussow, K., Quedenau, C., Sievert, V., Tischer, C., Scheich, H., Seitz, B., Hieke, F., Niesen, H., Gotz, F., Harttig, U., Lehrach, H. (2004). A catalog of human cDNA expression clones and its application to structural genomics. *Genome Biol* 5: R71.

Bussow, K., Scheich, C., Sievert, V., Harttig, U., Schultz, J., Simon, B., Bork, P., Lehrach, H., Heinemann, U. (2005). Structural genomics of human proteins – target selection and generation of a public catalogue of expression clones. *Microb Cell Fact* 4: 21.

Clackson, T., Hoogenboom, H.R., Griffiths, A.D., Winter, G. (1991). Making antibody fragments using phage display libraries. *Nature* 352: 624–628.

Cohen, S., Trinka, R.F., (2002). Fully automated screening systems. *Methods Mol Biol* 190: 213–228.

Coleman, M.A., Lao, V.H., Segelke, B.W., Beernink, P.T. (2004). High-throughput, fluorescence-based screening for soluble protein expression. *J Proteome Res* 3: 1024–1032.

De Masi, F., Chiarella, P., Wilhelm, H., Massimi, M., Bullard, B., Ansorge, W., Sawyer, A. (2005). High throughput production of mouse monoclonal antibodies using antigen microarrays. *Proteomics* 5: 4070–4081.

de Wildt, R.M., Mundy, C.R., Gorick, B.D., Tomlinson, I.M. (2000). Antibody arrays for high-throughput screening of antibody–antigen interactions. *Nat Biotechnol* 18: 989–994.

Frank, R. (2002). The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports–principles and applications. *J Immunol Methods* 267: 13–26.

Hallborn, J., Carlsson, R. (2002). Automated screening procedure for high-throughput generation of antibody fragments. *Biotechniques Suppl* 30–37.

Hamilton, S. (2002). Introduction to screening automation. *Methods Mol Biol* 190: 169–193.

Han, Z., Karatan, E., Scholle, M.D., McCafferty, J., Kay, B.K. (2004). Accelerated screening of phage-display output with alkaline phosphatase fusions. *Comb Chem High Throughput Screen* 7: 55–62.

Hedhammar, M., Stenvall, M., Lonneborg, R., Nord, O., Sjolin, O., Brismar, H., Uhlen, M., Ottosson, J., Hober, S. (2005). A novel flow cytometry-based method for analysis of expression levels in *Escherichia coli*, giving information about precipitated and soluble protein. *J Biotechnol* 119: 133–146.

Holz, C., Lang, C. (2004). High-throughput expression in microplate format in *Saccharomyces cerevisiae*. *Methods Mol Biol* 267: 267–276.

Hoogenboom, H.R., Griffiths, A.D., Johnson, K.S., Chiswell, D.J., Hudson, P., Winter, G. (1991). Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res* 19: 4133–4137.

Hultschig, C., Kreutzberger, J., Seitz, H., Konthur, Z., Bussow, K., Lehrach, H. (2006). Recent advances of protein microarrays. *Curr Opin Chem Biol* 10: 4–10.

Jia, X.C., Raya, R., Zhang, L., Foord, O., Walker, W.L., Gallo, M.L., Haak-Frendscho, M., Green, L.L., Davis, C.G. (2004). A novel method of Multiplexed Competitive Antibody Binning for the characterization of monoclonal antibodies. *J Immunol Methods* 288: 91–98.

Jostock, T., Vanhove, M., Brepoels, E., Van Gool, R., Daukandt, M., Wehnert, A., Van Hegelsom, R., Dransfield, D., Sexton, D., Devlin, M., Ley, A., Hoogenboom, H., Mullberg, J. (2004). Rapid generation of functional human IgG antibodies derived from Fab-on-phage display libraries. *J Immunol Methods* 289: 65–80.

Kersten, B., Feilner, T., Kramer, A., Wehrmeyer, S., Possling, A., Witt, I., Zanor, M.I., Stracke, R. Lueking, A., Kreutzberger, J., Lehrach H., Cahilll, D.J. (2003). Generation of *Arabidopsis* protein chips for antibody and serum screening. *Plant Mol Biol* 52: 999–1010.

Kigawa, T., Yabuki, T., Matsuda, N., Matsuda, T., Nakajima, R., Tanaka, A., Yokoyama, S. (2004). Preparation of *Escherichia coli* cell extract for highly productive cell-free protein expression. *J Struct Funct Genomics* 5: 63–68.

Köhler, G., Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.

Konthur, Z., Walter, G. (2002). Automation of phage display for high-throughput antibody development. *TARGETS* 1: 30–36.

Krebs, B., Rauchenberger, R., Reiffert, S., Rothe, C., Tesar, M., Thomassen, E., Cao, M., Dreier, T., Fischer, D., Hoss, A., Inge, L., Knappik, A., Marget, M., Pack, P., Meng, X.Q., Schier, R., Sohlemann, P., Winter, J., Wolle, J., Kretzschmar, T. (2001). High-throughput generation and engineering of recombinant human antibodies. *J Immunol Methods* 254: 67–84.

Li, M. (2000). Applications of display technology in protein analysis. *Nat Biotechnol* 18: 1251–1256.

Little, M., Kipriyanov, S.M., Le Gall, F., Moldenhauer, G. (2000). Of mice and men: hybridoma and recombinant antibodies. *Immunol Today* 21: 364–370.

Lou, J., Marzari, R., Verzillo, V., Ferrero, F., Pak, D., Sheng, M., Yang, C., Sblattero, D., Bradbury, A. (2001). Antibodies in haystacks: how selection strategy influences the outcome of selection from molecular diversity libraries. *J Immunol Methods* 253: 233–242.

Lueking, A., Possling, A., Huber, O., Beveridge, A., Horn, M., Eickhoff, H., Schuchardt, J., Lehrach, H., Cahill, D. J. (2003). A nonredundant human protein chip for antibody screening and serum profiling. *Mol Cell Proteomics* 2: 1342–1349.

McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348: 552–554.

Mechold, U., Gilbert, C., Ogryzko, V. (2005). Codon optimization of the BirA enzyme gene leads to higher expression and an improved efficiency of biotinylation of target proteins in mammalian cells. *J Biotechnol* 116: 245–249.

Menke, K.C. (2002). Unit automation in high throughput screening. *Methods Mol Biol* 190: 195–212.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., Erlich, H. (1986). Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. *Cold Spring Harb Symp Quant Biol* 51 (Pt 1): 263–273.

Poetz, O., Ostendorp, R., Brocks, B., Schwenk, J.M., Stoll, D., Joos, T.O., Templin, M.F. (2005). Protein microarrays for antibody profiling: specificity and affinity determination on a chip. *Proteomics* 5: 2402–2411.

Rhyner, C., Konthur, Z., Blaser, K., Crameri, R. (2003). High-throughput isolation of recombinant antibodies against recombinant allergens. *Biotechniques* 35: 672–676.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G.T., Erlich, H.A., Arnheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230: 1350–1354.

Schatz, P.J. (1993). Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in *Escherichia coli*. *Bio/Technology* 11: 1138–1143.

Scheich, C., Sievert, V., Bussow, K. (2003). An automated method for high-throughput protein purification applied to a comparison of His-tag and GST-tag affinity chromatography. *BMC Biotechnol* 3: 12.

Schweitzer, R.H., Abriola, L. (2002). Electrochemiluminescence. A technology evaluation and assay reformatting of the Stat6/P578 protein-peptide interaction. *Methods Mol Biol* 190: 87–106.

Smith, G.P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228: 1315–1317.

Stenvall, M., Steen, J., Uhlen, M., Hober, S., Ottosson, J. (2005). High-throughput solubility assay for purified recombinant protein immunogens. *Biochim Biophys Acta* 1752: 6–10.

Uhlen, M., Ponten, F. (2005). Antibody-based proteomics for human tissue profiling. *Mol Cell Proteomics* 4: 384–393.

van Werven, F.J., Timmers, H.T. (2006). The use of biotin tagging in Saccharomyces cerevisiae improves the sensitivity of chromatin immunoprecipitation. *Nucleic Acids Res* 34: e33.

Walter, G., Konthur, Z., Lehrach, H. (2001). High-throughput screening of surface displayed gene products. *Comb Chem High Throughput Screen* 4: 193–205.

Warford, A., Howat, W., McCafferty, J. (2004). Expression profiling by high-throughput immunohistochemistry. *J Immunol Methods* 290: 81–92.

Wassaf, D., Kuang, G., Kopacz, K., Wu, Q.L., Nguyen, Q., Toews, M., Cosic, J., Jacques, J., Wiltshire, S., Lambert, J. Pazmany, C. C., Hogan, S., Ladner, R.C., Nixon, A.E., Sexton, D.J. (2006). High-throughput affinity ranking of antibodies using surface plasmon resonance microarrays. *Anal Biochem* 351: 241–253.

Weiner, L.M. (2006). Fully human therapeutic monoclonal antibodies. *J Immunother* 29: 1–9.

Winter, G., Milstein, C. (1991). Man-made antibodies. *Nature* 349: 293–299.

5
Emerging Technologies for Antibody Selection

Mingyue He and Michael J. Taussig

5.1
Introduction

There is a continuing, intense demand for production of specific antibodies. As well as being the most widely used binding molecules in basic and medical research and their applications, antibodies are essential to the current rapid expansion of proteomic studies (Pandey and Mann 2000; Carter 2001; Nielsen et al. 2003; Holliger and Hudson 2005). For example, antibody microarrays (Mann and Jensen 2003), with their potential for rapid protein expression analysis and diagnostic biomarker detection, require thousands of antibodies to be immobilized on a solid surface. In order to meet the demands of such highly multiplexed systems, display technologies provide a means of producing recombinant monoclonal antibodies *in vitro* as a cost- and time-efficient alternative to conventional hybridomas. Through the physical linkage of genotype (DNA or RNA) with phenotype (antibody fragment), specific combining sites are selected from large combinatorial libraries (Hoogenboom 2005). Recombinant display technologies tap the exploitation potential of DNA diversity, creating and screening novel sequences that are inaccessible by *in vivo* processes. In addition, since selection takes place under defined *in vitro* conditions, the *in vivo* biological constraints on antibody production are exceeded, with affinities beyond the *in vivo* ceiling [Boder et al. 2000] as well as other required properties. Moreover, some of these technologies also provide a route to engineering of antibodies with enhanced properties through iterative cycles of mutation and selection.

A number of display methods have been established and are widely used (Hoogenboom 2005), among them phage display, cell surface display, and ribosome display. The cell-dependent methods (phage, cell surface display) have limitations which the fully cell-free system of ribosome display can overcome. In this chapter, we review the selection of antibodies by display *in vitro*, focusing particularly on ribosome display.

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5.2 Display Technologies

In these methods, the physical association of phenotype and genotype ensures the simultaneous selection of a functional binding protein and the genetic information that encodes it. Figure 5.1 illustrates the cell-based and cell-free methods which have been developed through display of proteins on phage, cell surfaces, or ribosome complexes. Even though the selection steps in the procedure are carried out *in vitro*, phage display and cell surface display require that individual proteins are first expressed intracellularly, followed by assembly on the surface of the phage or transfer to the cell wall or membrane. In contrast, completely cell-free systems such as ribosome display and mRNA display do not require cell transformation, relying on polymerase chain reaction (PCR) and cell-free expression to produce libraries of stable protein–ribosome–mRNA or protein–mRNA complexes respectively; after co-selection of nascent protein and its encoding mRNA, the latter is subsequently reverse transcribed to DNA.

Currently, phage, yeast surface, and ribosome display are the most frequently used methods for selection and evolution of antibodies and ligand-binding scaffold proteins *in vitro* (Hoogneboom 2005). Each has its own particular advantages. Phage display is widely employed for antibodies in both Fab and single-chain (sc) Fv formats; it has been used to select high-affinity antibodies against free antigenic targets as well as antigens in their native location and conformation in cells

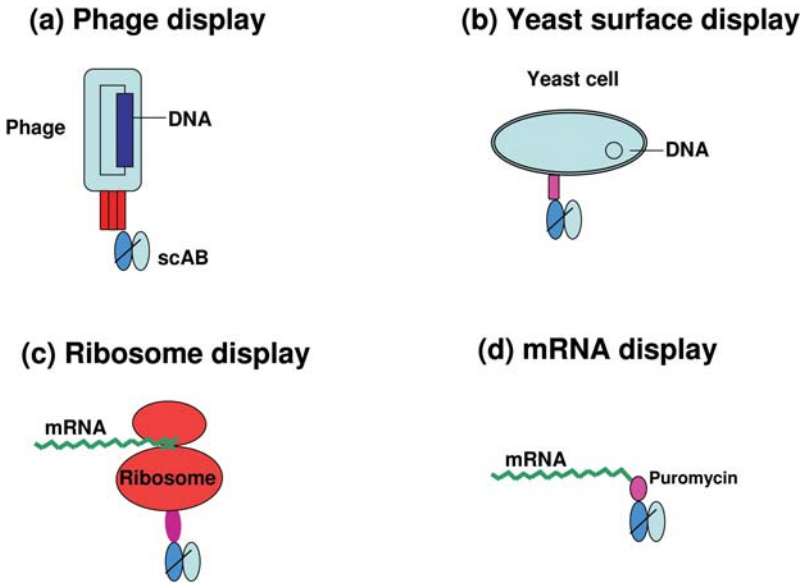

Fig. 5.1 Linkage of genotype and phenotype in four display technologies. scAB, single-chain antibody fragment carrying the antibody combining site.

Table 5.1 Comparison of phage display, yeast surface display and ribosome display.

	Phage display	***Yeast surface display***	***Ribosome display***
Maximum library size	10^{10}–10^{11}	10^{7}	10^{13}–10^{14}
Transformation required	Yes	Yes	No
Library form	Plasmid	Plasmid	PCR fragment or mRNA
Proteins to be displayed	Soluble, non-toxic, compatible with crossing membranes	Soluble, non-toxic, compatible with crossing membranes	Most proteins including cytotoxic, chemically modified and membrane proteins
Selection strategy	Panning	Sorting	Panning
Recovery	Elution, digestion or bacterial uptake. Strong binders may be lost	Cell sorting recovers strongest binders	RT-PCR potentially recovers all binders including strongest
Highest affinity antibody generated ($mol\,L^{-1}$)	10^{-11} [a]	10^{-14} [b]	10^{-12} [c]
Mutagenesis and protein evolution	DNA diversification followed by cloning	DNA diversification followed by cloning	DNA diversification without cloning

a Schier et al. 1996.
b Boder et al. 2000.
c Zahnd et al. 2004.

and tissues or through *in vivo* targeting. Yeast surface display allows direct screening by flow cytometry of individual antibody-displaying cells, providing a powerful tool for efficient sorting of antibody fragments with improved properties, and has produced affinities up to ~$10^{14}\,mol\,L^{-1}$ (Boder et al. 2000). However, these cell-dependent methods require cloning of DNA by cell transformation, restricting significantly the library size that can be displayed. Ribosome display (He and Khan 2005) overcomes this limitation through the use of PCR to create a DNA library encoding single-chain fragments which are then expressed in a cell-free system. Due to the ease with which very large PCR DNA libraries can be generated, ribosome display not only screens much larger populations but also allows continuous expansion of new diversity during the selection process; it is therefore suitable for rapid *in vitro* antibody evolution. Table 5.1 compares the properties of these three display systems.

5.3 Antibody Libraries

The diverse repertoire of antibody molecules is a result of the combination of six complementary determining regions (CDRs), three from the heavy (H) and three

from light (L) chain variable domains (V_H, V_L), and contained within V domain frameworks. In B cells, the complete V regions result from DNA rearrangement and combinatorial assembly of different gene segments followed, after cell activation, by somatic mutation (Milstein 1993; Ohlin and Zouali 2003). Display technologies use recombinant DNA methods to generate combinatorial DNA libraries for selection. This allows expanded diversity and creation of novel sequences that are not available to the immune system *in vivo*. Library size has been shown to influence the properties of selected antibodies, with larger libraries providing increased probability of finding specific, high-affinity binders to particular epitopes (Ling 2003).

Different types of combinatorial antibody library have been constructed from animals and humans (Hoogenboom 2005; He and Khan 2005). Randomized assembly of V_H and V_L domains from different lymphoid sources (e.g. peripheral blood, bone marrow, spleen or tonsils) has been used to make "naive" single-chain antibody (scAb) libraries (Winter and Milstein 1991). The combinations create new diversity, though some pairs may not be favorably recombined (de Wildt et al. 1999). Through the introduction of randomized codons into CDRs, "synthetic" antibody libraries with new diversity can be obtained (Hoogenboom and Winter 1992). Naive and synthetic libraries are both generally antigen independent and particularly useful for unbiased selection of antibodies against any target antigen.

A novel design has been used to construct libraries with single V_H and V_L domain frameworks or consensus sequences. Diversity was built up by shuffling native CDR repertoires onto the same V_H (or V_L) frameworks (Knappik et al. 2000; Söderlind et al. 2000). This approach permits random recombination of all six CDRs, providing a large potential for generating novel variants. An advantage of this method is that the library should be functional, as CDRs derived from natural sequences usually form folded molecules (Söderlind et al. 2000). The use of an appropriate single framework facilitates downstream expression, since some framework sequences are not expressed in heterologous systems (Söderlind et al. 2000). A very large human antibody library (HuCAL) was generated by using a few modular consensus frameworks to display a full set of CDR repertoires (Knappik et al. 2000). Recently, a focused library has been constructed, which created biased diversity specific for binding to small molecule antigens such as haptens (Persson et al. 2006).

In contrast to naive libraries, those from immunized animals or humans provide enriched sequences for rapid selection of antibodies against the antigens used for the immunization (He et al. 1999; Felding-Habermann et al. 2005). The combination of B-cell activation *in vivo* with antibody selection *in vitro* offers a more rapid route to isolation of specific antibodies (He et al. 1999; Persson et al. 2006).

5.4
Antibody Selection and Maturation *In Vitro*

Antibodies are selected from display libraries under defined *in vitro* conditions, which can be adjusted in a controllable direction to enrich for desirable molecules. A number of selection strategies have been developed, including off-rate, specificity, stability, and antibody-guided selection.

Off-rate selection is designed to enrich for antibodies with slower dissociation rates and hence higher affinity. One method is to equilibrate the displayed library with a biotinylated antigen, followed by addition of an excess of unlabeled antigen; antibodies with slow off-rates remain bound to the biotinylated antigen for a longer time and are subsequently captured by immobilized streptavidin, while those with faster dissociation rates are released and their reassociation blocked by the unlabeled antigen. This strategy has led to isolation of molecules with affinities of up to 10^{-12} mol L^{-1} (Jermutus et al. 2001).

For improved specificity, epitope-blocked panning is a method in which irrelevant epitopes are blocked with antibodies prior to selection (Tsui et al. 2002). Alternating a particular hapten conjugated on different protein carriers in selection cycles can reduce cross-reactivity to the carrier proteins (Yau et al. 2003a). Direct selection on whole cells or frozen tissue sections has also produced specific antibodies recognizing specific epitopes *in situ* (Tanaka et al. 2002).

Antibodies have also been selected under conditions of thermal or chemical denaturation, leading to highly stable and aggregation-resistant binding fragments (Wörn and Plückthun 2001; Jespers et al. 2004). Inclusion of a reducing reagent such as dithiothreitol (DTT) during selection has isolated those with enhanced solubility and stability (Jermutus et al. 2001).

Guided selection (Osbourn et al. 2005) is a useful approach to generating a human combining site equivalent of an existing rodent antibody with similar antigen specificity, serving as an alternative to humanization. In this approach, the H- or L-chain of the rodent antibody was displayed as a capturing reagent to select human antibody H- and L-chain partners on the same antigen (Osbourn et al. 2005). Alternatively, humanization of rodent antibodies can be carried out by "reshaping" (Verhoeyen et al. 1988), which involves changing solvent-exposed residues in a murine framework to their human homologs, followed by screening of all variants that best preserve the original antibody properties. This approach overcomes the drawback of other humanization methods that require separate construction and analysis of individual antibody mutants (Rosok et al. 1996; Wang et al. 2004).

De novo design of antigens enables selection of antibodies with novel combining sites (e.g. catalytic antibodies and conformation-specific antibodies can be selected by using designed antigen analogs) (Cesaro-Tadic et al. 2003).

Combined with DNA mutagenesis, display technologies are an efficient means of developing antibody properties through evolutionary approaches. By repeated cycles of mutation and functional selection, antibody variants with improved properties can be isolated (Plückthun et al. 2000). For example, an *in vitro* antibody

maturation strategy has selected mutants with affinities of up to 5×10^{-14} mol L^{-1} (Boder et al. 2000). Compared with *in vivo* somatic maturation processes, which usually provide antibodies of 10^{9}–10^{10} mol L^{-1}, the possibility of a 10^{3}-fold affinity improvement makes *in vitro* maturation a relevant approach for the selection of potential therapeutic antibodies.

5.5 Linking Antibodies to mRNA: Ribosome and mRNA Display

Ribosome and mRNA display are cell-free methods which overcome some of the limitations of cell-based systems, particularly in regard to library size, by directly expressing large PCR libraries without the need for cloning. In ribosome display, phenotype and genotype are linked as protein–ribosome–mRNA complexes, which are achieved by stalling the ribosome at the end of translation, usually as a result of stop codon deletion. Since the presence of a stop codon is required to engage release factors, its absence means that the nascent protein does not dissociate from the ribosome, remaining associated with the encoding mRNA (Fig. 5.1). The generation of a library of ribosome complexes permits affinity selection (e.g. by immobilized ligand) of a nascent antibody and its encoding mRNA, which can be recovered as DNA by reverse transcriptase (RT)-PCR (Fig. 5.2). Cyclical reiteration of this process leads progressively to enrichment of antibodies originally present as rare species in a large population, by a factor or 10^{3}–10^{5} per cycle (He and Taussig 1997). Both prokaryotic and eukaryotic ribosome display systems have been developed and applied successfully to antibody selection and evolution (He and Taussig 1997; Plückthun et al. 2000; He and Khan 2005).

In the method known as mRNA display, the protein and mRNA become covalently linked through a puromycin moiety attached to the 3′ end of the mRNA, which displaces the nascent protein at the end of translation (Roberts and Szostak 1997). The ribosome is no longer present in the complexes. mRNA display has been used for selection of alternative antibody mimics based on the fibronectin scaffold (Xu et al. 2002).

5.6 Advantages of Ribosome Display

Since there is no requirement for DNA cloning, ribosome display libraries of very large size can be generated with ease. For example, while a PCR library of $10^{12–14}$ members can be produced through a few reactions, up to 10^{5} transformations would be required for cell-based display methods, as each transformation usually generates $10^{7–9}$ clones (Lamla and Erdmann 2003). The restriction for a ribosome display library is perhaps only the number of functional ribosomes in the reaction, which can be scaled up to 10^{14} per mL in rabbit reticulocyte lysate (He and Taussig 1997). The use of PCR DNA templates also provides a simple

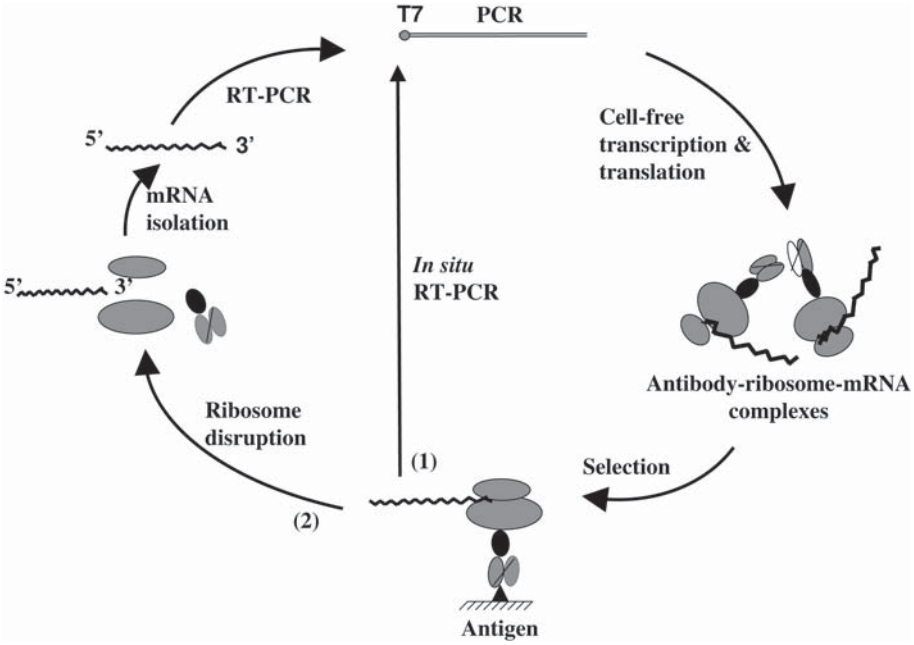

Fig. 5.2 Versions of the ribosome display cycle. (1) Rabbit reticulocyte lysate system with single-step recovery of DNA by RT-PCR. (2) *E. coli* S30 system with recovery by ribosome disruption and RT-PCR. T7, T7 promoter; RT-PCR, reverse transcriptase polymerase chain reaction.

tool for continuous introduction of additional diversity into the DNA pool for further selection cycles (Plückthun et al. 2000; He and Taussig 2002). Sequence changes can also be introduced at the mRNA level by inclusion of Qβ RNA-dependent RNA polymerases in the cell-free mixture (Irving et al. 2001). Thus, ribosome display provides an efficient system for antibody evolution *in vitro*, in contrast with phage display where such continuous "in-built" evolution is not possible.

5.7 Ribosome Display Systems

5.7.1 Prokaryotic: *E. coli* S30

The S30 cell-free lysate from *E. coli*, either with coupled or uncoupled transcription and translation, has been adapted for ribosome display (Mattheakis et al.

1994; Hanes and Plückthun 1997). In the coupled method, a DNA library is used to generate polyribosome complexes displaying proteins, which are captured with an immobilized ligand. To avoid any possible disruptive effect of DTT, prokaryotic ribosome display has more often been carried out in an uncoupled format (i.e the transcription and translation are performed separately) (Hanes and Plückthun 1997; Plückthun et al. 2000). In order to recover the associated mRNA, the polysome complexes are dissociated by chemical disruption and the released mRNA purified prior to RT-PCR (Fig. 5.2) (Mattheakis et al. 1994; Hanes and Plückthun 1997). The procedure has been modified to display folded antibody fragments by adding extra components such as protein disulfide isomerase, vanadyl ribonucleoside complexes, and anti-ssrA antisense oligonucleotide (Hanes and Plückthun 1997). Recent modification of *E. coli* ribosome display has included the generation of more stable ribosome complexes using a protein–mRNA interaction (Sawata et al. 2004).

5.7.2 Eukaryotic: Rabbit Reticulocyte

A eukaryotic system has also been developed for selection of antibody fragments using the coupled rabbit reticulocyte lysate. This technology was initially called ARM (antibody-ribosome-mRNA) display (He and Taussig 1997) (Fig. 5.2). In a modified version, oxidized/reduced glutathione and Qβ RNA-dependent RNA polymerase were included in the translation mixture to improve protein folding and introduce mutations (Irving et al. 2001). Wheatgerm cell-free lysate has also been adapted for ribosome display of folded proteins (Takahashi et al. 2002).

The main difference between the *E. coli* S30 and rabbit reticulocyte ribosome display systems lies in the DNA recovery step (Fig. 5.2). While the prokaryotic ribosome display method requires chemical disruption (e.g. EDTA chelation) to dissociate ribosome subunits and release mRNA prior to RT-PCR, rabbit reticulocyte lysate display employs an *in situ* recovery procedure in which RT-PCR is performed directly on the ribosome complexes without the need for prior dissociation. Successful *in situ* RT-PCR is achieved through the design of primers hybridizing slightly upstream of the 3′ end to avoid the region covered by the stalled ribosome [He and Taussig 1997]. *In situ* RT-PCR not only simplifies the recovery process but also avoids material losses incurred in disrupting complexes for mRNA isolation. It has been used to analyze the binding specificity of ribosome complexes through detection of the attached mRNA (He and Taussig 2005). *In situ* RT-PCR would also facilitate automation of the ribosome display process.

Interestingly, the prokaryotic ribosome disruption method seems to be a relatively poor procedure for releasing mRNA from rabbit reticulocyte ribosome complexes (Hanes et al. 1999; He and Taussig 2005; Douthwaite et al. 2006), possibly indicating a difference in stability between prokaryotic and eukaryotic complexes. A method has been described to disrupt rabbit reticulocyte complexes by heating above 70 °C (Bieberich et al. 2000).

5.7.3
Ribosome Display Constructs (Fig. 5.3)

DNA constructs for ribosome display should contain a T7 promoter and a translation initiation sequence such as Shine–Dalgarno for *E. coli* S30 or the Kozak sequence for eukaryotic systems. It is possible to generate a single prokaryotic/eukaryotic consensus sequence (Allen and Miller 1999). To enable the complete exit of the displayed portion of the nascent protein from the ribosome tunnel, a spacer domain of at least 23–30 amino acids is fused at the C-terminus (He and Taussig 1997; Plückthun et al. 2000). The DNA sequence of the spacer also provides a known region for designing an annealing primer for *in situ* RT-PCR recovery. Spacers reported for ribosome display of proteins include the constant region of Igκ L-chain (Cκ), the C_H3 domain of human IgM (Plückthun et al. 2000), gene III of M13 phage, streptavidin, and GST (Zhou et al. 2002). Spacer length has been shown to affect display efficiency, with a longer spacer being more efficient (Schaffitzel et al. 1999). Constructs for *E. coli* display also require incorporation of sequences containing stem–loop structures at both the 5′ and 3′ ends of the DNA to prevent mRNA degradation by the high RNase activities in the *E. coli* S30 system (Hanes and Plückthun 1997). The diverse library sequences are placed in-frame between the initiation codon ATG and the spacer. To form stable ribosome complexes, the stop codon should be deleted from the construct by using a 3′ primer without the stop codon.

5.7.4
Monosome versus Polysome Display

With the commonly used cell-free systems, both coupled and uncoupled methods have been developed for ribosome display of single-chain antibody fragments and

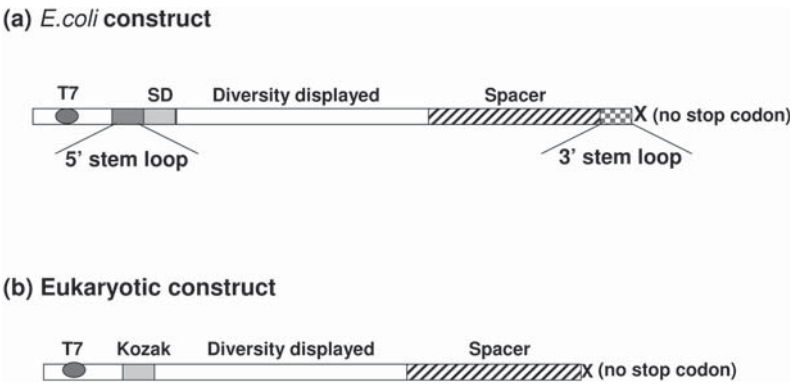

Fig. 5.3 Constructs used for prokaryotic and eukaryotic ribosome display. S/D, Shine–Dalgarno sequence for prokaryotic translation initiation; Kozak, eukaryotic translation initiation sequence.

other proteins (Plückthun et al. 2000; He and Taussig 2002). The choice of cell-free system depends mainly on the origins and properties of the proteins to be displayed and the downstream applications; some proteins may express better in one particular cell-free system than in another. One issue is whether the complexes are monoribosomal (monosome) or polyribosomal (polysome), the former having only one translatin ribosome per mRNA whereas the latter carry several. It has been shown that rabbit reticulocyte lysate produces mainly monosome complexes [Holet and Osbourn 2001], whereas *E. coli* S30 generates polysomes (Mattheakis et al. 1994). However, it is also possible to display monosomes in *E. coli* S30 by controlling the ratio of input mRNA to ribosome number (Fedorov and Baldwin 1998). The formation of polysome complexes raises the possibility of polyvalent display of incomplete nascent polypeptides and avidity effects which may lead to isolation of lower affinity combining sites (depending on the selection procedure used).

5.8
Antibody Generation by Ribosome Display

Ribosome display has been applied to selection, evolution, and humanization of antibodies *in vitro*, yielding molecules of high affinity and specificity (Schaffitzel et al. 1999; Plückthun et al. 2000; He and Taussig 2002). Single-domain mini-antibodies have also been successfully displayed (Yau et al. 2003). Through repeated rounds of mutation and *in vitro* selection, antibody variants with improved affinity (down to picomolar K_D), specificity, and stability have been isolated (Schaffitzel et al. 1999; He and Taussig 2002; Thom et al. 2006). Antibodies with novel binding sites recognizing conformation-specific epitopes or having catalytic activities have also been selected (Hanes et al. 2000; Amstutz et al. 2002). Recently, ribosome display has been utilized for antibody humanization by reshaping, which rapidly identifies humanized antibody variants from a shuffled DNA library (Wang et al. 2004), or through guided selection using the H- or L-chain of a rodent antibody as a capturing reagent to select human antibody partner chains with similar antigen specificity (Osbourn et al. 2005).

5.9
Summary

Display technologies provide powerful and versatile methods for selection and manipulation of recombinant antibodies. They are capable of generating binding molecules with optimized features or properties suitable for diagnostic and therapeutic applications, which may not be available through animal immunization. The operation of ribosome display is completely cell-free, overcoming the limitations of cell-based display methods by rapidly generating and screening very large libraries, which in turn increase the likelihood of finding high-affinity binding

molecules. Potentially, the selection power of ribosome display could be further enhanced by automation or combination with protein arrays, leading to high-throughput selections of antibodies against genome-coded targets for proteomic studies.

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References

Allen, S.V., Miller, E.S. (1999) RNA-binding properties of *in vitro* expressed histidine-tagged RB69 RegA translational repressor protein. *Anal Biochem* 269: 32–37.

Amstutz, P., Pelletier, J.N., Guggisberg, A. et al. (2002) In vitro selection for catalytic activity with ribosome display. *J Am Chem Soc* 124: 9396–9403.

Bieberich, E., Kapitonov, D., Tencomnao, T., Yu, R.K. (2000) Protein-ribosome-mRNA display: affinity isolation of enzyme-ribosome-mRNA complexes and cDNA cloning in a single-tube reaction. *Anal Biochem* 287: 294–298.

Boder, E.T., Midelfort, K.S., Wittrup, K.D. (2000) Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc Natl Acad Sci USA* 97: 10701–10705.

Carter, P. (2001) Improving the efficacy of antibody-based cancer therapies. *Nature Rev Cancer* 1: 118–129.

Cesaro-Tadic, S., Lagos, D., Honegger, A. et al. (2003) Turnover-based *in vitro* selection and evolution of biocatalysts from a fully synthetic antibody library *Nat Biotechnol* 21: 679–685.

de Wildt, R.M., Hoet, R.M., van Venrooij, W.J. et al. (1999) Analysis of heavy and light chain pairings indicates that receptor editing shapes the human antibody repertoire. *J Mol Biol* 285: 895–901.

Douthwaite, J.A., Groves, M.A., Dufner, P., Jermutus, L. (2006) An improved method for an efficient and easily accessible eukaryotic ribosome display technology. *Protein Eng Des Sel* 19: 85–90.

Fedorov, A.N., Baldwin, T.O. (1998) Protein folding and assembly in a cell-free expression system. *Methods Enzymol* 290: 1–17.

Felding-Habermann, B., Lerner, R.A., Lillo, A. et al. (2005) Combinatorial antibody libraries from cancer patients yield ligand-mimetic Arg-Gly-Asp-containing immunoglobulins that inhibit breast cancer metastasis *Proc Natl Acad Sci USA* 101: 17210–17215.

Hanes, J., Plückthun, A. (1997) *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc Natl Acad Sci USA* 94: 4937–4942.

Hanes, J., Jermutus, L., Schaffitzel, C., Plückthun, A. (1999) Comparison of *E. coli* and rabbit reticulocyte ribosome display systems. *FEBS Lett* 450: 105–110.

Hanes, J., Schaffitzel, C., Knappik, A., Plückthun, A. (2000) Picomolar affinity antibodies from a fully synthetic naïve library selected and evolved by ribosome display. *Nat Biotechnol* 18: 1287–1292.

He, M., Khan, F. (2005) Ribosome display: next generation display technologies for production of antibodies *in vitro*. *Expert Rev Proteomics* 2: 421–430.

He, M., Taussig, M.J. (1997) ARM complexes as efficient selection particles for *in vitro* display and evolution of antibody combining sites. *Nucleic Acids Res* 25: 5132–5134.

He, M., Taussig, M.J. (2002) Ribosome display: Cell-free protein display technology. *Briefings Functional Genomics Proteomics* 1: 204–212.

He, M., Taussig, M.J. (2005) Ribosome display of antibodies: expression, specificity and recovery in a eukaryotic system. *J Immunol Methods* 297: 73–82.

He, M., Menges, M., Groves, M.A. et al. (1999) Selection of a human anti-progesterone antibody fragment from a transgenic mouse library by ARM ribosome display. *J Immunol Methods* 231: 105–117.

Holet, T., Osbourn, J. (2001) Improvements to ribosome display. International patent application (PCT/ WO 0175097).

Holliger, P., Hudson, P. (2005) Review: Engineered antibody fragments and the rise of single domains. *Nat Biotechnol* 23: 1126–1136.

Hoogenboom, H.R. (2005) Review: selection and screening recombinant antibody libraries. *Nat Biotechnol* 23: 1105–1116.

Hoogenboom, H.R., Winter, G. (1992) By-passing immunisation: Human antibodies from synthetic repertoires of germline VH gene segments rearranged *in vitro*. *J Mol Biol* 227: 381–388.

Irving, R.A., Coia, G., Roberts, A., Nuttall, S.D., Hudson, P.L. (2001) Ribosome display and affinity maturation: from antibodies to single V-domains and steps towards cancer therapeutics. *J Immunol Methods* 248: 31–45.

Jermutus, L., Honegger, A., Schwesinger, F., Hanes, J., Plückthun, A.(2001) Tailoring *in vitro* evolution for protein affinity or stability. *Proc Natl Acad Sci USA* 98: 75–80.

Jespers, L., Schon, O., Famm, F., Winter, G. (2004) Aggregation-resistant domain antibodies selected on phage by heat denaturation. *Nat Biotechnol* 22: 1161–1165.

Knappik, A., Ge, L., Honegger, A. et al. (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J Mol Biol* 296: 57–86.

Lamla, T., Erdmann, V.A. (2003) Searching sequence space for high affinity binding peptides using ribosome display. *J Mol Biol* 329: 381–388.

Ling, M.M. (2003) Large antibody display libraries for isolation of high affinity antibodies. *Comb Chem High Throughput Screen* 6: 421–432.

Mann, M., Jensen, O.N. (2003) Proteomic analysis of post-translational modifications *Nat Biotechnol* 21: 255–261.

Mattheakis, L.C., Bhatt, R.R., Dower, W.J. (1994) An *in vitro* polysome display system for identifying ligands from very large peptide libraries. *Proc Natl Acad Sci USA* 91: 9022–9026.

Milstein, C. (1993) From the structure of antibodies to the diversification of immune response. *Scand J Immunol* 37: 385–395.

Nielsen, U.B., Cardone, M.H., Sinskey, A.J. et al. (2003) Profiling receptor tyrosine kinase activation by using Ab microarrays. *Proc Natl Acad Sci USA* 100: 9330–9335.

Ohlin, M., Zouali, M. (2003) The human antibody repertoire to infectious agents: implications for disease pathogenesis. *Mol Immunol* 40: 1–11.

Osbourn, J., Groves, M., Vaughan, T. (2005) From rodent reagents to human therapeutics using antibody guided selection. *Methods* 36: 61–68.

Pandey, A., Mann, M. (2000) Proteomics to study genes and genomes. *Nature* 405: 837–846.

Persson, H., Lantto, J., Ohlin, M. (2006) A focused antibody library for improved hapten recognition. *J Mol Biol* 357: 607–620.

Plückthun, A., Schaffitzel, C., Hanes, J., Jermutus, L. (2000) *In vitro* selection and evolution of proteins. *Adv Protein Chem* 55: 367–403.

Roberts, R.W., Szostak, J.W. (1997) RNA-peptide fusion for the in vitro selection of peptides and proteins. *Proc Natl Acad Sci USA* 94: 12297–12302.

Rosok, M.J., Yelton, D.E., Harris, L.J. *et al.* (1996) A combinatorial library strategy for the rapid humanisation of anticarcinoma BR96 Fab. *J Biol Chem* 271: 22611–22618.

Sawata, S.Y., Suyama, E., Taira, K. (2004) A system based on specific protein-RNA interactions for analysis of target protein-protein interactions *in vitro*: successful selection of membrane-bound bak-Bcl-xL

proteins *in vitro*. *Protein Eng Des Sel* 17: 501–508.

Schaffitzel, C., Hanes, J., Jermutus, L., Plückthun, A. (1999) Ribosome display: an *in vitro* method for selection and evolution of antibodies from libraries. *J Immunol Methods* 231: 119–135.

Schier, R., McCall, A., Adams, G.P. et al. (1996) Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity determining regions in the center of the antibody binding site. *J Mol Biol* 263: 551–567.

Söderlind, E., Strandberg, L., Jirholt, P. et al. (2000) Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. *Nature Biotechnol* 18: 852–856.

Takahashi, F., Ebihara, T., Mie, M. et al. (2002) Ribosome display for selection of active dihydrofolate reductase mutants using immobilized methotrexate on agarose beads. *FEBS Lett* 514: 106–110.

Tanaka, T., Ito, T., Furuta, M. et al. (2002) *In situ* phage screening: A method for identification of subnanogram tissue components *in situ*. *J Biol Chem* 277: 30382–30387.

Thom, G., Cockroft, A.C., Buchanan, A.G. et al. (2006) Probing a protein-protein interaction by *in vitro* evolution. *Proc Natl Acad Sci USA* 103: 7619–7624.

Tsui, P., Tornetta, M.A., Ames, R.S. et al. (2002) Progressive epitope-blocked panning of a phage librray for isolation of human RSV antibodies. *J Immunol Methods* 263: 123–132.

Verhoeyen, M., Milstein, C., Winter, G. (1988) Reshaping human antibodies: grafting an anti-lysozyme activity. *Science* 239: 1534–1536.

Wang, X.B., Zhou, B., Yin, C.C., Lin, Q., Huang, H.L. (2004) A new approach for rapidly reshaping single-chain antibody *in vitro* by combining DNA shuffling with ribosome display. *J Biochem* 136: 19–28.

Winter, G., Milstein, C. (1991) Man-made antibodies. *Nature* 349: 293–299.

Wörn, A., Plückthun, A. (2001) Stability engineering of antibody single-chain Fv fragments. *J Mol Biol* 305: 989–1010.

Xu, L., Aha, P., Gu, K. et al. (2002) Directed evolution of high-affinity antibody mimics using mRNA display. *Chem Biol* 9: 933–942.

Yau, K.Y., Groves, M.A., Li, S., Sheedy, C., Lee, H., Tanha, J., MacKenzie, C.R., Jermutus, L., Hall, J.C. (2003) Selection of hapten-specific single-domain antibodies from a non-immunized llama ribosome display library. *J Immunol Methods* 281: 161–175.

Zahnd, C., Spinelli, S., Luginbuhl, B., Amstutz, P., Cambillau, C., Plückthun, A. (2004) Directed *in vitro* evolution and crystallographic analysis of a peptide-binding single chain antibody fragment (scFv) with low picomolar affinity. *J Biol Chem* 279: 18870–18877.

Zhou, J.M., Fujita, S., Warashina, M., Baba, T., Taira, K. (2002) A novel strategy by the action of ricin that connects phenotype and genotype without loss of the diversity of libraries. *J Am Chem Soc* 124: 538–543.

6
Emerging Alternative Production Systems

Thomas Jostock

6.1
Introduction

The market for recombinant protein pharmaceuticals is growing rapidly and reached a volume of about US$47 billion in 2004. Among those, recombinant antibodies are the fastest growing group and a large number of new antibody products are in clinical and preclinical development, which will lead to an increased need for production capacities and expression systems in the near future. Since conventional cost-effective microbial expression systems are not yet applicable for the expression of fully functional IgG antibodies, all currently marketed therapeutic recombinant antibody products are produced from animal cell culture processes that are associated with relative high operating expenses. The production costs of recombinant antibodies result in significantly higher expense for antibody-based therapies compared with most chemical drugs. Thus, to make a broad use of recombinant antibody therapeutics more competitive and affordable for the healthcare systems, alternative production systems with improved efficiency and reduced operating expenses are highly desirable.

IgG currently is the dominant antibody format for therapeutics. As a heterotetrameric molecule with numerous disulfide bonds and several glycosylation sites, IgG makes itself a challenging candidate for heterologous expression, especially in prokaryotes. Glycosylation of the Fc region has been shown to be essential for mediating effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Boyd et al. 1995). Furthermore, the glycosylation pattern strongly influences the efficiency of the induction of effector functions by antibodies (Umana et al. 1999; Niwa et al. 2005). For applications that depend on the immunological effector functions of the antibody therefore, eukaryotic production systems with suitable glycosylation capabilities are necessary.

Antibody fragments such as single-chain fragment variable (scFv) and fragment antigen binding (Fab) are less complex and the antigen-binding activity is independent of glycosylation, which makes them qualified for prokaryotic expression systems. Certainly, these fragments are incapable of mediating

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immunological effector functions but for numerous applications this is not necessary or even unwanted.

Besides the two traditional expression systems – *E. coli* for antibody fragments and Chinese hamster ovary (CHO), baby hamster kidney (BHK), or myeloma cells for IgG antibodies – numerous alternative expression systems are currently under development. This includes the evaluation of new expression hosts such as filamentous fungi or transgenic plants as well as "tuning" of the more conventional expression hosts via genetic modifications. In the following section some emerging production systems for antibody expression are briefly described.

6.2 Production Systems

6.2.1 Prokaryotic Expression Systems

Fast growth, inexpensive culture media, and high resistance to shear forces usually are advantageous properties of prokaryotic organisms for recombinant protein production. The main disadvantages of prokaryotic expression systems, especially for antibody expression, are the limited glycosylation, folding, and secretion capabilities of the host cells. Currently the almost exclusive prokaryotic production system for antibodies is *E. coli*. Two members of the Gram-positive species *Bacillus* also have been reported to produce functional antibody fragments, *B. subtilis* (Inoue et al. 1997; Wu et al. 1998, 2002) and *B. brevis* (Bolhuis et al. 1999; Shiroza et al. 2001). The cell wall-less L-form of *Proteus mirabilis* was used to successfully produce scFv and mini-antibodies (Kujau et al. 1998; Rippmann et al. 1998). Using the prepro sequence of *S. hyicus* lipase, secretory production of a chimeric Fab was shown for *Staphylococcus carnosus* (Schnappinger et al. 1995). Several other prokaryotic expression systems are under development for the production of pharmaceutical proteins but to our knowledge have not been applied to produce antibodies yet, including *Ralstonia eutropha* and *Streptomyces* sp. with yields in the range of 100 mg L^{-1} to 2 g L^{-1} (Schmidt 2004). The following section describes the properties of some emerging prokaryotic systems.

6.2.1.1 Advanced and Alternative *E. coli* Expression Systems

The ease of handling, the well-characterized genetics, and the high transformation efficiency made the Gram-negative bacterium *E. coli* the most frequently used organism for recombinant protein expression. However, the lack of a eukaryotic-like folding apparatus and secretion machinery limits the applicability of *E. coli* as expression host for complex, multimeric, and glycosylated proteins like antibodies. Up to now there are only few examples of the successful production of entire IgG antibodies in *E. coli* (Simmons et al. 2002). In this report a careful balancing of the ratio of light to heavy chain expression and an adjustment of the translation levels to eliminate secretory blocks had to be performed to allow

the formation and accumulation of tetrameric IgG molecules in the periplasm. As expected, the aglycosylated IgG antibodies bound to the Fcγn receptor but not to C1q or to the FcγI receptor. Thus, a long *in vivo* plasma half-life is achieved with this antibody product but no ability to mediate effector functions, which limits the spectrum of possible therapeutic applications to indications depending on neutralization or agonistic activities of the antibody.

Several genetic modifications of *E. coli* strains have been undertaken in order to improve the folding and secretion capabilities for recombinant protein production. This includes strains over- or coexpressing chaperones for improved folding (Bothmann and Plückthun 2000; Zhang et al. 2003) as well as strains overexpressing disulfide isomerases for more efficient disulfide bond formation (Humphreys et al. 1996; Kurokawa et al. 2001). Further, protease-deficient strains have been developed to reduce periplasmic degradation of the product (Wulfing and Rappuoli 1997; Kandilogiannaki et al. 2001; Chen et al. 2004). With a triple mutant *E. coli* host strain yields of up to $2\,g\,L^{-1}$ for a $F(ab')_2$ molecule have been reported (Chen et al. 2004).

As an alternative to the traditionally used Sec-secretion pathway for periplasmic expression of antibody fragments, the TAT (twin arginine translocation) pathway can be used for exporting recombinant proteins to the periplasm (DeLisa et al. 2003). In contrast to the Sec-secretion pathway, where unfolded polypeptides are transferred to the periplasm (van Wely et al. 2001), the TAT system exports proteins only in a folded state (Berks et al. 2000), which could increase the yield of correctly folded and biologically active material. Since disulfide bond formation is efficient only in an oxidative milieu, strains with an oxidizing cytoplasm are used for efficient antibody expression (DeLisa et al. 2003).

Purification of proteins accumulated in the periplasm of *E. coli* requires lysis of the outer membrane and the cell wall, which complicates the purification process. *E. coli* strains lacking the cell wall allow secretion of functional antibody product to the culture medium (Kujau et al. 1998).

6.2.1.2 *Proteus mirabilis*

The L-form of *Proteus mirabilis* is without a cell wall and is thus able to secrete signal peptide-containing proteins to the growth medium. Furthermore this *P. mirabilis* strain is easy to transform and can be cultured in fermenters. Miniantibodies have been produced using *P. mirabilis* as production host with similar yields as with an L-form of *E. coli* (Kujau et al. 1998). In a comparative expression analysis of several scFv antibodies in *P. mirabilis* and the *E. coli* strain JM109, yields of 40–200 $mg\,L^{-1}$ active soluble antibody could be reached with *P. mirabilis*, which was about 60 times higher than with *E. coli* (Rippmann et al. 1998). An explanation could be the reduced toxic effects of the product which is released to the culture medium instead of accumulating in the periplasm.

6.2.1.3 *Bacillus subtilis* and *Bacillus brevis*

The lack of an outer membrane and endotoxins make Gram-positive bacteria attractive host cell candidates for the secretory expression of recombinant proteins

such as antibodies. N-terminal fusion of appropriate signal peptides or secretion domains facilitates export of the recombinant protein to the culture media which is advantageous for purification. Problems related to endogenous proteases can be avoided using protease-deficient mutant strains (Wu et al. 1993; Takimura et al. 1997; Wu et al. 1998; Wu et al. 2002).

Bacillus subtilis and *Bacillus brevis* both have been successfully used to produce scFv or Fab antibody fragments (Wu et al. 1993; Inoue et al. 1997; Wu et al. 1998; Bolhuis et al. 1999; Shiroza et al. 2001; Wu et al. 2002).

In *B. subtilis* the formation of inclusion bodies can limit the secretory production of antibody fragments. Overexpression of intracellular and extracytoplasmic molecular chaperones could substantially reduce the problem of inclusion body formation of a scFv fragment. The percentage of intracellular insoluble scFv protein dropped from 60% to 6%, while the percentage of secreted scFv increased from 23% to 43%, giving yields of up to $12\,mg\,L^{-1}$ (Wu et al. 1998).

Bacillus brevis has the advantage of naturally secreting much fewer proteases than *B. subtilis* and of being able to secrete heterologous proteins with disulfide bonds, like human epidermal growth factor (EGF) (Yamagata et al. 1989). In *B. brevis* an scFv antibody with a yield of $10\,mg\,L^{-1}$ (Shiroza et al. 2001) as well as a chimeric Fab fragment with an estimated yield of $100\,mg\,L^{-1}$ (Inoue et al. 1997) were produced. For Fab production the light chain and the heavy chain fragment of the Fab were expressed from a dicistronic construct, secretion was achieved by N-terminal fusion of the signal peptide of the middle-wall protein. The polypeptide chains were correctly processed and formed disulfide-linked heterodimeric Fab fragments.

Possible limitations of using *Bacillus* as expression host are relative low transformation efficiencies and plasmid stability. Further, for *Bacillus subtilis* bottlenecks for efficient secretion of functional proteins can be processing by the signal peptidase, passage through the cell wall, degradation in the wall and growth medium and misfolding due to incorrect disulfide bond formation (Bolhuis et al. 1999).

6.2.2 Eukaryotic Expression Systems

Eukaryotic systems are the method of choice if the antibody product needs to be glycosylated for its biological functions. Protein secretion in eukaryotes occurs via the endoplasmic reticulum and Golgi apparatus and is aided by chaperones and cofactors. This is a complex process but the principles are the same for all eukaryotic organisms. However, the pattern of protein glycosylation differs between different species, even if they are very closely related. For therapeutic antibodies it is aimed to have a glycosylation pattern as close to the human one as possible to minimize immunogenicity and, for some applications, to maximize induction of immunological effector functions. Besides production levels and cost, the glycosylation pattern of eukaryotic expression hosts therefore is one of the most noted rating characteristics of the production system. The set of eukary-

otic hosts that in the meantime have been developed for and applied to antibody expression ranges from simple organisms such as yeasts to transgenic mammals and plants.

6.2.2.1 Yeast

Yeasts share some advantageous properties with *E. coli*, such as rapid growth in cheap growth media, applicability for fermentation processes with high cell densities, and ease of genetic manipulation. In addition they provide a eukaryotic folding and secretion apparatus that allows secretion of functionally folded proteins to the culture media. This combination makes yeast an attractive expression system for large-scale production. Among the yeast species used for recombinant antibody expression are the well-characterized species *Saccaromyces cerevisiae*, as well as more unconventional hosts such as *Yarrowia lipolytica* and *Kluyveromyces lactis*, or the increasingly popular methylotrophic yeast *Pichia pastoris*, which has very promising secretion yields. The main issues for antibody expression in yeast are secretion rate, proteolytic activity, and glycosylation pattern.

Saccharomyces cerevisiae, which still is the most commonly used yeast species in pharmaceutical production (Schmidt 2004), has been used successfully for the expression of recombinant antibodies (Wood et al. 1985; Shusta et al. 1998). However, the secretion capabilities of *S. cerevisiae* for complex proteins seem to be limited (Rakestraw and Wittrup 2006) and hyperglycosylation of the recombinant proteins can lead to reduced pharmacological activity and further reduction of the secretion efficiency. Optimization of the gene expression and coexpression of chaperones or foldases lead to production yields of 20 mg L^{-1} for an scFv antibody (Shusta et al. 1998).

The two unconventional yeasts, *Y. lipolytica* and *K. lactis*, are regarded as safe (GRAS) organisms and are thought to have a good potential for the secretion of heterologous proteins. Swennen et al. (2002) report the production of an scFv antibody in *Y. lipolytica* and *K. lactis* with levels up to 10–20 mg L^{-1} showing the potential of both species for antibody production.

Within only a short period of time *Pichia pastoris* has become the most frequently used yeast species for recombinant protein production (Schmidt 2004; Gurkan and Ellar 2005; Macauley-Patrick et al. 2005). As a methylotrophic yeast *Pichia* is able to utilize methanol as sole carbon source. Since the alcohol oxidase enzyme of the methanol-assimilating pathway can reach up to 30% of the total protein of the cell, the endogenous alcohol oxidase promoter (AOX) provides a tightly regulated and powerful expression regulator. The very high homologous recombination rate of yeasts allows simple and efficient integration of the gene of interest into the AOX locus of the host cell genome, leading to production clones with high expression levels and long-term stability. By increasing the gene copy number an increasing expression of the recombinant protein can be achieved (Macauley-Patrick et al. 2005). However, in cases where folding and disulfide bond formation are the rate-limiting steps, a higher gene dose can also have no or even a negative effect (Hohenblum et al. 2004).

Pichia pastoris secretes only very low levels of endogenous proteins and can be cultured in well-defined minimal media with very low or no protein content (Hellwig et al. 2001), which predestines it for secretory expression and simplifies purification processes. Very high secretion levels for recombinant proteins have been reported using the *S. cerevisiae* alpha factor prepro peptide as secretion signal sequence for *P. pastoris*, reaching up to 15 g L^{-1} for murine collagen (Werten et al. 1999). However, heterologous proteins secreted via the alpha factor prepro leader have been shown to commonly contain variable N-termini with different numbers of N-terminal amino acids added to the protein of interest (Brocca et al. 1998). This is assumed to be due to influences of the N-terminal protein sequence on the efficiency of the two-step processing of the leader peptide. Initially, a part of the leader peptide is cleaved by the Kex2 protease before the Ste13 protease removes the remaining amino acids. If the cleavage efficiency of Ste13 is affected by the protein sequence, a mixture of partially processed and completely processed material can be found in the culture media.

There is little information on *P. pastoris* proteases, but proteolytic degradation of the recombinant product in the culture medium can be a problem. Three different types of proteases have been identified in *P. pastoris* cultures during the optimization of the expression of an scFv antibody: aspartic, cysteine, and serine type proteases (Shi et al. 2003). Strategies for the reduction of proteolysis include adaptation of culture conditions (e.g. pH and temperature), addition of protease inhibitors, use of protease-deficient strains, and adaptation of the sequence of the recombinant protein (Macauley-Patrick et al. 2005).

The glycosylation pattern of *P. pastoris* differs from that of *S. cerevisiae*, although both mainly produce *N*-linked glycosylation of the high-mannose type. In *P. pastoris*, there is less hyperglycosylation found and the average length of the added oligosaccharide chains is much shorter (Tschopp et al. 1987; Grinna and Tschopp 1989). The terminal α1,3 glycans found on the core oligosaccharides of *S. cerevisiae* are believed to be primarily responsible for the high antigenic nature of glycoproteins produced in *S. cerevisiae*. Since those α1,3 glycans are missing in *P. pastoris*-derived glycoproteins they are thought to be more suitable for therapeutic applications (Cregg et al. 1993). Nevertheless, a human-like glycosylation pattern with complex *N*-glycans is highly desirable for therapeutic applications and developments towards the engineering of *Pichia* strains capable of generating human-like glycosylation are ongoing (Choi et al. 2003; Hamilton et al. 2003; Gerngross 2004). After inactivation of an initial yeast-type glycosylation step by deleting the *OCH1* (α1,6 mannosyltransferase) gene, a combinatorial approach of fusing different mannosidase catalytic domains with different yeast localization domains was used to select a strain that efficiently trims core glycans *in vivo*, a prerequisite for the conversion of high mannose-tye glycans to hybrid or complex *N*-glycans. To further humanize the glycosylation pattern a second gene library of GnTI (β1,2-*N*-acetylglucosaminyltransferase I) catalytic domains combined with localization signals was screened and led to the identification of a strain producing human-like hybrid *N*-glycosylation structures (Choi et al. 2003). However, further removal of mannose and addition of β1,2-GlcNac would be

required to assemble complex *N*-glycans. Based on this work further adaptation of the glycosylation pattern was performed using a similar approach leading to the generation of a *Pichia* strain producing glycoproteins with uniform complex *N*-glycosylation (Hamilton et al. 2003). By analyzing Fc-receptor binding of antibodies produced in a set of *Pichia* strains with different glycosylation patterns, antibody glycoforms with improved ADCC efficiency could be identified (Li et al. 2006). *Pichia*-derived glycoforms with 10-fold improved binding affinity to the V158 allele of FcγRIIIa and 100-fold improved binding to the F158 allele of FCγRIIIa compared with commercial CHO-derived IgG (rituximab) were found. They were also shown to be more efficient than the mammalian material in a B-cell depletion assay.

The antibodies that so far have been produced in *Pichia* are mainly in the scFv format (Ridder et al. 1995; Luo et al. 1996; Eldin et al. 1997; Hellwig et al. 2001; Shi et al. 2003; Damasceno et al. 2004; Gurkan et al. 2004; Trentmann et al. 2004; Emberson et al. 2005), but production of Fab fragments (Lange et al. 2001; Ning et al. 2003), an scFv-Fc fusion (Powers et al. 2001), ds-diabodies (FitzGerald et al. 1997), and a bispecific tetravalent antibody fusion protein (Biburger et al. 2005) have also been reported. Expression of entire functional IgG antibodies in *Pichia* has been demonstrated but only a portion of the secreted antibody chains were assembled to intact tetrameric antibody (Ogunjimi et al. 1999). Recently, production of different glycoforms of IgG in *Pichia* has been reported, although there was no information on production yields (Li et al. 2006).

Using an online methanol control in fed-batch fermentation expression of an scFv antibody, yields of about $4\,g\,L^{-1}$ could be reached, which to our knowledge is the highest yield of a yeast-expressed antibody reported so far (Damasceno et al. 2004). This clearly demonstrates the potential of *Pichia* for high-level production of antibodies in fermentation processes. However, with proteins getting more complex, the expression levels of functional protein are decreasing. For a Fab fragment the portion of correctly assembled heteromeric protein in the culture medium was found to be 30%, which is primarily attributed to the failure of one of the chains to find a partner (Lange et al. 2001). Yeast-expressed scFv-Fc fusion proteins might be of special therapeutic value since they share some important features with intact IgG antibodies. Disulfide-linked dimeric scFv-Fc fusions could be produced in *Pichia* with yields of $2\,mg\,L^{-1}$ and were shown to efficiently mediate ADCC and to have a 12- to 30-fold prolonged *in vivo* plasma half-life compared with scFv alone (Powers et al. 2001).

6.2.2.2 **Filamenteous Fungi**

Filamenteous fungi such as *Aspergillus* or *Trichoderma* are known to be capable of secreting very high levels of homologous proteins ($30–40\,g\,L^{-1}$) and are well established in the industrial production of enzymes, antibiotics, etc. Further, fungi have a posttranslational modification apparatus that is more similar to mammalian cells than that of yeast, and several species possess the GRAS status (Schmidt 2004). For recombinant heterologous products, the secretion levels have so far failed to reach the levels achieved by endogenous proteins. While

heterologous fungal enzymes are produced with yields of up to 4 g L^{-1}, yields of mammalian proteins usually stay below the g L^{-1} range (Schmidt 2004). Antibody fragments have been produced in *Trichoderma reesei* (Nyyssonen et al. 1993) and in *Aspergillus niger* (Frenken et al. 1998). Recently, Ward et al. reported the expression of functional humanized IgG and Fab antibodies in *Aspergillus niger* (Ward et al. 2004). Both antibody chains were expressed with N-terminal fusion of the *Aspergillus* enzyme glucoamylase to improve the secretion efficiency. A KexB cleavage site was inserted between antibody chain and glucoamylase to mediate cleavage of the fusion protein in the Golgi apparatus by the native *Aspergillus* protease KexB. The cleavage efficiency did not reach 100% and the cleavage position varied, leading to heterogeneity of the N-termini. By adding a stuffer of three glycines between the antibody N-terminus and cleavage site the cleavage efficiency could be increased, leading to homogeneous N-termini. However, the three glycine residues thereby remain added to the N-termini of the processed antibody. The additional N-terminal amino acids had no influence on the binding activity of the antibodies and the *Aspergillus*-derived material was found to have similar affinities to antibodies derived from mammalian cell cultures. Approximately half of the secreted heavy chains were *N*-glycosylated with glycans of the high mannose type. Analysis of the *in vivo* serum half-life suggests that there is no significant difference to antibodies from mammalian production and the antibodies from *Aspergillus* production were shown to efficiently mediate ADCC. Secretion levels of up to 0.9 g L^{-1} were achieved in this study which clearly demonstrates that *Aspergillus niger* is a promising expression host for industrial full-length antibody production. For therapeutic applications improvement of the processing leading to native N-termini and a human-like glycosylation pattern to reduce imunogenecity are desirable.

6.2.2.3 **Insect Cells**

The baculovirus expression vector system for recombinant protein production in insect cells is a well-established system, especially in research and development. Gene transfer to the expression host in this system is based on the infection of insect cell lines that are usually derived from *Spodoptera frugiperda* (SF9 and SF-21) or *Trichoplusia ni* (High Five) with recombinant baculovirus. Advantages of the system are high transduction efficiencies, posttranslational modifications of higher eukaryotes, and high expression levels driven by strong baculoviral promoters (Hu 2005). There are numerous examples of recombinant antibody expression using baculovirus-infected insect cells including scFv (Kretzschmar et al. 1996; Brocks et al. 1997; Lemeulle et al. 1998; Ailor et al. 1999; Yoshida et al. 1999; Reavy et al. 2000; Demangel et al. 2005), Fab (Bes et al. 2001), scFv-Fc (Brocks et al. 1997), scFv-based immunotoxins (Choo et al. 2002), fluorescent scFv fusions (Peipp et al. 2004), full-length IgG (Hasemann and Capra 1990; Liang et al. 1997; Tan and Lam 1999; Liang et al. 2001), and IgA antibodies (Carayannopoulos et al. 1994). Yields of up to 200 mg L^{-1} for an scFv (Reavy et al. 2000) and 70 mg L^{-1} for entire IgG (Tan and Lam 1999) have been achieved.

One of the major disadvantages associated with the baculovirus expression system are cell death and lysis of the host insect cells within days after the infection. For secretory proteins, like antibodies, this can raise the problem of the release of unprocessed immature antibody polypeptides to the culture medium, especially since the highest expression levels driven by the strong polyhedrin promoter are noticed briefly prior to cell lysis. In that phase processing of proteins is already seriously affected by the damage of the secretory pathway (Jarvis and Summers 1989; Hu 2005). Another problem connected with a lytic expression system is protein degradation due to the release of proteases. To overcome these problems nonlytic transient and stable expression systems for insect cells have been developed that are based on cell transfection instead of baculovirus infection (Jarvis et al. 1990). Stably transformed insect cells have been used to produce full-length IgG antibodies, but the yields were considerably lower than those from transient expression after baculovirus infection (Guttieri et al. 2000, 2003).

The *N*-glycosylation pattern of insect cells differs from that of mammalian cells (Tomiya et al. 2004; Hu 2005; Kost et al. 2005). Insect cells can assemble *N*-glycans of the high mannose and paucimannose type but typically fail to produce *N*-glycans of the complex mammalian type with terminal galactose or sialic acid residues. This limits the suitability of the expression system for therapeutic approaches, which led to intensive research on the humanization of the glycosylation pattern of insect cells (Jarvis 2003). Transgenic insect cell lines were generated that produce *N*-glycan-processing enzymes of mammalian origin. Insect cells expressing bovine β1,4-galactosyltransferase have been shown to produce proteins with terminally galactosylated *N*-glycans (Hollister et al. 1998). The addition of a rat α2,6-sialyltransferase transgene to this cell line led to the generation of monoantennary terminally sialylated *N*-glycans (Hollister and Jarvis 2001). Further efforts led to the generation of a transgenic insect cell line that expresses five mammalian glycosyltransferases (Hollister et al. 2002) and finally a cell line that in addition contains transgenes for two murine enzymes to allow *de novo* glycoprotein syalization was created that produced a recombinant protein with highly homogeneous biantennary sialylated *N*-glycans (Aumiller et al. 2003).

6.2.2.4 **Mammalian Cells**

Despite rather high costs, currently 60–70% of all recombinant biopharmaceuticals and virtually all therapeutic monoclonal antibodies are produced in mammalian cell culture processes (Wurm 2004). During the last two decades a 100-fold improvement of production yields has been achieved for industrial large-scale processes. In 1986, when t-PA (tissue plaminogen activator) was the first recombinant pharmaceutical protein produced in CHO cells, the yield was about $50\,mg\,L^{-1}$. Nowadays, yields of up to $4.7\,g\,L^{-1}$, as reported for a recombinant antibody, are obtainable (Wurm 2004). This increase is mainly due to higher volumetric production yields based on much higher cell densities in the production process. Since the specific production rates of the cells during this period improved only moderately, there might be still potential to further improve production

yields by using alternative cell lines, genetic engineering of production cell lines, and optimization of expression vectors (Fussenegger et al. 1999; Wurm 2004; Dinnis and James 2005).

Currently, the most widely used cell line for antibody production is the CHO cell line. To a lesser extent the murine myeloma (NS0, SP2/0), BHK, and HEK293 (human embryonic kidney) cell lines are also used. Besides these more traditional host cell lines the human retinoblastoma cell line PER.C6 recently has been shown to efficiently produce recombinant IgG at high cell densitities (Jones et al. 2003). Among the advantages of this cell line are the human origin, leading to a nonimmunogenic glycosylation pattern, and the ability to adapt quickly to serum-free suspension culture conditions.

Gene transfer in mammalian systems for stable expression is usually based on integration of the expression cassette into the host cell genome. Gene integration in principal can occur in three different ways: random integration, homologous recombination, and recombinase-mediated targeted integration. It is widely accepted that the site of integration in the host genome has a dominant impact on expression level and stability of the recombinant cell line (position effect). Furthermore, even after integration into expression-competent euchromatin, expression of the transgene can be silenced rapidly (Mutskov and Felsenfeld 2004). Gene silencing might be influenced by surrounding endogenous condensed chromatin and is correlated with histone hypoacetylation, loss of methylation at H3 lysine 4, increase of histone H3 lysine 9 methylation and CpG DNA methylation at the promoter. The histone modifications thereby seem to be the primary events in gene silencing (Mutskov and Felsenfeld 2004). Using random integration-based methods, the generation of stable transfectants with high expression levels and high stability of transgene expression therefore often requires screening of a high number of clones. Alternatively, homologous recombination can be used, which allows targeted integration of the transgene at genomic sites that are known to allow high and stable expression. However, in the mammalian cell lines that are usually used for recombinant protein expression, homologous recombination is a much less frequent event than in yeast cells, which makes this strategy quite inefficient for biotechnological processes. To overcome this limitation site-specific targeted integration methods with higher recombination frequencies are developed (Bode et al. 2000). Site-directed targeted integration is mediated by recombinases that catalyze DNA strand exchange reactions between short target sequences. The first described site-specific recombination systems are based on the recombinases Cre from the phage P1 and Flp from the yeast *Saccaromyces cerevisiae* with their recognition sites LoxP and FRT, respectively (O'Gorman et al. 1991; Fukushige and Sauer 1992). Both systems can be used to perform a targeted integration driven by recognition sites localized on a circular targeting vector and the genomic DNA. However, the efficiency of the integration reaction is limited by competition with the thermodynamically favored re-excision reaction (Bode et al. 2000). Pairs of mutant recognition sites were developed that allow advanced gene exchange procedures (RMCE, recombinase mediated cassette exchange) for Cre (Araki et al. 1997) and Flp (Seibler

et al. 1998), like the recombinase-mediated exchange of a selection marker/reporter gene for the gene of interest in the genome of an acceptor cell line. Acceptor cell lines can be generated using reporter or selection genes or a combination of both to find and label genomic integration sites with high expression activity and long-term stability. Another advantage of the engineered recognition sites is the possibility to only insert the gene of interest without cointegration of prokaryotic vector components that might promote gene silencing. However, so far high-efficiency application of this principle is mainly restricted to embryonic stem (ES) cells.

A different approach to overcome gene silencing and position effects is the use of scaffold- or matrix-attachment regions (S/MAR elements) of chromosomal DNA flanking the expression cassette, which can augment transgene expression and reduce negative influence by surrounding chromatin (Klehr et al. 1991; Fussenegger et al. 1999). Genetic insulator elements, which naturally mark boundaries between open chromatin and constitutively condensed chromatin, can also be used to protect transgenes against silencing and position effects (Burgess-Beusse et al. 2002). However, the enhancer-blocking function of the insulator elements at the same time might reduce the transcriptional activity of the promoter.

Since the generation of stable recombinant mammalian cell lines is a time-consuming procedure, transient expression systems are gaining importance, especially in antibody production for research and development. Mainly host cells that are capable of episomal replication of suitable expression vectors are used, like COS monkey cell lines or the derivatives of the human HEK293 cell line HEK293-EBNA and HEK293-T (Fussenegger et al. 1999; Meissner et al. 2001; Jostock et al. 2004). HEK293 cells are very popular, since they allow high transfection efficiencies and constitutively express the adenoviral E1A transactivator which enhances transcription (Fussenegger et al. 1999). Vectors containing an SV40 origin of replication (ori) are highly amplified in cells expressing the T antigen of simian virus 40 (SV40), like COS and HEK293-T, reaching up to 200 000 copies per cell 48 h after transfection. This leads to very high expression levels but also finally culminates in cell death, which limits the system to transient production processes (Van Craenenbroeck et al. 2000). Recombinant IgG expression levels of 15–20 mg L^{-1} have been reported for transient transfections of HEK293T cells (Jostock et al. 2004).

Episomal replication driven by the EBNA1 transactivator and oriP from Epstein–Barr virus (EBV) leads to much lower copy numbers (5–100) but very high retention time of the plasmids (Van Craenenbroeck et al. 2000). Retention rates of 92–98% per cell generation are typical of EBV vectors in the absence of selection. Under selective pressure transgene expression is stable over a long period of time with the EBV expression system. Transient expression in HEK293-EBNA cells yielded up to 40 mg L^{-1} of recombinant IgG (Meissner et al. 2001).

Besides these viral replication systems, vectors for epsiomal replication driven by chromosomal S/MAR elements are also in development (Piechaczek et al. 1999; Jenke et al. 2004).

Upscaling of transient transfection of suspension-adapted HEK293-EBNA cells in bioreactors has been reported up to the 100 L scale with a production yield of 0.5 g IgG antibody (Girard et al. 2002). Thus, transient large-scale transfection might become a very time-efficient alternative for therapeutic antibody production in an early phase of development.

Several genetic engineering strategies, based on the overexpression of individual chaperones and foldases, have been applied to improve specific antibody production levels by mammalian host cells. However, although it is generally presumed that antibody production is limited by the posttranslational folding and assembly reactions, so far no reliable increase of production levels has been achieved with these engineered cell lines (Dinnis and James 2005). It is proposed that a global expansion of the complete secretory machinery, as is found during the differentiation of B cells to high-level antibody-producing plasma cells, would be necessary to generally improve the production capabilities of host cell lines (Dinnis and James 2005).

6.2.2.5 **Plants**

The almost unlimited upscaling potential of transgenic plants as production hosts for biopharmaceuticals is clearly a conceptional advantage compared with bioreactor-based production in microbial and mammalian cells. In 1990, human serum albumin was the first recombinant protein with pharmaceutical potential to be produced in transgenic plants (Sijmons et al. 1990; Ma et al. 2005). Since then, substantial improvements of transgenic plant technology have been achieved and currently several plant-derived recombinant proteins are in clinical studies, including antibodies (Ma et al. 2005).

The initial step of protein production in plants is the transfer of the gene of interest into the host (transformation). Plant transformation using physical methods is rather inefficient and usually done by biolistic gene delivery (gene gun) in the case of monocots, such as wheat, rice, and corn. In the case of dicots, such as tobacco and pea, *Agrobacterium*-mediated gene transfer is the method of choice (Schillberg et al. 2005). Both stable and transient plant expression systems have been developed. The generation of stable transgenic plants is quite time consuming and depends on the genomic integration of the transgene in the nuclear or plastid DNA. Integration in the genome of plastids, like chloroplasts in tobacco, has the advantage of high copy numbers of the transgene resulting in high expression levels of the recombinant protein but also an increased risk of proteolysis of the product (Ma et al. 2005).

Transient expression is less time consuming but limited in scale. Commonly used transient expression systems are agroinfiltration using recombinant *Agrobacterium tumefaciens* and viral vectors based on plant viruses like the tobacco mosaic virus (Schillberg et al. 2005). ScFv as well as full-size antibodies have been transiently produced in plants (Verch et al. 1998; Galeffi et al. 2005).

Tobacco is among the most commonly used species for molecular farming. Several other species, such as maize, potato, rice, wheat, pea, tomato, and banana, are also amenable to recombinant protein production. Comparison of different

production hosts with regards to production levels per kilogram of biomass of an scFv antibody revealed that the yields varied among different species and expression systems but were broadly comparable. In general, for all species tested, yields could be improved by accumulating the scFv in the endoplasmic reticulum via a KDEL ER-retention signal (Schillberg et al. 2005).

As higher eukaryotes, plants are able to synthesize and process even complex multi-subunit proteins such as full-size antibodies. Plant chaperones are homologous to those of mammalian cells, which ensures a high folding capacity, and the secretion apparatus of plants performs posttranslational modifications including *N*-linked glycosylation (Ma et al. 2005). However, plant and mammalian complex *N*-glycans differ in their structures, which might result in impaired effector functions of plant antibodies. Furthermore, antibodies produced in tobacco show a highly heterogeneous *N*-glycosylation pattern and plant *N*-glycans are potentially allergenic and immunogenic (Gomord et al. 2005). Several strategies are currently employed to eliminate unwanted sugar residues and to humanize the glycosylation pattern of host plants via genetically engineering (Gomord and Faye 2004).

For an IgA antibody a yield of 500 $mg\,kg^{-1}$ biomass from tobacco has been reported (Ma et al. 1998). Besides production rates for molecular farming several other key features, such as biomass yield, length of a production cycle and cost of processing of the host species have to be considered to evaluate the market potential (Schillberg et al. 2005). The cost for the production of recombinant proteins in transgenic plants is believed to be much lower than in mammalian cell culture (Hood et al. 2002). However, in the case of therapeutic antibodies, which are often administered intravenously, processing of the plant biomass to extract the antibody product in suitable quality and purity is likely to be cost intensive.

6.2.2.6 Transgenic Animals

Along with transgenic plants, transgenic animals might also become an alternative to bioreactor systems for the production of biopharmaceuticals. Mammary gland-specific expression of the transgene to target the recombinant protein to the milk of farm animals allows continuous recovery of the product during the lifespan of adult female animals. To achieve this, chimeric transgenes consisting of the regulatory elements of milk-specific genes and the coding regions of the gene of interest are used. Examples for such milk specific genes are the ovine β-lactoglobulin, rodent whey acid protein (WAP) and bovine α-s1-casein genes (Pollock et al. 1999). The chimeric transgenes are usually delivered to the host animals by pronuclei microinjection. Although well established, the efficiency of transgene integration into the host genome using this method is rather low and the time from initiating microinjection to full lactation is long (16–18 months for goats; Pollock et al. 1999).

The most commonly used animal species for transgenic protein expression are mouse, rabbit, pig, sheep, goat, and cattle. To date, recombinant antibodies have been produced in transgenic milk of mice and goats. The yields that are reported

for IgG are between 0.4 and 5 g L^{-1} in mice and up to 14 g L^{-1} in goats (Limonta et al. 1995; Castilla et al. 1998; Pollock et al. 1999). Small animals such as mice and rabbits have the advantage of good reproductive characteristics but the disadvantage of low milk yields, which makes them most suitable for the testing of expression systems. Goats have an average milk output of 600–800 L per 300-day lactation, thus, herds of transgenic goats should be able to produce kilogram amounts of product per year (Pollock et al. 1999).

It is expected that antibodies derived from transgenic mammals have a glycan pattern similar to material from mammalian cell culture. Since the antigen-binding properties have been shown to be similar or equivalent (Pollock et al. 1999), they may have a comparable clinical efficacy. Among the main advantages of transgenic animals for antibody production are the flexible scalability and cost-efficient maintenance of the production facilities. Disadvantages are time- and labor-intensive generation of founder animals as well as safety issues regarding the animal-derived material if intravenous application of the product is planned. Milk from transgenic animals also contains endogenous antibodies which might be difficult to separate from the product and the animals are potential hosts for pathogenic microorganisms, viruses, and prions.

6.3 Outlook

Mammalian cell lines are currently the dominant system for production processes of full-sized antibodies, and *E. coli* for antibody fragments. Both systems still may be further optimized by genetic engineering of the host cell lines for improved folding, secretion, and growth characteristics. Besides those, *P. pastoris* and insect cells are well established, especially in research. Due to high growth rates, high production levels, ease of handling and rapid generation of stable transformants, *P. pastoris* has good potential to reduce cost and time-lines of production processes. The ongoing humanization of the glycosylation pattern of *P. pastoris* might allow the production of antibody products with clinical efficacies and pharmacokinetics comparable to material from mammalian cell culture processes. However, high expression levels are mainly reported for antibody fragments rather than for entire IgG molecules. For insect cell lines developments towards the humanization of the glycolsylation pattern are also ongoing but there is still a demand for the development of high cell density large-scale production processes and efficient technologies to generate stable production cell lines. Because of their intrinsic high-performance secretion machineries, Gram-positive bacteria and filamentous fungi are well suited in general to production processes and several species are established production hosts in the food industry, but the application of these hosts for recombinant heterologous protein production is still in the development stage. Gram-positive bacteria are unable to perform eukaryote-like posttranslational modifications and thus might be suitable for the production of aglycosylated antibodies and antibody fragments only. The glycosylation pattern of filamentous fungi would have to be humanized to

obtain high-quality therapeutic products. Due to the virtually unlimited scalability and comparably low maintenance of the production facilities, transgenic plants and animals probably have the highest potential to reduce the costs of antibody production for applications with a high product demand. Long time-lines for the generation of the producer strains, complex and expensive downstream processes and finally unclear safety issues for the regulatory approval of the products and production facilities are the main hurdles that need to be cleared to make this approach state of the art.

In summary, substantial effort is currently underway to develop new alternative production systems for the growing market of recombinant antibody therapeutics. Some of the systems are close to market maturity while others are pretty much in an early phase of development. An overview of examples of the production systems presented in this chapter is given in Table 6.1. With the arrival of

Table 6.1 Examples of production levels for different expression systems.

Host	*Format*	*Yield*	*Citation*
Echerichia coli	F(ab′)2	2 g L^{-1}	Chen et al. 2004
	IgG	150 mg L^{-1}	Simmons et al. 2002
Proteus mirabilis	scFv	200 mg L^{-1}	Rippmann et al. 1998
	Mini-antibody	18 mg L^{-1}	Kujau et al. 1998
Bacillus brevis	scFv	10 mg L^{-1}	Shirazoa et al. 2001
	Fab	100 mg L^{-1}	Inoue et al. 1997
Bacillus subtilis	scFV	15 mg L^{-1}	Wu et al. 2002
Pichia pastoris	scFv	4 g L^{-1}	Damasceno et al. 2004
	Fab	40 mg/L	Lange et al. 2001
	ds-diabody	1 mg L^{-1}	FitzGerald et al. 1997
	scFv-Fc	2 mg L^{-1}	Powers et al. 2001
	IgG	n.s.	Ogunjimi et al. 1999 Li et al. 2006
Saccharomyces cerevisiae	scFv	20 mg L^{-1}	Shusta et al. 1998
	IgM	n.s.	Wood et al. 1985
Yarrowia lipolytica	scFv	20 mg L^{-1}	Swennen et al. 2002
Kluyveromyces lactis	scFv	10 mg L^{-1}	Swennen et al. 2002
Aspergillus niger	IgG	0.9 g L^{-1}	Ward et al. 2004
Insect cells (SF9)	IgG	18 mg L^{-1}	Liang et al. 2001
Insect cells (SF21)	IgG	40 mg L^{-1}	Tan et al. 1999
Insect cells (High Five)	IgG	70 mg L^{-1}	Tan et al. 1999
Mammalian cells (CHO)	IgG	4.7 g L^{-1}	Wurm F.M. 2004
Mammalian cells (PER.C6)	IgG	0.5–1 g L^{-1}	Jones et al. 2003
Tobacco	IgA	80 mg kg^{-1}	Ma et al. 1998
Transgenic mice	IgG	5 g L^{-1}	Castilla et al. 1998
Transgenic goat	IgG	14 g L^{-1}	Pollock et al. 1999

Volumetric productivity values for different expression hosts and antibody formats are shown. It should be noted that the comparability of the values to each other is very limited since no equalization of scale, conditions, and runtime of the expression or quality of the product was made.

biosimilars for therapeutics with expired patents, the pressure to reduce production costs will rise even further, since direct competition between different producers for the same biotherapeutic product will lead to significant market price reductions.

References

Ailor, E., Pathmanathan, J., Jongbloed, J.D., Betenbaugh, M.J. (1999) A bacterial signal peptidase enhances processing of a recombinant single chain antibody fragment in insect cells. *Biochem Biophys Res Commun* 255: 444–450.

Araki, K., Araki, M., Yamamura, K. (1997) Targeted integration of DNA using mutant lox sites in embryonic stem cells. *Nucleic Acids Res* 25: 868–872.

Aumiller, J.J., Hollister, J.R., Jarvis, D.L. (2003) A transgenic insect cell line engineered to produce CMP-sialic acid and sialylated glycoproteins. *Glycobiology* 13: 497–507.

Berks, B.C., Sargent, F., Palmer, T. (2000) The Tat protein export pathway. *Mol Microbiol* 35: 260–274.

Bes, C., Cerutti, M., Briant-Longuet, L., Bresson, D., Peraldi-Roux, S., Pugniere, M., Mani, J.C., Pau, B., Devaux, C., Granier, C., Devauchelle, G., Chardes, T. (2001) The chimeric mouse-human anti-CD4 Fab 13B8.2 expressed in baculovirus inhibits both antigen presentation and HIV-1 promoter activation. *Hum Antibodies* 10: 67–76.

Biburger, M., Weth, R., Wels, W.S. (2005) A novel bispecific tetravalent antibody fusion protein to target costimulatory activity for T-cell activation to tumor cells overexpressing ErbB2/HER2. *J Mol Biol* 346: 1299–311.

Bode, J., Schlake, T., Iber, M., Schubeler, D., Seibler, J., Snezhkov, E., Nikolaev, L. (2000) The transgeneticist's toolbox: novel methods for the targeted modification of eukaryotic genomes. *Biol Chem* 381: 801–813.

Bolhuis, A., Tjalsma, H., Smith, H.E., de Jong, A., Meima, R., Venema, G., Bron, S., van Dijl, J.M. (1999) Evaluation of bottlenecks in the late stages of protein secretion in *Bacillus subtilis*. *Appl Environ Microbiol* 65: 2934–2941.

Bothmann, H., Plückthun, A. (2000) The periplasmic *Escherichia coli* peptidylprolyl cis,trans-isomerase FkpA. I. Increased functional expression of antibody fragments with and without cis-prolines. *J Biol Chem* 275: 17100–17105.

Boyd, P.N., Lines, A.C., Patel, A.K. (1995) The effect of the removal of sialic acid, galactose and total carbohydrate on the functional activity of Campath-1H. *Mol Immunol* 32: 1311–1318.

Brocca, S., Schmidt-Dannert, C., Lotti, M., Alberghina, L., Schmid, R.D. (1998) Design, total synthesis, and functional overexpression of the *Candida rugosa* lip1 gene coding for a major industrial lipase. *Protein Sci* 7: 1415–1422.

Brocks, B., Rode, H.J., Klein, M., Gerlach, E., Dübel, S., Little, M., Pfizenmaier, K., Moosmayer, D. (1997) A TNF receptor antagonistic scFv, which is not secreted in mammalian cells, is expressed as a soluble mono- and bivalent scFv derivative in insect cells. *Immunotechnology* 3: 173–184.

Burgess-Beusse, B., Farrell, C., Gaszner, M., Litt, M., Mutskov, V., Recillas-Targa, F., Simpson, M., West, A., Felsenfeld, G. (2002) The insulation of genes from external enhancers and silencing chromatin. *Proc Natl Acad Sci USA* 99(Suppl 4): 16433–16437.

Carayannopoulos, L., Max, E.E., Capra, J.D. (1994) Recombinant human IgA expressed in insect cells. *Proc Natl Acad Sci USA* 91: 8348–8352.

Castilla, J., Pintado, B., Sola, I., Sanchez-Morgado, J.M., Enjuanes, L. (1998) Engineering passive immunity in transgenic mice secreting virus-neutralizing antibodies in milk. *Nat Biotechnol* 16: 349–354.

Chen, C., Snedecor, B., Nishihara, J.C., Joly, J.C., McFarland, N., Andersen, D.C., Battersby, J.E., Champion, K.M. (2004) High-level accumulation of a recombinant antibody fragment in the periplasm of *Escherichia coli* requires a triple-mutant (degP prc spr) host strain. *Biotechnol Bioeng* 85: 463–474.

Choi, B.K., Bobrowicz, P., Davidson, R.C., Hamilton, S.R., Kung, D.H., Li, H., Miele, R.G., Nett, J.H., Wildt, S., Gerngross, T.U. (2003) Use of combinatorial genetic libraries to humanize *N*-linked glycosylation in the yeast Pichia pastoris. *Proc Natl Acad Sci USA* 100: 5022–5027.

Choo, A.B., Dunn, R.D., Broady, K.W., Raison, R.L. (2002) Soluble expression of a functional recombinant cytolytic immunotoxin in insect cells. *Protein Expr Purif* 24: 338–347.

Cregg, J.M., Vedvick, T.S., Raschke, W.C. (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. *Biotechnology (NY)* 11: 905–910.

Damasceno, L.M., Pla, I., Chang, H.J., Cohen, L., Ritter, G., Old, L.J., Batt, C.A. (2004) An optimized fermentation process for high-level production of a single-chain Fv antibody fragment in *Pichia pastoris*. *Protein Expr Purif* 37: 18–26.

DeLisa, M.P., Tullman, D., Georgiou, G. (2003) Folding quality control in the export of proteins by the bacterial twin-arginine translocation pathway. *Proc Natl Acad Sci USA* 100: 6115–6120.

Demangel, C., Zhou, J., Choo, A.B., Shoebridge, G., Halliday, G.M., Britton, W. J. (2005) Single chain antibody fragments for the selective targeting of antigens to dendritic cells. *Mol Immunol* 42: 979–985.

Dinnis, D.M., James, D.C. (2005) Engineering mammalian cell factories for improved recombinant monoclonal antibody production: lessons from nature? *Biotechnol Bioeng* 91: 180–189.

Eldin, P., Pauza, M.E., Hieda, Y., Lin, G., Murtaugh, M.P., Pentel, P.R., Pennell, C.A. (1997) High-level secretion of two antibody single chain Fv fragments by *Pichia pastoris*. *J Immunol Methods* 201: 67–75.

Emberson, L.M., Trivett, A.J., Blower, P.J., Nicholls, P.J. (2005) Expression of an anti-CD33 single-chain antibody by *Pichia pastoris*. *J Immunol Methods* 305: 135–151.

FitzGerald, K., Holliger, P., Winter, G. (1997) Improved tumour targeting by disulphide stabilized diabodies expressed in *Pichia pastoris*. *Protein Eng* 10: 1221–1225.

Frenken, L.G., Hessing, J.G., Van den Hondel, C.A., Verrips, C.T. (1998) Recent advances in the large-scale production of antibody fragments using lower eukaryotic microorganisms. *Res Immunol* 149: 589–599.

Fukushige, S., Sauer, B. (1992) Genomic targeting with a positive-selection lox integration vector allows highly reproducible gene expression in mammalian cells. *Proc Natl Acad Sci USA* 89: 7905–7909.

Fussenegger, M., Bailey, J.E., Hauser, H., Mueller, P.P. (1999) Genetic optimization of recombinant glycoprotein production by mammalian cells. *Trends Biotechnol* 17: 35–42.

Galeffi, P., Lombardi, A., Donato, M.D., Latini, A., Sperandei, M., Cantale, C., Giacomini, P. (2005) Expression of single-chain antibodies in transgenic plants. *Vaccine* 23: 1823–1827.

Gerngross, T.U. (2004) Advances in the production of human therapeutic proteins in yeasts and filamentous fungi. *Nat Biotechnol* 22: 1409–1414.

Girard, P., Derouazi, M., Baumgartner, G., Bourgeois, M., Jordan, M., Jacko, B., Wurm, F.M. (2002) 100-liter transient transfection. *Cytotechnology* 38: 15–21.

Gomord, V., Faye, L. (2004) Posttranslational modification of therapeutic proteins in plants. *Curr Opin Plant Biol* 7: 171–181.

Gomord, V., Chamberlain, P., Jefferis, R., Faye, L. (2005) Biopharmaceutical production in plants: problems, solutions and opportunities. *Trends Biotechnol* 23: 559–565.

Grinna, L.S., Tschopp, J.F. (1989) Size distribution and general structural features of N-linked oligosaccharides from the methylotrophic yeast, *Pichia pastoris*. *Yeast* 5: 107–115.

Gurkan, C., Ellar, D.J. (2005) Recombinant production of bacterial toxins and their

derivatives in the methylotrophic yeast *Pichia pastoris*. *Microb Cell Fact* 4: 33.

Gurkan, C., Symeonides, S.N., Ellar, D.J. (2004) High-level production in Pichia pastoris of an anti-p185HER-2 single-chain antibody fragment using an alternative secretion expression vector. *Biotechnol Appl Biochem* 39: 115–122.

Guttieri, M.C., Bookwalter, C., Schmaljohn, C. (2000) Expression of a human, neutralizing monoclonal antibody specific to puumala virus G2-protein in stably-transformed insect cells. *J Immunol Methods* 246: 97–108.

Guttieri, M.C., Sinha, T., Bookwalter, C., Liang, M., Schmaljohn, C.S. (2003) Cassette vectors for conversion of Fab fragments into full-length human IgG1 monoclonal antibodies by expression in stably transformed insect cells. *Hybrid Hybridomics* 22: 135–145.

Hamilton, S.R., Bobrowicz, P., Bobrowicz, B., Davidson, R.C., Li, H., Mitchell, T., Nett, J.H., Rausch, S., Stadheim, T.A., Wischnewski, H., Wildt, S., Gerngross, T.U. (2003) Production of complex human glycoproteins in yeast. *Science* 301: 1244–1246.

Hasemann, C.A., Capra, J.D. (1990) High-level production of a functional immunoglobulin heterodimer in a baculovirus expression system. *Proc Natl Acad Sci USA* 87: 3942–3946.

Hellwig, S., Emde, F., Raven, N.P., Henke, M., van Der Logt, P., Fischer, R. (2001) Analysis of single-chain antibody production in *Pichia pastoris* using on-line methanol control in fed-batch and mixed-feed fermentations. *Biotechnol Bioeng* 74: 344–352.

Hohenblum, H., Gasser, B., Maurer, M., Borth, N., Mattanovich, D. (2004) Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. *Biotechnol Bioeng* 85: 367–375.

Hollister, J.R., Jarvis, D.L. (2001) Engineering lepidopteran insect cells for sialoglycoprotein production by genetic transformation with mammalian beta 1,4-galactosyltransferase and alpha 2,6-sialyltransferase genes. *Glycobiology* 11: 1–9.

Hollister, J.R., Shaper, J.H., Jarvis, D.L. (1998) Stable expression of mammalian beta 1,4-galactosyltransferase extends the *N*-glycosylation pathway in insect cells. *Glycobiology* 8: 473–480.

Hollister, J., Grabenhorst, E., Nimtz, M., Conradt, H., Jarvis, D.L. (2002) Engineering the protein *N*-glycosylation pathway in insect cells for production of biantennary, complex *N*-glycans. *Biochemistry* 41: 15093–15104.

Hood, E.E., Woodard, S.L., Horn, M.E. (2002) Monoclonal antibody manufacturing in transgenic plants–myths and realities. *Curr Opin Biotechnol* 13: 630–635.

Hu, Y.C. (2005) Baculovirus as a highly efficient expression vector in insect and mammalian cells. *Acta Pharmacol Sin* 26: 405–416.

Humphreys, D.P., Weir, N., Lawson, A., Mountain, A., Lund, P.A. (1996) Co-expression of human protein disulphide isomerase (PDI) can increase the yield of an antibody Fab' fragment expressed in *Escherichia coli*. *FEBS Lett* 380: 194–197.

Inoue, Y., Ohta, T., Tada, H., Iwasa, S., Udaka, S., Yamagata, H. (1997) Efficient production of a functional mouse/human chimeric Fab' against human urokinase-type plasminogen activator by *Bacillus brevis*. *Appl Microbiol Biotechnol* 48: 487–492.

Jarvis, D.L. (2003) Developing baculovirus-insect cell expression systems for humanized recombinant glycoprotein production. *Virology* 310: 1–7.

Jarvis, D.L., Summers, M.D. (1989) Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. *Mol Cell Biol* 9: 214–223.

Jarvis, D.L., Oker-Blom, C., Summers, M.D. (1990) Role of glycosylation in the transport of recombinant glycoproteins through the secretory pathway of lepidopteran insect cells. *J Cell Biochem* 42: 181–191.

Jenke, A.C., Stehle, I.M., Herrmann, F., Eisenberger, T., Baiker, A., Bode, J., Fackelmayer, F.O., Lipps, H.J. (2004) Nuclear scaffold/matrix attached region modules linked to a transcription unit are sufficient for replication and maintenance

of a mammalian episome. *Proc Natl Acad Sci USA* 101: 11322–11327.

Jones, D., Kroos, N., Anema, R., van Montfort, B., Vooys, A., van der Kraats, S., van der Helm, E., Smits, S., Schouten, J., Brouwer, K., Lagerwerf, F., van Berkel, P., Opstelten, D.J., Logtenberg, T., Bout, A. (2003) High-level expression of recombinant IgG in the human cell line PER.C6. *Biotechnol Prog* 19: 163–168.

Jostock, T., Vanhove, M., Brepoels, E., Van Gool, R., Daukandt, M., Wehnert, A., Van Hegelsom, R., Dransfield, D., Sexton, D., Devlin, M., Ley, A., Hoogenboom, H., Müllberg, J. (2004) Rapid generation of functional human IgG antibodies derived from Fab-on-phage display libraries. *J Immunol Methods* 289: 65–80.

Kandilogiannaki, M., Koutsoudakis, G., Zafiropoulos, A., Krambovitis, E. (2001) Expression of a recombinant human anti-MUC1 scFv fragment in protease-deficient *Escherichia coli* mutants. *Int J Mol Med* 7: 659–664.

Klehr, D., Maass, K., Bode, J. (1991) Scaffold-attached regions from the human interferon beta domain can be used to enhance the stable expression of genes under the control of various promoters. *Biochemistry* 30: 1264–1270.

Kost, T. A., Condreay, J.P., Jarvis, D.L. (2005) Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat Biotechnol* 23: 567–575.

Kretzschmar, T., Aoustin, L., Zingel, O., Marangi, M., Vonach, B., Towbin, H., Geiser, M. (1996) High-level expression in insect cells and purification of secreted monomeric single-chain Fv antibodies. *J Immunol Methods* 195: 93–101.

Kujau, M.J., Hoischen, C., Riesenberg, D., Gumpert, J. (1998) Expression and secretion of functional miniantibodies McPC603scFvDhlx in cell-wall-less L-form strains of *Proteus mirabilis* and *Escherichia coli*: a comparison of the synthesis capacities of L-form strains with an *E. coli* producer strain. *Appl Microbiol Biotechnol* 49: 51–58.

Kurokawa, Y., Yanagi, H., Yura, T. (2001) Overproduction of bacterial protein disulfide isomerase (DsbC) and its modulator (DsbD) markedly enhances periplasmic production of human nerve growth factor in *Escherichia coli*. *J Biol Chem* 276: 14393–14399.

Lange, S., Schmitt, J., Schmid, R.D. (2001) High-yield expression of the recombinant, atrazine-specific Fab fragment K411B by the methylotrophic yeast *Pichia pastoris*. *J Immunol Methods* 255: 103–114.

Lemeulle, C., Chardes, T., Montavon, C., Chaabihi, H., Mani, J.C., Pugniere, M., Cerutti, M., Devauchelle, G., Pau, B., Biard-Piechaczyk, M. (1998) Anti-digoxin scFv fragments expressed in bacteria and in insect cells have different antigen binding properties. *FEBS Lett* 423: 159–166.

Li, H., Sethuraman, N., Stadheim, T.A., Zha, D., Prinz, B., Ballew, N., Bobrowicz, P., Choi, B.K., Cook, W.J., Cukan, M., Houston-Cummings, N.R., Davidson, R., Gong, B., Hamilton, S.R., Hoopes, J.P., Jiang, Y., Kim, N., Mansfield, R., Nett, J. H., Rios, S., Strawbridge, R., Wildt, S., Gerngross, T.U. (2006) Optimization of humanized IgGs in glycoengineered *Pichia pastoris*. *Nat Biotechnol* 24: 210–215.

Liang, M., Guttieri, M., Lundkvist, A., Schmaljohn, C. (1997) Baculovirus expression of a human G2-specific, neutralizing IgG monoclonal antibody to Puumala virus. *Virology* 235: 252–260.

Liang, M., Dübel, S., Li, D., Queitsch, I., Li, W., Bautz, E.K. (2001) Baculovirus expression cassette vectors for rapid production of complete human IgG from phage display selected antibody fragments. *J Immunol Methods* 247: 119–130.

Limonta, J., Pedraza, A., Rodriguez, A., Freyre, F.M., Barral, A.M., Castro, F.O., Lleonart, R., Gracia, C.A., Gavilondo, J.V., de la Fuente, J. (1995) Production of active anti-CD6 mouse/human chimeric antibodies in the milk of transgenic mice. *Immunotechnology* 1: 107–113.

Luo, D., Mah, N., Wishart, D., Zhang, Y., Jacobs, F., Martin, L. (1996) Construction and expression of bi-functional proteins of single-chain Fv with effector domains. *J Biochem (Tokyo)* 120: 229–232.

Ma, J.K., Hikmat, B.Y., Wycoff, K., Vine, N.D., Chargelegue, D., Yu, L., Hein, M.B., Lehner, T. (1998) Characterization of a recombinant plant monoclonal secretory

antibody and preventive immunotherapy in humans. *Nat Med* 4: 601–606.

Ma, J.K., Barros, E., Bock, R., Christou, P., Dale, P.J., Dix, P.J., Fischer, R., Irwin, J., Mahoney, R., Pezzotti, M., Schillberg, S., Sparrow, P., Stoger, E., Twyman, R.M. (2005) Molecular farming for new drugs and vaccines. Current perspectives on the production of pharmaceuticals in transgenic plants. *EMBO Rep* 6: 593–599.

Macauley-Patrick, S., Fazenda, M.L., McNeil, B., Harvey, L.M. (2005) Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* 22: 249–270.

Meissner, P., Pick, H., Kulangara, A., Chatellard, P., Friedrich, K., Wurm, F.M. (2001) Transient gene expression: recombinant protein production with suspension-adapted HEK293-EBNA cells. *Biotechnol Bioeng* 75: 197–203.

Mutskov, V., Felsenfeld, G. (2004) Silencing of transgene transcription precedes methylation of promoter DNA and histone H3 lysine 9. *EMBO J* 23: 138–149.

Ning, D., Junjian, X., Xunzhang, W., Wenyin, C., Qing, Z., Kuanyuan, S., Guirong, R., Xiangrong, R., Qingxin, L., Zhouyao, Y. (2003) Expression, purification, and characterization of humanized anti-HBs Fab fragment. *J Biochem (Tokyo)* 134: 813–817.

Niwa, R., Natsume, A., Uehara, A., Wakitani, M., Iida, S., Uchida, K., Satoh, M., Shitara, K. (2005) IgG subclass-independent improvement of antibody-dependent cellular cytotoxicity by fucose removal from Asn297-linked oligosaccharides. *J Immunol Methods* 306: 151–160.

Nyyssonen, E., Penttila, M., Harkki, A., Saloheimo, A., Knowles, J.K., Keranen, S. (1993) Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*. *Biotechnology (NY)* 11: 591–595.

O'Gorman, S., Fox, D.T., Wahl, G.M. (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells. *Science* 251: 1351–1355.

Ogunjimi, A.A., Chandler, J.M., Gooding, C. M., Recinos III, A., Choudary, P.V. (1999) High-level secretory expression of immunologically active intact antibody from the yeast *Pichia pastoris*. *Biotechnol Lett* 21: 561–567.

Peipp, M., Saul, D., Barbin, K., Bruenke, J., Zunino, S.J., Niederweis, M., Fey, G.H. (2004) Efficient eukaryotic expression of fluorescent scFv fusion proteins directed against CD antigens for FACS applications. *J Immunol Methods* 285: 265–280.

Piechaczek, C., Fetzer, C., Baiker, A., Bode, J., Lipps, H.J. (1999) A vector based on the SV40 origin of replication and chromosomal S/MARs replicates episomally in CHO cells. *Nucleic Acids Res* 27: 426–428.

Pollock, D.P., Kutzko, J.P., Birck-Wilson, E., Williams, J.L., Echelard, Y., Meade, H.M. (1999) Transgenic milk as a method for the production of recombinant antibodies. *J Immunol Methods* 231: 147–157.

Powers, D.B., Amersdorfer, P., Poul, M., Nielsen, U.B., Shalaby, M.R., Adams, G.P., Weiner, L.M., Marks, J.D. (2001) Expression of single-chain Fv-Fc fusions in *Pichia pastoris*. *J Immunol Methods* 251: 123–135.

Rakestraw, A., Wittrup, K.D. (2006) Contrasting secretory processing of simultaneously expressed heterologous proteins in *Saccharomyces cerevisiae*. *Biotechnol Bioeng* 93: 896–905.

Reavy, B., Ziegler, A., Diplexcito, J., Macintosh, S.M., Torrance, L., Mayo, M. (2000) Expression of functional recombinant antibody molecules in insect cell expression systems. *Protein Expr Purif* 18: 221–228.

Ridder, R., Schmitz, R., Legay, F., Gram, H. (1995) Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast *Pichia pastoris*. *Biotechnology (NY)* 13: 255–260.

Rippmann, J.F., Klein, M., Hoischen, C., Brocks, B., Rettig, W.J., Gumpert, J., Pfizenmaier, K., Mattes, R., Moosmayer, D. (1998) Procaryotic expression of single-chain variable-fragment (scFv) antibodies: secretion in L-form cells of *Proteus mirabilis* leads to active product and overcomes the limitations of periplasmic expression in *Escherichia coli*. *Appl Environ Microbiol* 64: 4862–4869.

Schillberg, S., Twyman, R.M., Fischer, R. (2005) Opportunities for recombinant antigen and antibody expression in transgenic plants – technology assessment. *Vaccine* 23: 1764–1769.

Schmidt, F.R. (2004) Recombinant expression systems in the pharmaceutical industry. *Appl Microbiol Biotechnol* 65: 363–372.

Schnappinger, D., Geissdorfer, W., Sizemore, C., Hillen, W. (1995) Extracellular expression of native human anti-lysozyme fragments in *Staphylococcus carnosus*. *FEMS Microbiol Lett* 129: 121–127.

Seibler, J., Schubeler, D., Fiering, S., Groudine, M., Bode, J. (1998) DNA cassette exchange in ES cells mediated by Flp recombinase: an efficient strategy for repeated modification of tagged loci by marker-free constructs. *Biochemistry* 37: 6229–6234.

Shi, X., Karkut, T., Chamankhah, M., Alting-Mees, M., Hemmingsen, S.M., Hegedus, D. (2003) Optimal conditions for the expression of a single-chain antibody (scFv) gene in *Pichia pastoris*. *Protein Expr Purif* 28: 321–330.

Shiroza, T., Shibata, Y., Hayakawa, M., Shinozaki, N., Fukushima, K., Udaka, S., Abiko, Y. (2001) Construction of a chimeric shuttle plasmid via a heterodimer system: secretion of an scFv protein from *Bacillus brevis* cells capable of inhibiting hemagglutination. *Biosci Biotechnol Biochem* 65: 389–395.

Shusta, E.V., Raines, R.T., Plückthun, A., Wittrup, K.D. (1998) Increasing the secretory capacity of Saccharomyces cerevisiae for production of single-chain antibody fragments. *Nat Biotechnol* 16: 773–777.

Sijmons, P.C., Dekker, B.M., Schrammeijer, B., Verwoerd, T.C., van den Elzen, P.J., Hoekema, A. (1990) Production of correctly processed human serum albumin in transgenic plants. *Biotechnology (NY)* 8: 217–221.

Simmons, L.C., Reilly, D., Klimowski, L., Raju, T.S., Meng, G., Sims, P., Hong, K., Shields, R.L., Damico, L.A., Rancatore, P., Yansura, D.G. (2002) Expression of full-length immunoglobulins in *Escherichia coli*: rapid and efficient production of aglycosylated antibodies. *J Immunol Methods* 263: 133–147.

Swennen, D., Paul, M.F., Vernis, L., Beckerich, J.M., Fournier, A., Gaillardin, C. (2002) Secretion of active anti-Ras single-chain Fv antibody by the yeasts *Yarrowia lipolytica* and *Kluyveromyces lactis*. *Microbiology* 148: 41–50.

Takimura, Y., Kato, M., Ohta, T., Yamagata, H., Udaka, S. (1997) Secretion of human interleukin-2 in biologically active form by *Bacillus brevis* directly into culture medium. *Biosci Biotechnol Biochem* 61: 1858–1861.

Tan, W., Lam, P.H. (1999) Expression and purification of a secreted functional mouse/human chimaeric antibody against bacterial endotoxin in baculovirus-infected insect cells. *Biotechnol Appl Biochem* 30: 59–64.

Tomiya, N., Narang, S., Lee, Y.C., Betenbaugh, M.J. (2004) Comparing N-glycan processing in mammalian cell lines to native and engineered lepidopteran insect cell lines. *Glycoconj J* 21: 343–360.

Trentmann, O., Khatri, N.K., Hoffmann, F. (2004) Reduced oxygen supply increases process stability and product yield with recombinant *Pichia pastoris*. *Biotechnol Prog* 20: 1766–1775.

Tschopp, J.F., Brust, P.F., Cregg, J.M., Stillman, C.A., Gingeras, T.R. (1987) Expression of the lacZ gene from two methanol-regulated promoters in *Pichia pastoris*. *Nucleic Acids Res* 15: 3859–3876.

Umana, P., Jean-Mairet, J., Moudry, R., Amstutz, H., Bailey, J.E. (1999) Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat Biotechnol* 17: 176–180.

Van Craenenbroeck, K., Vanhoenacker, P., Haegeman, G. (2000) Episomal vectors for gene expression in mammalian cells. *Eur J Biochem* 267: 5665–5678.

van Wely, K.H., Swaving, J., Freudl, R., Driessen, A.J. (2001) Translocation of proteins across the cell envelope of Gram-positive bacteria. *FEMS Microbiol Rev* 25: 437–454.

Verch, T., Yusibov, V., Koprowski, H. (1998) Expression and assembly of a full-length monoclonal antibody in plants using a

plant virus vector. *J Immunol Methods* 220: 69–75.

Ward, M., Lin, C., Victoria, D.C., Fox, B.P., Fox, J.A., Wong, D.L., Meerman, H.J., Pucci, J.P., Fong, R.B., Heng, M.H., Tsurushita, N., Gieswein, C., Park, M., Wang, H. (2004) Characterization of humanized antibodies secreted by *Aspergillus niger*. *Appl Environ Microbiol* 70: 2567–2576.

Werten, M.W., van den Bosch, T.J., Wind, R. D., Mooibroek, H., de Wolf, F.A. (1999) High-yield secretion of recombinant gelatins by *Pichia pastoris*. *Yeast* 15: 1087–1096.

Wood, C.R., Boss, M.A., Kenten, J.H., Calvert, J.E., Roberts, N.A., Emtage, J.S. (1985) The synthesis and in vivo assembly of functional antibodies in yeast. *Nature* 314: 446–449.

Wu, S.C., Ye, R., Wu, X.C., Ng, S.C., Wong, S.L. (1998) Enhanced secretory production of a single-chain antibody fragment from *Bacillus subtilis* by coproduction of molecular chaperones. *J Bacteriol* 180: 2830–2835.

Wu, X.C., Ng, S.C., Near, R.I., Wong, S.L. (1993) Efficient production of a functional single-chain antidigoxin antibody via an engineered *Bacillus subtilis* expression-secretion system. *Biotechnology (NY)* 11: 71–76.

Wu, S.C., Yeung, J.C., Duan, Y., Ye, R., Szarka, S.J., Habibi, H.R., Wong, S.L. (2002) Functional production and characterization of a fibrin-specific single-chain antibody fragment from *Bacillus subtilis*: effects of molecular chaperones and a wall-bound protease on antibody fragment production. *Appl Environ Microbiol* 68: 3261–3269.

Wulfing, C., Rappuoli, R. (1997) Efficient production of heat-labile enterotoxin mutant proteins by overexpression of dsbA in a degP-deficient *Escherichia coli* strain. *Arch Microbiol* 167: 280–283.

Wurm, F.M. (2004) Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat Biotechnol* 22: 1393–1398.

Yamagata, H., Nakahama, K., Suzuki, Y., Kakinuma, A., Tsukagoshi, N., Udaka, S. (1989) Use of *Bacillus brevis* for efficient synthesis and secretion of human epidermal growth factor. *Proc Natl Acad Sci USA* 86: 3589–3593.

Yoshida, S., Matsuoka, H., Luo, E., Iwai, K., Arai, M., Sinden, R.E., Ishii, A. (1999) A single-chain antibody fragment specific for the *Plasmodium berghei* ookinete protein Pbs21 confers transmission blockade in the mosquito midgut. *Mol Biochem Parasitol* 104: 195–204.

Zhang, Z., Song, L.P., Fang, M., Wang, F., He, D., Zhao, R., Liu, J., Zhou, Z.Y., Yin, C.C., Lin, Q., Huang, H.L. (2003) Production of soluble and functional engineered antibodies in *Escherichia coli* improved by FkpA. *Biotechniques* 35: 1032–1038, 1041–1042.

7
Non-Antibody Scaffolds

Markus Fiedler and Arne Skerra

7.1
Introduction

The use of so-called protein scaffolds with engineered ligand specificities as antibody surrogates both in biomedical research and in medical therapy has gained increasing attention in recent years. This development started with the notion that immunoglobulins owe their biochemical function to the combination of a conserved framework region with a spatially well-defined antigen-binding site, whereby the latter is composed of peptide segments that are hypervariable in amino acid sequence and conformation. Based on the current advanced methods for antibody engineering, together with biomolecular library techniques which permit the selection of functional antibody fragments on a routine level, several other protein classes have been employed for the construction of practically useful ligand-binding reagents. Properties such as small size of the engineered receptor protein, stability, and ease of production were initially in the focus. Once it had been demonstrated that protein scaffolds can be engineered to yield novel biomolecules with ligand affinities and specificities comparable to antibodies, efforts started to make these novel proteins amenable to application for human therapy as well. To develop such innovative biopharmaceutical compounds, aspects such as bioavailability, serum half-life and stability, tissue penetration, and immunogenicity have to be considered. Data from preclinical research carried out to date suggest that engineered protein scaffolds have potential to yield superior drugs with beneficial functions for the molecular recognition and targeting of tissues, cells or pathogens in the future.

7.2
Motivation for Therapeutic Use of Alternative Binding Proteins

Since the development of monoclonal antibody technology three decades ago (Köhler and Milstein 1975) and the invention of bacterial expression systems for

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engineered antibody fragments in the late 1980s (Skerra and Plückthun 1988) recombinant immunoglobulins have made a tremendous impact on the field of modern biopharmaceuticals, with about 20 currently approved drugs for human therapy, as described in this monograph. Without doubt, this trend will accelerate even more in the near future as there are several hundred further antibody candidates in late preclinical and clinical development. However, despite this remarkable success there is also an increasing awareness of the limitations of antibody technology, both regarding the intrinsic molecular properties of immunoglobulins and their technological or even commercial aspects. Consequently, there is a growing demand for alternative reagents that can provide molecular recognition functions similar to those currently considered characteristic for antibodies, the products of the natural immune response (Skerra 2003).

The most problematic aspect for the broader application of intact (human or "humanized") antibodies in medical therapy is the large biotechnological effort and the associated high production cost for properly glycosylated, full-sized immunoglobulins, whose manufacturing requires elaborate cell culture fermentation systems, at least at the moment. Most of the currently approved antibody drugs make use of this format and, as significant therapeutic doses of the active compound are needed for many indications, the worldwide availability of sufficient production capacity has become an issue (Carson 2005). The complicated production is largely caused by the glycosylated Fc effector region of immunoglobulins, which mediates complement activation and antibody-dependent cellular cytotoxicity (ADCC) (Carter 2001) and is also responsible for the long serum half-life of antibodies (Ghetie and Ward 2002) as well as for their numerous interactions with immunological cell surface receptors (Gessner et al. 1998). However, for several medical applications the presence of this moiety is actually not needed, for example when merely binding or antagonistic inhibition of a target is desired. In these cases, bacterially produced antibody fragments can, in principle, be used, although many of them are expressed at low yields, have poor thermodynamic stability, and a propensity to oligomerize or aggregate (Binz et al. 2005).

Furthermore, with respect to the development and commercial exploitation of antibodies in biotech companies and the pharmaceutical industry the extremely complex intellectual property situation in this field has become a major obstacle (Owens 2005; Editor 2005; Van Brunt 2005). First, today there is a high burden involved in the protection of new antibody-based inventions. In particular, the US patent statutes §§ 102, 103, and 112, which demand novelty, non-obvious subject matter, and specification, greatly affect the patentability of new antibodies. A recent case (Smithkline Beecham vs. Aotex) cleary illustrates that novelty may be questioned for any antibody when considering the aspect of "inherent anticipation." Under this theory "a product that existed in trace amounts, although unknown and undetected and unisolated, is 'inherently anticipated' and barred from the patent system after it is discovered." As antibodies themselves and the ability to generate such antibodies is inherent to the immune system of a given species it is entirely possible that such a doctrine may prohibit the claim

of antibodies themselves or of the antigens with which they interact (Lu et al. 2005).

Second, the development of new antibody-based reagents for therapy, diagnosis, or even purification purposes is hampered by a multitude of existing patents covering methods of generation, optimization, production, formulation, delivery, etc., which leads to the obligation to reserve a significant portion of any potential revenue stream for paying other parties (Baker 2005). The dimension of this indirect cost can be visualized by the fact that in 2004 just 17 companies participated with direct sales in the antibody market (Evans and Das 2005). All the other over one hundred antibody-related companies grabbed a piece of the US$10.3 billion market by dealing with their intellectual property. These commercial considerations will increasingly hinder the development of therapeutic antibodies by dramatically cutting into the profit.

Using alternative binding proteins as a basis for biopharmaceutical drug development may help to avoid such restrictions at least to some degree (Jeong et al. 2005). Utilizing such innovative approaches might even be essential in enabling entry into the biologics market, especially for small and medium-sized companies. Their success is further facilitated by the fact that alternative binding proteins selected against validated target molecules will benefit from preclinical assays, animal models, and clinical study designs originally established for monoclonal antibodies. This synergy may help to save time in drug development, which tends to require a prolonged period for biologicals, typically 4 years in the last half of the 1980s but already more than 7 years in the first half of this decade (Lahteenmaki and Baker 2004).

Antibody surrogates derived from alternative protein scaffolds can circumvent many of these technological and strategic limitations (Table 7.1). Their concept is based on the notion that certain polypeptide folds are observed in nature to occur in different context and with varying biochemical function. Hence, such protein scaffolds may be utilized to selectively reshape their active sites via protein engineering in order to create novel ligand-binding functions without having to worry about the protein folding problem in general (Skerra 2000a). Candidates for protein scaffolds should possess intrinsic conformational stability and they should be able to present surface segments, preferably flexible loops of varying sequence and length, including exposed hydrophobic residues, without undergoing significant changes in the structural framework. Naturally, the ligand-binding properties of such artificial receptor proteins will depend on the number, spatial distribution, and diversity of the variable regions. According to practical demands they should further be based on monomeric polypeptides, which are small in size (Fig. 7.1), robust, easily engineered, and efficiently produced by inexpensive prokaryotic expression systems. Several examples for medically useful protein scaffolds have emerged in recent years and will be discussed in the following chapters. For a more complete overview the reader is referred to some recent review articles (Nygren and Skerra 2004; Binz and Plückthun 2005; Binz et al. 2005; Hey et al. 2005; Hosse et al. 2006).

Table 7.1 Properties of artificial binding proteins versus antibodies (or their conventional fragments) with relevance for therapeutic application.

Property	*Monoclonal antibodies (mABs)*	*Antibody fragments (Fab/scFv)*	*Scaffold-based proteins*
Size (kDa)	150	50/25	≤20[a]
Polypeptide chains	4	2/1	1
E. coli production	–	+/–	+
Ease of modification	+/–	+	+
Fusion proteins	–	–/(+)	+
High specificity	+	+	+
High affinity	+	+	+
High stability	+	–	+
Human origin	+/–	+/–	+/–
Ig effector function	+	–	–
IP situation	–	–	+
Neutralizing activity	+	+	+
Intracellular activity	–	+/–	+/–
Targeted delivery	+/–	+	+
Non-invasive delivery	–	–	(+)
Tissue penetration	–	+	+
Clearance	Slow	Fast	Fast

a Size of the binding protein depends on the scaffold chosen (see text).

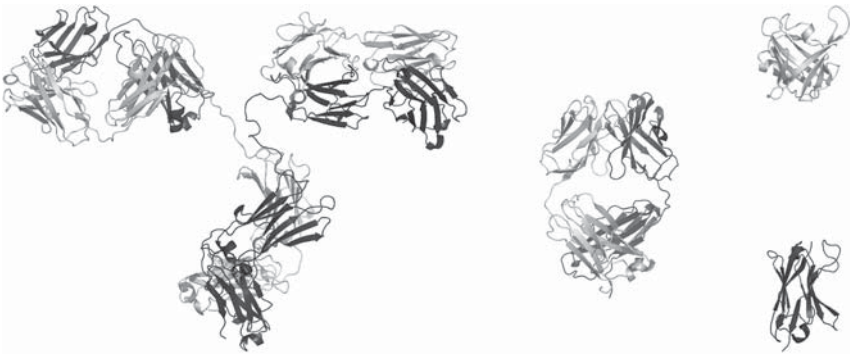

Fig. 7.1 Size comparison between an intact antibody (left), its Fab fragment (middle), a single immunoglobulin domain (right, bottom), and an anticalin as a typical scaffold protein (right, top).

In principle, engineered binding proteins offer a number of advantages, both when considered as drug candidates for therapy or for *in vivo* diagnostics and when applied for *in vitro* diagnostics or as laboratory research tools. First, the binding proteins are engineered entirely *in vitro*, without the need for animal immunization. Usually, a combinatorial library of the chosen scaffold protein is

created by selective random mutagenesis of appropriate surface residues (Skerra 2003) and variants with the desired target or "antigen" specificity are subsequently selected by well-established techniques such as phage display (Lowman and Wells 1991; Pini et al. 2002), ribosome display (Groves and Osbourn 2005), ELISA or colony screening (Schlehuber et al. 2000). This *in vitro* selection and screening methodology also offers numerous strategies to improve and fine-tune, under suitable conditions, the biomolecular properties of an initially identified scaffold-derived binding protein. In this regard there is usually a desire for exquisite target affinity and specificity (especially if discrimination against closely related targets is needed), thermodynamic folding stability (mostly against thermal denaturation), shelf-life and solubility as well as chemical stability and protease resistance.

The binding proteins resulting from this process are usually amenable to microbial production at high yields and with simplified downstream processing. Normally, it is intended to use protein scaffolds that lack glycosylation and which can thus be manufactured using conventional *E. coli* expression systems. If the scaffold is devoid of disulfide bonds the binding protein may be produced in the bacterial cytoplasm and directly extracted in a soluble and folded state, which is a clear advantage over antibody fragments that usually cannot fold in a reducing environment (Wörn and Plückthun 2001). Furthermore, intracellular applications may be possible, for example as antagonists of cytosolic protein interaction networks. Even scaffolds that carry disulfide bonds may be produced according to the cytoplasmic route, albeit a secretion strategy that directly yields correctly folded protein is often preferred, at least at the laboratory scale. However, if necessary, subsequent refolding from cytoplasmic inclusion bodies is usually much more efficient than for antibody fragments, which are composed of several subunits or domains, because almost all currently exploited scaffold proteins comprise a monomeric polypeptide chain and possess a robust globular fold.

Aiming at therapeutic application, a series of pharmaceutical aspects has also to be considered in conjunction with the intended medical indication when choosing an appropriate scaffold protein: serum half-life and stability, bioavailability, tissue penetration, and immunogenicity. Unfortunately, in practice some of the desirable biomolecular parameters tend to be of opposing nature. For example, a small protein size usually leads to better tissue penetration but also to a shorter serum half-life (Holliger and Hudson 2005). Nevertheless, owing to their simple biomolecular architecture, their robustness, and their rather small dimensions, scaffold proteins usually can be further engineered for improved pharmacokinetic and functional properties. Serum half-life can be extended by site-specific PEGylation (Harris and Chess 2003), preferentially via introduction of a free Cys side-chain into a sterically well accessible position remote from the active site (Rosendahl et al. 2005). Alternatively, fusion with serum albumin (Osborn et al. 2002), with albumin-binding peptides (Dennis et al. 2002) or domains (Lejon et al. 2004) or even the generation of a novel albumin-binding site (Connelly 2005) can be employed to achieve prolonged circulation.

Most of the scaffold-based binding proteins are ideally suited to construct fusion proteins in order to adopt novel effector functions. In the simplest case one could produce a fusion protein with the Fc portion of an antibody (Chamow and Ashkenazi 1996). This would not only serve to recruit the natural immunologic effector functions, but also bivalent binding properties should arise, thus leading to an enhanced avidity. Furthermore, this could even mediate crosslinking of target cell surface receptors and thus trigger associated intracellular signaling events. Nevertheless, the construction of such a hybrid protein would not give much advantage over the use of a conventional antibody possessing the same antigen specificity.

Rather, the use of a single domain scaffold permits the preparation of smarter fusion proteins, which are also easier to produce because heterologous chain assembly is usually not required – in contrast with multidomain antibody fragments. Hence, dimerization of such binding proteins can be achieved, for example, by simple tandem fusion (Schlehuber and Skerra 2001) or via fusion with a small pair-forming module such as a helix bundle (Pack and Plückthun 1992), a single constant Ig domain (Li et al. 1997), or a homodimeric enzyme (Tudyka and Skerra 1997).

Fusion proteins with enzymes should not only be useful to generate practically applicable diagnostic tools such as in combination with alkaline phosphatase (Schlehuber et al. 2000), they may also offer interesting biopharmaceutical agents, especially for enzyme prodrug activation approaches in tumor therapy (Florent et al. 1998; Carter 2001). Fusion proteins with enzyme toxins from bacteria (e.g. *Pseudomonas* exotoxin) (Pastan 2003), or from plants, such as saporin (Palmisano et al. 2004), have demonstrated the principal applicability of this approach, but at the expense of significant side effects due to the considerable immunogenicity and some intrinsic unspecific toxicity of the fusion partners. Combinations of nonimmunogenic alternative scaffolds with less toxic effectors of human origin, for example RNase (Zewe et al. 1997) (see Chapter 3, Vol II), will allow the development and higher yield production of promising "immunotoxin" drugs of a next generation, thereby eliminating all of the major roadblocks of the current antibody–heterologous toxin constructs.

Finally, the intrinsically small size and robustness of scaffold-based binding proteins may open novel routes of parenteral administration, apart from injection or infusion. In the light of recent developments on inhalable insulin (Powell 2004), pulmonary delivery has attracted particular attention because bioavailability through the lungs should be much higher for certain scaffold proteins than for large antibodies (Agu et al. 2001). Transdermal delivery could also be of interest (Barry 2004), and even oral administration has been envisaged for some of the engineered scaffold proteins (Goldberg and Gomez-Orellana 2003).

During the last 15 years several protein scaffolds have been investigated, mainly in an academic environment, for the tailoring of non-antibody binding proteins. With the currently available repertoire, artificial binding proteins with high affinities and specificities for molecules differing remarkably in size and shape have

been successfuly generated, thus addressing a variety of targets, ranging from low molecular weight compounds over carbohydrates and peptides to proteins. The different kinds of biomolecular architecture that underly the individual protein scaffolds can provide either cavities for small molecules, flexible loops which may enter substrate clefts in enzymes, for example, or extended interfaces for tight complex formation with larger proteins.

This review will focus on those examples that are already closest to medical application or that illustrate important structural and functional principles (Table 7.2, Fig. 7.2). Since this area has also seen increasing commercialization by small and medium-sized biotech companies in recent years, corresponding examples will be mentioned (Table 7.3). For a more complete compilation of the current scaffold protein approaches the reader should refer to recent review articles (Skerra 2000a; Nygren and Skerra 2004; Binz et al. 2005; Binz and Plückthun 2005; Hey et al. 2005; Hosse et al. 2006). Here, the various protein scaffold architectures will be grossly classified into four groups: single domain immunoglobulins, scaffold proteins presenting a contiguous hypervariable loop region, scaffold proteins for display of individual extended loops, and scaffold proteins providing a rigid secondary structure interface.

7.3 Single-Domain Immunoglobulins

Probably the best example of a protein scaffold that presents a set of structurally well-positioned hypervariable loops, giving rise to a contiguous interface for biomolecular interaction, is provided by the Ig class itself. While the immunological effector functions reside in the constant region of an antibody, its antigen-binding activity is exclusively provided by the pair of variable domains (V_H and V_L), which are located in close spatial neighborhood at the N-termini of each light and heavy chain (Padlan 1994). The fold of the mutually homologous variable domains is dominated by a sandwich of two antiparallel β-sheets that form a structurally conserved framework. Three loops at one end of the β-sandwich in each variable domain are thus brought together to form the so-called complementarity determining region (CDR) of an antibody. Due to the peculiar genetic mechanisms of the immune system each CDR loop is highly variable in its amino acid sequence such that altogether six peptide segments give rise to an extended antigen-combining site (Skerra 2003). The resulting structural diversity of the paratope explains the pronounced affinities and specificities that are typically observed for antibodies originating from a natural immune response.

Biotechnological methods for the generation of antibody fragments or even intact immunoglobulins with prescribed antigen specificities are well established and are described in accompanying chapters of this book. However, in spite of their still increasing use both as research tools and as protein therapeutics, the characteristic protein architecture of antibodies also causes some practical disadvantages. For example, immunoglobulins possess a rather large size, which

Table 7.2 Biomolecular characteristics of certain protein scaffolds that are being explored for medical purposes.

Name	*Scaffold*	*Origin*	*Fold*	*No. of residues/ crosslinks/domains*	*Randomized structural elements*	*Selected references*
Affibody	Three-helix bundle from Z-domain of Protein A	Bacterial	α_3	58/–/1	13 residues in 2 helices	Wikman et al. 2004
Affilin molecules	γ-Crystallin/ Ubiquitin	Human	β-Sandwich/α/β	74 and 174/–/1	8 residues in β-sheet	Hey et al. 2005
AdNectin	10th fibronectin type III domain	Human	β-Sandwich	94/–/1	2 to 3 loops	Xu et al. 2002
Anticalin	Lipocalins	Human and various species	β-Barrel	160–180/0,1,2 S-S/1	16 to 24 residues in 4 loops	Schlehuber and Skerra 2005
DARPin	Ankyrin repeat protein	Artificial consensus sequence	α_2/β_2 repeated	n × 33/–/combination of multiple domains	β-turn, 1 α-helix, 1 loop	Binz et al. 2003
Domain antibody (DAb)	Variable domain of antibody light or heavy chain	Human	β-Sandwich	ca. 120/1 S-S/1	3 loops (CDRs)	Holt et al. 2003
Evibody	Cytotoxic-associated antigen (CTLA-4)	Human	β-Sandwich	136/2 S-S/1	17.3 loops (CDR analogue)	Irving et al. 2001

Knottin	Different proteins from the knottin family	Multiple sources	β-Sheet or β-Sandwich	23–113/2 S-S/1	β-turn, β-sheet and/or loop	Craik et al. 2001
Kunitz-type domain	Trypsin inhibitor	Human and bovine	α/β	58/3 S-S/1	1 or 2 inserted loops	Dennis and Lazarus 1994
Maxibody/ Avimer	A-domain	Human	LDL receptor-like module, disulfide-rich calcium-binding fold	n × 35–45/3 S-S/ combination of multiple domains	mainly β-turns	Silverman et al. 2005
Nanobody	Variable domain of antibody heavy chain	Camelidae	β-Sandwich	ca. 120/1 S-S/1	3 loops (CDRs)	Conrath et al. 2005
Tetranectin	Monomeric or trimeric C-type lectin domain	Human	α/β	137/3 S-S/natural homo-trimer	2 loops	see Table 7.3
Trans-body	Serum transferrin	Human	α/β	329/8 S-S/2	1 or 2 inserted loops	see Table 7.3
V(NAR)	Variable domain of new antigen receptor	Shark	β-Sandwich	112/1 S-S/1	3 loops (CDRs)	Holliger and Hudson 2005
	β-Lactamase	Bacteria	α/β	263/1 S-S/1	1 or 2 inserted loops	Legendre et al. 2002

Table 7.3 List of companies and references to web sites for scaffold proteins that are in development for medical applications.

Company	*Scaffold name*	*Status/Indication*[a]
Ablynx (Ghent, Belgium) www.ablynx.com	Nanobody – single-domain antibodies from the camelid family	Preclinical[b]: rheumatoid arthritis (injectable), inflammatory bowel disease (oral administration), thrombosis associated with arterial stenosis (injectable)
Affibody (Bromma, Sweden) www.affibody.com	Affibody – Z-domain of Protein A from *S. aureus*	Preclinical[b]: breast cancer (*in vivo* diagnostics, radiotherapy)
Avidia (Mountain View, CA, USA) www.avidia.com	Maxibody – human A-domain	Preclinical[b]: inflammation
BioRexis (King of Prussia, PA, USA) www.biorexis.com	*Trans*-body – human transferrin	n.d.
Borean Pharma (Aarhus, Denmark) www.borean.com	Tetranectin monomeric or trimeric human C-type lectin domain	Preclinical[b]: inflammation
Compound Therapeutics (Waltham, MA, USA) www.compoundtherapeutics.com	AdNectin – human 10th fibronectin type III domain	Preclinical[b]: cancer, ophthalmology
Domantis (Cambridge, UK) www.domantis.com	Domain antibody-variable domain of human light or heavy chain	Preclinical[b]: inflammation, asthma, cancer
Dyax (Cambridge, MA, USA) www.dyax.com	Kunitz-type domain of human and bovine trypsin inhibitor	Clinical phase II: hereditary angioedema, on-pump heart surgery, cystic fibrosis
Evogenix (Sydney, Australia) www.evogenix.com	Evibody – CTLA-4	n.d.
Molecular Partners (Zürich, Switzerland) www.molecularpartners.com	DARPin – ankyrin repeat protein	n.d.
Pieris (Freising, Germany) www.pieris-ag.com	Anticalin – various human and insect lipocalins	Preclinical[b]: cancer, cardiovascular inflammatory diseases
Scil Proteins (Halle, Germany) www.scilproteins.com	Affilin molecules – human γ-crystallin/ human ubiquitin	Preclinical[b]: cancer, ophthalmology, inflammation

a As disclosed on the company home page (in February 2006).
b Preclinical studies with respect to biological activity in cell culture, serum half-life, (serum) stability, *in vivo* efficacy, toxicity in animal models, etc.

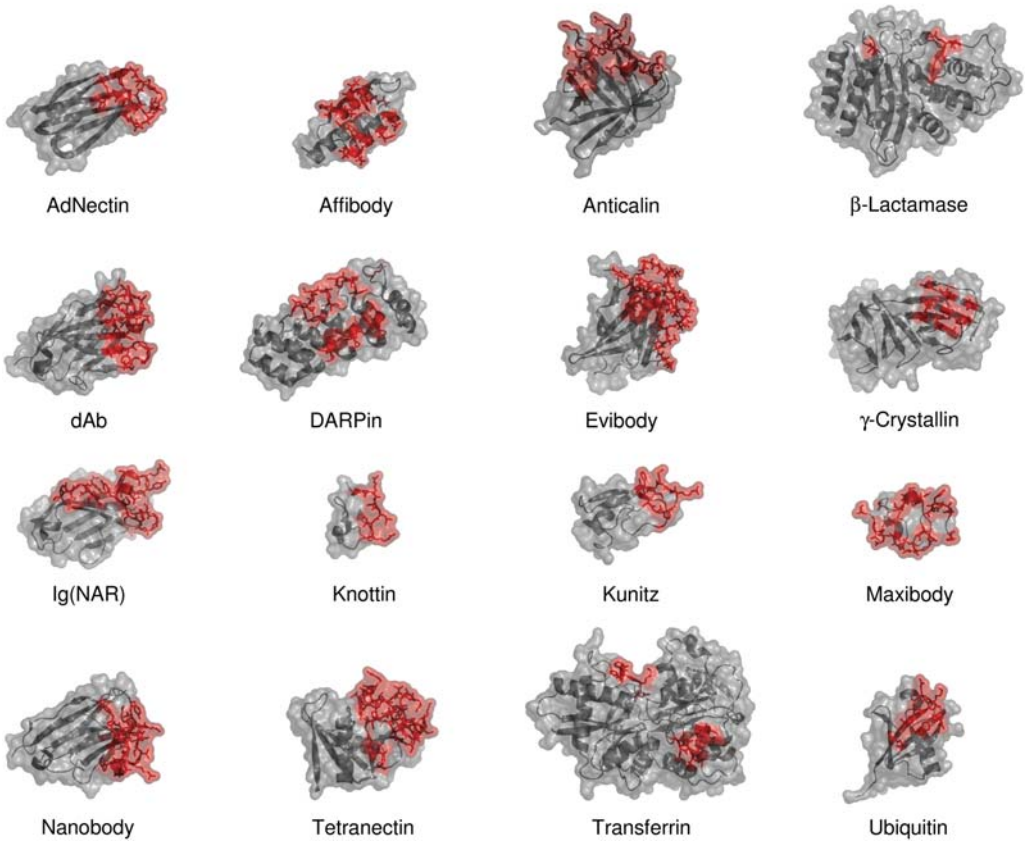

Fig. 7.2 Various scaffold proteins that are in development or considered for therapeutic application. Some of the examples shown are not discussed in the text, due to insufficient published data; their properties (as far as known) are listed in Tables 7.2 and 7.3.

results in poor production economy, not only with respect to their "specific antigen-binding activity" but also because the efficient production of intact antibodies usually requires eukaryotic cell culture. In addition, the voluminous molecular size results in poor tissue penetration. Even the smallest stable antibody fragment that carries an intact combining site, the scFv fragment, is composed of more than 250 residues. This is a disadvantage, especially when serving as an antigen recognition module, for example in a fusion protein with a reporter enzyme or a toxin.

Furthermore, the fact that the antigen-binding site is formed together by the light and heavy Ig chains turns out to be a problem in the biotechnological use of antibodies and, in particular, of their smaller antigen-binding fragments. On the one hand, two different coding regions have to be cloned and handled in

parallel and, on the other, extra measures must be taken to stabilize the heterodimerization of the two chains at the level of the biosynthetic protein. Especially in the Fv fragment, which merely comprises the two variable domains (Skerra and Plückthun 1988), the noncovalent association between both chains often leads to limited stability. In order to rigidify the fragment for practical applications, the two domains can be connected by a flexible peptide linker, resulting in so-called single-chain (sc) Fv fragments (Bird and Walker 1991). Yet, the format of the scFv fragment frequently results in other undesired properties, like low folding efficiency upon expression in *E. coli*, enhanced aggregation, and a pronounced tendency to form oligomers (Atwell et al. 1999).

Consequently, there is a generally recognized need for alternative scaffolds that offer the possibility of displaying structurally variable loops on a monomeric protein architecture, thus avoiding problems with chain pairing and insufficient folding stability. One example in this respect is provided by nature itself with a special Ig class whose antigen-binding region is formed by a single polypeptide chain. Normally, an unpaired variable domain exposes a significant area of hydrophobic surface to solvent, which is otherwise shielded by association either with the second variable domain or, as often seen for light chains, via formation of so-called Bence Jones dimers (Stevens et al. 1991). Especially in the case of isolated heavy chain variable domains (V_H) this fundamental property usually causes aggregation (Glockshuber et al. 1990; Davies and Riechmann 1994) and nonspecific adsorption.

Interestingly, a family of peculiar antibodies that is devoid of light chains has been identified as a natural subclass of the IgG pool in camelids (Hamers-Casterman et al. 1993; De Genst et al. 2006). In such "heavy-chain" antibodies, which are composed of a pair of heavy chains lacking the C_H1 domain, the V_H domains have apparently evolved to remain soluble without heterodimerization. Sequence analysis of the natural heavy-chain antibodies from camels in conjunction with X-ray structural analyses of corresponding V_H antibody fragments (dubbed VHH) from camels, dromedaries, and also from llamas revealed the reasons for the observed high solubility of this type of Ig domain (Muyldermans et al. 2001; Conrath et al. 2005). In essence, the camelid V_H domains have an increased surface hydrophilicity in the region that faces the V_L domain in ordinary Igs. In addition, they have a much longer CDR-H3, which often participates in a second disulfide bridge within the V_H domain, thus partially shielding the interface region from solvent.

Up to now, camel VHH domains were mainly generated by classical immunization of animals. Due to the simpler nature of their antigen-binding site compared with conventional antibodies, with just three CDR loops that protrude from one edge of the protein, they can no longer form deep pockets. Hence, the binding of haptens has only been established in special cases (e.g. with a rather large azo dye compound) (Spinelli et al. 2001), otherwise it is accompanied by rather poor affinity (Yau et al. 2003). However, their arrangement of three hypervariable loops, among which the CDR3 often adopts an extended conformation, seems to be particularly well suited for creating inhibitor proteins that can bind to the

active sites of enzymes (Lauwereys et al. 1998; Muyldermans et al. 2001; Conrath et al. 2001).

As a consequence of the loss of their hydrophobic interface VHH domains can often be expressed at high yields in *E. coli* – via secretion into the bacterial periplasm to ensure disulfide bond formation – and they possess favorable conformational stability with the feature of reversible denaturation, a property that distinguishes them from conventional V_H domains that usually show irreversible aggregation behavior (Dumoulin et al. 2002; Ewert et al. 2002). Currently, so-called "nanobodies" (cf. Tables 7.2 and 7.3) are in development against a number of medical targets, including enzymes, transcription factors, cytokines, and tumor markers, either for diagnostic purposes (Saerens et al. 2005) or for therapy in indications such as infectious diseases, rheumatoid arthritis, asthma, or solid tumors (Cortez-Retamozo et al. 2004).

Notably, there also exist some human or murine variable antibody domains whose natural functions seem not to depend on the association with a second variable domain – either V_L or V_H – so that they might be considered as human scaffolds for the engineering of single Ig domains. For example, a melanoma-specific "V_H antibody" was isolated from an scFv phage library derived from a patient that had been immunized with genetically modified autologous tumor cells (Cai and Garen 1996). In contrast, the monoclonal antibody NEMO, which was obtained by immunization of a mouse, consists solely of a κ light chain (Masat et al. 1994). Only the monomeric form of this Ig fragment was shown to be active and to recognize an antigen expressed by human cells of the melanocytic lineage. Furthermore, a natural V_H domain from a mouse hybridoma clone that carries a mutation at the interface with its V_L domain was successfully used as scaffold for the preparation of a phage display library after randomization of nine residues within CDR-H3, thus yielding soluble V_H domains with specific binding activities towards tumor necrosis factor (TNF) or immunoglobulins as targets (Reiter et al. 1999).

Thus, there seem to be structural solutions other than those originally found in camel VHH Igs which can confer solubility and specific binding properties onto isolated variable domains of human or murine antibodies. This notion is strengthened by the recent description of a conventional llama V_H domain that lacks the characteristic mutations of VHH antibodies within the interface region and is still highly soluble (Vranken et al. 2002). Moreover, the concept of selecting so-called Domain Antibodies (DAbs) from libraries of cloned human V_H or V_L domains has emerged (Holt et al. 2003; Holliger and Hudson 2005). DAbs (cf. Tables 7.2 and 7.3) have been successfully selected against targets such as TNF-α or human serum albumin, in the latter case resulting in so-called AlbuDabs, which can be fused to other therapeutic proteins to prolong their serum half-life (Connelly 2005). Pulmonary and oral routes of delivery are currently being explored for VHH domains of both human and cameloid origin.

Another source of single Ig domains that appear to be useful as scaffolds for the generation of artificial receptor proteins are cartilaginous fish, in particular wobbegong and nurse sharks (Holliger and Hudson 2005). The new antigen

receptor (NAR) of the nurse shark, *Ginglymostoma cirratum*, consists of two protein chains, each with one variable and five constant domains, and seems to be the representative of the Ig superfamily for adaptive immune response in this animal (Dooley et al. 2003). Based on this perception, a number of NAR variable domains from the spotted wobbegong shark, *Orectolobus maculatus*, were cloned and used for the construction of protein libraries in which the CDR3 loop was randomized (Nuttall et al. 2001). From this library mutant Ig domains with specificity for the protease gingipain K were identified via phage display.

Another phage display library was generated by incorporating synthetic CDR3 regions with 15–18 residues into the NAR domain. Thus, a variant with high affinity for the 60 kDa cytosolic domain of the outer membrane translocase receptor of human mitochondria (Tom70) was selected (Nuttall et al. 2003). In an attempt to generate IgNAR antibodies with anti-infective activity, V(NAR) domains directed against the malarial apical membrane antigen-1 (AMA1) from *Plasmodium falciparum* were selected, whose initial affinity of about 0.2 micromolar was approximately 10-fold enhanced by *in vitro* affinity maturation (Nuttall et al. 2004). More recently, a synthetic library of V(NAR) domains was successfully constructed on the basis of an anti-lysozyme IgNAR domain with known three-dimensional structure, which had been obtained from an immunized nurse shark, as a single well-defined scaffold (Shao et al. 2006).

Taken together, single domain Igs offer a somewhat conservative approach in so far as the methods for their generation as well as their biophysical properties are strongly related to conventionally engineered antibody fragments, except that they do not rely on the light/heavy chain association. Hence, intellectual property issues in this field also largely apply to the resulting binding proteins. In addition, it is not quite clear whether all single-domain Ig fragments behave as truly monomeric proteins. At least in some cases X-ray structural analysis has indicated a mode of dimerization in the crystal packing (Jespers et al. 2004; Streltsov et al. 2004) that resembles the well-known V_H/V_L pairing of antibodies (Padlan 1994) or the structurally analogous light-chain homo-association that is typically observed for Bence Jones proteins (Stevens et al. 1991; Pokkuluri et al. 1998).

7.4 Scaffold Proteins Presenting a Contiguous Hypervariable Loop Region

An obvious extension of the single-domain antibody concept is the use of more remote members of the Ig superfamily as protein scaffolds to generate novel binding proteins. This strategy can no longer benefit from the diversification mechanisms of the natural immune system – as, for example, in the case of cameloid VHH domains – instead, techniques of targeted randomization and subsequent *in vitro* selection of variants with the desired specificity must be applied.

For example, the human cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), a functionally important T-cell surface coreceptor (also described further

below), was explored as a potential protein scaffold. Its extracellular domain exhibits a V-like Ig fold, albeit with two intramolecular disulfide bonds instead of one. Normally, CTLA-4 is expressed as a membrane protein on activated T cells and exists as a homodimer that is crosslinked by a disulfide bridge within a stalk region just outside the transmembrane segment. Even though monomeric CTLA-4 V-domains were successfully synthesized in eukaryotic Chinese hamster ovary (CHO) or *Pichia pastoris* expression systems, attempts to produce active soluble protein in *E. coli* remained unsuccessful. However, when the loops corresponding either to CDR1 or to CDR3 of antibodies were substituted with somatostatin – a 14-residue intra-disulfide-linked peptide hormone – CTLA-4 variants with superior solubility were obtained and successfully produced in the periplasm of *E. coli* (Nuttall et al. 1999).

In a subsequent study, the nine-amino-acid CDR3-like loop was replaced by a random sequence surrounding a fixed central RGD motif and several CTLA-4-based variants capable of binding to the human $\alpha_v\beta_3$ integrin were selected using phage display (Hufton et al. 2000). A similar but nonbiased CTLA-4 library was later used to select variants with affinity towards lysozyme via ribosome display (Irving et al. 2001). Hence, at least in principle, the CTLA-4 extracellular domain may be utilized to generate globular binding proteins toward protein targets, despite the fact that a specificity with therapeutic relevance has so far not been described.

Tendamistat, a 74-amino-acid inhibitor of α-amylase with a β-sheet sandwich topology, which is stabilized by two rather than one disulfide bond as in Igs, was employed as a more distantly related Ig-type scaffold in order to present conformationally constrained random peptides (McConnell and Hoess 1995). In this case two loops, comprising residues 38–40 and 60–65, were randomly mutagenized and a phage display library was prepared and subjected to selection against the monoclonal antibody A8, which recognizes the 21-residue peptide hormone endothelin. As result, tendamistat variants exhibiting a variety of sequences in both of the loops, none of which matched a linear sequence from endothelin, were isolated. In another study loop no. 1 of tendamistat was used for the preparation of specialized random libraries containing the RGD motif and specific integrin-binding variants were identified via phage display (Li et al. 2003). Hence, the tendamistat scaffold seems to tolerate variegation of its CDR-like loops and it will be interesting to see whether any binding specificities of medical importance may be generated.

The 10th domain of the 15 repeating units in human fibronectin type III (FN3) provides another small, monomeric β-sandwich protein which shows structural resemblance to a trimmed Ig V_H domain. It consists of 94 amino acids and possesses seven β-strands (instead of nine for a normal Ig variable domain) with three loops connecting the strands at one end of the β-sandwich. In contrast with conventional members of the Ig superfamily, FN3 is devoid of disulfide bonds. The loop that carries the integrin-binding RGD sequence in the natural fibronectin domain is topologically equivalent to the CDR3 of a V_H domain. FN3-type domains are ubiquitous and occur in cell adhesion molecules, cell surface

hormone and cytokine receptors, chaperonins as well as in carbohydrate-binding domains, all of which are involved in molecular recognition.

Based on this FN3 domain a phagemid display library was prepared with 10 randomized residues, five in the BC loop (residues 26–30) and five in the FG loop (residues 77–81), and used for panning against ubiquitin. Indeed, one variant (initially called "monobody," later dubbed "Trinectin" or "AdNectin") with specific target-binding activity and a dissociation constant in the low micromolar range, was identified (Koide et al. 1998). Although this variant could be readily produced as a soluble protein in *E. coli*, its solubility was significantly lower than that of the recombinant wildtype FN3. In a subsequent study all three CDR-like loops were randomized at once and the mRNA display technique was applied for selection, leading to FN3 variants with high affinity towards TNF-α (Xu et al. 2002), a medically relevant target for the treatment of rheumatoid arthritis (Bang and Keating 2004). Recently, FN3-based binding proteins for the vascular endothelial growth factor receptor 2 (VEGF-R2), an important cell surface receptor in angiogenesis, have been described as well (Parker et al. 2005).

A different type of protein scaffold that presents a structurally hypervariable loop region on top of a rigid β-sheet secondary structure is derived from the lipocalin protein family (Skerra 2000b). The lipocalins represent a class of small, robust proteins that share a rigid β-barrel of eight antiparallel strands winding around a central axis as their central folding motif. Lipocalins are functionally diverse polypeptides of 160–180 residues, with rather weak sequence homology but high similarity at the tertiary structural level (Flower 1996). The family comprises several hundred members, which are found in almost all vertebrates, including humans (Breustedt et al. 2006), but also in insects and in bacteria. In most cases, their physiological role lies in the storage or transport of hydrophobic and/or chemically sensitive organic compounds. At the open end of the conical structure the β-strands are connected in a pair-wise fashion by four loops, which form the entrance to the ligand-binding pocket (Fig. 7.2). In contrast to the highly conserved β-barrel topology, this loop region differs considerably among individual lipocalins, both in conformation and length of the corresponding polypeptide segments. Hence, there appears to be a functional resemblance with the antigen-binding region of immunoglobulins (Skerra 2003).

Initially, the 174-residue bilin-binding protein (BBP) from *Pieris brassicae* with its rather wide and shallow ligand pocket – where biliverdin IX_γ is complexed as a natural ligand – served as scaffold for the generation of novel binding proteins towards several low molecular weight molecules (Beste et al. 1999). Sixteen residues distributed across all four loop segments and commonly located at the center of the binding site were identified by molecular modeling and subjected to concerted random mutagenesis, followed by phagemid display selection. In the case of the ligand fluorescein, which was chosen as a well-known immunological hapten, several variants with high specificity for this compound and dissociation constants as low as 35.2 nanomolar were identified. Following X-ray analysis of the complex between the corresponding artificial binding protein and its cognate ligand (Korndörfer et al. 2003a), improved variants with K_D values for fluorescein

around 1 nanomolar were constructed just by optimizing two side-chains in the binding pocket (Vopel et al. 2005). Thus, it has been demonstrated that engineered lipocalins with novel specificities – so-called "anticalins" – can provide hapten-binding proteins with affinities as they are typical for antibodies. Notably, the BBP variants recognize fluorescein or other small molecule targets as true haptens, without measurable context dependence concerning the carrier protein that was employed during selection. With their capability to provide deep and highly complementary ligand pockets anticalins distinguish themselves from most other scaffolds currently under investigation.

From the BBP mutant library an anticalin with specificity for the cardiac steroid digoxigenin was also selected (Schlehuber et al. 2000). Its initially rather moderate affinity was subsequently raised by selective random mutagenesis of the first hypervariable loop, followed by phagemid display and colony screening, resulting in a 10-fold lower K_D value of 30.2 nanomolar. Attempts to improve the affinity for digoxigenin even further were made with a combinatorial approach using the previously employed "loop-walking" randomization strategy (Schlehuber and Skerra 2002), and also by rational protein design based on the crystal structure of the anticalin (Korndörfer et al. 2003b). These approaches allowed the identification of several point mutations that led to K_D values as low as 800 picomolar for digoxin (i.e. the natural glycosylated derivative of digoxigenin) (Schlehuber and Skerra 2005).

The resulting anticalin, called Digical, may be directly suitable as a therapeutic agent for the treatment of digitalis intoxication. Although digitalis is widely applied in conjunction with heart insufficiency and arrhythmias (Hauptman and Kelly 1999), this drug has a very narrow therapeutic window and precise adjustment of digoxin plasma levels is essential to prevent intoxication with fatal outcome. When Digical was used in studies with a guinea-pig animal model of digitalis intoxication the anticalin appeared to be effective in reversing the digoxin-induced toxicity after administering just a moderate stoichiometric excess (Schlehuber and Skerra 2005). The neutralizing effect of the anticalin was likewise confirmed in preclinical studies with farmyard pigs, which more closely reflect the physiological situation in humans, thus demonstrating the acute protective effect of this anticalin on the cardiovascular system and its suitability as an antidote against digoxin.

Recently, the anticalin concept was extended in two directions. First, some human lipocalins, in particular tear lipocalin, siderocalin (also known as NGAL), and apolipoprotein D (Breustedt et al. 2006), were recruited as protein scaffolds for the generation of anticalins in order to reduce the risk of immunogenic side effects upon chronic medical treatment of patients. Second, specialized random libraries were constructed by mutagenizing residues at more exposed positions within the four hypervariable loops to yield anticalins with specificities for larger proteins (i.e. the more relevant class of targets in human therapy) instead of haptens (Schlehuber and Skerra 2005). In an initial proof of concept, an anticalin based on the ApoD scaffold was selected against hemoglobin (Vogt and Skerra 2004).

Another "human" anticalin with specificity for a protein target, based on the NGAL scaffold, was recently selected against CTLA-4 (cf. above). CTLA-4 (CD152) is known as an "immune brake" because it reverses CD28-dependent costimulation of T cells after initial activation. Hence, CTLA-4 has emerged as an attractive target for immunomodulatory drugs that can block its inhibitory function and concomitantly enhance T-cell activity, hence offering potential for cancer immunotherapy (Leach et al. 1996). Neutralizing antibodies against the extracellular domain of human CTLA-4 have been shown to be effective for cancer treatment in several preclinical and clinical studies (Keler et al. 2003). However, an Fc region is not required for the antagonistic function, and in fact it may even lead to undesired side effects. Therefore, an anticalin with high affinity, in the single-digit nanomolar range, was successfully selected against the extracellular region of CTLA-4. The CTLA-4-specific anticalin recognizes the intact target receptor protein on cells, both in immunohistochemistry and in fluorocytometry, and it was shown to bind in an antagonistic manner with the natural counter-receptors B7.1 and B7.2 (Schlehuber and Skerra 2005). Most importantly, it effectively blocks CTLA-4 in cell culture assays, thus providing a promising biopharmaceutical drug candidate for cancer therapy.

7.5 Scaffold Proteins for Display of Individual Extended Loops

Probably the simplest approach for the generation of alternative binding proteins is the modification of a single pre-existing exposed loop that is presented by a stable scaffold. Thus, the peptide loop acquires new binding properties and maintains conformationally fixed on the protein surface. Because of their typically small size and robust nature protease inhibitors provide attractive candidates in this respect. Their natural protease-binding site is mostly formed by a peptide loop of varying length and sequence, which predestines them to insert there novel peptide segments and thus create artificial activities.

Kunitz-type protease inhibitors are small α/β-proteins of about 60 amino acids with few secondary structure elements but stabilized by three disulfide bonds. They naturally act as slow but tight-binding, reversible inhibitors of serine proteases. Bovine pancreatic trypsin inhibitor (BPTI) (Roberts et al. 1992), Alzheimer's amyloid β-protein precursor inhibitor (APPI) (Dennis and Lazarus 1994), tendamistat (McConnell and Hoess 1995), human pancreatic secretory trypsin inhibitor (PSTI) (Röttgen and Collins 1995), and human lipoprotein-associated coagulation inhibitor D1 (LACI-D1) (Markland et al. 1996b) are examples of proteins that have been employed as scaffolds for the presentation of binding peptides.

The selection of novel binding proteins based on the first Kunitz domain of LACI-D1 from a corresponding phage display library yielded particularly promising results. Using procedures of iterative variegation it was possible to select potent inhibitors of human plasma kallikrein (pKAL), a serine protease that is an

important mediator in the pathophysiology of hereditary angioedema (HAE), in two steps: randomization of five positions in the P1 loop of LACI-D1, followed by randomization of another four residues in a neighboring second loop, yielded highly pKAL-specific variants with inhibitory constants (K_i) in the picomolar range, which also exhibit excellent stability (Markland et al. 1996a).

Meanwhile, this approach has led to a first drug candidate (Williams and Baird 2003), DX-88, with potent and selective inhibitory activity towards human plasma kallikrein. In a phase II clinical trial DX-88 provided substantial therapeutic benefit in HAE patients who had experienced acute attacks, including life-threatening laryngeal attacks, irrespective of whether administered through the intravenous or subcutaneous route. Clinical response, defined as the beginning of improvement of HAE symptoms within four hours of dosing with DX-88, was observed at all dose levels. For the intravenous dosing ($5\,mg\,m^{-2}$, $10\,mg\,m^{-2}$, $20\,mg\,m^{-2}$), the response rates ranged from 86 to 100%, while the subcutaneous outcome (30 mg fixed dose) showed a 100% response rate. A corresponding clinical phase III trial is ongoing (http://www.dyax.com).

A second drug candidate derived from a Kunitz-type domain scaffold, DX-890 or EPI-hNE4, is an engineered inhibitor for human neutrophil elastase (hNE). EPI-hNE4 was derived from the second Kunitz domain of inter-α-inhibitor protein (ITI-D2) and is a highly specific and potent inhibitor ($K_i = 4 \times 10^{-12}\,mol\,L^{-1}$) of hNE. The protein of 56 amino acids is produced by fermentation in *Pichia pastoris* and is resistant to oxidative and proteolytic inactivation. EPI-hNE4 has completed two phase IIa trials and is currently subject to a phase IIb trial in Europe for the treatment of cystic fibrosis (http://www.debio.com). Both compounds have obtained orphan drug designation in the US and EU.

Another straightforward strategy for the construction of proteins with new binding properties is the fusion or insertion of a peptide which *per se* shows intrinsic target recognition into a permissible site. Thus, the peptide becomes incorporated into a larger carrier protein that can provide beneficial properties, for example with respect to prolonged serum half-life, and it ideally retains its pre-existing affinity and specificity for the target. This strategy can be extended such that instead of a predefined peptide being inserted, a library of sequences with varying amino acid composition and possibly length is generated in the context of the scaffold protein, followed by selection of variants with the desired target specificity.

Thioredoxin (TrxA) was employed as a first scaffold for the display of single conformationally constrained peptide sequences which were inserted into its active site loop. Naturally, TrxA is a small enzyme involved in the cytosolic thiol/disulfide equilibrium of *E. coli*. It is highly soluble, structurally rigid, can be overexpressed at elevated levels and has been exploited as a generic fusion partner for the bacterial production of recombinant proteins (LaVallie et al. 1993). In the oxidized state of the enzyme its short active site sequence Cys-Gly-Pro-Cys forms a tight, disulfide-constrained and solvent-accessible loop. This segment permits the insertion of diverse peptide sequences, whereby the enzymatic activity gets lost.

When a 20-residue random sequence was inserted into the active site loop, TrxA variants – dubbed "aptamers" – with affinity towards human cyclin-dependent kinase 2 (Cdk2) could be selected by means of the yeast two-hybrid system (Colas et al. 1996). The same selection strategy was applied towards the DNA-binding and dimerization domains of the E2F transcription factors as a target (Fabbrizio et al. 1999). In this case, the variegated segment alone appeared to remain active as a synthetic peptide with respect to blocking cell proliferation in the G_1 phase, hence suggesting that this peptide aptamer is sufficiently structured even in the absence of the thioredoxin framework. Since then several specific peptide aptamers have been selected as inhibitors of individual signaling components that are essential in cancer development and progression, thus providing potential new lead structures for drug development (Borghouts et al. 2005).

Members of the so-called "knottin family" represent small, 25- to 35-residue proteins, some of which are protease inhibitors while others function as sugar- or lipid-binding molecules (Craik et al. 2001). Even though naturally occurring knottins mutually share little sequence homology, they typically contain a small, triple-stranded antiparallel β-sheet and a cysteine-knot motif that arises from three interlocking disulfide bridges. Their small size and their high stability make them attractive as scaffold proteins for presenting an inserted peptide loop that may be highly variable both in sequence and in length. The general suitability of some members of the knottin family for the generation of specific binding proteins was demonstrated with the C-terminal cellulose-binding domain (CBD) of cellobiohydrolase I from the fungus *Trichoderma reesei* (Smith et al. 1998; Lehtio et al. 2000) and with the *Ecballium elaterium* trypsin inhibitor II (EETI-II) (Le Nguyen et al. 1990; Christmann et al. 1999; Hilpert et al. 2003; Souriau et al. 2005). CBD variants with novel binding activities were isolated from a library constructed by randomization of seven residues clustered either close to the N-terminus or close to the C-terminus of the protein. Selection via phagemid display led to the isolation of variants with specificity for alkaline phosphatase, revealing moderate K_D values in the micromolar range (Smith et al. 1998).

Certain classes of neurotoxins possessing specificities for voltage-gated ion channels represent another type of small scaffold that provides exposed loops which may be engineered to achieve novel ligand-binding activities. The structural motif of charybdotoxin, a scorpion toxin that also belongs to the knottin protein family (Norton and Pallaghy 1998), was used in order to transfer other functional sites onto its β-sheet, for instance the CDR2-like loop of human CD4, which conformationally resembles the corresponding β-hairpin in natural charybdotoxin (Vita et al. 1998). The chemically synthesized toxin variant was able to inhibit binding between soluble recombinant gp120 and a HSA-CD4 hybrid protein with an apparent IC_{50} of 20 micromolar (compared with 0.8 nanomolar for HSA-CD4 itself). More recently, the structurally related scyllatoxin, which gives higher yields and purity after chemical peptide synthesis, was employed as an alternative scaffold for CD4-mimetic peptides, which were even able to inhibit HIV-1 infection in cell culture (Dowd et al. 2002; Martin et al. 2003; Huang et al. 2005).

Another principle, originally devised for the biological delivery of peptide drugs, relies on a transport protein from blood, human serum transferrin (HST). This monomeric glycoprotein with a molecular mass of about 80 kDa was used for the functional insertion of a peptide sequence cleavable by HIV-1 protease (VSQNYPIVL) into a permissible surface loop, without altering its overall structure and physiological function (Ali et al. 1999b). HST possesses favorable intrinsic properties such as a long circulatory half-life, which makes it suitable as a therapeutic peptide drug carrier. Interestingly, the insertion of the otherwise highly immunogenic HIV-1 protease substrate peptide into the HST scaffold resulted in an attenuated antipeptide immune response in rabbits (Ali et al. 1999a), which might be an advantage for clinical applications.

An interesting extension to this approach is the use of a scaffold protein with intrinsic enzymatic activity to insert a peptide motif that confers targeting function. The class of bacterial TEM-1 β-lactamases provides extremely efficient enzymes, with high turnover numbers when catalyzing the nucleophilic cleavage of the amide bond in β-lactam antibiotics (Christensen et al. 1990). Notably, β-lactamase has no mammalian counterpart, which makes it of interest for selective prodrug activation in tumor chemotherapy (McDonagh et al. 2003; Cortez-Retamozo et al. 2004). Due to the uniqueness of the catalyzed reaction, a variety of specific substrates have been synthesized (Hakimelahi et al. 2002a,b).

Different combinatorial libraries of TEM-1 β-lactamase were constructed introducing one or two insertions of six random residues into permissible surface loops that surround the catalytic site of the enzyme. The libraries were first selected on ampicillin to remove inactive clones and then used for isolating variants with novel binding activities via phage display (Legendre et al. 1999). Variants were successfully selected for binding to monoclonal antibodies against the prostate-specific antigen (PSA) or to streptavidin. These engineered enzymes with their integrated target-binding activities were applied in homogeneous immunoassays as binding of the antigen markedly interfered with β-lactamase activity. In a following study β-lactamase variants with specificities towards ferritin and β-galactosidase could be isolated and were shown to retain nearly native enzyme activity (Legendre et al. 2002). Bifunctional proteins of this kind might prove useful to replace enzyme-conjugated antibodies in antibody-directed enzyme prodrug therapy (ADEPT), especially when further engineered for reduced immunogenicity (Harding et al. 2005).

Taken together, although the molecular diversity that may be obtained with a single peptide segment is more restricted than in the case of randomizing several loops at once, novel binding proteins with advantageous properties can be identified by this strategy. This is especially the case if the target protein offers a narrow cleft which provides access to a slim peptide motif for molecular interaction, as exemplified with the successful selection of novel inhbitors of serine proteases. In general, the presentation of a peptide by a protein scaffold offers an advantage with respect to the achievable affinity compared with the isolated peptide. While the latter usually assumes a flexible conformation in solution, fixation of the

peptide backbone by the scaffold leads to a lower entropic cost upon complex formation. On the other hand, a peptide with pre-existing target affinity may lose its binding activity when presented by a protein scaffold as a consequence of sterical restriction. Indeed, the context dependence of such a peptide was recently shown when attempts were made to transfer peptide aptamers that were initially selected on the TrxA scaffold onto a different protein (e.g. to green fluorescent protein (GFP) or staphylococcal nuclease), resulting in loss of binding activity (Klevenz et al. 2002).

7.6 Scaffold Proteins Providing a Rigid Secondary Structure Interface

A different class of protein scaffolds does not make use of the loop-mediated binding mechanisms described in the previous sections. Rather, target recognition is accomplished through amino acids that are mostly situated within rigid secondary structure elements. In this case, solvent-exposed side-chains on the surface of an α-helix bundle or of a β-sheet are randomized in order to modify a pre-existed binding site or to generate an entirely novel interface for molecular recognition.

One of the first scaffold proteins investigated in this context was derived from protein Z and later dubbed "affibody." Protein Z represents the engineered domain B of the IgG-binding "Protein A" on the cell surface of *Staphylococcus aureus*, a small three-helix bundle of 58 amino acids (Uhlen et al. 1992; Nygren and Skerra 2004). Affibody libraries were generated by randomization of up to 13 exposed amino acids on the surface of the two α-helices that are naturally involved in binding to the Fc part of antibodies (Nord et al. 1995). Interestingly, this extensive randomization of secondary structure elements was possible without affecting the overall structure of the parental scaffold, even though some of the resulting variants may adopt molten globule structures in the absence of their target proteins (Wahlberg et al. 2003).

According to this strategy, protein-binding affibodies displaying micromolar affinities for *Taq* DNA polymerase, human insulin, and human apolipoprotein A-1, were selected by phage display (Nord et al. 1997) and, in one case, subsequently improved to achieve a dissociation constant in the nanomolar range (Gunneriusson et al. 1999). Affibodies were also selected against several therapeutically relevant targets, including human CD28, whereby the affibody was shown to block the interaction with the immunological counter-receptor CD80 that is involved in T-cell stimulation (Sandstrom et al. 2003). Similarly, an affibody was raised against the breast cancer target HER2/neu, exhibiting nanomolar affinity and, after radiolabeling, showing specific binding to the native receptor on HER2-expressing cells (Wikman et al. 2004). To take advantage of an avidity effect a dimeric version of this affibody was constructed, which seems to be a promising candidate for radionuclide-based detection of HER-2 expression in tumors *in vivo* (Steffen et al. 2005, 2006).

The ankyrin repeat proteins (ARPs) offer another rigid and also modular architecture that has been employed for the generation of artificial binding proteins. The ARP family comprises a variety of natural receptor proteins that are directed against other proteins, mostly in the cellular cytoplasm but also in the extracellular environment (Mosavi et al. 2004). Each of their characteristic repeats of 33 amino acids exhibits a β-turn and two antiparallel α-helices. This fold provided the basis for the generation of so-called "designed ankyrin repeat proteins" (DARPins) which contain up to three of such repeats flanked by N- and C-terminal capping modules (Binz et al. 2003).

Libraries of DARPins were generated starting from a consensus module as building block and randomizing six positions per repeat, whereby in principle a varying number of units may be employed (Binz et al. 2003; Forrer et al. 2004). Substitutions were mainly allowed within the β-turn and the first α-helix of each repeat. Using ribosome display, DARPins with nanomolar affinities and specificities for the *E. coli* maltose-binding protein as well as for the eukaryotic mitogen-activated kinases JNK2 and p38 were isolated (Binz et al. 2004). The beneficial biophysical properties of the parental ankyrin scaffold (high-level expression, solubility, and stability) were largely retained in these DARPins.

Recently, high-affinity inhibitors of aminoglycoside phosphotransferase (3′)-IIIa (APH) were also isolated from a DARPin library. *In vitro* and *in vivo* assays showed complete enzyme inhibition, thus underlining the potential of DARPins for modulation of intracellular protein function (Amstutz et al. 2005). One of these inhibitors was co-crystallized with the target protein and its allosteric inhibition mechanism was elucidated (Kohl et al. 2005). Interestingly, so-called leucine-rich repeat (LRP) proteins, which share a certain structural resemblance with ARPs, were found to mediate the adaptive immune response of the sea lamprey (Pancer et al. 2004), a jawless vertebrate, indicating that protein architectures other than the immunoglobulin fold may be utilized in higher organisms for diversification and selection under natural conditions.

"Avimers" are artificial multidomain proteins derived from the so-called A-domain scaffold. This concept makes use of multiple interactions with the target protein, thus involving an avidity effect (Silverman et al. 2005). A native A-domain comprises just 35 amino acids and folds efficiently into a defined conformation that is stabilized by disulfide bond formation and complexation of calcium ions. A conserved sequence motif of merely 12 residues seems to be required to adopt this structure. Random libraries of individual A-domains were generated by taking advantage of their natural diversity, whereas positions with 90% sequence identity in an alignment of 197 homologous sequences were kept fixed. Degenerate codons at the approx. 28 variable positions were chosen to allow only amino acids that occur in natural family members.

Selection of multimers with novel binding activities was achieved by means of phage display, using selection of different binding domains which each recognize another epitope on the target protein and subsequent combination in a single fusion protein. According to this strategy avimers with subnanomolar affinities were finally obtained for IL-6, CD28, and CD40L (Silverman et al. 2005). Trimeric

avimers directed against IL-6 were subjected to preclinical studies. To prolong their circulation, a domain with IgG-binding activity was added to the N-terminus, resulting in an expected half-life of around 178 h in human serum as predicted from pharmacokinetic studies in cynomolgus monkeys. A subpicomolar IC_{50} value was demonstrated for the best of these avimers in cell-based proliferation assays. Furthermore, this avimer completely abrogated acute phase protein induction by human IL-6 (but not by IL-1) in a dose-dependent manner in mice (Silverman et al. 2005).

Further to these scaffold examples, which were based on protein domains with certain natural binding functions, some proteins with rigid secondary structure that do not possess a binding activity of their own were engineered to implement molecular recognition. In this strategy, which has led to so-called "affilin" proteins, the choice of suitable frameworks was primarily driven by biotechnological aspects such as protein stability, solubility, and ease of recombinant production. For example, human γ-crystallin, a protein of 176 amino acids, folds into an overall β-sheet structure with extraordinary stability (Jaenicke and Slingsby 2001). Once deposited at high protein concentration in the eye lens during embryogenesis it fulfils its function (i.e. providing the refractory power necessary for vision) during the entire lifetime without any turnover. By randomization of eight surface-exposed amino acids on two adjacent β-strands it was possible to create a *de novo* binding site for predefined targets, for example steroid hormones (Fiedler and Rudolph 2001). Also human ubiquitin, a rather small 76-amino-acid protein that exhibits an exposed β-sheet, possesses remarkable stability against chemical and physical denaturation and has served to generate affilins in a similar approach (Fiedler et al. 2004). Affilins with binding activities in the nanomolar K_D range towards steroids and towards disease-relevant proteins were successfully generated using both protein scaffolds.

Whereas the binding sites of antibodies as well as of their surrogates that utilize flexible loops, as described in the sections above, can undergo substantial changes upon binding of a molecular target (called induced fit), artificial binding proteins that are based on secondary structure elements are likely to act more as rigid bodies. Nevertheless, many examples of such a classical lock-and-key mechanism of protein–protein interaction are known from nature and can indeed lead to very specific and tight complex formation. In fact, if both partners already present a geometry with mutual complementary, no structural adaptation is required. It is speculated (Binz et al. 2004) that such a rigid body interaction might be advantageous both for affinity, because of low entropic costs upon binding, and specificity, due to conformational restriction. However, in cases where no geometric complementarity exists in the first place the antigen itself may be forced to respond with an induced fit to complex formation, in particular if the binding protein exhibits a stiff fold. A nice example for such an inverse mechanism of conformational adaptation is seen in the crystal structure of a complex between a cognate DARPin with aminoglycoside phosphotransferase, where a whole loop is pulled out of the target protein and eventually leads to loss of enzymatic function via a conformational mechanism (Kohl et al. 2005).

7.7 Conclusions and Outlook: Therapeutic Potential and Ongoing Developments

Up to now the concept of engineering novel scaffold-based binding proteins with high medical target specificity and affinity was proven in various approaches, leading in some cases to convincing preclinical data packages or even to investigational drug candidates for clinical trials. Concerning their newly acquired binding functions, these engineered proteins resemble classical antibodies with their exquisite abilities to recognize antigens, but lack their immunological effector functions. Nevertheless, in order to gain wider acceptance as putative therapeutic agents in the future artificial binding proteins will have to fulfil not only the criteria in terms of specificity and high affinity but they also have to offer further advantages.

In this respect the worldwide limited manufacturing capacity for full-sized (humanized) antibodies and their high production cost (Werner 2004) probably provides one of the strongest arguments for the development and clinical application of simpler surrogates in order to serve the public with an innovative and beneficial class of biotherapeutics without overstraining current healthcare systems. In contrast to antibodies, most of the alternative scaffolds described so far are single-domain proteins and do not require posttranslational modification, which facilitates their production in microbial organisms at the industrial scale. When produced in *E. coli* the yields of some scaffold proteins can be in the gram per liter range at the fermenter scale. Furthermore, they may even fold properly under reducing conditions, thus permitting cytoplasmic expression in a soluble form, which might also open new therapeutic approaches by using intracellular antagonistic or inhibitory activities as an alternative to gene-based knockout techniques (Amstutz et al. 2005).

Another important point that has to be considered concerns pharmacokinetic and pharmacodynamic parameters such as clearance rate, serum stability, bioavailability, and tissue penetration. Because of their small size, most of the alternative scaffolds exhibit rapid clearance from the bloodstream, but for the same reason they show improved tissue penetration, making alternative binding proteins ideally suited for drug targeting applications. In this respect their amenability to genetic fusion with protein toxins or to the deliberate introduction of specific coupling sites for payloads such as radioisotopes or photosensitizers provides an additional benefit. However, a short serum half-life is disadvantageous if high drug levels have to be maintained for a longer period without using constant infusion. In this case, the rate of renal clearance can be slowed down by enlarging the size of the binding protein (e.g. by PEGylation) or by enabling complex formation with abundant serum proteins, such as HSA or Ig, which possess a much longer half-life.

As the appearance of neutralizing host antibodies to therapeutic proteins is an issue of general concern (Koren et al. 2002; Schellekens 2002), the immunogenicity of scaffold-based biopharmaceuticals deserves careful examination. To reduce the risk of eliciting an immune response in patients, efforts are taken to make

non-human therapeutic proteins as similar to their human counterparts as possible. For antibodies, numerous examples of "humanization" by grafting rodent CDR residues onto human acceptor scaffolds, deimmunization by removing T-cell epitopes, and production of human antibodies in transgenic mice have been described (see Chapter 4, 6, 11, Vol I). On the other hand, an endogenous human scaffold protein that is employed for library construction should be less immunogenic right from the start even though novel T-cell epitopes might arise during the generation of the target-binding site. However, these may be identified and removed using bioinformatics methods (Schirle et al. 2001; Flower 2003; Bian and Hammer 2004). Nevertheless, even administration of entirely human proteins, for example insulin, can elicit antibodies without preventing clinical use. In the end, only clinical trials can give a reliable picture of a protein's behavior in humans and the next few years will certainly provide a lot of insight into the medical benefits, but possibly also the caveats, of currently emerging therapeutic antibody surrogates.

References

Agu, R.U., Ugwoke, M.I., Armand, M., Kinget, R., Verbeke, N. (2001) The lung as a route for systemic delivery of therapeutic proteins and peptides. *Respir Res* 2: 198–209.

Ali, S.A., Joao, H.C., Hammerschmid, F., Eder, J., Steinkasserer, A. (1999a) An antigenic HIV-1 peptide sequence engineered into the surface structure of transferrin does not elicit an antibody response. *FEBS Lett* 459: 230–232.

Ali, S.A., Joao, H.C., Hammerschmid, F., Eder, J., Steinkasserer, A. (1999b) Transferrin trojan horses as a rational approach for the biological delivery of therapeutic peptide domains. *J Biol Chem* 274: 24066–24073.

Amstutz, P., Binz, H.K., Parizek, P., Stumpp, M.T., Kohl, A., Grutter, M.G., Forrer, P., Plückthun, A. (2005) Intracellular kinase inhibitors selected from combinatorial libraries of designed ankyrin repeat proteins. *J Biol Chem* 280: 24715–24722.

Atwell, J.L., Breheney, K.A., Lawrence, L.J., McCoy, A.J., Kortt, A.A., Hudson, P.J. (1999) scFv multimers of the anti-neuraminidase antibody NC10: length of the linker between VH and VL domains dictates precisely the transition between diabodies and triabodies. *Protein Eng* 12: 597–604.

Baker, M. (2005) Upping the ante on antibodies. *Nat Biotechnol* 23: 1065–1072.

Bang, L.M., Keating, G.M. (2004) Adalimumab: a review of its use in rheumatoid arthritis. *BioDrugs* 18: 121–139.

Barry, B.W. (2004) Breaching the skin's barrier to drugs. *Nat Biotechnol* 22: 165–167.

Beste, G., Schmidt, F.S., Stibora, T., Skerra, A. (1999) Small antibody-like proteins with prescribed ligand specificities derived from the lipocalin fold. *Proc Natl Acad Sci USA* 96: 1898–1903.

Bian, H., Hammer, J. (2004) Discovery of promiscuous HLA-II-restricted T cell epitopes with TEPITOPE. *Methods* 34: 468–475.

Binz, H.K., Plückthun, A. (2005) Engineered proteins as specific binding reagents. *Curr Opin Biotechnol* 16: 459–469.

Binz, H.K., Stumpp, M.T., Forrer, P., Amstutz, P., Plückthun, A. (2003) Designing repeat proteins: well-expressed, soluble and stable proteins from combinatorial libraries of consensus ankyrin repeat proteins. *J Mol Biol* 332: 489–503.

Binz, H.K., Amstutz, P., Kohl, A., Stumpp, M.T., Briand, C., Forrer, P., Grutter, M.G., Plückthun, A. (2004) High-affinity binders selected from designed ankyrin repeat protein libraries. *Nat Biotechnol* 22: 575–582.

Binz, H.K., Amstutz, P., Plückthun, A. (2005) Engineering novel binding proteins from nonimmunoglobulin domains. *Nat Biotechnol* 23: 1257–1268.

Bird, R.E.and Walker, B.W. (1991) Single chain antibody variable regions. *Trends Biotechnol* 9: 132–137.

Borghouts, C., Kunz, C., Groner, B. (2005) Peptide aptamers: recent developments for cancer therapy. *Expert Opin Biol Ther* 5: 783–797.

Breustedt, D.A., Schönfeld, D.L., Skerra, A. (2006) Comparative ligand-binding analysis of ten human lipocalins. *Biochim Biophys Acta* 1764: 161–173.

Cai, X., Garen, A. (1996) A melanoma-specific VH antibody cloned from a fusion phage library of a vaccinated melanoma patient. *Proc Natl Acad Sci USA* 93: 6280–6285.

Carson, K.L. (2005) Flexibility – the guiding principle for antibody manufacturing. *Nat Biotechnol* 23: 1054–1058.

Carter, P. (2001) Improving the efficacy of antibody-based cancer therapies. *Nat Rev Cancer* 1: 118–129.

Chamow, S.M., Ashkenazi, A. (1996) Immunoadhesins: principles and applications. *Trends Biotechnol* 14: 52–60.

Christensen, H., Martin, M.T., Waley, S.G. (1990) Beta-lactamases as fully efficient enzymes. Determination of all the rate constants in the acyl-enzyme mechanism. *Biochem J* 266: 853–861.

Christmann, A., Walter, K., Wentzel, A., Kratzner, R., Kolmar, H. (1999) The cystine knot of a squash-type protease inhibitor as a structural scaffold for *Escherichia coli* cell surface display of conformationally constrained peptides. *Protein Eng* 12: 797–806.

Colas, P., Cohen, B., Jessen, T., Grishina, I., McCoy, J., Brent, R. (1996) Genetic selection of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2. *Nature* 380: 548–550.

Connelly, R. (2005) Fully human domain antibody therapeutics: the best of both worlds. *Innovations Pharmac Technol* 42–45.

Conrath, K.E., Lauwereys, M., Galleni, M., Matagne, A., Frere, J.M., Kinne, J., Wyns, L., Muyldermans, S. (2001) β-Lactamase inhibitors derived from single-domain antibody fragments elicited in the Camelidae. *Antimicrob Agents Chemother* 45: 2807–2812.

Conrath, K., Vincke, C., Stijlemans, B., Schymkowitz, J., Decanniere, K., Wyns, L., Muyldermans, S., Loris, R. (2005) Antigen binding and solubility effects upon the veneering of a camel VHH in framework-2 to mimic a VH. *J Mol Biol* 350: 112–125.

Cortez-Retamozo, V., Backmann, N., Senter, P.D., Wernery, U., De Baetselier, P., Muyldermans, S., Revets, H. (2004) Efficient cancer therapy with a nanobody-based conjugate. *Cancer Res* 64: 2853–2857.

Craik, D. J., Daly, N.L., Waine, C. (2001) The cystine knot motif in toxins and implications for drug design. *Toxicon* 39: 43–60.

Davies, J., Riechmann, L. (1994) "Camelising" human antibody fragments: NMR studies on VH domains. *FEBS Lett* 339: 285–290.

De Genst, E., Saerens, D., Muyldermans, S., Conrath, K. (2006) Antibody repertoire development in camelids. *Dev Comp Immunol* 30: 187–198.

Dennis, M.S., Lazarus, R.A. (1994) Kunitz domain inhibitors of tissue factor-factor VIIa. I. Potent inhibitors selected from libraries by phage display. *J Biol Chem* 269: 22129–22136.

Dennis, M.S., Zhang, M., Meng, Y.G., Kadkhodayan, M., Kirchhofer, D., Combs, D., Damico, L.A. (2002) Albumin binding as a general strategy for improving the pharmacokinetics of proteins. *J Biol Chem* 277: 35035–35043.

Dooley, H., Flajnik, M.F., Porter, A.J. (2003) Selection and characterization of naturally occurring single-domain (IgNAR) antibody fragments from immunized sharks by phage display. *Mol Immunol* 40: 25–33.

Dowd, C.S., Leavitt, S., Babcock, G., Godillot, A.P., Van Ryk, D., Canziani, G. A., Sodroski, J., Freire, E., Chaiken, I.M. (2002) Beta-turn Phe in HIV-1 Env binding site of CD4 and CD4 mimetic miniprotein enhances Env binding affinity

but is not required for activation of co-receptor/17b site. *Biochemistry* 41: 7038–7046.
Dumoulin, M., Conrath, K., Van Meirhaeghe, A., Meersman, F., Heremans, K., Frenken, L.G., Muyldermans, S., Wyns, L., Matagne, A. (2002) Single-domain antibody fragments with high conformational stability. *Protein Sci* 11: 500–515.
Editor (2005) King in the kingdom of uncertainty. *Nat Biotechnol* 23: 1025.
Evans, D., Das, R. (2005) *Monoclonal Antibodies: Evolving into a $30 Billion Market*. www.datamonitor.com.
Ewert, S., Cambillau, C., Conrath, K., Plückthun, A. (2002) Biophysical properties of camelid V(HH) domains compared to those of human V(H)3 domains. *Biochemistry* 41: 3628–3636.
Fabbrizio, E., Le Cam, L., Polanowska, J., Kaczorek, M., Lamb, N., Brent, R., Sardet, C. (1999) Inhibition of mammalian cell proliferation by genetically selected peptide aptamers that functionally antagonize E2F activity. *Oncogene* 18: 4357–4363.
Fiedler, U., Rudolph, R. (2001) Fabrication of beta-pleated sheet proteins with specific binding properties. International patent publication W00104144 (A3).
Fiedler, M., Fiedler, U., Rudolph, R. (2004) Generation of artificial binding proteins based on ubiquitin proteins. International patent publication W02004106368 (A1).
Florent, J.C., Dong, X., Gaudel, G., Mitaku, S., Monneret, C., Gesson, J.P., Jacquesy, J.C., Mondon, M., Renoux, B., Andrianomenjanahary, S., Michel, S., Koch, M., Tillequin, F., Gerken, M., Czech, J., Straub, R., Bosslet, K. (1998) Prodrugs of anthracyclines for use in antibody-directed enzyme prodrug therapy. *J Med Chem* 41: 3572–3581.
Flower, D.R. (1996) The lipocalin protein family: structure and function. *Biochem J* 318: 1–14.
Flower, D.R. (2003) Towards in silico prediction of immunogenic epitopes. *Trends Immunol* 24: 667–674.
Forrer, P., Binz, H.K., Stumpp, M.T., Plückthun, A. (2004) Consensus design of repeat proteins. *Chembiochem* 5: 183–189.
Gessner, J.E., Heiken, H., Tamm, A., Schmidt, R.E. (1998) The IgG Fc receptor family. *Ann Hematol* 76: 231–248.
Ghetie, V., Ward, E.S. (2002) Transcytosis and catabolism of antibody. *Immunol Res* 25: 97–113.
Glockshuber, R., Malia, M., Pfitzinger, I., Plückthun, A. (1990) A comparison of strategies to stabilize immunoglobulin F_v-fragments. *Biochemistry* 29: 1362–1367.
Goldberg, M., Gomez-Orellana, I. (2003) Challenges for the oral delivery of macromolecules. *Nat Rev Drug Discov* 2: 289–295.
Groves, M.A., Osbourn, J.K. (2005) Applications of ribosome display to antibody drug discovery. *Expert Opin Biol Ther* 5: 125–135.
Gunneriusson, E., Nord, K., Uhlen, M., Nygren, P. (1999) Affinity maturation of a Taq DNA polymerase specific affibody by helix shuffling. *Protein Eng* 12: 873–878.
Hakimelahi, G.H., Shia, K.S., Pasdar, M., Hakimelahi, S., Khalafi-Nezhad, A., Soltani, M.N., Mei, N.W., Mei, H.C., Saboury, A.A., Rezaei-Tavirani, M., Moosavi-Movahedi, A.A. (2002a) Design, synthesis, and biological evaluation of a cephalosporin-monohydroguaiaretic acid prodrug activated by a monoclonal antibody-beta-lactamase conjugate. *Bioorg Med Chem* 10: 2927–2932.
Hakimelahi, G.H., Shia, K.S., Xue, C., Hakimelahi, S., Moosavi-Movahedi, A.A., Saboury, A.A., Khalafi-Nezhad, A., Soltani-Rad, M.N., Osyetrov, V., Wang, K., Liao, J. H., Luo, F.T. (2002b) Design, synthesis, and biological evaluation of a series of beta-lactam-based prodrugs. *Bioorg Med Chem* 10: 3489–3498.
Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E.B., Bendahman, N., Hamers, R. (1993) Naturally occurring antibodies devoid of light chains. *Nature* 363: 446–448.
Harding, F.A., Liu, A.D., Stickler, M., Razo, O.J., Chin, R., Faravashi, N., Viola, W., Graycar, T., Yeung, V.P., Aehle, W., Meijer, D., Wong, S., Rashid, M.H., Valdes, A.M., Schellenberger, V. (2005) A beta-lactamase with reduced immunogenicity for the targeted delivery of chemotherapeutics

using antibody-directed enzyme prodrug therapy. *Mol Cancer Ther* 4: 1791–1800.
Harris, J.M., Chess, R.B. (2003) Effect of pegylation on pharmaceuticals. *Nat Rev Drug Discov* 2: 214–221.
Hauptman, P.J., Kelly, R.A. (1999) Digitalis. *Circulation* 99: 1265–1270.
Hey, T., Fiedler, E., Rudolph, R., Fiedler, M. (2005) Artificial, non-antibody binding proteins for pharmaceutical and industrial applications. *Trends Biotechnol* 23: 514–522.
Hilpert, K., Wessner, H., Schneider-Mergener, J., Welfle, K., Misselwitz, R., Welfle, H., Hocke, A.C., Hippenstiel, S., Höhne, W. (2003) Design and characterization of a hybrid miniprotein that specifically inhibits porcine pancreatic elastase. *J Biol Chem* 278: 24986–24993.
Holliger, P., Hudson, P.J. (2005) Engineered antibody fragments and the rise of single domains. *Nat Biotechnol* 23: 1126–1136.
Holt, L.J., Herring, C., Jespers, L.S., Woolven, B.P., Tomlinson, I.M. (2003) Domain antibodies: proteins for therapy. *Trends Biotechnol* 21: 484–490.
Hosse, R.J., Rothe, A., Power, B.E. (2006) A new generation of protein display scaffolds for molecular recognition. *Protein Sci* 15: 14–27.
Huang, C.C., Stricher, F., Martin, L., Decker, J.M., Majeed, S., Barthe, P., Hendrickson, W.A., Robinson, J., Roumestand, C., Sodroski, J., Wyatt, R., Shaw, G.M., Vita, C., Kwong, P.D. (2005) Scorpion-toxin mimics of CD4 in complex with human immunodeficiency virus gp120 crystal structures, molecular mimicry, and neutralization breadth. *Structure* 13: 755–768.
Hufton, S.E., van Neer, N., van den Beuken, T., Desmet, J., Sablon, E., Hoogenboom, H.R. (2000) Development and application of cytotoxic T lymphocyte-associated antigen 4 as a protein scaffold for the generation of novel binding ligands. *FEBS Lett* 475: 225–231.
Irving, R.A., Coia, G., Roberts, A., Nuttall, S.D., Hudson, P.J. (2001) Ribosome display and affinity maturation: from antibodies to single V-domains and steps towards cancer therapeutics. *J Immunol Methods* 248: 31–45.
Jaenicke, R., Slingsby, C. (2001) Lens crystallins and their microbial homologs: structure, stability, and function. *Crit Rev Biochem Mol Biol* 36: 435–499.
Jeong, K.J., Mabry, R., Georgiou, G. (2005) Avimers hold their own. *Nat Biotechnol* 23: 1493–1494.
Jespers, L., Schon, O., James, L.C., Veprintsev, D., Winter, G. (2004) Crystal structure of HEL4: a soluble, refoldable human V(H) single domain with a germ-line scaffold. *J Mol Biol* 337: 893–903.
Keler, T., Halk, E., Vitale, L., O'Neill, T., Blanset, D., Lee, S., Srinivasan, M., Graziano, R.F., Davis, T., Lonberg, N., Korman, A. (2003) Activity and safety of CTLA-4 blockade combined with vaccines in cynomolgus macaques. *J Immunol* 171: 6251–6259.
Klevenz, B., Butz, K., Hoppe-Seyler, F. (2002) Peptide aptamers: exchange of the thioredoxin-A scaffold by alternative platform proteins and its influence on target protein binding. *Cell Mol Life Sci* 59 1993–1998.
Kohl, A., Amstutz, P., Parizek, P., Binz, H. K., Briand, C., Capitani, G., Forrer, P., Pluckthun, A., Grutter, M.G. (2005) Allosteric inhibition of aminoglycoside phosphotransferase by a designed ankyrin repeat protein. *Structure (Camb)* 13: 1131–1141.
Köhler, G., Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.
Koide, A., Bailey, C.W., Huang, X., Koide, S. (1998) The fibronectin type III domain as a scaffold for novel binding proteins. *J Mol Biol* 284: 1141–1151.
Koren, E., Zuckerman, L.A., Mire-Sluis, A.R. (2002) Immune responses to therapeutic proteins in humans – clinical significance, assessment and prediction. *Curr Pharm Biotechnol* 3: 349–360.
Korndörfer, I.P., Beste, G., Skerra, A. (2003a) Crystallographic analysis of an "anticalin" with tailored specificity for fluorescein reveals high structural plasticity of the lipocalin loop region. *Proteins Struct Funct Genet* 53: 121–129.
Korndörfer, I.P., Schlehuber, S., Skerra, A. (2003b) Structural mechanism of specific ligand recognition by a lipocalin tailored

for the complexation of digoxigenin. *J Mol Biol* 330: 385–396.
Lahteenmaki, R., Baker, M. (2004) Public biotechnology 2003 – the numbers. *Nat Biotechnol* 22: 665–670.
Lauwereys, M., Ghahroudi, M.A., Desmyter, A., Kinne, J., Hölzer, W., De Genst, E., Wyns, L., Muyldermans, S. (1998) Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *EMBO J* 17: 3512–3520.
LaVallie, E.R., DiBlasio, E.A., Kovacic, S., Grant, K.L., Schendel, P.F., McCoy, J.M. (1993) A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E. coli cytoplasm. *Biotechnology (NY)* 11: 187–193.
Le Nguyen, D., Heitz, A., Chiche, L., Castro, B., Boigegrain, R.A., Favel, A., Coletti-Previero, M.A. (1990) Molecular recognition between serine proteases and new bioactive microproteins with a knotted structure. *Biochimie* 72: 431–435.
Leach, D.R., Krummel, M.F.and Allison, J.P. (1996) Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 271: 1734–1736.
Legendre, D., Soumillion, P., Fastrez, J. (1999) Engineering a regulatable enzyme for homogeneous immunoassays. *Nat Biotechnol* 17: 67–72.
Legendre, D., Vucic, B., Hougardy, V., Girboux, A.L., Henrioul, C., Van Haute, J., Soumillion, P., Fastrez, J. (2002) TEM-1 beta-lactamase as a scaffold for protein recognition and assay. *Protein Sci* 11: 1506–1518.
Lehtio, J., Teeri, T.T., Nygren, P.A. (2000) Alpha-amylase inhibitors selected from a combinatorial library of a cellulose binding domain scaffold. *Proteins* 41: 316–322.
Lejon, S., Frick, I.M., Bjorck, L., Wikstrom, M., Svensson, S. (2004) Crystal structure and biological implications of a bacterial albumin binding module in complex with human serum albumin. *J Biol Chem* 279: 42924–42928.
Li, E., Pedraza, A., Bestagno, M., Mancardi, S., Sanchez, R., Burrone, O. (1997) Mammalian cell expression of dimeric small immune proteins (SIP). *Protein Eng* 10: 731–736.
Li, R., Hoess, R.H., Bennett, J.S., DeGrado, W.F. (2003) Use of phage display to probe the evolution of binding specificity and affinity in integrins. *Protein Eng* 16: 65–72.
Lowman, H.B., Wells, J.A. (1991) Monovalent phage display: a method for selecting variant proteins from random libraries. *Methods* 3 205–216.
Lu, D.L., Collison, A.M., Kowalski, T.J. (2005) The patentability of antibodies in the United States. *Nat Biotechnol* 23: 1079–1080.
Markland, W., Ley, A.C., Ladner, R.C. (1996a) Iterative optimization of high-affinity protease inhibitors using phage display. 2. Plasma kallikrein and thrombin. *Biochemistry* 35: 8058–8067.
Markland, W., Ley, A.C., Lee, S.W., Ladner, R.C. (1996b) Iterative optimization of high-affinity proteases inhibitors using phage display. 1. Plasmin. *Biochemistry* 35: 8045–8057.
Martin, L., Stricher, F., Misse, D., Sironi, F., Pugniere, M., Barthe, P., Prado-Gotor, R., Freulon, I., Magne, X., Roumestand, C., Menez, A., Lusso, P., Veas, F., Vita, C. (2003) Rational design of a CD4 mimic that inhibits HIV-1 entry and exposes cryptic neutralization epitopes. *Nat Biotechnol* 21: 71–76.
Masat, L., Wabl, M., Johnson, J.P. (1994) A simpler sort of antibody. *Proc Natl Acad Sci USA* 91: 893–896.
McConnell, S.J., Hoess, R.H. (1995) Tendamistat as a scaffold for conformationally constrained phage peptide libraries. *J Mol Biol* 250: 460–470.
McDonagh, C.F., Beam, K.S., Wu, G.J., Chen, J.H., Chace, D.F., Senter, P.D., Francisco, J.A. (2003) Improved yield and stability of L49-sFv-beta-lactamase, a single-chain antibody fusion protein for anticancer prodrug activation, by protein engineering. *Bioconjug Chem* 14: 860–869.
Mosavi, L.K., Cammett, T.J., Desrosiers, D. C., Peng, Z.Y. (2004) The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci* 13: 1435–1448.
Muyldermans, S., Cambillau, C., Wyns, L. (2001) Recognition of antigens by single-domain antibody fragments: the superfluous luxury of paired domains. *Trends Biochem Sci* 26: 230–235.
Nord, K., Nilsson, J., Nilsson, B., Uhlen, M., Nygren, P.A. (1995) A combinatorial

library of an alpha-helical bacterial receptor domain. *Protein Eng* 8: 601–608.

Nord, K., Gunneriusson, E., Ringdahl, J., Stahl, S., Uhlen, M., Nygren, P.A. (1997) Binding proteins selected from combinatorial libraries of an alpha-helical bacterial receptor domain. *Nat Biotechnol* 15: 772–777.

Norton, R.S., Pallaghy, P.K. (1998) The cystine knot structure of ion channel toxins and related polypeptides. *Toxicon* 36: 1573–1583.

Nuttall, S.D., Rousch, M.J., Irving, R.A., Hufton, S.E., Hoogenboom, H.R., Hudson, P.J. (1999) Design and expression of soluble CTLA-4 variable domain as a scaffold for the display of functional polypeptides. *Proteins* 36: 217–227.

Nuttall, S.D., Krishnan, U.V., Hattarki, M., De Gori, R., Irving, R.A., Hudson, P.J. (2001) Isolation of the new antigen receptor from wobbegong sharks, and use as a scaffold for the display of protein loop libraries. *Mol Immunol* 38: 313–326.

Nuttall, S.D., Krishnan, U.V., Doughty, L., Pearson, K., Ryan, M.T., Hoogenraad, N.J., Hattarki, M., Carmichael, J.A., Irving, R. A., Hudson, P.J. (2003) Isolation and characterization of an IgNAR variable domain specific for the human mitochondrial translocase receptor Tom70. *Eur J Biochem* 270: 3543–3554.

Nuttall, S.D., Humberstone, K.S., Krishnan, U.V., Carmichael, J.A., Doughty, L., Hattarki, M., Coley, A.M., Casey, J.L., Anders, R.F., Foley, M., Irving, R.A., Hudson, P.J. (2004) Selection and affinity maturation of IgNAR variable domains targeting Plasmodium falciparum AMA1. *Proteins* 55: 187–197.

Nygren, P.A., Skerra, A. (2004) Binding proteins from alternative scaffolds. *J Immunol Methods* 290: 3–28.

Osborn, B.L., Olsen, H.S., Nardelli, B., Murray, J.H., Zhou, J.X., Garcia, A., Moody, G., Zaritskaya, L.S., Sung, C. (2002) Pharmacokinetic and pharmacodynamic studies of a human serum albumin-interferon-alpha fusion protein in cynomolgus monkeys. *J Pharmacol Exp Ther* 303: 540–548.

Owens, J. (2005) Genentech calm Cabilly storm. *Nat Rev Drug Discov* 4: 876.

Pack, P., Plückthun, A. (1992) Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric F_V fragments with high avidity in *Escherichia coli*. *Biochemistry* 31: 1579–1584.

Padlan, E.A. (1994) Anatomy of the antibody molecule. *Mol Immunol* 31: 169–217.

Palmisano, G.L., Tazzari, P.L., Cozzi, E., Bolognesi, A., Polito, L., Seveso, M., Ancona, E., Ricci, F., Conte, R., Stirpe, F., Ferrara, G.B., Pistillo, M.P. (2004) Expression of CTLA-4 in nonhuman primate lymphocytes and its use as a potential target for specific immunotoxin-mediated apoptosis: results of in vitro studies. *Clin Exp Immunol* 135: 259–266.

Pancer, Z., Amemiya, C.T., Ehrhardt, G.R., Ceitlin, J., Gartland, G.L., Cooper, M.D. (2004) Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. *Nature* 430: 174–180.

Parker, M.H., Chen, Y., Danehy, F., Dufu, K., Ekstrom, J., Getmanova, E., Gokemeijer, J., Xu, L., Lipovsek, D. (2005) Antibody mimics based on human fibronectin type three domain engineered for thermostability and high-affinity binding to vascular endothelial growth factor receptor two. *Protein Eng Des Sel* 18: 435–444.

Pastan, I. (2003) Immunotoxins containing Pseudomonas exotoxin A: a short history. *Cancer Immunol Immunother* 52: 338–341.

Pini, A., Ricci, C., Bracci, L. (2002) Phage display and colony filter screening for high-throughput selection of antibody libraries. *Comb Chem High Throughput Screen* 5: 503–510.

Pokkuluri, P.R., Huang, D.B., Raffen, R., Cai, X., Johnson, G., Stevens, P.W., Stevens, F.J., Schiffer, M. (1998) A domain flip as a result of a single amino-acid substitution. *Structure* 6: 1067–1073.

Powell, K. (2004) Inhaled insulin products puff along. *Nat Biotechnol* 22: 1195–1196.

Reiter, Y., Schuck, P., Boyd, L.F., Plaksin, D. (1999) An antibody single-domain phage display library of a native heavy chain variable region: isolation of functional single-domain VH molecules with a unique interface. *J Mol Biol* 290: 685–698.

Roberts, B.L., Markland, W., Ley, A.C., Kent, R.B., White, D.W., Guterman, S.K., Ladner, R.C. (1992) Directed evolution of a protein: selection of potent neutrophil elastase inhibitors displayed on M13 fusion phage. *Proc Natl Acad Sci USA* 89: 2429–2433.

Rosendahl, M.S., Doherty, D.H., Smith, D.J., Bendele, A.M., Cox, G.N. (2005) Site-specific protein PEGylation – Application to cysteine analogs of recombinant human granulocyte colony-stimulating factor. *BioProcess Int* 52–60.

Röttgen, P., Collins, J. (1995) A human pancreatic secretory trypsin inhibitor presenting a hypervariable highly constrained epitope via monovalent phagemid display. *Gene* 164: 243–250.

Saerens, D., Frederix, F., Reekmans, G., Conrath, K., Jans, K., Brys, L., Huang, L., Bosmans, E., Maes, G., Borghs, G., Muyldermans, S. (2005) Engineering camel single-domain antibodies and immobilization chemistry for human prostate-specific antigen sensing. *Anal Chem* 77: 7547–7555.

Sandstrom, K., Xu, Z., Forsberg, G., Nygren, P.A. (2003) Inhibition of the CD28-CD80 co-stimulation signal by a CD28-binding affibody ligand developed by combinatorial protein engineering. *Protein Eng* 16: 691–697.

Schellekens, H. (2002) Immunogenicity of therapeutic proteins: clinical implications and future prospects. *Clin Ther* 24: 1720–1740; discussion 1719.

Schirle, M., Weinschenk, T., Stevanovic, S. (2001) Combining computer algorithms with experimental approaches permits the rapid and accurate identification of T cell epitopes from defined antigens. *J Immunol Methods* 257: 1–16.

Schlehuber, S., Skerra, A. (2001) Duocalins: engineered ligand-binding proteins with dual specificity derived from the lipocalin fold. *Biol Chem* 382: 1335–1342.

Schlehuber, S., Skerra, A. (2002) Tuning ligand affinity, specificity, and folding stability of an engineered lipocalin variant – a so-called "anticalin" – using a molecular random approach. *Biophys Chem* 96: 213–228.

Schlehuber, S., Skerra, A. (2005) Lipocalins in drug discovery: from natural ligand-binding proteins to "anticalins". *Drug Discov Today* 10: 23–33.

Schlehuber, S., Beste, G., Skerra, A. (2000) A novel type of receptor protein, based on the lipocalin scaffold, with specificity for digoxigenin. *J Mol Biol* 297: 1105–1120.

Shao, C.Y., Secombes, C.J., Porter, A.J. (2006) Rapid isolation of IgNAR variable single-domain antibody fragments from a shark synthetic library. *Mol Immunol* 44: 656–665.

Silverman, J., Lu, Q., Bakker, A., To, W., Duguay, A., Alba, B.M., Smith, R., Rivas, A., Li, P., Le, H., Whitehorn, E., Moore, K.W., Swimmer, C., Perlroth, V., Vogt, M., Kolkman, J., Stemmer, W.P. (2005) Multivalent avimer proteins evolved by exon shuffling of a family of human receptor domains. *Nat Biotechnol* 23: 1556–1561.

Skerra, A. (2000a) Engineered protein scaffolds for molecular recognition. *J Mol Recognit* 13: 167–187.

Skerra, A. (2000b) Lipocalins as a scaffold. *Biochim Biophys Acta* 1482: 337–350.

Skerra, A. (2003) Imitating the humoral immune response. *Curr Opin Chem Biol* 7: 683–693.

Skerra, A., Plückthun, A. (1988) Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. *Science* 240: 1038–1041.

Smith, G.P., Patel, S.U., Windass, J.D., Thornton, J.M., Winter, G., Griffiths, A.D. (1998) Small binding proteins selected from a combinatorial repertoire of knottins displayed on phage. *J Mol Biol* 277: 317–332.

Souriau, C., Chiche, L., Irving, R., Hudson, P. (2005) New binding specificities derived from Min-23: a small cystine-stabilized peptidic scaffold. *Biochemistry* 44: 7143–7155.

Spinelli, S., Tegoni, M., Frenken, L., van Vliet, C., Cambillau, C. (2001) Lateral recognition of a dye hapten by a llama VHH domain. *J Mol Biol* 311: 123–129.

Steffen, A.C., Orlova, A., Wikman, M., Nilsson, F.Y., Stahl, S., Adams, G.P., Tolmachev, V., Carlsson, J. (2006) Affibody-mediated tumour targeting of HER-2 expressing xenografts in mice. *Eur J Nucl Med Mol Imaging* 33: 631–638.

Steffen, A.C., Wikman, M., Tolmachev, V., Adams, G.P., Nilsson, F.Y., Stahl, S., Carlsson, J. (2005) In vitro characterization of a bivalent anti-HER-2 affibody with potential for radionuclide-based diagnostics. *Cancer Biother Radiopharm* 20: 239–248.

Stevens, F.J., Solomon, A., Schiffer, M. (1991) Bence Jones proteins: a powerful tool for the fundamental study of protein chemistry and pathophysiology. *Biochemistry* 30: 6803–6805.

Streltsov, V.A., Varghese, J.N., Carmichael, J.A., Irving, R.A., Hudson, P.J., Nuttall, S.D. (2004) Structural evidence for evolution of shark Ig new antigen receptor variable domain antibodies from a cell-surface receptor. *Proc Natl Acad Sci USA* 101: 12444–12449.

Tudyka, T., Skerra, A. (1997) Glutathione S-transferase can be used as a C-terminal, enzymatically active dimerization module for a recombinant protease inhibitor, and functionally secreted into the periplasm of *Escherichia coli*. *Protein Sci* 6: 2180–2187.

Uhlen, M., Forsberg, G., Moks, T., Hartmanis, M., Nilsson, B. (1992) Fusion proteins in biotechnology. *Curr Opin Biotechnol* 3: 363–369.

Van Brunt, J. (2005) The monoclonal maze. *SIGNALS* www.signalsmag.com.

Vita, C., Vizzavona, J., Drakopoulou, E., Zinn-Justin, S., Gilquin, B., Menez, A. (1998) Novel miniproteins engineered by the transfer of active sites to small natural scaffolds. *Biopolymers* 47: 93–100.

Vogt, M., Skerra, A. (2004) Construction of an artificial receptor protein ("anticalin") based on the human apolipoprotein D. *Chembiochem* 5: 191–199.

Vopel, S., Mühlbach, H., Skerra, A. (2005) Rational engineering of a fluorescein-binding anticalin for improved ligand affinity. *Biol Chem* 386: 1097–1104.

Vranken, W., Tolkatchev, D., Xu, P., Tanha, J., Chen, Z., Narang, S., Ni, F. (2002) Solution structure of a llama single-domain antibody with hydrophobic residues typical of the VH/VL interface. *Biochemistry* 41: 8570–8579.

Wahlberg, E., Lendel, C., Helgstrand, M., Allard, P., Dincbas-Renqvist, V., Hedqvist, A., Berglund, H., Nygren, P.A., Hard, T. (2003) An affibody in complex with a target protein: structure and coupled folding. *Proc Natl Acad Sci USA* 100: 3185–3190.

Werner, R.G. (2004) Economic aspects of commercial manufacture of biopharmaceuticals. *J Biotechnol* 113: 171–182.

Wikman, M., Steffen, A.C., Gunneriusson, E., Tolmachev, V., Adams, G.P., Carlsson, J., Stahl, S. (2004) Selection and characterization of HER2/neu-binding affibody ligands. *Protein Eng Des Sel* 17: 455–462.

Williams, A., Baird, L.G. (2003) DX-88 and HAE: a developmental perspective. *Transfus Apher Sci* 29: 255–258.

Wörn, A., Plückthun, A. (2001) Stability engineering of antibody single-chain Fv fragments. *J Mol Biol* 305: 989–1010.

Xu, L., Aha, P., Gu, K., Kuimelis, R.G., Kurz, M., Lam, T., Lim, A.C., Liu, H., Lohse, P.A., Sun, L., Weng, S., Wagner, R.W., Lipovsek, D. (2002) Directed evolution of high-affinity antibody mimics using mRNA display. *Chem Biol* 9: 933–942.

Yau, K.Y., Groves, M.A., Li, S., Sheedy, C., Lee, H., Tanha, J., MacKenzie, C.R., Jermutus, L., Hall, J.C. (2003) Selection of hapten-specific single-domain antibodies from a non-immunized llama ribosome display library. *J Immunol Methods* 281: 161–175.

Zewe, M., Rybak, S.M., Dubel, S., Coy, J.F., Welschof, M., Newton, D.L., Little, M. (1997) Cloning and cytotoxicity of a human pancreatic RNase immunofusion. *Immunotechnology* 3: 127–136.

8
Emerging Therapeutic Concepts I: ADEPT

Surinder K. Sharma, Kerry A. Chester, and Kenneth D. Bagshawe

8.1
Introduction and Basic Principles of ADEPT

Although the production of antisera in various species was well underway in the 1930s it was not until the late 1950s that the idea of using these as a diagnostic tool took root. Following the discovery of carcinoembryonic antigen (CEA) (Gold and Freedman 1965), pathologists began to use antisera to identify antigens on tissue sections. If antibodies could bind to antigens on tissue sections and to antigens in solution the question arose whether, when injected intravenously, they would localize for instance on CEA expressed on colorectal cancers. That this could be achieved was demonstrated by Mach et al. (1974) and Goldenberg et al. (1974). The advent of hybridoma technology followed by the introduction of monoclonal antibodies (Köhler and Milstein 1975) focused attention on their enormous potential in diagnosis and therapy.

So could antibodies deliver cytotoxic agents with less toxicity than by conventional means? Several approaches had been developed to target cytotoxic agents (Levy et al. 1975; Pimm et al. 1982) and toxins (Thorpe et al. 1978; Gilliard et al. 1980; Pastan and Kreitman 2002) by linking to monoclonal antibodies. One of the limitations of this antibody–drug conjugate approach was that an intact antibody could only carry about 10–15 molecules of a cytotoxic drug without losing its antigen-binding ability. Moreover large molecules could gain entry to tumor sites but diffused poorly (Pedley et al. 1993) so that they failed to deliver their pay load where it was wanted. This failure was compounded by the fact that in carcinomas, as opposed to the lymphomas, there is marked heterogeneity in the distribution of the known antigenic targets so that many viable tumor cells fail to express the target antigen (Edwards et al. 1985).

It was recognized that if cytotoxic agents could be generated and restricted to cancer sites this would have the potential to deliver more drug to tumors and avoid normal tissue toxicity. The idea of relatively nontoxic prodrugs that would be converted by tumor-located enzymes into potent cell killing agents had been explored for several years but the failure to find enzymes exclusively located in

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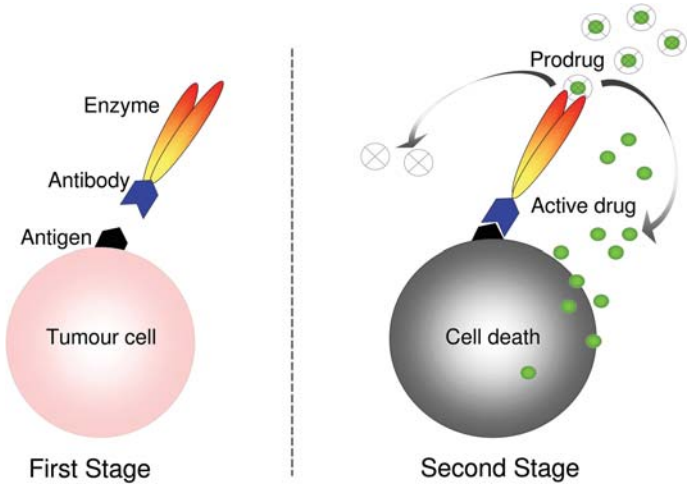

Fig. 8.1 Basic principle of ADEPT. An antibody–enzyme construct (AEC) is injected and allowed to localize to tumors. After blood clearance of enzyme activity, a nontoxic prodrug is given. The enzyme cleaves the protective moiety to release the cytotoxic active drug. The drug is generated extracellularly and can diffuse throughout the tumor killing both antigen-positive as well as antigen-negative cells, thus providing a "bystander" effect.

cancer cells had proved the stumbling block. So it was suggested that antibodies directed at tumor antigens could be used to vector enzymes not found in human tissues to tumor sites where they could activate appropriate prodrugs (Bagshawe 1987). This formed the basis of what is described as ADEPT, or Antibody Directed Enzyme Prodrug Therapy (Bagshawe et al. 1988; Senter et al. 1988) (Fig. 8.1).

It was clear from the outset that there would be several obstacles to overcome. It was recognized that if foreign enzymes were used, and those of bacterial origin seemed the most likely, there would be an immunogenicity issue that would have to be addressed. The same consideration also applied to the murine monoclonals that were then the norm (Reilly et al. 1995), although there were good reasons to believe that human or humanized antibodies would resolve that issue and so it has proved (Jones et al. 2004; Winter and Harris 1993). Second, it was known from work with radiolabeled intact IgG antibodies in humans that they remain in the blood for many days and it would not be useful to give prodrug whilst antibody–enzyme conjugate (AEC) was still in the blood since to do so would simply mimic conventional therapy. Similarly, it would be necessary for the drug generated at tumor sites to have a short half-life so that it did not leak out of tumors on a scale big enough to produce systemic toxicity.

Although it was evident that not all these requirements could be achieved quickly, the approach had big potential advantages. Each enzyme molecule located within a tumor mass would be able to activate a large number of prodrug molecules, thereby providing an amplification factor. The drugs generated would be

small molecules that would diffuse through the tumor mass more readily than the AEC. The drug molecules would be able to attack not only cells expressing the target antigen but also those that failed to express the antigen, the so-called bystander effect.

8.2 Preclinical Studies

8.2.1 CPG2 and Benzoic Mustard Prodrugs

It seemed important to test the system as soon as possible. In our studies, we have utilized a bacterial enzyme carboxypeptidase G2 (CPG2), isolated from a *Pseudomonas* species (Sherwood et al. 1985). At the Trophoblastic Disease Centre at Charing Cross Hospital, London, one of the tumor cell lines under regular study was a choriocarcinoma xenograft that was resistant to the cytotoxic agents (Bagshawe 1989) available when used singly or in combination. The serum of nude mice bearing these xenografts contained quite high levels of human chorionic gonadotrophin (hCG). This model was selected for testing the ADEPT approach *in vivo* (Bagshawe et al. 1988). Nude mice with established choriocarcinoma xenografts (CC3) were given an AEC comprising a murine monoclonal IgG antibody directed at hCG chemically conjugated (Searle et al. 1986) to the bacterial enzyme carboxypeptidase (CPG2). It was known from previous studies (Begent et al. 1986) that antibodies could localize in the tumors despite the high level of hCG in blood. In the CC3 xenograft model, a prodrug was given at 48 h after the AEC when there was no detectable enzyme in the blood. This resulted in the elimination of 9 out of 12 tumors without regrowth of these tumors followed up to 1 year (Springer et al. 1991).

This first custom-made prodrug was glutamated benzoic acid mustard (Springer et al. 1990), which was activated by cleavage of the glutamate by the CPG2. Its most immediate limitation was poor aqueous solubility so that it could only be administered in dimethyl sulfoxide (DMSO).

The next xenograft target was a poorly differentiated colon cancer model LS174T. Although it expressed CEA at the cellular level there was no detectable CEA in the blood of xenograft-bearing mice. Mice bearing established xenografts received antibody directed at CEA conjugated to CPG2 followed at 72 h by the prodrug. All the mice died. It was evident that in the CC3 experiment the AEC had cleared quickly from the blood through immune complex formation with the hCG in the blood and that in the LS174T model there was still enzyme in blood at 72 h, which activated the prodrug and caused fatal toxicity. The experiment was repeated with a time interval of 7 days between giving the AEC and the prodrug. This time there was no toxicity but little tumor response.

These results had been anticipated and the use of second antibodies directed at another antibody had been used in radioimmunoassay for many years. One of

the antienzyme antibodies produced (SB43) inactivated the enzyme (Sharma et al. 1990) and this was used to clear enzyme activity from blood in the next experiment and marked growth delay was then obtained with the LS174T xenografts (Sharma et al. 1991). To prevent this antibody inactivating enzyme at tumor sites it was galactosylated to ensure its rapid clearance. Hence a three-component system was developed: (1) the AEC, (2) the galactosylated enzyme-inactivating antibody, and (3) the prodrug (Sharma et al. 1991, 1994a).

Another approach to accelerate clearance of CPG2 from blood was also explored. This involved galactosylation of the AEC so that it cleared from blood and tissues via the carbohydrate receptors in the liver. Localization in tumors was achieved by blocking carbohydrate receptors in the liver for a period of time using an asialo-bovine submaxillary gland mucin. As this blocking agent cleared from the liver, AEC rapidly cleared from blood and other tissues but was retained in the tumor, allowing prodrug to be given safely in the LS174T xenograft model and resulting in a growth delay that was comparable with that achieved in the three-component ADEPT system (Sharma et al. 1994a). However, this approach was not used clinically as the blocking agent was likely to be immunogenic and its safety and side effects were not known. The three-component system subsequently studied in the clinic utilized the galactosylated SB43 as it had the advantage of inactivating CPG2 in blood within minutes without affecting tumor enzyme levels and without immune complex toxicity. ADEPT studies with the same antibody and enzyme/prodrug system resulted in growth delay of a drug resistant ovarian xenograft model (Sharma et al. 1994b) and using a different antibody produced regressions and cures in a human breast xenograft model (Eccles et al. 1994).

8.2.2 Other Enzyme/Prodrug Systems

A number of preclinical studies of ADEPT have been reported since the ADEPT approach was first proposed (Bagshawe et al. 1988; Senter et al. 1988). These include enzymes of mammalian and nonmammalian origin in combination with prodrugs of current chemotherapy agents (reviewed in Senter and Springer 2001; Bagshawe et al. 2004). The mammalian enzymes, including human, studied were alkaline phosphatase (Senter et al. 1988), carboxypeptidase A (Smith et al. 1997), and β-glucuronidase (Bosslet et al. 1994). The nonmammalian enzymes with mammalian homolog include bacterial nitroreductase (Mauger et al. 1994) and β-glucuronidase (Bignami et al. 1992), which has a substrate specificity different from that of its human counterpart (Houba et al. 1996). Both these are potentially immunogenic in humans. The nonmammalian enzymes with no human analog include CPG2 (described earlier), β-lactamase (Alexander et al. 1991; Meyer et al. 1993, 1995; Cortez-Retamozo et al. 2004; Roberge et al. 2006), cytosine deaminase (Wallace et al. 1994), and penicillin G-amidase (Bignami et al. 1992). Virtually all of these studies have used prodrug versions of existing, approved cytotoxic agents. Many of these studies give no data on the half-life of the drugs and the importance of this characteristic is not widely appreciated.

Various subsequent studies have reported the use of human enzymes but none have yet been reported in clinical trials.

8.2.3
Catalytic Antibodies

The first catalytic antibodies (or abzymes) were made by immunizing rabbits, and later mice, with transition state analogs (Nevinsky and Buneva 2003; Xu et al. 2004). The resulting antibodies acted as catalysts by interacting to stabilize the transition state in a similar manner to enzymes. The potential of using these antibodies to catalyze a prodrug had been anticipated in the early days of ADEPT (Bagshawe 1989). Although progress in this field has been initially relatively slow and the catalytic antibodies generated remained less efficient than naturally occurring enzymes (Wentworth et al. 1996; Shabat et al. 1999). However, recent advances in the development of efficient catalytic antibodies (Rader et al. 2003; Shamis et al. 2004; Sinha et al. 2004) are more encouraging and catalytic antibodies are now being employed in enzyme–prodrug therapy of cancer (Kakinuma et al. 2002), for example in neuroblastoma where *in vivo* tumor growth was delayed and there did not appear to be conversion of prodrug by endogenous enzymes (Shabat et al. 2001).

Indeed, the potential of current antibody technology to create human catalytic antibodies is enormous. Active human catalytic antibodies can be selected from synthetic antibody libraries (Cesaro-Tadic et al. 2003) and directed evolution of enzymes can be achieved using phage display (Fernandez-Gacio et al. 2003). Thus, it is possible that bispecific antitumor/abzyme molecules could eventually provide an entirely nonimmunogenic approach for targeted cancer chemotherapy.

8.3
Clinical Studies

8.3.1
$F(ab)_2$ Fragments Conjugated to CPG2

Following our extensive preclinical studies in xenograft models, a pilot clinical study in patients with advanced metastatic colorectal cancer followed. It was necessary in this exploratory study to incorporate prodrug dose escalation and exploratory dose levels of the AEC and the enzyme-inactivating antibody. A total of 17 patients entered the study and of the eight that received the highest doses of prodrug four achieved partial responses and one a mixed response (Bagshawe et al. 1991, 1995; Bagshawe and Begent 1996).

A second, small-scale clinical study with the same three-component system followed but with a lower dose of the AEC. This was an important mechanistic study (Napier et al. 2000) which resulted in one partial response and most of the other patients had stable disease for several months. An important result of this

study was that biopsies of liver metastases, after the inactivating antibody had been given, showed tumor-to-blood ratios far in excess of 10 000 : 1, demonstrating the efficiency of the inactivating antibody and confirming that the myelosuppressive effects seen in both clinical studies resulted not from enzyme in blood but from the long half-life of the drug generated at tumor sites (Martin et al. 1997).

In response to these findings a bis-iodo phenol mustard prodrug was developed (Springer et al. 1995). This prodrug is also an alkylating agent and is activated by CPG2 to generate a drug with very short half-life. In combination with AEC this was shown to be highly potent *in vivo* (Blakey et al. 1996) and the mechanism of cell death to be mainly apoptosis (Monks et al. 2001). This bis-iodo phenol mustard prodrug in combination with AEC was used in the next clinical study but the enzyme inactivation step was omitted in the cause of simplicity. There were no responses in any of the 28 patients entered (Francis et al. 2002).

8.3.2
Recombinant scFv-CPG2 Fusion Protein

The clinical studies highlighted a need for better enzyme delivery systems and at this time advances in molecular manipulation offered the opportunity to end the limitations of chemical conjugation and to be able to build fusion proteins of antibody and enzyme with characteristics to order. A new anti-CEA single-chain Fv (scFv) antibody (MFE23) with higher binding affinity than the antibody used previously had been developed (Chester et al. 2000). The gene for MFE was fused with the gene for CPG2 to result in a fusion protein MFE23-CPG2 (Michael et al. 1996). Expressed in *E. coli* this showed improved pharmacokinetics *in vivo* as compared with the AEC (Bhatia et al. 2000) but the yield was low. The subsequent expression of this fusion protein (MFECP) in *Pichia pastoris* resulted in high yield of a stable protein which was also glycosylated (Medzihradszky et al. 2004). MFECP localized effectively in colon carcinoma xenografts with rapid clearance of enzyme activity in blood, resulting in tumor-to-plasma ratios of 1400 : 1 within 6 h after injection. Therapy studies in combination with the bis-iodo phenol mustard prodrug showed growth delay of the LS174T and regressions in the SW1222 colon carcinoma xenografts without apparent toxicity (Sharma et al. 2005).

A phase I clinical trial with a single cycle of ADEPT comprising MFECP1 and the bis-iodo phenol prodrug has been completed successfully (Mayer et al. 2004a; Mayer and Begent, unpublished data) and a repeated cycle ADEPT study is in progress.

8.4
Immunogenicity

Successful cancer therapy requires multiple cycles of treatment and one of the limitations of the ADEPT approach using a murine monoclonal antibody conjugated to a bacterial enzyme was that of immunogenicity and as such was anti-

cipated from the first (Bagshawe 1989). All patients receiving AEC showed the presence of human anti-mouse antibodies (HAMA) and human anti-CPG2 antibodies (HACA) (Sharma et al. 1992). However, using an immunosuppressive agent, up to three ADEPT cycles could be given within a 21-day period (Bagshawe et al. 1995; Bagshawe and Sharma 1996).

The clinical studies with MFECP1 have shown a lower frequency of HACA than with AEC (Mayer et al. 2004b). This may be due to the addition of a hexa-histidine tag (His-tag) to the C-terminus of CPG2 where an immunodominant B-cell epitope had been identified (Spencer et al. 2002).

The B-cell modification approach to reduce immunogenicity may be limited in that removing antigenic epitopes may not necessarily reduce overall immunogenicity because repeated administration with the modified protein can elicit an antibody response to a different set of epitopes on the same molecule. Another approach, modification of T-cell epitopes, may be more successful in creating nonimmunogenic enzymes for ADEPT, because T cell help is required to mount a long-lived, isotype-switched, and high-affinity antibody response (see review, Chester et al. 2005).

Enzymes present a challenge for the T-cell epitope modification strategy, because changes in amino acid sequence can readily lead to loss of catalytic activity. However, the potential effectiveness of this approach makes it very attractive and it has already met with (*in vitro*) success with beta-lactamase from *Enterobacter cloacae* (Harding et al. 2005). Lactamases are useful enzymes for ADEPT as they can activate a wide variety of prodrugs, but they are immunogenic due to their bacterial origin. Harding et al. mutated T-cell epitopes in beta-lactamase to create a variant which retained enzyme activity but which induced significantly less T-cell stimulation in human and mouse cell assays. The results are promising although it remains to be seen to what extent its immunogenicity has been overcome in humans.

The possibility of removing T-cell epitopes from CPG2 is also being explored. Eight potential immunogenic regions have been identified using T-cell proliferation assays and *in silico* analysis (Chester et al. 2005). It is proposed that suitable amino acid substitutions in these regions will lead to a CPG2 molecule with reduced immunogenicity *in vivo*.

The immunological response may also be addressed by using human, rather than foreign, enzymes for ADEPT. However, this increases the risk of unwanted activation of prodrug by endogenous human enzyme in nontarget organs. One way of preventing this is to mutate human enzymes so they will activate prodrugs not recognized by their wildtype human equivalent. This has been shown to be possible in principle with a mutant of human carboxypeptidase A1 which has been modified to activate prodrugs of methotrexate *in vitro* (Wolfe et al. 1999), although as yet this system does not appear to be effective *in vivo*. Another approach is to use a human enzyme which has little or no activity in human blood, for example human beta-glucuronidase (de Graaf et al. 2002; Biela et al. 2003). An ADEPT system using a recombinant thermostable human prolyl endopeptidase is also promising (Heinis et al. 2004).

New, nonimmunogenic enzymatic activities may also be obtained from combinatorial libraries of designed amino acid sequences (Wei and Hecht 2004) or by novel screening and selection technologies from enzyme-encoding gene repertoires (Aharoni et al. 2005). These approches all indicate that progress is underway in the development of less immunogenic ADEPT enzymes. However, further work is clearly necessary to overcome the immunogenicity hurdle, whether by further elimination of troublesome epitopes, mutated human enzymes, abzymes, or new recombinant enzymes.

8.5 Important Considerations/Outlook

It has been recognized that the drug generated in an ADEPT system should have a very short half-life to avoid leak back into blood where it can result in toxicity. It has also been recognized that it is necessary to continue to eliminate immunogenic epitopes on the bacterial enzymes used in ADEPT.

It is evident from numerous studies that the concentration of enzyme, as AEC, in tumors tends to equilibrate with that in blood. If the AEC clears quickly from blood the concentration in tumors never attains the peak concentration in blood and also clears quickly. It is therefore necessary to maintain a high concentration of AEC in blood for a prolonged period. Studies are needed to determine the optimum duration of that period.

Prolonged residence of AEC in tumors also has the benefit of delayed escape of AEC from tumors when the blood level falls. The fall in concentration in tumors is then slower than that in blood and provides a basis for a time window in which there is adequate enzyme in tumors and zero enzyme in blood. It is now known how to construct AECs with the necessary characteristics.

For an ADEPT system to be a practical procedure in the clinic it is useful to minimize the effects of individual variation on the clearance of antibody–enzyme constructs from blood. Early studies with a galactosylated enzyme inactivating antibody proved safe and precisely defined a time window for prodrug administration and this may be a way forward for future ADEPT systems.

Understanding of ADEPT has increased with the successful development of preclinical therapies and knowledge of essential requirements from clinical trials. Tools to address the limitations of ADEPT are available and it now remains for ADEPT to fulfil its potential and promise in the clinic.

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References

Aharoni, A., Amitai, G., Bernath, K., Magdassi, S., Tawfik, D.S. (2005) High-throughput screening of enzyme libraries: thiolactonases evolved by fluorescence-activated sorting of single cells in emulsion compartments. *Chem Biol* 12: 1281–1289.

Alexander, R.P., Beeley, N.R.A., Odriscoll, M. et al. (1991) Cephalosporin nitrogen mustard carbamate prodrugs for ADEPT. *Tetrahedron Lett* 32: 3269–3272.

Bagshawe, K.D. (1987) Antibody directed enzymes revive anti-cancer prodrugs concept. *Br J Cancer* 56: 531–532.

Bagshawe, K.D. (1989) The First Bagshawe lecture. Towards generating cytotoxic agents at cancer sites. *Br J Cancer* 60: 275–281.

Bagshawe, K.D., Begent, R.H.J. (1996) First clinical experience with ADEPT. *Adv Drug Deliv Rev* 22: 365–367.

Bagshawe, K.D., Springer, C.J., Searle, F., Antoniw, P., Sharma, S.K., Melton, R.G., Sherwood, R.F. (1988) A cytotoxic agent can be generated selectively at cancer sites. *Br J Cancer* 58: 700–703.

Bagshawe, K.D., Sharma, S.K., Springer, C. J., Antoniw, P., Boden, J.A., Rogers, G.T., Burke, P.J., Melton, R.G., Sherwood, R.F. (1991) Antibody directed enzyme prodrug therapy (ADEPT): clinical report. *Dis Markers* 9: 233–238.

Bagshawe, K.D., Sharma, S.K., Springer, C. J., Antoniw, P. (1995) Antibody-directed enzyme prodrug therapy: a pilot scale clinical trial. *Tumor Targeting* 1: 17–29.

Bagshawe, K.D., Sharma, S.K. (1996) Cyclosporine delays host immune response to antibody enzyme conjugate in ADEPT. *Transplant Proc* 28: 3156–3158.

Bagshawe, K.D., Sharma, S.K., Begent, R.H. (2004) Antibody-directed enzyme prodrug therapy (ADEPT) for cancer. *Expert Opin Biol Ther* 4: 1777–1789.

Begent, R.H., Keep, P.A., Searle, F., Green, A.J., Mitchell, H.D., Jones, B.E., Dent, J., Pendower, J.E., Parkins, R.A., Reynolds, K.W. (1986) Radioimmunolocalization and selection for surgery in recurrent colorectal cancer. *Br J Surg* 73: 64–67.

Bhatia, J., Sharma, S.K., Chester, K.A., Pedley, R.B., Boden, R.W., Read, D.A., Boxer, G.M., Michael, N.P., Begent, R.H. (2000) Catalytic activity of an in vivo tumor targeted anti-CEA scFv: carboxypeptidase G2 fusion protein. *Int J Cancer* 85: 571–577.

Biela, B.H., Khawli, L.A., Hu, P., Epstein, A.L. (2003) Chimeric TNT-3/human beta-glucuronidase fusion proteins for antibody-directed enzyme prodrug therapy (ADEPT). *Cancer Biother Radiopharm* 18: 339–353.

Bignami, G.S., Senter, P.D., Grothaus, P.G., Fischer, K.J., Humphreys, T., Wallace, P.M. (1992) *N*-(4′-hydroxyphenylacetyl)palytoxin: a palytoxin prodrug that can be activated by a monoclonal antibody-penicillin G amidase conjugate. *Cancer Res* 52: 5759–5764.

Blakey, D.C., Burke, P.J., Davies, D.H., Dowell, R.I., East, S.J., Eckersley, K.P., Fitton, J.E., McDaid, J., Melton, R.G., Niculescu-Duvaz, I.A., Pinder, P.E., Sharma, S.K., Wright, A.F., Springer, C.J. (1996) ZD2767: an improved system for antibody-directed enzyme prodrug therapy that results in tumor regressions in colorectal tumor xenografts. *Cancer Res* 56: 3287–3292.

Bosslet, K., Czech, J., Hoffmann, D. (1994) Tumor-selective prodrug activation by fusion protein-mediated catalysis. *Cancer Res* 54: 2151–2159.

Cesaro-Tadic, S., Lagos, D., Honegger, A., Rickard, J.H., Partridge, L.J., Blackburn, G.M., Plückthun, A. (2003) Turnover-based in vitro selection and evolution of biocatalysts from a fully synthetic antibody library. *Nat Biotechnol* 21: 679–685.

Chester, K.A., Bhatia, J., Boxer, G., Cooke, S.P., Flynn, A.A., Huhalov, A., Mayer, A., Pedley, R.B., Robson, L., Sharma, S.K., Spencer, D.I., Begent, R.H. (2000) Clinical applications of phage-derived sFvs and sFv fusion proteins. *Dis Markers* 16: 53–62.

Chester, K.A., Baker, M., Mayer, A. (2005) Overcoming the immonological response to foreign enzymes in cancer therapy. *Expert Rev Clin Immunol* 1: 549–559.

Cortez-Retamozo, V., Backmann, N., Senter, P.D., Wernery, U., De Baetselier, P., Muyldermans, S., Revets, H. (2004) Efficient cancer therapy with a nanobody-based conjugate. *Cancer Res* 64: 2853–2857.

de Graaf, M., Boven, E., Oosterhoff, D., van der Meulen-Muileman, I.H., Huls, G.A., Gerritsen, W.R., Haisma, H.J., Pinedo, H. M. (2002) A fully human anti-Ep-CAM scFv-beta-glucuronidase fusion protein for selective chemotherapy with a glucuronide prodrug. *Br J Cancer* 86: 811–818.

Eccles, S.A., Court, W.J., Box, G.A., Dean, C.J., Melton, R.G., Springer, C.J. (1994) Regression of established breast carcinoma xenografts with antibody-directed enzyme prodrug therapy against c-erbB2 p185. *Cancer Res* 54: 5171–5177.

Edwards, P.A., Skilton, R.A., Payne, A.W., Ormerod, M.G. (1985) Antigenic heterogeneity of breast cell lines detected by monoclonal antibodies and its relationship with the *Cell Cycle J Cell Sci* 73: 321–333.

Fernandez-Gacio, A., Uguen, M., Fastrez, J. (2003) Phage display as a tool for the directed evolution of enzymes. *Trends Biotechnol* 21: 408–414.

Francis, R.J., Sharma, S.K., Springer, C., Green, A.J., Hope-Stone, L.D., Sena, L., Martin, J., Adamson, K.L., Robbins, A., Gumbrell, L., O'Malley, D., Tsiompanou, E., Shahbakhti, H., Webley, S., Hochhauser, D., Hilson, A.J., Blakey, D., Begent, R.H. (2002) A phase I trial of antibody directed enzyme prodrug therapy (ADEPT) in patients with advanced colorectal carcinoma or other CEA producing tumours. *Br J Cancer* 87: 600–607.

Gilliland, D.G., Steplewski, Z., Collier, R.J., Mitchell, K.F., Chang, T.H., Koprowski, H. (1980) Antibody-directed cytotoxic agents: use of monoclonal antibody to direct the action of toxin A chains to colorectal carcinoma cells. *Proc Natl Acad Sci USA* 77: 4539–4543.

Gold, P., Freedman, S.O. (1965) Specific carcinoembryonic antigens of the human digestive system. *J Exp Med* 122: 467–481.

Goldenberg, D.M., Preston, D.F., Primus, F.J., Hansen, H.J. (1974) Photoscan localization of GW-39 tumors in hamsters using radiolabeled anticarcinoembryonic antigen immunoglobulin G. *Cancer Res* 34: 1–9.

Harding, F.A., Liu, A.D., Stickler, M., Razo, O.J., Chin, R., Faravashi, N., Viola, W., Graycar, T., Yeung, V.P., Aehle, W., Meijer, D., Wong, S., Rashid, M.H., Valdes, A.M., Schellenberger, V. (2005) A beta-lactamase with reduced immunogenicity for the targeted delivery of chemotherapeutics using antibody-directed enzyme prodrug therapy. *Mol Cancer Ther* 4: 1791–1800.

Heinis, C., Alessi, P., Neri, D. (2004) Engineering a thermostable human prolyl endopeptidase for antibody-directed enzyme prodrug therapy. *Biochemistry* 43: 6293–6303.

Houba, P.H., Boven, E., Haisma, H.J. (1996) Improved characteristics of a human beta-glucuronidase-antibody conjugate after deglycosylation for use in antibody-directed enzyme prodrug therapy. *Bioconjug Chem* 7: 606–611.

Jones, R.L., Smith, I.E. (2004) Efficacy and safety of trastuzumab. *Expert Opin Drug Saf* 3: 317–327.

Kakinuma, H., Fujii, I., Nishi, Y. (2002) Selective chemotherapeutic strategies using catalytic antibodies: a common pro-moiety for antibody-directed abzyme prodrug therapy. *J Immunol Methods* 269: 269–281.

Köhler, G., and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.

Levy, R., Hurwitz, E., Maron, R., Arnon, R., Sela, M. (1975) The specific cytotoxic effects of daunomycin conjugated to antitumor antibodies. *Cancer Res* 35: 1182–1186.

Mach, J.P., Carrel, S., Merenda, C., Sordat, B., Cerottini, J.C. (1974) In vivo localisation of radiolabelled antibodies to carcinoembryonic antigen in human colon carcinoma grafted into nude mice. *Nature* 248: 704–706.

Martin, J., Stribbling, S.M., Poon, G.K. et al. (1997) Antibody-directed enzyme prodrug therapy: Pharmacokinetics and plasma levels of prodrug and drug in a phase I clinical trial. *Cancer Chemother Pharmacol* 40: 189–201.

Mauger, A.B., Burke, P.J., Somani, H.H., Friedlos, F., Knox, R.J. (1994) Self-immolative prodrugs: candidates for antibody-directed enzyme prodrug therapy in conjunction with a nitroreductase enzyme. *J Med Chem* 37: 3452–3458.

Mayer, A., Francis, R., Sharma, S.K. et al. (2004a) A phase I/II trial of antibody directed enzyme prodrug therapy (ADEPT) with MFECP1 and ZD2767P. *Br J Cancer* 91(Suppl 1): S8.

Mayer, A., Sharma, S.K., Tolner, B. et al. (2004b) Modifying an immunogenic epitope on a therapeutic protein: a step towards an improved system for antibody-directed enzyme prodrug therapy (ADEPT). *Br J Cancer* 90: 2402–2410.

Medzihradszky, K.F., Spencer, D.I., Sharma, S.K., Bhatia, J., Pedley, R.B., Read, D.A., Begent, R.H., Chester, K.A. (2004) Glycoforms obtained by expression in Pichia pastoris improve cancer targeting potential of a recombinant antibody-enzyme fusion protein. *Glycobiology* 14: 27–37.

Meyer, D.L., Jungheim, L.N., Law, K.L., Mikolajczyk, S.D., Shepherd, T.A., Mackensen, D.G., Briggs, S.L., Starling, J.J. (1993) Site-specific prodrug activation by antibody-beta-lactamase conjugates: regression and long-term growth inhibition of human colon carcinoma xenograft models. *Cancer Res* 53: 3956–3963.

Meyer, D.L., Law, K.L., Payne, J.K., Mikolajczyk, S.D., Zarrinmayeh, H., Jungheim, L.N., Kling, J.K., Shepherd, T. A., Starling, J.J. (1995) Site-specific prodrug activation by antibody-beta-lactamase conjugates: preclinical investigation of the efficacy and toxicity of doxorubicin delivered by antibody directed catalysis. *Bioconjug Chem* 6: 440–446.

Michael, N.P., Chester, K.A., Melton, R.G., Robson, L., Nicholas, W., Boden, J.A., Pedley, R.B., Begent, R.H., Sherwood, R.F., Minton, N.P. (1996) In vitro and in vivo characterisation of a recombinant carboxypeptidase G2::anti-CEA scFv fusion protein. *Immunotechnology* 2: 47–57.

Monks, N.R., Blakey, D.C., Curtin, N.J., East, S.J., Heuze, A., Newellm, D.R. (2001) Induction of apoptosis by the ADEPT agent ZD2767: comparison with the classical nitrogen mustard chlorambucil and a monofunctional ZD2767 analogue. *Br J Cancer* 85: 764–771.

Napier, M.P., Sharma, S.K., Springer, C.J., Bagshawe, K.D., Green, A.J., Martin, J., Stribbling, S.M., Cushen, N., O'Malley, D., Begent, R.H. (2000) Antibody-directed enzyme prodrug therapy: efficacy and mechanism of action in colorectal carcinoma. *Clin Cancer Res* 6: 765–772.

Nevinsky, G.A., Buneva, V.N. (2003) Catalytic antibodies in healthy humans and patients with autoimmune and viral diseases. *J Cell Mol Med* 7: 265–276.

Pastan, I., Kreitman, R.J. (2002) Immunotoxins in cancer therapy. *Curr Opin Investig Drugs* 3: 1089–1091.

Pedley, R.B., Boden, J.A., Boden, R., Dale, R., Begent, R.H. (1993) Comparative radioimmunotherapy using intact or F(ab')2 fragments of 131I anti-CEA antibody in a colonic xenograft model. *Br J Cancer* 68: 69–73.

Pimm, M.V., Embleton, M.J., Perkins, A.C., Price, M.R., Robins, R.A., Robinson, G.R., Baldwin, R.W. (1982) In vivo localization of anti-osteogenic sarcoma 791T monoclonal antibody in osteogenic sarcoma xenografts. *Int J Cancer* 30: 75–85.

Rader, C., Turner, J.M., Heine, A., Shabat, D., Sinha, S.C., Wilson, I.A., Lerner, R.A., Barbas, C.F. (2003) A humanized aldolase antibody for selective chemotherapy and adaptor immunotherapy. *J Mol Biol* 332: 889–899.

Reilly, R.M., Sandhu, J., Alvarez-Diez, T.M., Gallinger, S., Kirsh, J., Stern, H. (1995) Problems of delivery of monoclonal antibodies. Pharmaceutical and pharmacokinetic solutions. *Clin Pharmacokinet* 28: 126–142.

Roberge, M., Estabrook, M., Basler, J., Chin, R., Gualfetti, P., Liu, A., Wong, S.B., Rashid, M.H., Graycar, T., Babe, L., Schellenberger, V. (2006) Construction and optimization of a CC49-based scFv-beta-lactamase fusion protein for ADEPT. *Protein Eng Des Sel* 19: 141–145.

Searle, F., Bier, C., Buckley, R.G., Newman, S., Pedley, R.B., Bagshawe, K.D., Melton, R.G., Alwan, S.M., Sherwood, R.F. (1986) The potential of carboxypeptidase G2-antibody conjugates as anti-tumour agents.

I. Preparation of antihuman chorionic gonadotrophin-carboxypeptidase G2 and cytotoxicity of the conjugate against JAR choriocarcinoma cells in vitro. *Br J Cancer* 53: 377–384.

Senter, P.D., Springer, C.J. (2001) Selective activation of anticancer prodrugs by monoclonal antibody-enzyme conjugates. *Adv Drug Deliv Rev* 53: 247–264.

Senter, P.D., Saulnier, M.G., Schreiber, G.J., Hirschberg, D.L., Brown, J.P., Hellstrom, I., Hellstrom, K.E. (1988) Anti-tumor effects of antibody-alkaline phosphatase conjugates in combination with etoposide phosphate. *Proc Natl Acad Sci USA* 85: 4842–4846.

Shabat, D., Rader, C., List, B., Lerner, R.A., Barbas, C.F., III (1999) Multiple event activation of a generic prodrug trigger by antibody catalysis. *Proc Natl Acad Sci USA* 96: 6925–6930.

Shabat, D., Lode, H.N., Pertl, U., Reisfeld, R.A., Rader, C., Lerner, R.A., Barbas, C.F., III (2001) In vivo activity in a catalytic antibody-prodrug system: Antibody catalyzed etoposide prodrug activation for selective chemotherapy. *Proc Natl Acad Sci USA* 98: 7528–7533.

Shamis, M., Lode, H.N., Shabat, D. (2004) Bioactivation of self-immolative dendritic prodrugs by catalytic antibody 38C2. *J Am Chem Soc* 126: 1726–1731.

Sharma, S.K., Bagshawe, K.D., Burke, P.J., Boden, R.W., Rogers, G.T. (1990) Inactivation and clearance of an anti-CEA carboxypeptidase G2 conjugate in *Blood* after localisation in a xenograft model. *Br J Cancer* 61: 659–662.

Sharma, S.K., Bagshawe, K.D., Springer, C. J., Burke, P.J., Rogers, G.T., Boden, J.A., Antoniw, P., Melton, R.G., Sherwood, R.F. (1991) Antibody directed enzyme prodrug therapy (ADEPT): a three phase system. *Dis Markers* 9: 225–231.

Sharma, S.K., Bagshawe, K.D., Melton, R.G., Sherwood, R.F. (1992) Human immune response to monoclonal antibody-enzyme conjugates in ADEPT pilot clinical trial. *Cell Biophys* 21: 109–120.

Sharma, S.K., Bagshawe, K.D., Burke, P.J., Boden, J.A., Rogers, G.T., Springer, C.J., Melton, R.G., Sherwood, R.F. (1994a) Galactosylated antibodies and antibody-enzyme conjugates in antibody-directed enzyme prodrug therapy. *Cancer* 73: 1114–1120.

Sharma, S.K., Boden, J.A., Springer, C.J., Burke, P.J., Bagshawe, K.D. (1994b) Antibody-directed enzyme prodrug therapy (ADEPT). A three-phase study in ovarian tumor xenografts. *Cell Biophys* 24–25: 219–228.

Sharma, S.K., Pedley, R.B., Bhatia, J., Boxer, G.M., El Emir, E., Qureshi, U., Tolner, B., Lowe, H., Michael, N.P., Minton, N., Begent, R.H., Chester, K.A. (2005) Sustained tumor regression of human colorectal cancer xenografts using a multifunctional mannosylated fusion protein in antibody-directed enzyme prodrug therapy. *Clin Cancer Res* 11: 814–825.

Sherwood, R.F., Melton, R.G., Alwan, S.M., Hughes, P. (1985) Purification and properties of carboxypeptidase G2 from Pseudomonas sp. strain RS-16. Use of a novel triazine dye affinity method. *Eur J Biochem* 148: 447–453.

Sinha, S.C., Li, L.S., Miller, G.P., Dutta, S., Rader, C., Lerner, R.A. (2004) Prodrugs of dynemicin analogs for selective chemotherapy mediated by an aldolase catalytic Ab. *Proc Natl Acad Sci USA* 101: 3095–3099.

Smith, G.K., Banks, S., Blumenkopf, T.A., Cory, M., Humphreys, J., Laethem, R.M., Miller, J., Moxham, C.P., Mullin, R., Ray, P.H., Walton, L.M., Wolfe, L.A., III (1997) Toward antibody-directed enzyme prodrug therapy with the T268G mutant of human carboxypeptidase A1 and novel in vivo stable prodrugs of methotrexate. *J Biol Chem* 272: 15804–15816.

Spencer, D.I., Robson, L., Purdy, D., Whitelegg, N.R., Michael, N.P., Bhatia, J., Sharma, S.K., Rees, A.R., Minton, N.P., Begent, R.H., Chester, K.A. (2002) A strategy for mapping and neutralizing conformational immunogenic sites on protein therapeutics. *Proteomics* 2: 271–279.

Springer, C.J., Antoniw, P., Bagshawe, K.D., Searle, F., Bisset, G.M., Jarman, M. (1990) Novel prodrugs which are activated to cytotoxic alkylating agents by carboxypeptidase G2. *J Med Chem* 33: 677–681.

Springer, C.J., Bagshawe, K.D., Sharma, S. K., Searle, F., Boden, J.A., Antoniw, P., Burke, P.J., Rogers, G.T., Sherwood, R.F., Melton, R.G. (1991) Ablation of human choriocarcinoma xenografts in nude mice by antibody-directed enzyme prodrug therapy (ADEPT) with three novel compounds. *Eur J Cancer* 27: 1361–1366.

Springer, C.J., Dowell, R., Burke, P.J., Hadley, E., Davis, D.H., Blakey, D.C., Melton, R.G., Niculescu-Duvaz, I. (1995) Optimization of alkylating agent prodrugs derived from phenol and aniline mustards: a new clinical candidate prodrug (ZD2767) for antibody-directed enzyme prodrug therapy (ADEPT). *J Med Chem* 38: 5051–5065.

Thorpe, P.E., Ross, W.C., Cumber, A.J., Hinson, C.A., Edwards, D.C., Davies, A.J. (1978) Toxicity of diphtheria toxin for lymphoblastoid cells is increased by conjugation to antilymphocytic globulin. *Nature* 271: 752–755.

Wallace, P.M., MacMaster, J.F., Smith, V.F., Kerr, D.E., Senter, P.D., Cosand, W.L. (1994) Intratumoral generation of 5-fluorouracil mediated by an antibody-cytosine deaminase conjugate in combination with 5-fluorocytosine. *Cancer Res* 54: 2719–2723.

Wei, Y., Hecht, M.H. (2004) Enzyme-like proteins from an unselected library of designed amino acid sequences. *Protein Eng Des Sel* 17: 67–75.

Wentworth, P., Datta, A., Blakey, D., Boyle, T., Partridge, L.J., Blackburn, G.M. (1996) Toward antibody-directed "abzyme" prodrug therapy, ADAPT: carbamate prodrug activation by a catalytic antibody and its in vitro application to human tumor cell killing. *Proc Natl Acad Sci USA* 93: 799–803.

Winter, G., Harris, W.J. (1993) Humanized antibodies. *Trends Pharmacol Sci* 14: 139–143.

Wolfe, L.A., Mullin, R.J., Laethem, R., Blumenkopf, T.A., Cory, M., Miller, J.F., Keith, B.R., Humphreys, J., Smith, G.K. (1999) Antibody-directed enzyme prodrug therapy with the T268G mutant of human carboxypeptidase A1: in vitro and in vivo studies with prodrugs of methotrexate and the thymidylate synthase inhibitors GW1031 and GW1843. *Bioconjug Chem* 10: 38–48.

Xu, Y., Yamamoto, N., Janda, K.D. (2004) Catalytic antibodies: hapten design strategies and screening methods. *Bioorg Med Chem* 12: 5247–5268.

9
Emerging Therapeutic Concepts II: Nanotechnology

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9.1
Introduction

It has been known for decades that in colloid systems solid particles, liquid droplets, gas bubbles, and liquid films between them possess different properties from those of the same material in bulk when their size becomes smaller than 100–1000 nm (reviewed in Dimitrov 1983). Recent advances in physics, chemistry, materials sciences, engineering, and molecular biology have allowed the development of nanoparticles (size 1–100 nm and rarely up to 1000 nm) by combining atoms or molecules one at a time, and in arrangements that do not occur in nature (reviewed in Ferrari 2005). Such particles have attracted much attention because of their unique mechanical, electrical, and optical properties. This has resulted in a renewed interest in various nanoparticles already known about for many years (e.g. liposomes) and the development of new ones (e.g. quantum dots and gold nanoshells).

For biomedical applications, any particle with a size typically in the range from 1 to 100 nm can be referred to as a nanoparticle. A multifunctional nanoparticle has important additional properties and contains a synthetic component. Thus biological molecules of this size or their assemblies alone are excluded from this definition. To distinguish from generic liposomes (vesicles from a bilayer lipid membrane), such as cell-size liposomes, we will call a liposome in the nanosize range a nanoliposome, which is approximately equivalent to a small unilamellar vesicle (SUV) (Fig. 9.1). If this nanoliposome contains a drug encapsulated by its membrane, and directing molecules, for example a targeting antibody, on the surface, it becomes a multifunctional nanoparticle because it: (1) increases the half-life and decreases the systemic toxicity and potential immunogenicity of the drug, and (2) specifically binds to molecules of choice, in particular, cell surface receptors, that can ensure directed delivery of the drug to targeted cancer cells. An ideal multifunctional nanoparticle for use against cancer would also have a signaling component so it can diagnose cancer and assess the therapeutic

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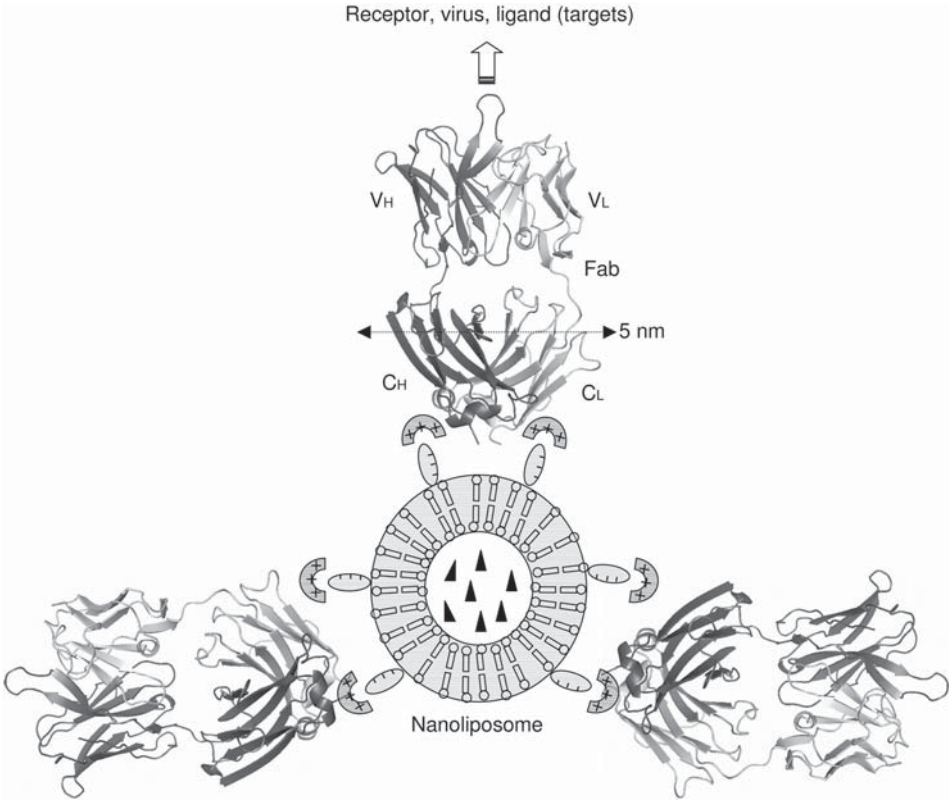

Fig. 9.1 Sketch of a nanoliposome–antibody conjugate. The nanoliposome with a diameter typically in the range from 20 to 100 nm is not in scale. Three Fab molecules are shown with positive charges interacting with patches of negative charges on the liposome surface. Dark triangles indicate drug molecules.

effect. In addition, it could have a triggering property so it can be made to release the drug only after reaching the target.

9.2 Nanoliposomes, Gold Nanoshells, Quantum Dots, and Other Nanoparticles with Biomedical Potential

Many nanoparticles, including carbon nanotubes (Sinha and Yeow 2005), nanodroplets (Garcia-Fuentes et al. 2005; Prego et al. 2005) including lipid emulsions (Hashida et al. 2005), solid lipid nanoparticles (Manjunath et al. 2005), and polymer nanoparticles (Soppimath et al. 2001; Ravi et al. 2004; Csaba et al. 2005) have potential biomedical applications. Although nanoliposomes are perhaps best investigated as vehicles for drug delivery, recently quantum dots have shown

potential for imaging, and gold nanoshells for triggered drug delivery and imaging. These nanoparticles are briefly reviewed below.

Liposomes were discovered by Alec D. Bangham 40 years ago (Bangham et al. 1965a,b,c) and have been developed subsequently as vehicles for the sustained delivery of various chemotherapeutic agents, genes, and vaccines and their enhanced targeting (Szoka and Papahadjopoulos 1980; Szoka 1990; Gregoriadis 1995; Gregoriadis et al. 2002; Park et al. 2004; Hashida et al. 2005; Torchilin 2005). The basic structure of a liposome is either a small or large unilamellar, multilamellar, or multivesicular membrane consisting of alternating aqueous and lipid bilayers. This structural design consequently permits the incorporation of either water-soluble or lipophilic drugs. Amphipathic drugs are actually encapsulated within the aqueous and/or the lipid bilayers; the weakly lipophilic components are partially distributed within the aqueous compartment. The lipid bilayer typically consists of naturally occurring biocompatible materials such as phospholipids (derived from soy or egg sources) and cholesterol. Liposomes therefore resemble biological membranes, which should confer them with low immunogenicity and toxicity.

Another system with low, if any, immunogenicity and toxicity that allows separation of compounds from the environment, and provides a skeleton for attachment of functional groups hence does not occur in nature or resemble any biological entity, is the nanoshell. Nanoshells are either hollow or consist of a dielectric core surrounded by a thin metal (typically gold) shell and offer significant advantages over conventional imaging probes including continuous and broad wavelength tunability, far greater scattering and absorption coefficients, increased chemical stability, and improved biocompatibility (Loo et al. 2005a,b). Based on the relative dimensions of the shell thickness and core radius, nanoshells may be designed to scatter and/or absorb light over a broad spectral range, including the near-infrared (NIR), a wavelength region that provides maximal penetration of light through tissue. The ability to control both wavelength-dependent scattering and absorption of nanoshells offers the opportunity to design nanoshells which provide, in a single nanoparticle, both diagnostic and therapeutic capabilities. Nanoshells can be engineered to both scatter light in the NIR, enabling optical molecular cancer imaging, and to absorb light, allowing destruction of targeted cells through photothermal therapy (Loo et al. 2005b).

Hollow nanoshells can be also made, allowing encapsulation of compounds, as in nanoliposomes. For example, hollow nanoshells of gold (diameter below 100 nm) entrapping an enzyme, horseradish peroxidase (HRP), in the cavity have been prepared in reverse micelles by leaching out silver chloride (AgCl) from Au(shell)AgCl(core) nanoparticles with diluted ammonia solution (Kumar et al. 2005). Small substrate molecules such as *o*-dianisidine can easily enter through the pores of the nanoshell and can undergo enzymatic oxidation by H_2O_2. When the substrate is chemically conjugated with dextran molecule (10 kDa), the enzymatic reaction is practically completely prevented perhaps by the inability of dextran–*o*-dianisidine conjugate to penetrate the pores of the nanoshells; HRP does not show any activity when trapped inside solid gold nanoparticles.

Quantum dots (qdots) cannot encapsulate compounds but offer unique imaging possibilities that have generated a great deal of interest recently. They are fluorescent semiconductor nanocrystals combining high brilliance, photostability, broad excitation but very narrow emission spectra, size-tunable emission (from the UV to the IR), narrow spectral linewidths, continuous absorption profiles, and stability against photobleaching. Their large surface area-to-volume ratio also makes them appealing for the design of more complex nanosystems and their surface chemistry is compatible with biomolecular conjugation (Han et al. 2001; Lidke and Arndt-Jovin 2004; Lidke et al. 2004; Ozkan 2004; Arya et al. 2005; Gao et al. 2005; Hotz 2005; Michalet et al. 2005). Research on qdots has evolved over the past two decades from electronic materials science to biological applications. Recent examples of their experimental use include the observation of diffusion of individual glycine receptors in living neurons and the identification of lymph nodes in live animals by NIR emission during surgery (Michalet et al. 2005).

New generations of qdots have far-reaching potential for the study of intracellular processes at the single-molecule level, high-resolution cellular imaging, long-term *in vivo* observation of cell trafficking, and as tumor targeting agents, and in diagnostics.

9.3 Conjugation of Antibodies to Nanoparticles

We briefly review here several approaches for conjugation of antibodies to liposomes, including nanoliposomes, that could be also used for other nanoparticles although with major modifications. Conjugation methodologies can be divided into two major groups: through noncovalent interactions or through covalent chemical bonds. A major noncovalent approach for conjugation is simply mixing liposomes and antibodies which can bind to each other if appropriate charge complementarity, polar interactions, and hydrophobic interactions are available. We have developed a model which may help to design liposomes and antibodies that can spontaneously associate (Prabakaran and Dimitrov, in preparation). The noncovalent interactions have not been utilized much due to the nature of the weak interactions between liposome and other ligands, and uncontrolled distribution of the ligands on the liposome surface (Nobs et al. 2004). In the case of antibodies, we considered the possibility of protein engineering for the structurally well-studied antibody templates to provide a charged surface at the constant regions of heavy and light chains of antibody. Antibodies are in the typical nanodimension with a width of 5 nm for a Fab (Fig. 9.1).

The liposome surface can be readily altered with various modifications to introduce surface charges. To achieve noncovalent interactions between liposomes and antibodies based on electrostatic properties, we have to incorporate a negative charge through chemical modification such as carboxylate, phosphate, or sulfate moieties on the liposome surface, or by modulating the liposome membrane composition to form rafts of negatively charged lipids. On the antibody surface,

amino acid residues of constant domains were selected based on the knowledge of crystal structures; residues that are not involved in the structural stability and are highly exposed were mutated to basic residues such as lysine, arginine, or histidine. This methodology offers the possibility of conjugation similar to that observed with other covalent linkage methods and also gives control over the orientation of the antibodies. A simple model of this approach is given in Fig. 9.1, in which the liposome particle with a diameter of about 20 nm holds three Fabs. The Fabs shown are from an anti-HIV antibody (m18) in which the heavy and light chains are shown by dark and light gray colors, respectively. Each of the constant domains (C_H and C_L) has a cluster of several residues bearing significant positive charges as indicated by plus signs. The negative charges residing on the liposome are shown by the minus signs. Depending on the size and charge of the liposome molecule, the bulkiness of the mutated residues in the antibody, and charge complementarities, the noncovalent interactions between liposome and antibody are stabilized, leading to the formation of an antibody–nanoliposome conjugate.

Another noncovalent antibody–liposome conjugation approach is based on the use of hydrophobic anchors, including long-chain fatty acids such as palmitic acid and phospholipids such as phosphatidylethanolamine (PE) and phosphatidylinositol (PI). These anchors provide long spacer arms to prevent steric hindrance. Typically these anchors are precoupled with the antibodies and then mixed with the other liposome components to form liposomes. In this case antibodies can be also trapped inside the liposomes. To avoid such problems anchors can be incorporated in the liposomes and used for conjugation with the antibodies.

The covalent conjugation group of approaches are based on the use of five types of covalent bonds: thiolether, disulfide, amide, hydrazone, and amine-amine (Nobs et al. 2004). The thiolether bond is formed by antibody thiol groups and maleimide groups on liposomes. Native thiol groups are readily available in antibody molecules, and can be also engineered by placing cysteines far from the antigen-binding site. The partner on the liposomes for this covalent bond is often *N*-[4-(*p*-maleimidophenyl)butyryl]phosphatidylethanolamine (or MPB-PE). Coupling can be achieved by adding antibodies with available thiol groups to preformed liposomes with incorporated maleimide. No crosslinker is required. This method has also been extended to couple antibodies to stealth (PEGylated) liposomes at the distal end of PEG (Nobs et al. 2004). Studies have been reported to use reversed function groups: a thiol function on liposomes with maleimide-containing antibodies. In this case, antibodies should be treated with a bifunctional crosslinker such as succinimidyl 4-(*p*-maleimidopheny)butyrate (or SPMB).

The coupling chemistry through disulfide bonds is efficient, rapid, and easy to carry out. The thiol group on the antibody molecule can react with a pyridyldithio group on anchor molecules. A drawback of this method is the relative instability of disulfide bonds in circulation.

Coupling through amide bonds is based on the use of anchoring molecules with a free carboxy group, which can form an amide bond with primary amines

of antibodies. The reaction is mediated by 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC) and *N*-hydroxysulfosuccinimide (NHS). No pretreatment is required.

The hydrazone bond is formed between carbohydrate group and hydrazide groups incorporated in liposomes. The carbohydrate on the constant region of the antibody heavy chain is oxidated by galactose oxidase or sodium periodate into aldehyde groups. Lauric acid hydrazide is often used as anchor on liposomes for this coupling. The obvious advantadge of this technique is that antibodies are favorably orientated, leaving the antigen-binding region free. The shortfall of this method is the relatively low coupling efficiency.

Coupling though primary amine–amine bonds is based on the use of primary amine groups on antibodies that are crosslinked with anchors such as glutaldehyde or suberimidate. The only pretreatment required is the activation of the primary amine group on liposomes; no manipulation of antibodies is needed. This otherwise efficient method has not been widely used because of the difficulty of controlling the process of homopolymerization of antibodies and liposomes.

9.4 Nanoparticle–Antibody Conjugates for the Treatment of Cancer and Other Diseases

Liposomes conjugated with antibodies, also termed immunoliposomes, have been evaluated for their potential as drug delivery systems mostly for the treatment of cancer and gene delivery (Felnerova et al. 2004; Noble et al. 2004; Park et al. 2004; Schnyder and Huwyler 2005; Torchilin 2005). Several liposomal preparations, including PEGylated liposomal doxorubucin, are in clinical use (Tsatalas et al. 2003). PEGylated liposomes exhibit increased plasma half-life (several days) compared with pure lipid systems (several hours). They can extravasate in tumor tissue (Fig. 9.2) in accordance with an enhanced permeability and retention effect. Due to their relatively long half-life, the PEGylated (sterically stabilized) liposomes can gradually accumulate in tumors but do not bind and enter tumor cells directly. Because these molecules rather stay within tumor stroma and release drug for diffusion into the tumor cells, relative specificity is provided by enhanced permeability and retention of tumor tissue. However, truly targeted immunoliposomes are usually developed by conjugating them with antibody fragments.

Immunoliposomes have been used successfully to target cancer cells. For example, anti-HER2 immunoliposomes were developed for drug delivery to cancer cells overexpressing the oncogenic receptor tyrosine kinase HER2 (Park et al. 2004). These immunoliposomes were optimized for clinical trials and such trials are ongoing. A similar approach has been used for other targets, including EGFR (Mamot et al. 2003). Although immunoliposomes specifically target cells expressing the respective receptor they do not fuse with the plasma or endosomal membrane but gradually release drugs when becoming degraded. The fusion of

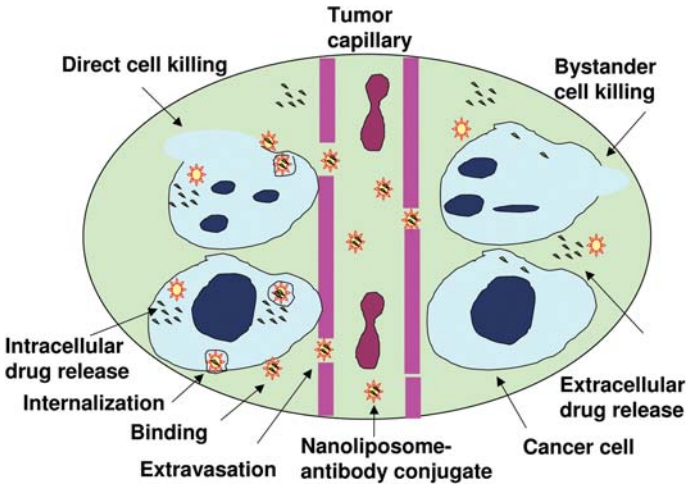

Fig. 9.2 Nanoliposome–antibody conjugates for the treatment of cancer. Liposomes extravasate in tumors and kill cancer cells either by entering the cells and releasing the drug intracellularly (direct killing) or by releasing the drug in the extracellular space but close to cancer cells, which then diffuses into the cancer cells (bystander cell killing). Targeting cancer cells by antibodies specific for cancer-related antigens enhances direct cell killing by an enhanced intracellular delivery of the toxic drug.

such liposomes could increase their efficiency. In this respect reconstitution of viral fusogenic proteins in liposomes (virosomes) (Sarkar et al. 2002) can lead to liposome–cell fusion. However, viral proteins are immunogenic and target only their own receptor. In an attempt to combine specificity of targeting and fusion ability, we have recently constructed a fusion protein of an antibody targeting the IGF-IR and a nonspecific fusogenic protein, and are currently evaluating its properties (Dimitrov et al., in preparation). Such nanoliposome systems could have an increased efficiency of delivery. However, they could fuse nonspecifically with any cell to which they bind. A more sophisticated system should include a trigger that would allow fusion only after interaction of the targeting antibody with the targeted receptor. Such systems are under development.

Gold nanoshells, gold nanocapes, and gold nanoparticles in general, conjugated to antibodies have been recently developed for various purposes, including imaging and therapy of cancer (Chen et al. 2005; El Sayed et al. 2005; Loo et al. 2005a,b), and as a substrate for an immunoassay that is capable of detecting subnanogram levels of analyte within whole blood on the order of minutes (Hirsch et al. 2005). Recently, qdots have also been used as conjugates with antibodies for imaging and detection of cancer (Kaul et al. 2003; Arya et al. 2005; Nida et al. 2005). In general, conjugation of nanoparticles to antibodies allows their targeting to appropriate receptors. In the next section an example of such targeting of macromolecules (protein toxins), not nanoparticles, is discussed in detail to allow a comparison between protein–antibody fusions and nanoparticle–antibody conjugates.

9.5
Comparison Between Nanoparticle–Antibody Conjugates and Fusion Proteins for Cancer Diagnosis and Treatment

9.5.1
Fusion Proteins

Various proteins have been fused to antibodies for improvement of their therapeutic activity by targeting to appropriate receptors. Members of the ribonuclease (RNase) A superfamily have been linked to antibodies against tumor-associated antigens to selectively kill the tumor cell (Rybak and Newton 1999) without the associated toxicities of current strategies employing plant and bacterial toxins (Rybak and Youle 1991; Frankel et al. 2000). Angiogenin (ANG) is an RNase that is a normal component of human plasma (Shapiro 1987), is not itself cytotoxic to cultured tumor cells yet is a potent inhibitor of protein synthesis in both cell-free systems (St Clair 1988) and when injected into a living cell (Saxena et al. 1992). These studies prompted the development of methods to specifically internalize ANG into tumor cells.

Recombinant derivatives comprising robust, small-sized monovalent and bivalent scFvs (Arndt et al. 2003, 2004; Krauss et al. 2003) derived from the anti-CD22 monoclonal antibodies LL2 (Pawlak-Byczkowska 1989) and RFB4 (Amlot et al. 1993) were fused to ANG (Fig. 9.3). Two monomeric fusion proteins carrying ANG either at the N- or C-terminal end were created (Fig. 9.3b,d), expressed as soluble proteins in *E. coli* and purified to homogeneity (Arndt et al. 2005). The orientation of the RNase relative to the antibody moiety played a significant role towards susceptibility of the fusion protein to degradation from bacterial proteases. Only the ANG-spacer-scFv aligned fusion protein (Fig. 9.3b) was cleaved. This phenomenon was also observed for other bacterially produced scFv-RNase fusion proteins when generated in the same orientation (Krauss, unpublished observations). This problem could be solved by producing these fusion proteins in mammalian cells (Fig. 9.3c) (Krauss et al. 2005). To create a dimeric fusion protein carrying two ribonuclease effector domains, ANG was fused via a $(G_4S)_3$ spacer peptide to the C-terminal end of the stable dimeric anti-CD22 V_L-V_H zero-linker scFv MLT-7 (Arndt et al. 2004, 2005) (Fig. 9.3e). Comparative studies with the homogenously purified dimeric fusion protein (Fig. 9.3e) and its monovalent counterpart (Fig. 9.3d) revealed that both constructs specifically bound to the target antigen and retained ribonucleolytic activity. However, they exhibited a markedly different capability for killing CD22-positive tumor cells. The monomeric construct (ANG-scFv$_{LL2}$) inhibited protein synthesis of target cells in a dose-dependent manner but 50% inhibition (IC_{50}) could only be achieved at concentrations of >360 nmol L^{-1}. In contrast, the dimeric fusion protein efficiently killed CD22-positive Raji and Daudi tumor cell lines with IC_{50} values of 74 nmol L^{-1} and 118 nmol L^{-1}, respectively (Table 9.1). These results show that the therapeutic potential of scFv-ANG fusion proteins can be markedly enhanced by engineering dimeric derivatives.

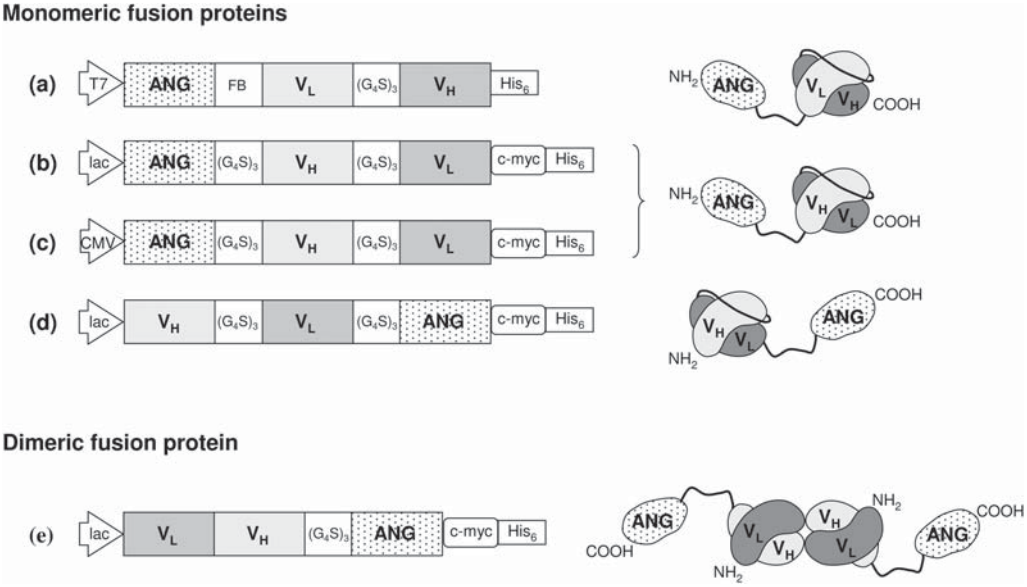

Fig. 9.3 Single-chain Fv–angiogenin fusion proteins. (a–d) Monovalent scFv-ANG fusion proteins with angiogenin expressed either at the N-terminal or the C-terminal end of the antibody. (a) Antitransferrin receptor scFv-ANG fusion protein refolded from inclusion bodies in *E. coli*; (b–d) anti-CD22 scFv-ANG fusion proteins expressed (b,d) as soluble protein into the periplasm of *E. coli*; (c) from Chinese hamster ovary cells. (e) Non-covalent dimers forming fusion protein with ANG at the C-terminal of the monoclonal antibody LL2-derived anti-CD22 zero-linker scFv MJ7. C-myc and His_6-tags for purification are indicated. ANG, human RNase angiogenin; FB (staphylococcal protein A residues 48–60, AKKLNDAQAPKSD) and $(G_4S)_3$ spacer peptides are separating ANG from the scFv.

9.5.2
Nanoparticle-conjugated Antibodies

Compared with protein–antibody fusions such as the previously described examples the conjugation of antibodies to nanoparticles provides a number of advantages (Table 9.2). Hollow nanoparticles have the key property of being able to isolate the internal content from the environment. On the one hand the encapsulated material is protected from a hostile environment and on the other hand the environment is protected from the adverse effects of the enclosed component. The modular organization of the nanoparticle technology also enables a combinatorial approach in which a repertoire of monoclonal antibody fragments can be used in conjunction with a series of encapsulated drugs to yield a new generation of molecularly targeted agents. Possible disadvantages in some cases are the larger size of the nanoparticles, which can decrease their penetration into solid tumors and their increased cost of production.

Table 9.1 In vitro potency of angogenin–scFv fusion proteins.

Fusion protein	*Target antigen*	*Cell line*[a]	*IC_{50} ($nmol\,L^{-1}$)*[b]	*Reference*
$F(ab')_2CH2ANG$	Transferrin receptor	K562	0.05	Rybak et al. 1992
ANG-FB-scFv	Transferrin receptor	MDA-MB-231^{mdr1}	10	Newton et al. 1996
		HT-29^{mdr1}	7	
		HS578T	>100	
		ACHN	4	
		SF539	17	
		Malme	>100	
$scFv_{LL2}$-$(G_4S)_3$-ANG (monomer)	CD22	Raji	360	Arndt et al. 2005
		Daudi	>380	
$scFv_{LL2}$-$(G_4S)_3$-ANG (dimer)	CD22	Raji	74	Arndt et al. 2005
		Daudi	118	
ANG-$(G_4S)_3$-$scFv_{RFB4}$	CD22	Daudi	56	Krauss et al. 2005

a All cell lines are of human origin and are as follows: K562, erythroleukemia; HT-29^{par}, colon carcinoma; HT-29^{mdr1}, multidrug-resistant colon carcinoma; MDA-MB-231^{mdr1}, multidrug-resistant breast carcinoma; HS578T, breast carcinoma; ACHN, renal carcinoma; SF539, CNS carcinoma; MALME, melanoma; Daudi and Raji, B cell lymphoma.

b IC_{50}, concentration of fusion protein required to inhibit protein synthesis or cause cell death by 50%.

To compare the efficacy of targeted liposome-encapsulated doxorubicin (DOX) versus DOX alone, a simple model was recently developed that assumes that the cytotoxic potency of a drug is a function of the intracellular drug level in a critical compartment (Eliaz et al. 2004). Upon exposure to drug, cell death commences after a lag time, and the cell killing rate is dependent on the amount of drug in the critical intracellular compartment. The calculated number of cells in the culture at any time after exposure to the drug takes into account the cell proliferation rate, the cell killing rate, the average intracellular drug concentration, and a lag time for cell killing. This model was applied to DOX encapsulated in liposomes targeted to CD44 on B16F10 melanoma cells in culture. CD44 is the surface receptor that binds to hyaluronan and is overexpressed on various cancer cells, including B16F10. The results demonstrated that the enhanced potency of the encapsulated drug could stem from its enhanced uptake. However, in certain cases, where larger amounts of the free drug were added, such that the intracellular amounts of drug exceeded those obtained from the encapsulated drug, the numbers of viable cells were still significantly smaller for the encapsulated drug.

This finding demonstrated that for given amounts of intracellular DOX, the encapsulated form was more efficient in killing B16F10 cells than the free drug. The outcome was expressed in the kinetic model as a 5- to 6-fold larger rate constant of cell killing potency for the encapsulated drug versus the free drug. The model provided a quantitative framework for comparing the cytotoxic effect

Table 9.2 Comparison between nanoliposome–antibody conjugates and antibody conjugates with small molecules and proteins used as drugs and imaging agents currently under development for diagnosis and treatment of cancer.

Property	*Small molecule–antibody*	*Protein–antibody fusion*	*Nanoliposome–antibody*
Protection from environment	None	None	Yes
Carrying capacity	Several molecules	One	High
Toxicity	High	High	Low
Specific penetration of solid tumors	Moderate	Moderate to low	low
Bystander killing of cancer cells	No	No	Yes
Antibody format	Usually IgG but also others	Usually IgG but also others	scFv, Fab or IgG
Valency of binding	1 or 2	1 or 2	Much higher
Requirement for high-affinity antibody	Yes	Yes	No
Conjugation	Chemical	Fusion protein or chemical	Direct conjugation not required
Stability	Variable	Variable	High
Immunogenicity	Variable	High	None
Half-life in circulation	Long if stable	Long if not immunogenic	Long
Design	Direct	Direct	Modular
Production	Depends on the small molecule	Depends on the protein	Standard for liposomes, antibody

in cultured cells when applying the drug in the free form or in a delivery system.

We have developed a somewhat similar model to compare the toxicity of encapsulated versus free drugs to normal cells and to targeted cancer cells. As we discussed above, toxins and their derivatives as antibody–toxin fusion proteins have shown antitumor activity in humans. However, their efficacy is limited by nonspecific toxicity caused by binding to and killing of normal cells. Encapsulation of toxins in liposomes could decrease the extent of nonspecific toxicity. Major differences between binding of liposome encapsulated and free toxins relate to half-life, penetration, avidity, and capacity (ratio of toxin to targeting molecules – for free single-chain antibody–toxin fusion protein this ratio equals 1). In addition, intracellular delivery of toxin by liposomes may not depend on the use of internalizing antibodies. In an initial attempt to quantify the contribution of each of these factors we developed a model based on the assumption that the targeting molecule (e.g. scFv) is the same, and the distribution in the body is the same for both formulations – toxin-loaded liposome-conjugated antibody and toxin-

conjugated antibody (see Appendix, Sidorov, Blumenthal and Dimitrov, in preparation). The results, which are being validated in experimental systems, could be useful in the design of toxin-encapsulating antibody-conjugated nanoliposomes with reduced toxicity compared with free toxin–antibody fusion proteins.

9.6 Conclusions

In recent years the interest in antibody-guided nanoparticles for cancer imaging and treatment has increased significantly due to successes in both nanotechnology and engineered therapeutic antibodies. The combination of these two promising technologies in antibody–nanoparticle conjugates offers new possibilities for early diagnosis, prevention, and treatment of cancer. The major direction of research appears to continue with the development of various improved systems based on drug-bearing nanoliposomes being conjugated with antibodies that target various surface-associated molecules, including growth factor receptors. In addition, imaging promises to be another major area of research where the marriage of nanotechnology and antibodies could produce entirely new possibilities.

9.7 Summary

Solid particles, liquid droplets, gas bubbles, and liquid films between them in colloid systems have been known for decades to possess size-dependent properties that differ from those of the same material in bulk when size is smaller than about 100 nm. Recent advances in physics, chemistry, materials sciences, engineering, and molecular biology have allowed the development of nanoparticles (size 1–100 nm) by combining atoms or molecules one at a time in arrangements that do not occur in nature. Such particles have attracted much attention because of their unique mechanical, electrical, and optical properties. This resulted in a renewed interest in various nanoparticles already known about for many years (e.g. liposomes) and the development of new ones (e.g. quantum dots and gold nanoshells). Such particles conjugated to antibodies can improve their binding or/and effector functions or confer new functions (e.g. cytotoxicity, size-dependent fluorescence, and light scattering).

Compared with engineered antibodies based on fusion proteins or chemical conjugates made with other compounds, nanoparticle–antibody conjugates have the fundamental capacity to separate compounds loaded inside the particle (e.g. inside liposomes) from the environment, thus avoiding possible toxicity and immunogenicity. In addition, high local concentrations of loaded active substances (e.g. imaging agents) can be achieved. Nanoparticles can also serve as skeletons for the construction of multifunctional nanoparticle–antibody conju-

gates that combine targeting, imaging, and therapeutic properties. Thus nanoparticle–antibody conjugates can complement existing diagnostic tools and treatment protocols and offer entirely new possibilities for the diagnosis and treatment of diseases.

References

Amlot, P.L., Stone, M.J., Cunningham, D., Fay, J., Newman, J., Collins, R., May, R., McCarthy, M., Richardson, J., Ghetie, V. et al. (1993) A phase I study of an anti-CD22-deglycosylated ricin A chain immunotoxin in the treatment of B-cell lymphomas resistant to conventional therapy. *Blood* 82: 2624–2633.

Arndt, M.A.E., Krauss, J., Schwarzenbacher, R., Vu, B.K., Green, S., Rybak, S.M. (2003) Generation of a highly stable internalizing anti-CD22 single-chain Fv fragment for targeting non-Hodgkin's lymphoma. *Int J Cancer* 107: 822–829.

Arndt, M.A.E., Krauss, J., Rybak, S.M. (2004) Antigen binding and stability properties of non-covalently linked anti-CD22 single-chain Fv dimers. *FEBS Lett* 578: 257–261.

Arndt, M.A., Krauss, J., Vu, B.K., Newton, D.L., Rybak, S.M. (2005) A dimeric angiogenin immunofusion protein mediates selective toxicity toward CD22+ tumor cells. *J Immunother* 28: 245–251.

Arya, H., Kaul, Z., Wadhwa, R., Taira, K., Hirano, T., Kaul, S.C. (2005) Quantum dots in bio-imaging: Revolution by the small. *Biochem Biophys Res Commun* 329: 1173–1177.

Bangham, A.D., Standish, M.M., Miller, N. (1965a) Cation permeability of phospholipid model membranes: effect of narcotics. *Nature* 208: 1295–1297.

Bangham, A.D., Standish, M.M., Watkins, J.C. (1965b) Diffusion of univalent ions across the lamellae of swollen phospholipids. *J Mol Biol* 13: 238–252.

Bangham, A.D., Standish, M.M., Weissmann, G. (1965c) The action of steroids and streptolysin S on the permeability of phospholipid structures to cations. *J Mol Biol* 13: 253–259.

Chen, J., Saeki, F., Wiley, B.J., Cang, H., Cobb, M.J., Li, Z.Y., Au, L., Zhang, H., Kimmey, M.B., Li, X., Xia, Y. (2005) Gold nanocages: bioconjugation and their potential use as optical imaging contrast agents. *Nano Lett* 5: 473–477.

Csaba, N., Caamano, P., Sanchez, A., Dominguez, F., Alonso, M.J. (2005) PLGA: poloxamer and PLGA: poloxamine blend nanoparticles: new carriers for gene delivery. *Biomacromolecules* 6: 271–278.

Dimitrov, D.S. (1983) Dynamic interactions between approaching surfaces of biological interest. *Progr Surface Sci* 14: 295–424.

El Sayed, I.H., Huang, X., El Sayed, M.A. (2005) Surface plasmon resonance scattering and absorption of anti-EGFR antibody conjugated gold nanoparticles in cancer diagnostics: applications in oral cancer. *Nano Lett* 5: 829–834.

Eliaz, R.E., Nir, S., Marty, C., Szoka, F.C., Jr. (2004) Determination and modeling of kinetics of cancer cell killing by doxorubicin and doxorubicin encapsulated in targeted liposomes. *Cancer Res* 64: 711–718.

Felnerova, D., Viret, J.F., Gluck, R., Moser, C. (2004) Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs. *Curr Opin Biotechnol* 15: 518–529.

Ferrari, M. (2005) Cancer nanotechnology: opportunities and challenges. *Nat Rev Cancer* 5: 161–171.

Frankel, A.E., Kreitman, R.J., Sausville, E.A. (2000) Targeted toxins. *Clin Can Res* 6: 326–334.

Gao, X., Yang, L., Petros, J.A., Marshall, F.F., Simons, J.W., Nie, S. (2005) In vivo molecular and cellular imaging with quantum dots. *Curr Opin Biotechnol* 16: 63–72.

Garcia-Fuentes, M., Alonso, M.J., Torres, D. (2005) Design and characterization of a new drug nanocarrier made from solid-

liquid lipid mixtures. *J Colloid Interface Sci* 285: 590–598.
Gregoriadis, G. (1995) Engineering liposomes for drug delivery: progress and problems. *Trends Biotechnol* 13: 527–537.
Gregoriadis, G., Bacon, A., Caparros-Wanderley, W., McCormack, B. (2002) A role for liposomes in genetic vaccination. *Vaccine* 20(Suppl 5): B1–B9.
Han, M., Gao, X., Su, J.Z., Nie, S. (2001) Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. *Nat Biotechnol* 19: 631–635.
Hashida, M., Kawakami, S., Yamashita, F. (2005) Lipid carrier systems for targeted drug and gene delivery. *Chem Pharm Bull (Tokyo)* 53: 871–880.
Hirsch, L.R., Halas, N.J., West, J.L. (2005) Whole-blood immunoassay facilitated by gold nanoshell-conjugate antibodies. *Methods Mol Biol* 303: 101–111.
Hotz, C.Z. (2005) Applications of quantum dots in biology: an overview. *Methods Mol Biol* 303: 1–17.
Kaul, Z., Yaguchi, T., Kaul, S.C., Hirano, T., Wadhwa, R., Taira, K. (2003) Mortalin imaging in normal and cancer cells with quantum dot immuno-conjugates. *Cell Res* 13: 503–507.
Krauss, J., Arndt, M.A., Martin, A.C., Liu, H., Rybak, S.M. (2003) Specificity grafting of human antibody frameworks selected from a phage display library: generation of a highly stable humanized anti-CD22 single-chain Fv fragment. *Protein Eng* 16: 753–759.
Krauss, J., Arndt, M.A., Vu, B.K., Newton, D.L., Rybak, S.M. (2005) Targeting malignant B-cell lymphoma with a humanized anti-CD22 scFv-angiogenin immunoenzyme. *Br J Haematol* 128: 602–609.
Kumar, R., Maitra, A.N., Patanjali, P.K., Sharma, P. (2005) Hollow gold nanoparticles encapsulating horseradish peroxidase. *Biomaterials* 26: 6743–6753.
Lidke, D.S., Arndt-Jovin, D.J. (2004) Imaging takes a quantum leap. *Physiology (Bethesda)* 19: 322–325.
Lidke, D.S., Nagy, P., Heintzmann, R., Arndt-Jovin, D.J., Post, J.N., Grecco, H.E., Jares-Erijman, E.A., Jovin, T.M. (2004) Quantum dot ligands provide new insights into erbB/HER receptor-mediated signal transduction. *Nat Biotechnol* 22: 198–203.
Loo, C., Hirsch, L., Lee, M.H., Chang, E., West, J., Halas, N., Drezek, R. (2005a) Gold nanoshell bioconjugates for molecular imaging in living cells. *Opt Lett* 30: 1012–1014.
Loo, C., Lowery, A., Halas, N., West, J., Drezek, R. (2005b) Immunotargeted nanoshells for integrated cancer imaging and therapy. *Nano Lett* 5: 709–711.
Mamot, C., Drummond, D.C., Greiser, U., Hong, K., Kirpotin, D.B., Marks, J.D., Park, J.W. (2003) Epidermal growth factor receptor (EGFR)-targeted immunoliposomes mediate specific and efficient drug delivery to. *Cancer Res* 63: 3154–3161.
Manjunath, K., Reddy, J.S., Venkateswarlu, V. (2005) Solid lipid nanoparticles as drug delivery systems. *Methods Find Exp Clin Pharmacol* 27: 127–144.
Michalet, X. and others (2005) Quantum dots for live cells, in vivo imaging, and diagnostics. *Science* 307: 538–544.
Newton, D.L., Xue, Y., Olson, K.A., Fett, J.W., Rybak, S.M. (1996) Angiogenin single-chain immunofusions: influence of peptide linkers and spacers between fusion protein domains. *Biochemistry* 35: 545–553.
Nida, D.L., Rahman, M.S., Carlson, K.D., Richards-Kortum, R., Follen, M. (2005) Fluorescent nanocrystals for use in early cervical cancer detection. *Gynecol Oncol* 99(3 Suppl 1): S89–94.
Noble, C.O., Kirpotin, D.B., Hayes, M.E., Mamot, C., Hong, K., Park, J.W., Benz, C.C., Marks, J.D., Drummond, D.C. (2004) Development of ligand-targeted liposomes for cancer therapy. *Expert Opin Ther Targets* 8: 335–353.
Nobs, L., Buchegger, F., Gurny, R., Allemann, E. (2004) Current methods for attaching targeting ligands to liposomes and nanoparticles. *J Pharm Sci* 93: 1980–1992.
Ozkan, M. (2004) Quantum dots and other nanoparticles: what can they offer to drug discovery? *Drug Discov Today* 9: 1065–1071.
Park, J.W., Benz, C.C., Martin, F.J. (2004) Future directions of liposome- and immunoliposome-based cancer therapeutics. *Semin Oncol* 31: 196–205.

Pawlak-Byczkowska, E.J. (1989) Two new monoclonal antibodies, EPB-1 and EPB-2, reactive with human lymphoma. *Cancer Res* 49: 4568–4577.
Prego, C., Garcia, M., Torres, D., Alonso, M.J. (2005) Transmucosal macromolecular drug delivery. *J Control Release* 101: 151–162.
Ravi, K.M., Hellermann, G., Lockey, R.F., Mohapatra, S.S. (2004) Nanoparticle-mediated gene delivery: state of the art. *Expert Opin Biol Ther* 4: 1213–1224.
Rybak, S.M., Newton, D.L. (1999) Natural and engineered cytotoxic ribonucleases: therapeutic potential. *Exp Cell Res* 253: 325–335.
Rybak, S.M., Youle, R.J. (1991) Clinical use of immunotoxins: monoclonal antibodies conjugated to protein. *Immunol Allergy Clin N Am* 11: 359–380.
Rybak, S.M., Hoogenboom, H.R., Meade, H.M., Raus, J.C.M., Schwartz, D., Youle, R.J. (1992) Humanization of immunotoxins. *Proc Natl Acad Sci USA* 89: 3165–3169.
Sarkar, D.P., Ramani, K., Tyagi, S.K. (2002) Targeted gene delivery by virosomes. *Methods Mol Biol* 199: 163–173.
Saxena, S.K., Rybak, S.M., Davey, R.T., Jr., Youle, R.J., Ackerman, E.J. (1992) Angiogenin is a cytotoxic, tRNA-specific ribonuclease in the RNase A superfamily. *J Biol Chem* 267: 21982–21986.
Schnyder, A., Huwyler, J. (2005) Drug transport to brain with targeted liposomes. *Neurorx* 2: 99–107.
Shapiro, R. (1987) Isolation of angiogenin from normal human plasma. *Biochemistry* 26: 5141–5146.
Sinha, N., Yeow, J.T. (2005) Carbon nanotubes for biomedical applications. *IEEE Trans NanobioSci* 4: 180–195.
Soppimath, K.S., Aminabhavi, T.M., Kulkarni, A.R., Rudzinski, W.E. (2001) Biodegradable polymeric nanoparticles as drug delivery devices. *J Control Release* 70: 1–20.
St Clair, D.K. (1988) Angiogenin abolishes cell-free protein synthesis by specific ribonucleolytic inactivation of 40S ribosomes. *Biochemistry* 27: 7263–7268.
Szoka, F.C., Jr. (1990) The future of liposomal drug delivery. *Biotechnol Appl Biochem* 12: 496–500.
Szoka, F., Jr., Papahadjopoulos, D. (1980) Comparative properties and methods of preparation of lipid vesicles (liposomes). *Annu Rev Biophys Bioeng* 9: 467–508.
Torchilin, V.P. (2005) Recent advances with liposomes as pharmaceutical carriers. *Nat Rev Drug Discov* 4: 145–160.
Tsatalas, C., Martinis, G., Margaritis, D., Spanoudakis, E., Kotsianidis, I., Karpouzis, A., Bourikas, G. (2003) Long-term remission of recalcitrant tumour-stage mycosis fungoides following chemotherapy with pegylated liposomal doxorubicin. *J Eur Acad Dermatol Venereol* 17: 80–82.

Appendix

A model of cancer cell killing by toxin-loaded liposomes conjugated with targeting antibodies.

Model equations To estimate quantitatively the efficacy of killing normal and tumor cells by toxin-encapsulating liposome and free drug molecules we have developed a dynamic model. The model consists of four equations and describes the dynamics of total intracellular toxin (I, μg) and concentrations of toxin encapsulated in liposomes (L, $\mu g\,mL^{-1}$), free toxin (T, $\mu g\,mL^{-1}$), and tumor or normal cells (C, cells mL^{-1}):

$$\frac{dL}{dt} = -k_L CL - a_L L$$

$$\frac{dT}{dt} = -k_T CT + a_L nL - a_T T$$

$$\frac{dI}{dt} = k_T T + k_L L - a_I I$$

$$\frac{dC}{dt} = gC - dC$$

where:

$$d = d_0 + d_I \frac{I}{I + K}$$

k_L and k_T characterize the avidity of binding liposome and free toxin molecules to cells and transition into cell (mL per cell h^{-1}); n is the number of toxin molecules encapsulated in one liposome; a_L and a_T are the rate constants of liposome leaking and free toxin molecules destruction (h^{-1}), respectively; a_I is the rate constant of intracellular toxin destruction; d_0 is the death rate for normal or tumor cells (h^{-1}); d_I is the maximum increase in value cell death rate for high intracellular toxin concentration (h^{-1}); K is the amount of intracellular toxin giving an increase of 50% of maximum in death rate (μg); and g is the rate of cell growth (h^{-1}). In this mode we assume that cell death rate d depends nonlinearly on total amount of intracellular toxin.

Parameter values Using the data for doxorubicin (DOX) uptake by the B16F10 cells (1.6×10^5 cells mL^{-1}) after 3 h of exposure to five different concentrations of free and liposome-encapsulated toxin (Eliaz et al. 2004) we can estimate parameters $k_L = 5.5 \times 10^{-8}$ mL $cells^{-1}$ h^{-1} and $k_T = 1.8 \times 10^{-8}$ mL $cells^{-1}$ day^{-1} as the result of data fitting. These values of these constants can provide uptake of femtomoles (from 0.6 to 32) of DOX per cell after 3 h by cells with concentration of 1.6×10^5 cells mL^{-1} in presence of micromoles (from 0.5 to 20) of DOX in media as reported by Eliaz et al. (2004). Rate constant of B16F10 cells proliferation was also estimated in this work: $g = 0.030\,h^{-1}$. Let us assume that $d_0 = 0.028\,h^{-1}$ and d_I is 5 times larger ($= 0.14\,h^{-1}$). A constant value of $K = 200$ μg can be estimated using the dose–response curves and the data about intracellular toxin amount. Here we assume that there is no liposome leakage and intracellular/extracellular toxin molecules are stable ($a_L = a_T = d_I = 0$).

Simulations Figure 9.4 shows the results of simulation for the experiments represented in (Eliaz et al. 2004) with the parameter values described above. B16F10 cells were treated with HAL liposomes encapsulating DOX and free DOX molecules. Cytotoxic effect was determined either immediately at 3, 6, 12, 24, 48, 72, and 96 h after treatment or with a 96-h delay irrespective of treatment duration

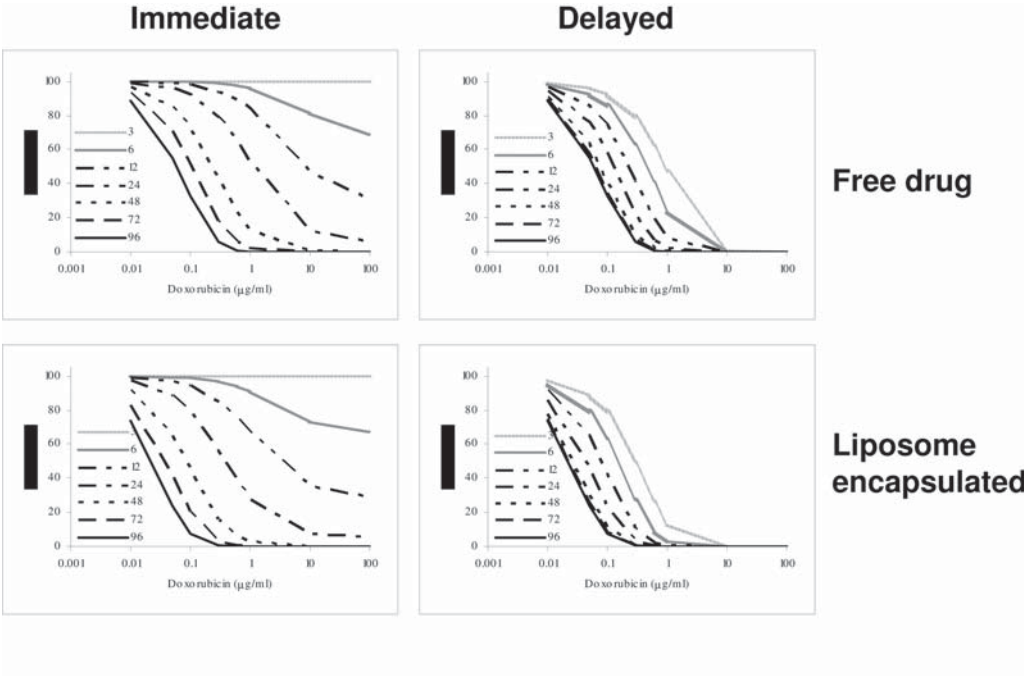

Fig. 9.4 Simulations of immediate and delayed cytotoxic effect of free and liposome-encapsulated DOX (experimental conditions as described in Eliaz et al. 2004) (see Appendix). B16F10 cell were treated with HAL liposome-encapsulated and free DOX molecules. Cytotoxic effect was determined either immediately at 3, 6, 12, 24, 48, 72, and 96 h after treatment or measured following a 96-h delay. The model parameters are described in the text.

(3, 6, 12, 24, 48, 72, and 96 h). It was noted that there is a lag-time in drug effect with no cell killing for about 3 h for very large drug concentration. We modeled this effect by holding the cell death rate at a value of d_0 for the first 3 h of treatment ($d = d_0$, for $0 \le t < 3$ h). The results of the simulations represented in Fig. 9.3 describe the main features of immediate and delayed treatment: sigmoidal dose–response; increase in effect with prolonged treatment; increase in cell killing with the increase of free or liposome-encapsulated drug concentration; increased effect for delayed treatment compared with immediate one and for liposome-encapsulted drug compared with the free drug.

We used the model with the parameters described above to simulate an *in vitro* experiment: treatment of three different mixtures of normal and tumor cells (10%, 50%, and 80% of normal cells, respectively) by the liposome-encapsulated antibody-conjugated liposomes. It was assumed that normal and tumor cells (total concentration 10^7 cells mL^{-1}) have different avidity to the liposomes (different number of receptors specific to the liposome-conjugated antibodies): k_L for normal cells is 10 times less than for tumor cells (5.5×10^{-8} and 5.5×10^{-7} mL $cells^{-1}$ h^{-1},

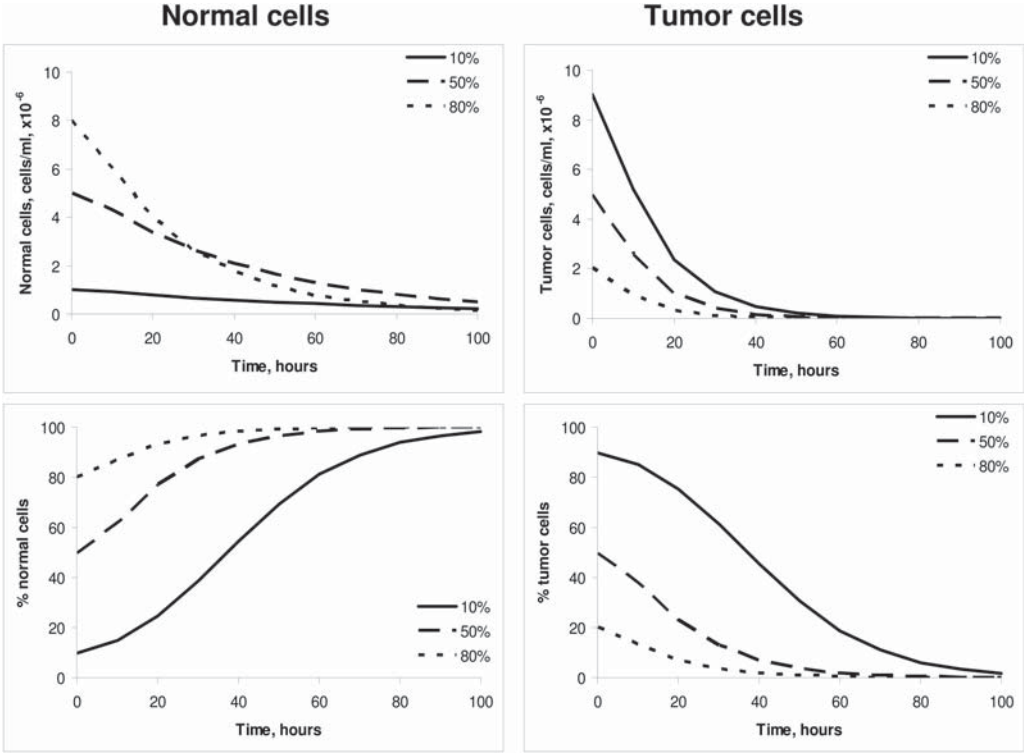

Fig. 9.5 Treatment of mixtures of tumor and normal cells (10%, 50%, and 80% of normal cells) with 10% $\mu g\,mL^{-1}$ of liposome-encapsulated toxin. The model parameters are described in the Appendix.

respectively). All other parameters are the same as described above. Liposome-encapsulated toxin concentration was 10 µg/ml. Dynamics normal cells, tumor cells and percentage of cell in the population were calculated. As shown in Fig. 9.5 the toxic effect for normal cells was delayed comparing to the tumor cells for all initial ratios. Although concentrations of both normal and tumor cells decreased with time after treatment, it was about 100% of live normal cells and after 80 h of treatment for all tested ratios. These results show that the therapeutic effect of a liposome-encapsulated drug can be higher for normal cells than for tumor cells.

10
Emerging Therapeutic Concepts III: Chimeric Immunoglobulin T Cell Receptors, T-Bodies

Thomas Schirrmann and Gabriele Pecher

10.1
Introduction

The potential of monoclonal antibodies as targeted therapeutics has attracted a huge amount of interest over recent decades. However, there are still some obstacles to overcome. One is that antibodies mostly do not mediate effector functions themselves against virus-infected or tumor cells. Only in rare cases antibodies directly induce antiproliferatory, apoptotic, or cytotoxic effects against target cells, depending on the signaling properties of the antigen. Antibodies also need to recruit effector components or cells of the immune system. They are also rapidly removed from the tumor cell surface by processes such as capping, shedding, or endocytosis. To overcome the requirement of additional immunological effectors, therapeutic antibodies are usually fused to toxins, prodrug-activating enzymes, or radionuclides. Nevertheless, the inefficient access of large macromolecules such as antibodies or immunotoxins to poorly vascularized tumors as well as those protected by endothelia, surrounding tissue, or the blood–brain barrier limits their therapeutic efficacy.

In contrast to antibodies, immunological effector lymphocytes such as cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells are capable of migrating into solid tissues or tumors and of mediating potent cytotoxic effector functions. However, the effectiveness and specificity of patient's lymphocytes is not efficient enough to completely destroy all tumor cells. Furthermore, the isolation and expansion of tumor-specific T cells from individual tumor patients has been proven to be problematic (Hoffman et al. 2000). Therefore, a strategy combining the antigen specificity of antibodies with the effector properties of cytotoxic lymphocytes is needed to achieve more efficient cancer immunotherapy.

Two major strategies have been directed towards this objective. First, bispecific antibodies binding to a tumor-associated antigen (TAA) and signal molecules such as CD3 on T cells or CD16 on NK cells have been successfully used to specifically redirect and activate effector lymphocytes against tumor cells (Perez

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et al. 1985; Canevari et al. 1995). However, bispecific antibodies suffer from several limitations, such as the need for large amounts of recombinant protein, their inefficient migration into solid tumors (Jain 1990) or through endothelial barriers and their limited presence on the targeted effector lymphocytes due to dissociation. In addition, T cells stimulated by anti-(CD3 × TAA) bispecific antibodies can lose their signal transduction and effector properties following target cell recognition (Blank-Voorthuis et al. 1993) and require an additional costimulatory signal for full activation, for instance by ligation of CD28 using $CD80^+$ cells or certain anti-CD28 antibodies (Kipriyanov et al. 2002).

The second approach, which is discussed in this chapter, is based on adoptive cellular immunotherapy employing effector lymphocytes gene modified with tumor-specific chimeric receptor genes consisting of an antigen recognition domain of antibodies and a signal domain, triggering their cytolytic mechanisms. These chimeric immunoglobulin TCRs (cIgTCRs) are also termed "T-bodies" (Eshhar et al. 1996) because most of the studies employed T cells and signaling components of the T-cell receptor (TCR). In this review, we describe the development and design of cIgTCR constructs. Furthermore, we take a look at the genetic modification of effector lymphocytes and preclinical studies using cIgTCR gene-modified effector lymphocytes. Finally, we consider the therapeutic aspects of cIgTCR gene-modified effector lymphocytes in the context of other adoptive immunotherapy approaches and the first clinical studies.

10.2
Chimeric Immunoglobulin T-Cell Receptors – "T-Bodies"

10.2.1
Antigen Recognition of Antibodies and T-Cell Receptors

Adaptive immunity has evolved into two major recognition systems for non-self antigens which are expressed by T cells and B cells (Table 10.1). Both lymphocyte types express individual specific antigen recognition molecules that are not determined in the germline. T cells are responsible for the cellular arm of adaptive immunity and are the main protagonists of immune regulation. T cells expressing the α/β TCRs recognize short peptide antigen molecules displayed in noncovalent association of major histocompatibility complexes (MHC) on the surface of antigen-presenting cells and target cells. Antigenic peptides of 9 or 13–17 amino acids are generated by proteolytic digestion of proteins from either intracellular or extracellular origin presented in MHC classes I or II, respectively. TCR binding to the MHC–antigen complex is of low affinity and supported by the coreceptors CD8 or CD4. These coreceptors do not influence the antigen specificity of the TCR complex but enhance its signal over three magnitudes, that the recognition of about 100 antigen–MHC complexes is sufficient to activate the T cell.

B cells are the major players of the humoral arm of adaptive immunity. In contrast to TCRs, antibodies recognize a broad spectrum of antigens independent of cellular or MHC context and often with very high affinity.

Table 10.1 Comparison of antibody and TCR antigen recognition.

	Antibodies	***α/β-TCR***
Cell type	B lymphocytes, plasma cells	T lymphocytes
Expression	Membrane-bound (IgM, IgD), soluble (all isotypes)	Membrane-bound
Class switch	Yes	No
Antigen epitopes	Linear peptides, structural epitopes, haptens, soluble and cell-bound antigens	Linear peptides in MHC context, cell-bound[a]
MHC restriction	No[b]	Yes[a]
Antigen origin	Extracellular	Intracellular (MHC class I) Extracellular (MHC class II)[c]
Antigen on target cells	Usually 10 000–1 000 000 molecules per cell	Usually 10–1000 peptide antigen–MHC complexes per cell
Inhibition by soluble antigen	Yes	No[d]
Coreceptor	No	Th: CD4 for MHC class II CTL: CD8 for MHC class I
Antigen-driven affinity maturation	Yes	No
Affinity	IgG: very high (10^{-9} mol L^{-1}) IgM: usually median (10^{-7} mol L^{-1})	Low (10^{-5}–5×10^{-6} mol L^{-1})
Valency	IgG: bivalent; IgA: tetravalent IgM: 10 or 12 binding sites	Monovalent

a MHC-unrestricted recognition demonstrated for haptens.
b Antibody fragments with TCR-like MHC-restricted peptide antigen recognition obtained by phage display.
c Dendritic cells are able to present extracellular peptides also in MHC-I.
d Artificial antagonistic peptides can block TCR binding.

Despite their fundamentally different antigen recognition systems, antibodies and TCRs are very similar in molecular structure. They are formed by two different disulfide-linked chains, light (L) and heavy (H) chains for antibodies and α/β chains for TCRs. Each chain consists of immunoglobulin domains forming a constant (C) and a variable (V) region. The V region is responsible for the antigen recognition of antibodies as well as of TCRs. The C domains stabilize the antigen recognition domain, assemble the different chains, mediate effector or signal functions and thus transmembrane association. Immunoglobulin and TCR gene loci are similarly organized. During the early phases of T or B cell development both antibody and TCR chains are formed from separate genetic germline V(D)J elements organized in large and diverse clusters by somatic recombination. This mechanism provides the major part of the tremendous repertoire of antigen recognition of adaptive immunity. In contrast to TCRs, however,

immunoglobulin genes undergo an additional process of somatic hypermutation and antigen-driven affinity maturation.

At the end of the 1980s, investigators made use of the structural similarity of immunoglobulins and TCRs by constructing chimeric TCR variants with antibody V regions to facilitate their studies of the TCR complex (Kuwana et al. 1987). They could also demonstrate that the antigen recognition of antibodies and the TCR signaling properties can be combined in chimeric receptor constructs. Based on these results efforts have been made to develop a technology towards the generation of T cells grafted with antibody specificity. Immunotherapy employing T cells recognizing antigens in an antibody-like fashion might overcome the MHC downregulation frequently seen in virus-infected and tumor cells.

10.2.2
General Design of Chimeric Immunoglobulin T-cell Receptors

Chimeric immunoglobulin TCRs generally consist of an appropriate N-terminal signal peptide followed by an extracellular antibody-derived antigen recognition domain, a transmembrane domain, and a cytoplasmatic signal domain (Fig. 10.1). These domains are required to enable the cell surface expression of the receptor and to couple the antibody-mediated antigen recognition with the effector function of the gene-modified immune cell. Additional domains, in particular extracellular spacer domains between antigen recognition domain and transmembrane domain, can dramatically improve the expression, antigen binding, and signal function of the cIgTCR. Novel cIgTCR designs contain additional motifs of costimulatory receptors or utilize downstream signal molecules to enhance the effector functions of the gene modified immune cells.

10.2.3
Antibody Fv and Fab Fragments in Double Chain T-Bodies

Initially, chimeric TCR variants were constructed by the substitution of both TCR V regions by those of antibodies to facilitate the study of the TCR in T cells. These studies did not reveal any structural advantage of the combination V_H with C_α and V_L with C_β or vice versa (Kuwana et al. 1987; Goverman et al. 1990). The chimeric TCR chains also interacted with endogenous TCR chains, which often resulted in nonfunctional TCR hybrid complexes. Interestingly, chimeric TCR chains with the V_H region of hapten-specific antibodies were often sufficient for hapten binding after association with V_α or V_β of the endogenous TCR chains (Gross et al. 1989a; Gorochov et al. 1992). Most antibodies recognize more complex epitopes and need the assembly of both antibody V regions to form a functional Fv fragment. Another problem with this approach of double chain T-bodies is that the chimeric TCR chains must assemble correctly with endogenous TCR signal molecules for signal transduction, thus limiting this approach to T cells.

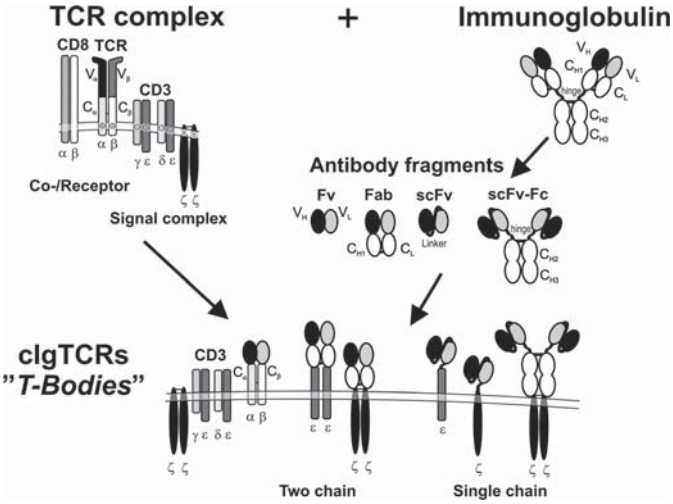

Fig. 10.1 Chimeric immunoglobulin T-cell receptors – "T-bodies." Effector lymphocytes can be grafted with antibody specificity by gene transfer of cIgTCRs. These chimeric receptors are also called "T-bodies," because they combine both the antigen recognition of antibodies with the signal properties of the T-cell receptor (TCR) complex. TCR V regions can be substituted by those of antibodies which require the assembly with the TCR signal complex, limiting this approach to T cells. To circumvent the assembly with the TCR complex, antibody Fv or Fab fragments were directly fused to TCR signal chains such as the CD3 ε or ζ chain (or the FcεRI γ chain, not shown). The employment of scFv antibody fragments allowed single-chain receptor constructs, which is the basis for most currently used cIgTCR designs.

The fusion of antibody V regions to one of the TCR signal chains like the CD3ε or ζ chain or the FcεRI γ chain allowed the extension of T-body approach to other immune cells than T cells. The fusion of CD4 to the cytoplasmatic domains of the ζ chain resulted in a chimeric single-chain receptor gene which could be expressed in T cells (Roberts et al. 1994) as well as in NK cells (Tran et al. 1995). Effector lymphocytes transfected with the chimeric CD4-ζ receptor showed a specific cytotoxicity against $gp120^+$ or HIV-infected target cells. Moreover, the transduction of stem cells with the CD4-ζ construct allowed the subsequent differentiation to NK cells and neutrophils expressing the chimeric receptor (Roberts et al. 1998). Accordingly, the introduction of an appropriate signal chain into cIgTCR constructs can extend the T-body approach to a broad set of immune cell types, including T cells, NK cells, neutrophils, mast cells (Bach et al. 1994) and monocytes (Biglari et al. 2006). In contrast to antibody Fv fragments, the introduction of Fab fragments fused to CD3ε, ζ or FcεRI γ chain enhanced the formation of functional antigen-binding domains stabilized by the disulfide bridge between C_L and C_H1 domains and reduced the interaction with endogenous TCR signal chains (Nolan et al. 1999; Yun et al. 2000). However, cIgTCR constructs

employing Fab as well as Fv fragments still require the transfection of two genes.

10.2.4 Single-Chain Antibody Fragments in Single-Chain T-Bodies

First, the use of single-chain Fv (scFv) antibody fragments consisting of antibody V domains joined by a flexible peptide linker (Bird et al. 1988) allowed cIgTCR constructs consisting of one polypeptide chain. Single-chain Fv fragments can be directly obtained by phage display from human antibody gene libraries, whereas V regions derived from hybridoma antibodies must be genetically engineered and do not always result in functional scFv fragments (Asano et al. 2000). It should be noted that scFv fragments constructed of antibody V regions of mammalian origin cannot always be expressed in *E. coli*. To circumvent problems of bacterial expression scFv fragments can be fused with the human IgG1 Fc part (Schirrmann and Pecher 2002, 2005). To achieve the secretory expression of these scFv-Fc proteins in mammalian cell lines an N-terminal secretory signal peptide was introduced. The scFv-Fc fusion proteins are secreted as homodimers and are more stable than scFv fragments. Finally, the complete scFv-Fc fragment, including its secretory signal peptide, can be directly used in cIgTCR constructs with the Fc domain as spacer domain between antibody fragment and transmembrane moiety (see also Section 10.2.9). For this receptor design, the scFv-Fc protein represents the complete extracellular part of the cIgTCR, thus allowing more accurate analysis of the antigen-binding properties of the receptor in comparison to scFv fragments.

10.2.5 Signal Chains

CTLs and NK cells can mediate their cytotoxicity by two different mechanisms (Fig. 10.2). owever, enhanced target cell binding by receptor-grafted effector lymphocytes alone is not sufficient to obtain an efficient tumor targeting (Schirrmann and Pecher 2001). The effector cell requires a signal for the release of its cytotoxic factors upon the target cell binding. Therefore, an appropriate signal chain must be included in the cIgTCR construct or the receptor must assemble with cellular signal molecules triggering the cytotoxic mechanisms of the effector cell. The TCR on T cells and most of the activatory and cytotoxic immune receptors on T and NK cells (Moretta et al. 2001) are noncovalently associated with transmembrane signal molecules which can serve as signal domains for cIgTCR constructs. In most studies, the ζ chain of the TCR complex and the FcεRI γ chain have been used as signal domains. These are disulfide-linked homodimers with a very short extraplasmatic domain and their signal transduction is mainly mediated by cytoplasmic activatory sequence motifs containing two tyrosine phosphorylation sites (YxxL/I) separated by 6–8 amino acids. These immunoreceptor tyrosine activation motifs (ITAMs) recruit nonreceptor

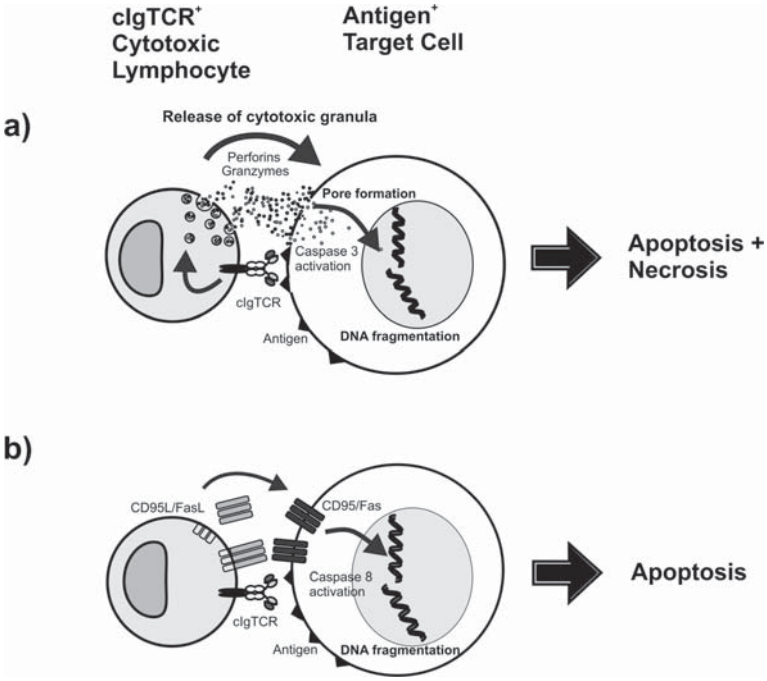

Fig. 10.2 Effector mechanisms of cIgTCR$^+$ cytotoxic lymphocytes (CTLs). CTLs and NK cells are able to mediate the cytotoxicity by two different mechanisms, mediated by cIgTCR gene-modified effector lymphocytes. (a) Cytotoxic factors (perforins, granzymes) stored in specialized granules are released by exocytosis upon target cell binding. Released perforins form pores in the target cell membrane. Granzymes penetrate through these pores and activate the apoptosis cascade in the target cell. The membrane disintegration can also lead to a direct target cell lysis (necrosis). (b) The second mechanism is mediated by Fas ligand (FasL), which is expressed on most activated cytotoxic lymphocytes. FasL is a homotrimer secreted by proteolytic cleavage from the effector cell surface with a short half-life in solution. It binds to Fas$^+$ (CD95, Apo-1) target cells and induces apoptosis.

protein tyrosine kinases (PTKs) of the Src family such as Syk and ZAP70 (ζ-associated protein 70) via their SH2 (Src homology 2) domains. The PTKs activate a multitude of signal pathways (Eshhar and Fitzer-Attas 1998). Figure 10.3 shows the activation of the phosphatidylinositol-3-kinase (PI3K) in the center of the cytotoxicity, which activates the mitogen activated protein kinase (MAPK) pathway. Finally, the activation of the extracellular signal regulatory kinase 2 (ERK-2) induces the mobilization of cytotoxic granules toward the bound target cell (Jiang et al. 2000).

The ζ chain contains three ITAMs whereas the FcεRI γ chain and the other signal molecules of the TCR complex contain only one ITAM. The high number of activatory sequence motifs in the ζ chain is not essential for signal transduction since CD3 ε, FcεRI γ chain, and artificial ζ chain variants with one ITAM also

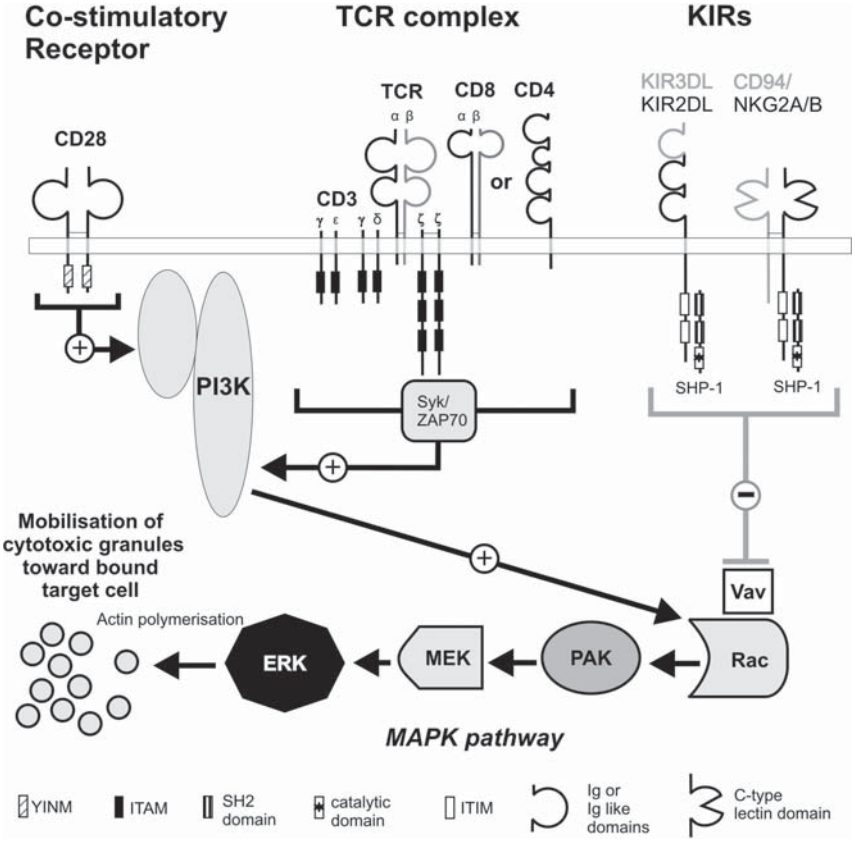

Fig. 10.3 Signal transduction in T cells. The α/β-TCR assembles with a signal complex consisting of CD3 molecules and the ζ chain. The coreceptors CD8 and CD4 enhance the sensitivity of the TCR complex. CD3 signal molecules and the ζ chain recruit nonreceptor protein tyrosine kinases (PTKs) of the Src family, such as Syk and ZAP70. These PTKs activate a multitude of signal pathways. The activation of phosphoinositide-3 kinase (PI3K) central to cytotoxicity. PI3K activates the MAPK pathway and finally ERK-2, which induces the mobilization of cytotoxic granules towards the bound target cell. The costimulatory receptor CD28 binds directly to the p85 subunit of PI3K and supports the T-cell activation. On the other hand, NK cells and T cells (at least subsets) express killer inhibitory receptors (KIRs) for HLA-C and nonclassical major histocompatibility complex (MHC) class Ib molecules. After ligand binding, KIRs recruit via their cytoplasmatic immunoreceptor tyrosine inhibitory motifs (ITIMs) the SH2-containing protein tyrosine phosphatase SHP-1, which dephosphorylates the guanosine nucleotide exchange factor Vav-1. The inactivation of Vav-1 inhibits the MAPK pathway and the granule-mediated cytotoxicity.

trigger the cytolytic function of T cells (Romeo et al. 1992; van Oers et al. 1998; Nolan et al. 1999). But the individual ITAMs of the ζ chain are recognized by different downstream signal molecules (Zenner et al. 1996) suggesting not only additional signaling events but also stronger regulation. The positive selection

and T-cell maturation is more efficiently supported by the ζ chain than by the γ chain (Shores et al. 1997), whereas both signal chains seem to equally mediate T-cell development and function. T cells transfected with cIgTCR gene constructs containing the ζ chain instead of the FcεRI γ chain showed a greater capacity for tumor control (Haynes et al. 2001).

10.2.6
Signal Domains Employing Downstream Signal Molecules

Although sporadic human tumors show significant changes in their antigen expression which should activate adaptive immunity, they frequently develop mechanisms to evade or to actively suppress the tumor-directed immune response (Finke et al. 1999). T cells of tumor patients often have an altered TCR composition, blunted calcium responses, as well as functional deficiencies in the CD3 ζ chain, the PTKs $p56^{lck}$ and $p59^{fyn}$ and the transcription factor NFκB p65 (Salvadori et al. 1994; Correa et al. 1997; Bukowski et al. 1998; Whiteside 1999). The CD3 ζ chain seems to be proteolytically degraded by caspases 3 and 7, since no change in the mRNA level was observed. Chronic antigen stimulation without costimulation also leads to a downregulation of the ζ chain and to a substitution by the γ chain (Mizoguchi et al. 1992). To bypass receptor proximal signaling defects and to overcome the problems with tumor recognition frequently seen in cancer patients, novel approaches have been developed introducing downstream signal molecules of the TCR signal transduction pathway into cIgTCR constructs (Eshhar and Fitzer-Attas 1998). Since none of the TCR complex subunits possess their own intrinsic enzymatic activity, the activation of cytoplasmatic nonreceptor PTKs is one of the earliest signal transduction events of the TCR.

Receptor clustering is thought to stimulate PTKs of the Src family to phosphorylate the ITAMs of CD3 subunits and ζ chain, creating docking sites for the SH2 domains of ZAP70 or Syk. These Syk family PTKs are subsequently activated by phosphorylation. Together with Src family PTKs they phosphorylate downstream signal proteins involved in the PI3K activation and in the stimulation of the Ras pathway.

ZAP70 and Syk have been studied as signal domains of cIgTCR constructs (Eshhar and Fitzer-Attas 1998). Chimeras consisting of scFv-ZAP70 were totally ineffective in transducing signals for IL-2 secretion or target cell lysis (Fitzer Attas et al. 1998). In contrast, cIgTCRs utilizing Syk instead of ZAP70 were able trigger the IL-2 production and cytolysis in T cells (Fitzer Attas et al. 1997). The employment of Syk may overcome signaling defects and anergic states of the TCR as well as the need for costimulatory signals to activate T cells.

10.2.7
The Transmembrane Domain – More than Just a Membrane Anchor?

The transmembrane domain of the cIgTCR constructs is not just a membrane anchor but also plays an important role in the association with other signal

components. Activating cytotoxic receptors on T or NK cells usually have a positive charge on their transmembrane domain, whereas signal chains such as the ζ or γ chain contain a corresponding negative charge. It has been noted that transmembrane moieties of CD3 components may negatively affect cIgTCR responses (Willemsen et al. 2003), but there are also results showing that the exchange of transmembrane domain did not affect the signal transduction capacity of the ζ chain (Romeo et al. 1992).

Independent studies have described different effects of transmembrane domains chosen from CD4 and CD8 in cIgTCR constructs (Roberts et al. 1994; Fitzer Attas et al. 1998; McGuinness et al. 1999). For example, the transmembrane domain of CD8α was crucial for cIgTCRs containing cytoplasmatic nonreceptor PTKs as signal domain, probably caused by different assembly and clustering of the chimeric receptor (Kolanus et al. 1993; Eshhar and Fitzer-Attas 1998). The choice of the optimal transmembrane domain may therefore depend on the respective cIgTCR construct.

10.2.8
Extracellular Spacer Domains Promote cIgTCR Expression and Function

Many cIgTCR constructs were gained with the introduction of extracellular domains between scFv fragment and transmembrane moiety. They served as spacers to increase the distance between the antigen recognition domain and the plasma membrane. Furthermore, hinge-like properties improved the accessibility of the antigen-binding region to the antigen (Moritz and Groner 1995).

Several groups have introduced the human IgG1 Fc fragment (hinge-CH_2-CH_3 domains) as a spacer domain into the cIgTCR constructs (Schirrmann and Pecher 2002, 2005). In addition to its hinge and spacer function, the Fc fragment also promotes and stabilizes cIgTCR homodimerization and suppresses an interaction with the endogenous ζ chain (Hombach et al. 1998, 1999). In contrast, cIgTCR constructs without a dimerization domain are often associated with the endogenous ζ chain. Heterodimeric complexes with the endogenous ζ chain are able to mediate cIgTCR functions but their antigen recognition and signaling may be reduced (Hombach et al. 2000a). Heterodimers formed by an scFv-γ receptor and the endogenous ζ chain reduced the antigen response of T-cell hybridomas (Annenkov et al. 1998). Other groups have employed the D3/D4 Ig-like domains of CD4, the hinge region of CD8α (Moritz and Groner 1995; Ren Heidenreich et al. 2000), and domains of CD28 (Eshhar et al. 2001). In some cIgTCR constructs the introduction of extracellular domains stabilized cell surface expression (Patel et al. 1999) and were necessary for an efficient antigen recognition and T-cell activation (Moritz and Groner 1995; Fitzer Attas et al. 1998).

In general, chimeric receptor constructs containing the ζ chain took more advantage from spacer domains than receptor constructs containing the γ chain (Patel et al. 1999). This is probably due to the stabilization of cIgTCR expression

on the cell surface, preventing intracellular proteolytic breakdown of the ζ chain.

10.2.9
Accessory and Cosignaling Elements

In addition to the TCR-mediated signal, T cells require a costimulatory signal to become fully activated and to prevent anergy after TCR crosslinking. One major costimulatory receptor on T cells is CD28. The introduction of CD28 signal domains resulted in cIgTCR constructs with costimulatory properties. Double transfectants with scFv-CD28 and scFv-ζ showed synergistic signal effects (Alvarez-Vallina and Hawkins 1996). Novel strategies introduced signal domains of CD28 and ζ chain into one receptor construct. These cIgTCR constructs mediated both cytotoxic and costimulatory signals in T cells (Eshhar et al. 2001; Haynes et al. 2002a,b). Nevertheless, there are also controversial results regarding the requirement of CD28 costimulation in cIgTCR-transfected T cells (Hombach et al. 2001c; Ren-Heidenreich et al. 2002). The introduction of CD28 and CD134 (OX40) domains into one cIgTCR construct showed a more complex activation of T cells after antigen stimulation (Pule et al. 2005).

10.3
Preclinical Studies

10.3.1
Retroviral Gene Transfer into T Lymphocytes

Most studies used retroviral systems for the gene transfer of cIgTCRs into primary effector lymphocytes and effector cell lines. Optimized retroviral gene delivery systems allow the transduction of primary T cells with high efficiency (Finer et al. 1994; Weijtens et al. 1998a; Engels et al. 2003). Most of these systems are based on the Moloney murine leuke mia virus (MoMuLV), the amphotropic murine leukemia virus (A-MLV), or lentiviruses. The pseudotyping of the viral surface of MoMuLV vectors with the envelop of A-MLV or gibbon ape leukemia virus (GaLV) significantly improved the transfection efficiencies of human T cells (Gladow et al. 2000; Stitz et al. 2000; Uckert et al. 2000). In contrast, pseudotyping of HIV (human immunodeficiency virus)-derived lentiviral vectors did not improve the transfection of $CD4^+$ T lymphocytes (Muhlebach et al. 2003). Coculturing primary T lymphocytes with the retrovirus resulted in transduction efficiencies of over 75% (Altenschmidt et al. 1996, 1997). Retroviruses and derived vectors integrate into the chromosome of the host cell allowing stable, long-term transgene expression.

The tendency of retroviruses to integrate in transcription-active genomic loci, however, can also lead to interference or destruction of host genes. Studies have

revealed a nonrandom preference of MoMuLV integration into fragile chromosomal sites (Bester et al. 2006). In addition, retroviral long terminal repeats (LTR) include enhancer elements that can upregulate cellular genes over long distances of more than 10 kb (Williams and Baum 2003).

Considering that there are over 100 proto-oncogenes in the human genome, oncogene dysregulation may occur in about 0.1–1% of all retroviral gene transfer events (Baum et al. 2003). The upregulation of oncogenes located near a retroviral chromosomal integration site may cause uncontrolled proliferation of the transduced cells, as was shown in a retroviral gene therapy study of X-SCID (X chromosomal linked severe cellular immunodeficiency) in young children with a defective gene of the common γ chain of the IL-2 receptor (Hacein-Bey-Abina et al. 2003a,b). However, extensive studies with retroviral transduced T cells did not reveal similar side effects after infusion into patients (Rosenberg et al. 1990; Rosenberg 1991). Therefore, the transfection of undifferentiated hematopoietic stem and precursor cells may have side effects different to those produced by gene modification of differentiated lymphocytes.

Another shortcoming of retroviral transduction is that only proliferating cells can be transduced. Accordingly, peripheral blood lymphocytes must be activated by IL-2 or mitogens like phytohemagglutinin (PHA) before retroviral transfection. This unspecific activation may lead to nonspecific activity or activation-induced cell death.

10.3.2 "Naked" DNA Gene Delivery Systems

In contrast to retroviral transduction, most plasmid vector-based gene delivery systems do not achieve high transfection efficiencies in primary lymphocytes and many lymphoid cell lines. Furthermore, they generally suffer from an ineffective chromosomal integration in human cells. The selection of transfected cells using antibiotics according to the selection marker gene does not ensure the cIgTCR expression in all transfected cells. In particular, cDNA-based transgenes are often downregulated or inactivated. Therefore, the use of genomic sequences instead of pure cDNA sequences should be considered to stabilize and increase transgene expression (McKnight et al. 1995). Alternatively, the insertion of intron sequences into the nontranslated 5′ sequence or into the transgene can stabilize expression (Yew et al. 1997).

The chromosomal integration site, methylation, and chromatin accessibility of a transgene have a great influence on the level and stability of its expression (Garrick et al. 1996; Schubeler et al. 2000). Homologous genome sequences (Li and Baker 2000) or certain viral sequences (Kogure et al. 2001) flanking the transgene can enhance the probability and localization of chromosomal integration. The cIgTCR gene transfer of primary T cells using plasmid vectors has been demonstrated by electroporation (Jensen et al. 2000, 2003). Novel "naked" DNA transfection protocols such as the Nucleofector technique promise very high

transfection rates, comparable to those of retroviral vectors for primary cells as well as effector cell lines (Gresch et al. 2004; Maasho et al. 2004).

10.3.3
Enrichment of cIgTCR-Transfected Effector Cells

The clinical employment of cIgTCR-grafted effector lymphocytes requires stable and high expression of the receptor. Therefore, enrichment of cIgTCR-expressing cells is often required. For example, 2 days after electroporation only 1–5% cells of human NK cell line YT showed cIgTCR expression, and this did not increase to more than 10–15% cIgTCR$^+$ YT cells after antibiotic selection. However, after enrichment using the magnetic activated cell sorting (MACS) system and antibodies against the Fc spacer domain, levels of over 90% cIgTCR$^+$ YT cells could be obtained (Schirrmann and Pecher 2002). Immunological enrichment was also described for cIgTCR-transfected T cells (Weijtens et al. 1998a; Nolan et al. 1999; Beecham et al. 2000; Hombach et al. 2001c). However, crosslinking of cIgTCRs by antibodies may lead to apoptosis or reciprocal lysis of cIgTCR$^+$ lymphocytes. Therefore, antibodies for cell sorting should be carefully chosen and tested. Importantly, T cells undergo activation-induced cell death if their TCR or CD3 complex is stimulated without costimulation (Li et al. 2000) as well as NK cells stimulated with IL-2 or IL-12 after CD16 crosslinking (Ortaldo et al. 1995; Jewett 2001).

10.3.4
Effector Functions of cIgTCR Gene-modified Effector Lymphocytes

The specific effector response of T and NK cells and cell lines transfected with different cIgTCR gene constructs has been demonstrated in many independent studies as summarized in Table 10.2. After cIgTCR gene transfer, T cell lines and hybridomas, peripheral blood T lymphocytes, tumor-infiltrating lymphocytes (TILs), and NK cell lines are capable of intracellular Ca^{2+} mobilization and the secretion of different cytokines such as IL-2 and IFNγ upon stimulation by anti-idiotypic antibodies as well as antigen bound on plastic surfaces or expressed on target cells. Thus, cIgTCR crosslinking specifically induces signal transduction in different gene-modified effector cell types. Furthermore, cIgTCR$^+$ cytotoxic lymphocytes specifically lyse target cells expressing the corresponding antigen in an MHC-unrestricted antibody-like manner. The lysis is specifically mediated against target cells expressing the antigen without major bystander killing. However, cIgTCR-transfected peripheral T cells often show an unspecific lysis caused by T-cell activation with IL-2 or phytohemagglutinin (PHA) required for retroviral transduction (Beecham et al. 2000; Eshhar et al. 2001). Gene-modified T cells with high cIgTCR expression were able to lyse target cells with low antigen expression, in contrast to T cells with a low cIgTCR expression (Weijtens et al. 2000). Therefore, high cIgTCR expression on gene-modified effector cells can overcome problems caused by low antigen expression on target cells. In addition,

Table 10.2 Studies with chimeric immunoglobulin receptors.

Recognition domain[a] *(specificity)*	*Spacer*	*TM*	*Signal domain*	*Effector cell*	*Response and remarks*	*References*
Two-chain Fv (PC)	Cα/Cβ	Cα/Cβ	Cα/Cβ	Murine EL4 T cell line	Intracellular calcium release	Kuwana et al. 1987
Two-chain Fv (TNP)	Cα/Cβ	Cα/Cβ	Cα/Cβ	Murine CTL hybridoma MD45	Lysis of hapten-modified target cells	Gross et al. 1989b
Only VH (PC)	Cα/Cβ	Cα/Cβ	Cα/Cβ	EL4	VHCα/VHCβ and VHCβ/VHCβ dimers	Goverman et al. 1990
Sp6 (TNP)	No	ζ; γ	ζ; γ	MD45	Lysis of hapten-modified target cells	Eshhar et al. 1993
N29 (ErbB-2)	No	ζ	ζ	MD45	IL-2 secretion; lysis of ErbB-2$^+$ cells	Stancovski et al. 1993
Sp6; N29; MOv18 (TNP; ErbB-2; FBP)	No	ζ or γ	ζ or γ	Rat basophilic leukemia mast cell line RBL-2H3	Ag-specific degranulation	Bach et al. 1994
84.1c (mouse IgE)	No	Cβ	Cβ	Human leukemia line Jurkat; MD45	Lysis of IgE-secreting BC; suppression of IgE production	Lustgarten and Eshhar 1995
Sp6 (TNP), MOV18 (FBP)	No	ζ or γ	ζ or γ	Human CD8$^+$ TIL	GM-CSF secretion; target cell lysis	Hwu et al. 1993a
TR66 (human CD3e)	CD8α hinge	ζ	ζ	Murine T cell hybridoma BW5147	Ag-specific production of IL-2 and IL-3	Brocker et al. 1993
TR66 (human CD3e)	No	Cβ	Cβ	BW5147	Assembly with endogenous TCR α chain	Brocker et al. 1996

CD4; 98.6 (gp120; gp41)	CD4; Fc	CD4; CD4	ζ; ζ	Jurkat; human CTL	IL-2 secretion; proliferation and target lysis (only PBL)	Roberts et al. 1994
FRP5 (ErbB-2)	CD8α	ζ	ζ	Murine CTL line CI96 (C57/BL6 mice)	Target lysis; localisation at tumor sites; retarded ErbB-2$^+$ tumor growth in nude mice	Moritz et al. 1994
CD4 (gp120, HIV)	CD4	CD4	ζ	Human NK cell clone NK3.3	Transfection of NK cells, lysis of gp120$^+$ B cell line	Tran et al. 1995
MOv18 (FBP)	No	γ	γ	Murine CD8$^+$ TILs	*In vivo* model	Hwu et al. 1995
FRP5 (ErbB-2)	CD4 or CD8α	ζ	ζ	Jurkat; CI96	IFNγ production; target lysis; requirement of spacer domain	Moritz and Groner 1995
1.1ASML (CD44 exon 6 variant)	CD8α	ζ	ζ	CI96	Target lysis; suppression of tumor cell xenografts	Hekele et al. 1996
C11; FRP5 (rat; human ErbB2)		ζ	ζ	Murine and rat TC; TC lines	Optimized retroviral gene transfer into TC	Altenschmidt et al. 1996
G250 (CAIX)	No	CD4; γ	γ	Human activated PBLs	Target lysis 4.5 months receptor expression; recycling of lytic activity	Weijtens et al. 1996
B1.8; NQ10, 12.5 (NIP;) Hawkins 1996	CD28; no	CD28; ζ	CD28; ζ	Jurkat	CD28 like costimulation, enhanced IL-2 production, synergy with scFvζ receptor	Alvarez-Vallina and phOx
Sp6 (TNP)	CD8α	CD8α; CD4	Syk; ZAP70	CTL hybridoma MD45.27J	IL-2 production; target lysis; Syk promotes CBL but not PLCγ phosphorylation	Fitzer Attas et al. 1997 1998
B72.3 (TAG72)	No	γ; ζ	γ; ζ	MD45	Only scFvγ expression	Hombach et al. 1997
CC49 (TAG72)	Fc	γ; ζ	γ; ζ		scFv-Fcγ/ζ expression	Hombach et al. 1998

Table 10.2 *Continued*

Recognition domain[a] *(specificity)*	*Spacer*	*TM*	*Signal domain*	*Effector cell*	*Response and remarks*	*References*
FRP5 (ErbB2)	No	ζ	ζ	Murine TC	Tumor regression in syn-geneic mice; no antibody response to modified TC	Altenschmidt et al. 1997
C2 (Kollagen-II)	No	γ	γ	Murine CTL hybridomas	Heterodimers with ζ chain reduce antigen response	Annenkov et al. 1998
CD4 (gp120)	CD4	CD4	ζ	Hematopoietic stem and precursor cells	Differentiation to transgeneic NK cells and neutrophils	Roberts et al. 1998
G250 (CAIX)	Diverse	Div.	γ; ζ; ε	Human CTL	STITCH retroviral vector	Weijtens et al. 1998a
G250 (CAIX)	Fc	γ	γ	Human TC	Lysis supported by high cIgTCR expression and adhesion molecules	Weijtens et al. 1998b, Weijtens et al. 2000
447D (gp120)	Fc; CD4; CD7; CD8α	CD4; CD8α	ζ	Human TC	Impact of extracellular domains	Patel et al. 1999
BW431 (CEA)	Fc	γ; ζ	γ; ζ	MD45; human TC	No inhibition by 25 μg mL^{-1} soluble CEA	Hombach et al. 1999, 2000b
763.74 (HMW-MAA)	No	γ	γ	MD45	Melanoma	Reinhold et al. 1999
MN14 as scFv and Fab' (CEA)	CD8α; no	ζ; ε	ζ; ε	Human TC	scFv- and Fab-ζ/ε	Nolan et al. 1999
ErbB2	No	γ	γ	MD45	FasL-mediated cytotoxicity	Haynes et al. 1999
CC49 (TAG72)	Fc:hinge -CH3	CD4	ζ	Human TC	No "bystander killing," xenograft mouse model	McGuinness et al. 1999

CO17.1A, GA733) (EGP40	no	γ	γ	Human PBL, murine 14.1 cell line	Only GA733-cIgTCR with specific cytokine response	Daly et al. 2000
TR66 (human CD3ε	No; no	ζ; ε	ζ; ε	cIgTCR-transgeneic mice	IL-2 stimulation required; syngeneic tumor model	Brocker 2000
Col1; CC49; 98.6, 447D, CD4 (CEA; TAG72; HIV)	CEA:a3b3; Fc; CD4	CD4	ζ	Human TC	Bispecific cIgTCRs; dual specificity	Patel et al. 2000
GA733.2 (EGP-2)	No; CD8α	γ; γ or ζ	γ;. γ or ζ	Human TC	Hinge domain improves properties, costimulation not necessary	Ren Heidenreich et al. 2000, 2002
MB3.6 (GD3)	CD8α; no	ζ; ε	ζ; ε	Human TC	Target lysis, no inhibition by 100 μg mL^{-1} soluble GD3	Yun et al. 2000
MN14 (CEA)	CD8α	ζ	ζ	Human TC	Target lysis; selection of CEA-tumor cell population	Beecham et al. 2000
HRS3 (CD30)	No; Fc	γ	γ	Human TC	Impact of Fc spacer domain	Hombach et al. 2000a,b
BW431; HRS3 (CEA; CD30)	Fc; no	γ; ζ	γ; ζ	Human $CD4^+$ TC	Target lysis by cIgTCR+ $CD4^+$ TC	Hombach et al. 2001a
BW431; HRS3 (CEA; CD30)	Fc	γ; ζ	γ; ζ	Human $CD4^+$ TC; PBL	ext. CD28 costimulation enhances IL-2 but not IFNγ, proliferation and cytotoxicity	Hombach et al. 2001c
A5B7? (CEA)	CD8α	γ; ζ	γ; ζ	MD45	ζ stronger signal than γ	Haynes et al. 2001
14.G2a (GD2)	No	ζ	ζ	Human TC; EBV specific TC	Lysis of neuroblastoma; dual specificity against EBV^+ and $GD2^+$ targets	Rossig et al. 2001; Roessig et al. 2002
A5B7?; FRP5? (CEA; ErbB2)	CD8α	CD28	$CD28^+ζ$	Murine TC	Enhanced IFNγ secretion; syn-/xenogeneic model	Haynes et al. 2002a,b

Table 10.2 *Continued*

Recognition domain[a] *(specificity)*	*Spacer*	*TM*	*Signal domain*	*Effector cell*	*Response and remarks*	*References*
FRP5 (ErbB2)	CD8α	ζ	ζ	Human NK cell line NK92	Lysis of Erb^+ tumor cell lines	Uherek et al. 2002
BW431/26 (CEA)	Fc	ζ	ζ	Human NK cell line YT	Target lysis; tumor growth inhibition by irradiated $cIgTCR^+$ YT cells in vivo	Schirrmann and Pecher 2002
C2 (Collagen-II)	No; CD8α	γ; ζ	γ; ζ	Murine KLH-specific Th1 and Th2 cells	cIgTCR stimulation optimal for IFNγ but not for IL-4	Annenkov et al. 2003
FRP5? (ErbB2)	CD8α	CD28	$CD28^+$ζ	Murine TC	Functional role of internal CD28 domain	Moeller et al. 2004
HuM195 (CD33)	Fc	ζ	ζ	YT	Lysis of AML cell line	Schirrmann and Pecher 2005
Hu3S193 (LeY)	?	CD28	$CD28^+$ ζ	Human TC	Lysis of ovarian tumor	Westwood et al. 2005
14g2a (GD2)	IgG1-hinge	CD28	$CD28^+$ $CD134^+$ ζ	Human PBL	$CD28^+$CD134 domains augment TC activation	Pule et al. 2005

a scFv fragments if not otherwise indicated.

Ag, antigen; AML, acute myeloid leukemia; BC, B cells; Cα/Cβ, constant TCR domains; CAIX, carboanhydrase IX on RCC; CD4, TCR coreceptor, binds to HIV gp120; CD7, antigen on TC; CD8α, TCR coreceptor, hinge (aa 105–165); CD19, B-cell lineage differentiation marker; CD28, costimulatory receptor, binds to CD80 (B7.1); CD30, Hodgkin lymphoma marker; CD33, myeloid leukemia marker; CD134 (OX40), costimulatory receptor; CEA, carcinoembryonic antigen; CTL, cytotoxic T lymphocyte; EGP, epithelial glycoprotein; ErbB-2 (Neu/HER2), human breast cancer-associated antigen; FasL, Fas or CD95 ligand; FBP, folate-binding protein; Fc, IgG1 Fc domains (hinge, CH2CH3); GD2, ganglioside on neuroblastoma; GD3, ganglioside on melanoma; gp41 and gp120, glycoprotein 41 and 120 of HIV; HIV, human immunodeficiency virus; HMW-MAA, high molecular weight melanoma-associated antigen; KLH, keyhole limpet hemocyanin; NIP, 4-hydroxy-5-iodo-3-nitrophenyl-acetyl; NK, natural killer; PBL, peripheral blood lymphocytes; PC, phosphorylcholin; phOx, 2-phenyl-2-oxazolin-5-one; PLCγ, phospholipase C γ; PTK, protein tyrosine kinase; RCC, renal cell carcinoma; Syk, non-receptor PTK; TAG72, mucin antigen on adenocarcinoma; TC, T cells; Th, T helper cells; TIL, tumor infiltrating lymphocyte; TM, transmembrane; TNP, 2,4,6-trinitrophenyl; ZAP70, ζ associated protein 70, nonreceptor PTK.

the cytotoxicity of cIgTCR$^+$ effector lymphocytes is also supported by adhesion molecules (Weijtens et al. 1998b).

10.3.5
Influence of Soluble Antigen

One major limitation of antibody-mediated antigen recognition is the interference with soluble antigen. This must also be considered for cIgTCR-transfected effector cells (Goverman et al. 1990; Eshhar et al. 1993; Daly et al. 2000), which is of particular interest if the tumor antigen occurs in soluble form in the serum. For example, patients with colorectal carcinomas frequently show serum levels of $1\,\mu g\,mL^{-1}$ carcinoembryonic antigen (CEA) (Moertel et al. 1986). Higher concentrations at the tumor site must be expected. In studies using the NK cell line YT transfected with a CEA-specific cIgTCR construct, no inhibition of cytotoxicity against CEA$^+$ target cells was detected in the presence of $10\,\mu g\,mL^{-1}$ soluble CEA (Schirrmann and Pecher 2002). Similar results for T cells transfected with CEA-specific cIgTCR constructs were demonstrated by other groups (Hombach et al. 1999; Beecham et al. 2000).

The cytotoxicity of T cells transfected with a GD3-specific cIgTCR was not blocked in the presence of $100\,\mu g\,mL^{-1}$ soluble GD3 ganglioside (Yun et al. 2000). The large number of receptor–antigen interactions between cIgTCR$^+$ effector cells and antigen-expressing target cells seem to prevent in most cases efficient inhibition of target cell lysis by physiologically relevant concentrations of soluble antigen. It was approximated that over 80% of the cIgTCRs on the transfected effector lymphocytes can be blocked by soluble antigen without inhibiting cytotoxicity (Beecham et al. 2000). The cytotoxicity of effector cells transfected with a cIgTCR construct containing an Fc spacer domain was inhibited at about 5–10 times lower concentrations of soluble antigen than cIgTCR constructs without an Fc spacer domain (Hombach et al. 2000a). But in this study, a homodimeric Fc fusion protein of the antigen was used, which binds more efficiently to Fc spacer-containing homodimeric cIgTCR constructs than to Fc spacer-less monomeric cIgTCR variants. Thus the introduction of multimerization domains into cIgTCR constructs should be considered if the antigen occurs as multimeric form in the serum.

10.3.6
Animal Models

Several studies demonstrated that cIgTCR gene-modified effector lymphocytes can inhibit the development of tumors *in vivo* if injected together with tumor cells in mice. For example, the simultaneous injection of CEA$^+$ tumor cells and irradiated cIgTCR gene-modified cells of the NK cell line YT significantly inhibited tumor growth in NOD/SCID (nonobese diabetic/severe cellular immunodeficient) mice (Schirrmann and Pecher 2002). After subcutaneous coinjection of human T cells transduced with a TAG72-specific cIgTCR construct and TAG72$^+$

tumor cells into NOD/SCID mice three out of four mice survived and did not develop tumors. Furthermore, in four of four mice the intraperitoneal coinjection of these cIgTCR$^+$ T cells inhibited the development of TAG72$^+$ KLE B cell tumors (McGuinness et al. 1999).

However, the simultaneously injection of tumor cells and effector cells is an artificial approach that is far away from describing the situation for the therapy of tumor patients. Tumors are usually diagnosed when the tumor has already reached a certain size. Therefore, experimental approaches should be adapted to this situation. So far, only a few studies have been published investigating the treatment of established tumors with cIgTCR-grafted effector cells. Hanson et al. (2000) showed a kinetic dependency between tumor size and number of adoptively transferred murine CTLs expressing a tumor-specific TCR in a syngeneic mouse tumor model. They could even control bigger tumors. The dependency of tumor size and the number of effector cells required for adoptive immunotherapy was also shown for irradiated cells of the cytotoxic T cell line TALL-104. Multiple intratumoral injections of these MHC nonrestricted TALL-104 cells reduced smaller implanted human tumors (<150 mg) about 50–75% and suppressed the formation of lung metastasis (Cesano et al. 1998).

10.4
Therapeutic Considerations

10.4.1
Adoptive Cellular Immunotherapy

The basic concept of adoptive cellular immunotherapy can be dated back to the early 1960s when J.L. Gowans identified lymphocytes as most important mediators of immune response and as the carriers of immunological memory (Gowans and Uhr 1966). He also demonstrated that immunological competence can be transferred from one individual to another by the adoptive transfer of lymphocytes. In animal experiments, the adoptive immunotherapy of tumor diseases was successfully demonstrated (Rosenstein et al. 1984; Hanson et al. 2000). However, adoptive immunotherapy strongly depends on the availability of a large number of immunological effector cells and their tumor specificity. In contrast to animal models, it is difficult, time-consuming, and expensive to provide sufficient numbers of tumor-specific lymphocytes for the individual human patient. These effector cell resources could be improved by gene modification with tumor-specific cIgTCR gene constructs.

10.4.2
Autologous Approaches

Human autologous lymphocytes have been extensively studied for use in adoptive immunotherapy (Table 10.3). Peripheral blood lymphocytes can be activated to

Table 10.3 Characteristics of cytotoxic lymphocytes useful for adoptive immunotherapy.

Effector type	*CTLs*	*TILs*	*NK cells*	*LAK cells*	*CIK cells*
Source	Peripheral blood lymphocytes	Tumors, metastatic or tumor draining lymph nodes	Peripheral blood and bone marrow	NK cells and CTL activated by IL-2	Subset of T lymphocytes activated by cytokines
In vitro expansion					
Tumor stimulation	Yes	None	None	None	None
Requirement of IL-2 for response	++++	++++	++++ (CD56 dim) + (CD56 bright)	++++	++++[a]
Duration of culture	6 weeks	4 weeks	2–3 weeks	2–5 days	2–5 weeks
Target cells *in vitro*	Allogeneic cells	Autologous tumor cells	K562	Raji, Daudi	Autologous and allogeneic tumor cells
In vitro cytotoxicity					
Specificity	MHC restricted to allogeneic cells	MHC–TAA restricted to autologous tumor	Spontaneous lysis of virus infected and certain tumor cells	Lyse a wide spectrum of tumor cells (also NK resistant cells)	Cytotoxic activity superior to LAK; lyse autologous or allogeneic CML blasts
ADCC	CD16+ subsets	None	Yes	CD16+ subsets	Reverse ADCC
Effector phenotype	CD3+/4+,CD3+/8+,CD3+/8+/16+	CD3+/8+/56+	CD3-/16+/56+	CD56+/25+	CD3+/56+

a Additional stimulation by IFNγ, IL-12, and anti-CD3 monoclonal antibody.
b ADCC, Antibody dependent cellular cytotoxicity; CIK, Cytokine-induced killer; CML, Chronic myeloid leukemia; CTL, cytotoxic T lymphocytes; LAK, lymphokine-activated killer; NK, natural killer; TIL, tumor infiltrating lymphocytes.

produce lymphokin-activated killer (LAK) cells in the presence of high concentrations of IL-2. Although these lyse a broad range of tumor cell lines *in vitro*, therapies employing LAK cells showed only limited success in clinical trials due to their low tumor specificity and temporary activation (Bordignon et al. 1999). Peripheral blood lymphocytes were also activated with IL-2, IL-12, IFNγ, and anti-CD3 monoclonal antibodies to cytokine-induced killer (CIK) cells, mediating

enhanced cytotoxic properties but still with limited specificity (Schmidt-Wolf et al. 1993; Kornacker et al. 2006). Tumor-infiltrating lymphocytes (TILs) isolated from solid tumors and tumor-draining lymph nodes frequently contain tumor-specific T cells that can be reactivated *ex vivo* with IL-2. The combination of TIL therapy and IL-2 treatment has shown some therapeutical effects against metastatic melanoma and renal cell carcinoma (Yannelli et al. 1996; Bordignon et al. 1999), but in contrast to LAK cells, their preparation is complicated and unreliable for individual patients (Hoffman et al. 2000).

Autologous T cells can also be sensitized *in vitro* (Lipshy et al. 1997). Human CTLs have been stimulated with Epstein–Barr virus (EBV)-immortalized autologous B cells *in vitro* and used for the prevention of EBV-associated lymphoproliferative diseases after bone marrow transplantation (Rooney et al. 1995, 1998).

NK cells are another type of effector lymphocytes. Unlike T cells, they do not express individual antigen receptors. They mediate an MHC-unrestricted cytotoxicity against a broad spectrum of virus-transformed cells or tumor cells without prior sensitization (Cervantes et al. 1996; Uharek et al. 1996). The ability of NK cells to eliminate tumor cells suggests that they are also involved in the control of cancer and that their presence and state of activation could be important for the outcome of the disease and finally in the treatment of tumors (Barlozzari et al. 1983). In the presence of high doses of IL-2, adherent NK cells can be generated with an advanced activity against tumor and metastatic cells (Vujanovic et al. 1995). Although there are many hopeful data from animal experiments, only few clinical studies using autologous NK cells have been published so far (Lister et al. 1995; Nalesnik et al. 1997).

10.4.3 Allogeneic Approaches

Autologous approaches generally suffer from the requirement to isolate, modify, and expand lymphocytes from each patient, which is unreliable in the context of a clinical routine application. Furthermore, the production of sufficient autologous tumor-specific lymphocytes is time-consuming and cannot be guaranteed because advanced tumor diseases are often accompanied by immune suppression (Finke et al. 1999). Contamination with the patient's tumor cells must also be excluded. In view of these limitations, the employment of allogeneic donor lymphocytes could be advantageous because their provision does not depend on a single patient. Allogeneic donor lymphocytes can mediate a graft-versus-tumor effect. However, allogeneic T lymphocytes are also responsible for graft-versus-host disease (GvHD), which may cause the death of the recipient (Bordignon et al. 1999). The control of GvHD is still a major problem in allogeneic stem cell transplantations and donor lymphocyte infusions. In recent years, allogeneic NK cells have become the focus of attention for allogeneic stem cell transplantation. Allogeneic NK cells are able to mediate an antitumor effect and reduce rejection of the transplant by eliminating residual immune cells of the recipient. But unlike allogeneic T cells, they do not cause GvHD (Ruggeri et al. 2001, 2002).

10.4.4
Gene Modification of Lymphocytes to Enhance Specificity

The specificity and effector properties of cytotoxic lymphocytes can be improved by gene modification. The development of recombinant TCR gene constructs (Eshhar 1997; Calogero et al. 2000), together with efficient retroviral gene transfer systems for activated human T cells and TILs (Finer et al. 1994; Uckert et al. 1998; Weijtens et al. 1998a; Engels et al. 2003), could overcome the limitations of the LAK and TIL therapies, such as the lack of specificity or complicated isolation and expansion of the effector lymphocytes, since these technologies allow a large number of tumor-specific T cells to be generated within a short time-scale. Human T cells transfected with recombinant TCR genes have been successfully targeted to virus-infected and tumor cells *in vitro* and *in vivo* (Table 10.2).

10.4.5
Effector Cell Lines – The Way to Cell-based Therapeutics?

Therapeutic strategies employing primary autologous or allogeneic lymphocytes can be performed only for individual patients or small patient groups. The "individualized" provision of tumor-specific lymphocytes is time-consuming, expensive, and success cannot be guaranteed. Therefore, allogeneic effector cell lines with antitumor properties could be an alternative source for adoptive immunotherapy. Allogeneic effector cell lines can be expanded without limitation. Furthermore, their employment is not limited to the individual patient. The production of effector cell lines is independent of patients or donors and can be performed in large-scale processes, allowing a high degree of standardization combined with a reduction of technical effort, time, and costs. Tumor-specific effector cell lines may open the way for the broad clinical use of adoptive immunotherapy comparable to immunopharmaceuticals such as antibodies or cytokines.

In clinical trials, however, the unlimited growth of effector cell line must be prevented, for example, by irradiation. Allogeneic cytotoxic cell lines with antitumor properties have been investigated in preclinical and clinical studies. The MHC nonrestricted cytotoxic T cell line TALL-104 was examined for adoptive immunotherapy of tumor diseases in mice models (Cesano et al. 1996b, 1997b), in dogs (Cesano et al. 1996a, Visonneau et al. 1999), and in a phase I trial in patients with advanced breast cancer (Visonneau et al. 2000). TALL-104 cells were well tolerated without any cellular immunization. Moreover, the human NK cell line NK92 irradiated with 1000 rad was used for the purging of bone marrow samples in leukemia patients (Maki et al. 2003) and has been tested in a phase I clinical trial in patients with advanced cancer (Tonn et al. 2001). The expansion and production of standardized lots of human effector cell lines TALL-104 (Visonneau et al. 2000) and NK92 (Tam et al. 2003) has been described.

Nevertheless, there are only a small number of human effector cell lines available. Therefore, it would be interesting to extend the spectrum of recognized tumor types of established effector cells by gene modification with tumor-specific

cIgTCR genes. Schirrmann and Pecher (2002, 2005) were able to successfully redirect the IL-2-independent growing human NK cell line YT by cIgTCR gene transfer against CEA^+ and $CD33^+$ tumor cells without the need for retroviral gene delivery systems. After simultaneous transfer of the irradiated $cIgTCR^+$ YT cells and CEA^+ MC32A tumor cells tumor growth was significantly inhibited in NOD/SCID mice. Irradiation decreased the vitality and cytotoxicity of the $cIgTCR^+$ YT cells within days, but cytotoxicity was still sufficient. The gene modification of this effector cell line with suicide genes that convert a nontoxic prodrug into a cytotoxic compound could be an alternative approach to irradiation to prevent unlimited growth (Lal et al. 2000).

10.4.6
Clinical Studies with cIgTCR Gene-modified T Lymphocytes

Table 10.4 gives an overview of clinical trials with receptor gene-modified autologous peripheral T lymphocytes, although only the results of single studies have been published so far. Walker et al. (2000) studied the transfer of T cells obtained from a syngeneic twin and gene modified with a CD4-ζ receptor. Single and multiple infusions of 10^{10} gene-modified $CD8^+$ T cells resulted in a peak fraction of 10^4–10^5 gene-modified cells per 10^6 mononuclear cells after 24–48 h, which declined 100–1000 times within 8 weeks. In order to provide a longer, high-level persistence of the transferred gene-modified T cells, a second series of infusions containing gene-modified $CD4^+$ and $CD8^+$ T cells were used and costimulated *ex vivo* with anti-CD3- and anti-CD28-coated beads. Sustained fractions of about 10^3–10^4 gene-modified T cells per 10^6 total T cells persisted for at least a year. The cell infusions were well tolerated and were not associated with substantive immunological or virological changes. Subsequent studies with CD4-ζ gene-modified T cells achieved prolonged survival in HIV-infected adults (Mitsuyasu et al. 2000).

A phase I/II trial of adoptive immunotherapy against metastatic renal cell cancer (RCC) using autologous T lymphocytes transduced *ex vivo* with a gene encoding a cIgTCR containing an scFv fragment derived from the carbonic anhydrase IX (CAIX)-specific murine monoclonal antibody G250, the CD4 transmembrane domains and the FcεRI γ signal chain were initiated (Bolhuis et al. 1998). The results for the first three patients have been published (Lamers et al. 2006). After retroviral gene transfer 52–76% scFv(G250)-γ expression was achieved on the transduced T cells with a mean number of 2–7 copies per scFv(G250)-γ^+ T cell. All patients were treated with the transduced T cells in an inpatient dose-escalation scheme of intravenous doses of 2×10^7 cells at day 1, 2×10^8 cells at day 2, 2×10^9 cells at days 3–5 (cycle 1) and 2×10^9 cells at days 17–19 (cycle 2), in combination with IL-2 (5×10^5 U m^{-2} twice daily administered subcutaneously at days 1–10 and days 17–26). The time period during which the transduced cells could be detected in the circulation were up to 32 days by flow cytometry and up to 53 days by PCR, depending on the sensitivity of the method of 14 copies per 100 ng DNA or 0.008% and 0.06%, respectively (Lamers et al. 2005). Infusions

Table 10.4 Clinical trials with receptor gene-modified lymphocytes.

Phase	*Disease*	*Antigen*	*Receptor construct*	*Effector cells*	*References and remarks*
I[a]	Metastatic melanoma	MART-1 (HLA-A2)	TCR	Allogeneic CTL line C Cure 709	Gene-modified effector cell line; intratumoral injections (Duval et al. 2006)
I/II[b]	RCC	CAIX	scFv (G250)-TM (CD4)-γ	Autologous TC	(Lamers et al. 2005, 2006)
I[c]	$CD20^+$ B cell lymphoma	CD20	scFv-Fc-ζ	Autologous PBL	Naked plasmid DNA gene transfer (Jensen et al. 2000, 2003)
I/II[a]	HIV	gp120	CD4-ζ	Syngeneic/autologous PBL	(Mitsuyasu et al. 2000, Walker et al. 2000)
I[a]	CEA^+ tumors	CEA	scFv-ζ	Autologous PBL	(Referred in Ma et al. 2002)
I[c]	Ovarian cancer	FBP	scFv-γ	Autologous PBL	Trial number 96C0011
I[d]	Prostate cancer	PSMA	scFv-ζ	Autologous PBL	Preclinical data in (Gade et al. 2005)
I/II[d]	Neuroblastoma	GD2	scFv(14g2a)-CD8α/hinge-CD28-CD134-ζ	Autologous PBL	Texas Children's Hospital, Houston, USA (NCT00085930)

Abbreviations as in Table 10.2 except: PSMA, prostate-specific membrane antigen.
a Clinical study performed, published.
b Clinical study in progress, preliminary data published.
c Clinical study performed, no report available.
d Intended studies.

of these gene-modified T cells were initially well-tolerated. However, after four or five infusions, liver enzyme disturbances developed, reaching National Cancer Institute Common Toxicity Criteria grades 2 to 4, probably caused by a specific attack of the scFv(G250)-γ^+ T cells against $CAIX^+$ bile duct epithelial cells. Furthermore, all three patients developed low levels of anti-scFv(G250)-γ antibodies between 37 and 100 days after the start of T cell therapy, which were directed against the G250 idiotype. Remarkably, these responses were less frequent in RCC patients treated with weekly infusions of 50 mg chimeric G250 mAb (6–30% of patients), indicating that the expression of scFv(G250)-γ on the cell membrane of T cells elicits a relatively efficient immune response against the murine G250 idiotype of the cIgTCR. In order to prevent liver toxicity in future patients, the infusion of 5 mg chimeric G250 antibody 3 days before the first infusion of gene-modified T cells will be included into the clinical protocol.

Recently, the results of a phase I dose-escalation trial with C Cure 709 cells, an allogeneic CTL cell line transduced with an HLA-A2-specific TCR encoding gene

recognizing MART-1$^+$ tumor cells, have been published (Duval et al. 2006). Fifteen patients received a total of 24 intratumoral injection cycles of C Cure 709 cells. Toxicity was minor to moderate and most common injection site reactions were fever, fatigue, nausea/vomiting, and arthralgia/myalgia. Side effects disappeared in general within 24 h. Toxicity was not dose-dependent. One patient obtained a partial response, encompassing both metastases used and not used for intratumoral injections. The remaining patients did not achieve an overall response. In addition, local regression of metastases used for injection in two patients and of metastases not used for injection in one patient was observed. Intratumoral injections of C Cure 709 are feasible, safe, and capable of inducing tumor regression.

10.5 Perspectives

10.5.1 Multispecific Approaches

The treatment of sporadic human tumors by monospecific cIgTCR$^+$ effector cells results in a selection of antigen-negative tumor cells because of their heterogenEous antigen expression (Beecham et al. 2000). The employment of multispecific effector cells could reduce the probability of selection of antigen-negative tumor cells. Allogeneic immunized T cells transfected with tumor-specific cIgTCR showed antigen specificity paired with alloreactivity and antitumor activity *in vivo* (Kershaw et al. 2002). The transfection of EBV-specific T cells with a CD19- or GD2-specific cIgTCR construct led to a dual specificity (Roessig et al. 2002; Rossig et al. 2002). Furthermore, only EBV-specific T cells but not primary T cells could be maintained long term in the presence of EBV-infected B cells. The gene transfer of bispecific cIgTCR constructs into T cells expanded their recognition properties to two different antigens (Patel et al. 2000). The transfection of two or more cIgTCR constructs into cytotoxic lymphocytes could also enhance their target recognition. However, the transfection and expression of several cIgTCRs in one cell may cause conflicts because of heterodimer formation and the competition for the same signal transduction pathways. Hence, it seems to be more practicable to use a mixture of different effector lymphocytes transfected with one cIgTCR gene construct.

The effector properties of effector lymphocytes could be enhanced by transfection with cytokine genes (Hwu et al. 1993b), but cytokines such as GM-CSF (granulocyte-macrophage colony-stimulating factor) and TNF-α (tumor necrosis factor α) can also promote the growth of tumors *in vivo* (Bordignon et al. 1999). In addition, TNF-α gene transfer induced apoptosis in transfected T cells (Ebert et al. 1997).

10.5.2
Tumor Taxis and Application of cIgTCR$^+$ Effector Cells

Another important property of effector cells in adoptive immunotherapy is the ability to accumulate in the tumor, a prerequisite for the systemic application of cIgTCR$^+$ effector cells. After injection into the tail vein of mice the murine CTL line CI96 transfected with an ErbB2-specific cIgTCR specifically accumulated in established ErbB2-transfected NIH3T3 tumors. The tumor growth was even slightly retarded (Moritz et al. 1994). Tumor-specific chemotaxis mediated by the ζ signal chain of the cIgTCR seems to be doubtful and other mechanisms of the CI96 cell have to be considered. Biodistribution experiments with radioactive labeled cells of the MHC nonrestricted CTL line TALL-104 injected intravenously into mice revealed primary accumulation in the lung and later in the liver, spleen, and kidney. There was also continuous accumulation of TALL-104 cells in the tumor and its metastasis (Cesano et al. 1999).

Effector cells can also be transfected with chemokine receptor genes. Chemokine receptors specifically influence the polarity and motility of cells depending on the concentration of their ligand (Mellado et al. 2001). Tumors secrete different chemokines, for example IL-8 by melanoma (Payne and Cornelius 2002). However, chemokines are not tumor specific. Fusion proteins consisting of an antibody fragment and a chemokine portion could combine the recognition of tumor specific antibodies with the chemotactic properties of chemokines (Challita-Eid et al. 1998).

10.5.3
Neovascularization of Solid Tumors – Barrier or Target?

After reaching a size of 1–2 mm solid tumors require their own blood vessels to ensure an adequate supply of oxygen and nutrients. Tumor-associated vessels are formed by the recruitment of endothelial cells and prevent the direct contact of immune cells or antibodies from blood and lymph with the tumor. In contrast to normal endothelium, endothelial cells from neovascular vessels express vascular endothelial growth factor (VEGF) receptor complexes (Brekken and Thorpe 2001). The targeting of effector lymphocytes to tumor-associated vessels could be accomplished by gene modification with recombinant receptors. Lymphocytes transfected with a chimeric receptor specific for the angiogenic endothelial receptor KDR achieved the lysis of KDR$^+$ cells. Furthermore, the secretion of the chemokine IL-8 and the expression of vascular cell adhesion molecule (VCAM) and E-selectin was induced in human umbilical vein endothelial cells (HUVECs), playing an important role in the tumor immune response (Kershaw et al. 2000). Lymphocytes transfected with a chimeric receptor gene consisting of the disintegrin kistrin, which binds to the integrin $\alpha_V\beta_3$ on angiogenic endothelial cells, and the adhesion molecule CD31 (PECAM-1) accumulated in the tumor (Wiedle et al. 1999).

10.5.4
Rejection of Receptor Gene-modified Effector Lymphocytes

The clinical efficacy and safety of receptor gene-modified T cells still needs to be subject to comprehensive studies since only few long-term data are available regarding their *in vivo* behavior in patients (see Section 10.4.6). The adoptive transfer of gene-modified T cells did not induce any humoral response in a syngeneic mouse model (Altenschmidt et al. 1997). Against that, human autologous T cells transfected with an HIV-specific receptor were eliminated by receptor-specific CTLs (Riddell et al. 1996). The widely used retroviral vector pLXSN sensibilized transduced T cells against autologous NK cells and antigen-specific CTLs caused by the selection marker neo (Bordignon et al. 1999; Liberatore et al. 1999), which could be bypassed by selection marker-less vector systems. Murine antibody recognition domains of murine antibodies can induce a human anti-mouse antibody (HAMA) response, which can block but also activate cIgTCR$^+$ effector cells (Lamers et al. 2006). Therefore non-human domains should be avoided. However, anti-idiotypic responses against human antibody V regions are not excluded.

In particular for allogeneic effector cells and cell lines, rejection by the patient's immune system might be the major drawback during adoptive immunotherapy. Interestingly, studies with the CTL line TALL-104 demonstrated that allogeneic effector cell lines can be well-tolerated by different patients. The adoptive transfer of up to 10^8 TALL-104 cells per kg body weight into patients with advanced breast cancer was well tolerated and no cellular immunization against this cell line was observed, even after repeated administrations (Visonneau et al. 2000).

10.5.5
Combination of Conventional Tumor Therapies and Adoptive Immunotherapy

The synergistic effects of chemotherapy and the adoptive transfer of LAK cells (+ IL-2) have been demonstrated (Kawata et al. 1990, 1995). The surgical excision of human tumors subcutaneously implanted in mice improved the efficacy of adoptive transferred, irradiated TALL-104 cells (Cesano et al. 1998). Chemotherapy with adriamycin gave a better prognosis in combination with adoptive immunotherapy using TALL-104 cells (Cesano et al. 1997a). Treatment with cyclophosphamide reduced tumor-induced suppression of T cells (North 1982; Awwad and North 1988). T cells transfected with an ErbB2-specific cIgTCR achieved a dramatic increase in the survival of mice in an adjuvant setting of day 8 metastatic disease that was significantly greater than that afforded by either doxorubicin, 5-fluorouracil, or herceptin (Kershaw et al. 2004).

10.6 Conclusions

Despite past therapeutic failures, novel immunotherapy approaches such as adoptive immunotherapy employing receptor gene-modified effector lymphocytes grafted with the tumor specificity of antibodies are showing promise. There has been recent progress in the technological development of gene modification of primary T lymphocytes. However, individualized approaches are expensive, time-consuming, and still some way from routine application. Strategies to produce tumor-specific effector lymphocytes may need to be developed in new directions. The employment of effector cell lines could overcome some of the limitations, but they are also connected with problems regarding their allogenicity and unlimited growth. In the next few years more results of clinical studies employing receptor gene-modified effector lymphocytes should be expected.

References

Altenschmidt, U., Kahl, R., Moritz, D., Schnierle, B.S., Gerstmayer, B., Wels, W., Groner, B. (1996) Cytolysis of tumor cells expressing the Neu/erbB-2, erbB-3, and erbB-4 receptors by genetically targeted naive T lymphocytes. *Clin Cancer Res* 2: 1001–1008.

Altenschmidt, U., Klundt, E., Groner, B. (1997) Adoptive transfer of in vitro-targeted, activated T lymphocytes results in total tumor regression. *J Immunol* 159: 5509–5515.

Alvarez-Vallina, L., Hawkins, R.E. (1996) Antigen-specific targeting of CD28-mediated T cell co-stimulation using chimeric single-chain antibody variable fragment-CD28 receptors. *Eur J Immunol* 26: 2304–2309.

Annenkov, A.E., Moyes, S.P., Eshhar, Z., Mageed, R.A., Chernajovsky, Y. (1998) Loss of original antigenic specificity in T cell hybridomas transduced with a chimeric receptor containing single-chain Fv of an anti-collagen antibody and Fc epsilonRI-signaling gamma subunit. *J Immunol* 161: 6604–6613.

Annenkov, A.E., Daly, G.M., Brocker, T., Chernajovsky, Y. (2003) Clustering of immunoreceptor tyrosine-based activation motif-containing signalling subunits in CD4(+) T cells is an optimal signal for IFN-gamma production, but not for the production of IL-4. *Int Immunol* 15: 665–677.

Asano, R., Takemura, S., Tsumoto, K., Sakurai, N., Teramae, A., Ebara, S., Katayose, Y., Shinoda, M., Suzuki, M., Imai, K., Matsuno, S., Kudo, T., Kumagai, I. (2000) Functional construction of the anti-mucin core protein (MUC1) antibody MUSE11 variable regions in a bacterial expression system. *J Biochem (Tokyo)* 127: 673–679.

Awwad, M., North, R.J. (1988) Cyclophosphamide (Cy)-facilitated adoptive immunotherapy of a Cy-resistant tumour. Evidence that Cy permits the expression of adoptive T-cell mediated immunity by removing suppressor T cells rather than by reducing tumour burden. *Immunology* 65: 87–92.

Bach, N.L., Waks, T., Schindler, D.G., Eshhar, Z. (1994) Functional expression in mast cells of chimeric receptors with antibody specificity. *Cell Biophys* 24–25: 229–236.

Barlozzari, T., Reynolds, C.W., Herberman, R.B. (1983) In vivo role of natural killer cells: involvement of large granular lymphocytes in the clearance of tumor cells in anti-asialo GM1-treated rats. *J Immunol* 131: 1024–1027.

Baum, C., Dullmann, J., Li, Z., Fehse, B., Meyer, J., Williams, D.A., von Kalle, C. (2003) Side effects of retroviral gene transfer into hematopoietic stem cells. *Blood* 101 2099–2114.

Beecham, E.J., Ortiz Pujols, S., Junghans, R.P. (2000) Dynamics of tumor cell killing by human T lymphocytes armed with an anti-carcinoembryonic antigen chimeric immunoglobulin T-cell receptor. *J Immunother* 23: 332–343.

Bester, A.C., Schwartz, M., Schmidt, M., Garrigue, A., Hacein-Bey-Abina, S., Cavazzana-Calvo, M., Ben-Porat, N., Von Kalle, C., Fischer, A., Kerem, B. (2006) Fragile sites are preferential targets for integrations of MLV vectors in gene therapy. *Gene Ther* 3: 1057–1059.

Biglari, A., Southgate, T.D., Fairbairn, L.J., Gilham, D.E. (2006) Human monocytes expressing a CEA-specific chimeric CD64 receptor specifically target CEA-expressing tumour cells in vitro and in vivo. *Gene Ther* 13: 602–610.

Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee, S.M., Lee, T., Pope, S.H., Riordan, G.S., Whitlow, M. (1988) Single-chain antigen-binding proteins. *Science* 242: 423–426.

Blank-Voorthuis, C.J., Braakman, E., Ronteltap, C.P., Tilly, B.C., Sturm, E., Warnaar, S.O., Bolhuis, R.L. (1993) Clustered CD3/TCR complexes do not transduce activation signals after bispecific monoclonal antibody-triggered lysis by cytotoxic T lymphocytes via CD3. *J Immunol* 151: 2904–2914.

Bolhuis, R.L., Willemsen, R.A., Lamers, C.H., Stam, K., Gratama, J.W., Weijtens, M.E. (1998) Preparation for a phase I/II study using autologous gene modified T lymphocytes for treatment of metastatic renal cancer patients. *Adv Exp Med Biol* 451: 547–555.

Bordignon, C., Carlo-Stella, C., Colombo, M.P., De Vincentiis, A., Lanata, L., Lemoli, R.M., Locatelli, F., Olivieri, A., Rondelli, D., Zanon, P., Tura, S. (1999) Cell therapy: achievements and perspectives. *Haematologica* 84: 1110–1149.

Brekken, R.A., Thorpe, P.E. (2001) VEGF-VEGF receptor complexes as markers of tumor vascular endothelium. *J Control Release* 74: 173–181.

Brocker, T. (2000) Chimeric Fv-zeta or Fv-epsilon receptors are not sufficient to induce activation or cytokine production in peripheral T cells. *Blood* 96: 1999–2001.

Brocker, T., Peter, A., Traunecker, A., Karjalainen, K. (1993) New simplified molecular design for functional T cell receptor. *Eur J Immunol* 23: 1435–1439.

Brocker, T., Riedinger, M., Karjalainen, K. (1996) Redirecting the complete T cell receptor/CD3 signaling machinery towards native antigen via modified T cell receptor. *Eur J Immunol* 26: 1770–1774.

Bukowski, R.M., Rayman, P., Uzzo, R., Bloom, T., Sandstrom, K., Peereboom, D., Olencki, T., Budd, G.T., McLain, D., Elson, P., Novick, A., Finke, J.H. (1998) Signal transduction abnormalities in T lymphocytes from patients with advanced renal carcinoma: clinical relevance and effects of cytokine therapy. *Clin Cancer Res* 4: 2337–2347.

Calogero, A., de Leij, L.F., Mulder, N.H., Hospers, G.A. (2000) Recombinant T-cell receptors: an immunologic link to cancer therapy. *J Immunother* 23: 393–400.

Canevari, S., Stoter, G., Arienti, F., Bolis, G., Colnaghi, M.I., Di Re, E.M., Eggermont, A.M., Goey, S.H., Gratama, J.W., Lamers, C.H. et al. (1995) Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody. *J Natl Cancer Inst* 87: 1463–1469.

Cervantes, F., Pierson, B.A., McGlave, P.B., Verfaillie, C.M., Miller, J.S. (1996) Autologous activated natural killer cells suppress primitive chronic myelogenous leukemia progenitors in long-term culture. *Blood* 87: 2476–2485.

Cesano, A., Visonneau, S., Jeglum, K.A., Owen, J., Wilkinson, K., Carner, K., Reese, L., Santoli, D. (1996a) Phase I clinical trial with a human major histocompatibility complex nonrestricted cytotoxic T-cell line (TALL-104) in dogs with advanced tumors. *Cancer Res* 56: 3021–3029.

Cesano, A., Visonneau, S., Pasquini, S., Rovera, G., Santoli, D. (1996b) Antitumor efficacy of a human major histocompatibility complex nonrestricted cytotoxic T-cell line (TALL-104) in immunocompetent mice bearing

syngeneic leukemia. *Cancer Res* 56: 4444–4452.

Cesano, A., Visonneau, S., Rovera, G., Santoli, D. (1997a) Synergistic effects of adriamycin and TALL-104 cell therapy against a human gastric carcinoma in vivo. *AntiCancer Res* 17: 1887–1892.

Cesano, A., Visonneau, S., Wolfe, J.H., Jeglum, K.A., Fernandez, J., Gillio, A., O'Reilly, R.J., Santoli, D. (1997b) Toxicological and immunological evaluation of the MHC-non-restricted cytotoxic T cell line TALL-104. *Cancer Immunol Immunother* 44: 125–136.

Cesano, A., Visonneau, S., Santoli, D. (1998) TALL-104 cell therapy of human solid tumors implanted in immunodeficient (SCID) mice. *AntiCancer Res* 18: 2289–2295.

Cesano, A., Visonneau, S., Tran, T., Santoli, D. (1999) Biodistribution of human MHC non-restricted TALL-104 killer cells in healthy and tumor bearing mice. *Int J Oncol* 14: 245–251.

Challita-Eid, P.M., Abboud, C.N., Morrison, S.L., Penichet, M.L., Rosell, K.E., Poles, T., Hilchey, S.P., Planelles, V., Rosenblatt, J.D. (1998) A RANTES-antibody fusion protein retains antigen specificity and chemokine function. *J Immunol* 161: 3729–3736.

Correa, M.R., Ochoa, A.C., Ghosh, P., Mizoguchi, H., Harvey, L., Longo, D.L. (1997) Sequential development of structural and functional alterations in T cells from tumor-bearing mice. *J Immunol* 158: 5292–5296.

Daly, T., Royal, R.E., Kershaw, M.H., Treisman, J., Wang, G., Li, W., Herlyn, D., Eshhar, Z., Hwu, P. (2000) Recognition of human colon cancer by T cells transduced with a chimeric receptor gene. *Cancer Gene Ther* 7: 284–291.

Duval, L., Schmidt, H., Kaltoft, K., Fode, K., Jensen, J.J., Sorensen, S.M., Nishimura, M. I., von der Maase, H. (2006) Adoptive transfer of allogeneic cytotoxic T lymphocytes equipped with a HLA-A2 restricted MART-1 T-cell receptor: a phase I trial in metastatic melanoma. *Clin Cancer Res* 12: 1229–1236.

Ebert, O., Finke, S., Salahi, A., Herrmann, M., Trojaneck, B., Lefterova, P., Wagner, E., Kircheis, R., Huhn, D., Schriever, F., Schmidt-Wolf, I.G. (1997) Lymphocyte apoptosis: induction by gene transfer techniques. *Gene Ther* 4: 296–302.

Engels, B., Cam, H., Schuler, T., Indraccolo, S., Gladow, M., Baum, C., Blankenstein, T., Uckert, W. (2003) Retroviral vectors for high-level transgene expression in T lymphocytes. *Hum Gene Ther* 14: 1155–1168.

Eshhar, Z. (1997) Tumor-specific T-bodies: towards clinical application. *Cancer Immunol Immunother* 45: 131–136.

Eshhar, Z., Fitzer-Attas, C.J. (1998) Tyrosine kinase chimeras for antigen-selective T-body therapy. *Adv Drug Deliv Rev* 31: 171–182.

Eshhar, Z., Waks, T., Gross, G., Schindler, D.G. (1993) Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc Natl Acad Sci USA* 90: 720–724.

Eshhar, Z., Bach, N., Fitzer-Attas, C.J., Gross, G., Lustgarten, J., Waks, T., Schindler, D.G. (1996) The T-body approach: potential for cancer immunotherapy. *Springer Semin Immunopathol* 18: 199–209.

Eshhar, Z., Waks, T., Bendavid, A., Schindler, D.G. (2001) Functional expression of chimeric receptor genes in human T cells. *J Immunol Methods* 248: 67–76.

Finer, M.H., Dull, T.J., Qin, L., Farson, D., Roberts, M.R. (1994) kat: a high-efficiency retroviral transduction system for primary human T lymphocytes. *Blood* 83: 43–50.

Finke, J., Ferrone, S., Frey, A., Mufson, A., Ochoa, A. (1999) Where have all the T cells gone? Mechanisms of immune evasion by tumors. *Immunol Today* 20: 158–160.

Fitzer Attas, C.J., Schindler, D.G., Waks, T., Eshhar, Z. (1997) Direct T cell activation by chimeric single chain Fv-Syk promotes Syk-Cbl association and Cbl phosphorylation. *J Biol Chem* 272: 8551–8557.

Fitzer Attas, C.J., Schindler, D.G., Waks, T., Eshhar, Z. (1998) Harnessing Syk family tyrosine kinases as signaling domains for chimeric single chain of the variable domain receptors: optimal design for T cell activation. *J Immunol* 160: 145–154.

Gade, T.P., Hassen, W., Santos, E., Gunset, G., Saudemont, A., Gong, M.C., Brentjens, R., Zhong, X.S., Stephan, M., Stefanski, J., Lyddane, C., Osborne, J.R., Buchanan, I.M., Hall, S.J., Heston, W.D., Riviere, I., Larson, S.M., Koutcher, J.A., Sadelain, M. (2005) Targeted elimination of prostate cancer by genetically directed human T lymphocytes. *Cancer Res* 65: 9080–9088.

Garrick, D., Sutherland, H., Robertson, G., Whitelaw, E. (1996) Variegated expression of a globin transgene correlates with chromatin accessibility but not methylation status. *Nucleic Acids Res* 24: 4902–4909.

Gladow, M., Becker, C., Blankenstein, T., Uckert, W. (2000) MLV-10A1 retrovirus pseudotype efficiently transduces primary human CD4+ T lymphocytes. *J Gene Med* 2: 409–415.

Gorochov, G., Lustgarten, J., Waks, T., Gross, G., Eshhar, Z. (1992) Functional assembly of chimeric T-cell receptor chains. *Int J Cancer* Suppl. 7: 53–57.

Goverman, J., Gomez, S.M., Segesman, K.D., Hunkapiller, T., Laug, W.E., Hood, L. (1990) Chimeric immunoglobulin-T cell receptor proteins form functional receptors: implications for T cell receptor complex formation and activation. *Cell* 60: 929–939.

Gowans, J.L., Uhr, J.W. (1966) The carriage of immunological memory by small lymphocytes in the rat. *J Exp Med* 124: 1017–1030.

Gresch, O., Engel, F.B., Nesic, D., Tran, T.T., England, H.M., Hickman, E.S., Korner, I., Gan, L., Chen, S., Castro-Obregon, S., Hammermann, R., Wolf, J., Muller-Hartmann, H., Nix, M., Siebenkotten, G., Kraus, G., Lun, K. (2004) New non-viral method for gene transfer into primary cells. *Methods* 33: 151–163.

Gross, G., Gorochov, G., Waks, T., Eshhar, Z. (1989a) Generation of effector T cells expressing chimeric T cell receptor with antibody type-specificity. *Transplant Proc* 21: 127–130.

Gross, G., Waks, T., Eshhar, Z. (1989b) Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc Natl Acad Sci USA* 86: 10024–10028.

Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J.L., Fraser, C.C., Cavazzana-Calvo, M., Fischer, A. (2003a) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 348: 255–256.

Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M.P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C.S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J.I., de Saint Basile, G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa-Lyonnet, D., Romana, S., Radford-Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L.E., Wissler, M., Prinz, C., Rabbitts, T.H., Le Deist, F., Fischer, A., Cavazzana-Calvo, M. (2003b) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302: 415–419.

Hanson, H.L., Donermeyer, D.L., Ikeda, H., White, J.M., Shankaran, V., Old, L.J., Shiku, H., Schreiber, R.D., Allen, P.M. (2000) Eradication of established tumors by CD8+ T cell adoptive immunotherapy. *Immunity* 13: 265–276.

Haynes, N.M., Smyth, M.J., Kershaw, M.H., Trapani, J.A., Darcy, P.K. (1999) Fas-ligand-mediated lysis of erbB-2-expressing tumour cells by redirected cytotoxic T lymphocytes. *Cancer Immunol Immunother* 47: 278–286.

Haynes, N.M., Snook, M.B., Trapani, J.A., Cerruti, L., Jane, S.M., Smyth, M.J., Darcy, P.K. (2001) Redirecting mouse CTL against colon carcinoma: superior signaling efficacy of single-chain variable domain chimeras containing TCR-zeta vs Fc epsilon RI-gamma. *J Immunol* 166: 182–187.

Haynes, N.M., Trapani, J.A., Teng, M.W., Jackson, J.T., Cerruti, L., Jane, S.M., Kershaw, M.H., Smyth, M.J., Darcy, P.K. (2002a) Rejection of syngeneic colon carcinoma by CTLs expressing single-chain antibody receptors codelivering CD28 costimulation. *J Immunol* 169: 5780–5786.

Haynes, N.M., Trapani, J.A., Teng, M.W., Jackson, J.T., Cerruti, L., Jane, S.M., Kershaw, M.H., Smyth, M.J., Darcy, P.K. (2002b) Single-chain antigen recognition receptors that costimulate potent rejection

of established experimental tumors. *Blood* 100: 3155–3163.

Hekele, A., Dall, P., Moritz, D., Wels, W., Groner, B., Herrlich, P., Ponta, H. (1996) Growth retardation of tumors by adoptive transfer of cytotoxic T lymphocytes reprogrammed by CD44v6-specific scFv: zeta-chimera. *Int J Cancer*. 68: 232–238.

Hoffman, D.M., Gitlitz, B.J., Belldegrun, A., Figlin, R.A. (2000) Adoptive cellular therapy. *Semin Oncol* 27: 221–233.

Hombach, A., Heuser, C., Sircar, R., Tillmann, T., Diehl, V., Kruis, W., Pohl, C., Abken, H. (1997) T cell targeting of TAG72+ tumor cells by a chimeric receptor with antibody-like specificity for a carbohydrate epitope. *Gastroenterology* 113: 1163–1170.

Hombach, A., Sircar, R., Heuser, C., Tillmann, T., Diehl, V., Kruis, W., Pohl, C., Abken, H. (1998) Chimeric anti-TAG72 receptors with immunoglobulin constant Fc domains and gamma or zeta signalling chains. *Int J Mol Med* 2: 99–103.

Hombach, A., Koch, D., Sircar, R., Heuser, C., Diehl, V., Kruis, W., Pohl, C., Abken, H. (1999) A chimeric receptor that selectively targets membrane-bound carcinoembryonic antigen (mCEA) in the presence of soluble CEA. *Gene Ther* 6: 300–304.

Hombach, A., Heuser, C., Gerken, M., Fischer, B., Lewalter, K., Diehl, V., Pohl, C., Abken, H. (2000a) T cell activation by recombinant FcepsilonRI gamma-chain immune receptors: an extracellular spacer domain impairs antigen-dependent T cell activation but not antigen recognition. *Gene Ther* 7: 1067–1075.

Hombach, A., Schneider, C., Sent, D., Koch, D., Willemsen, R.A., Diehl, V., Kruis, W., Bolhuis, R.L., Pohl, C., Abken, H. (2000b) An entirely humanized CD3 zeta-chain signaling receptor that directs peripheral blood t cells to specific lysis of carcinoembryonic antigen-positive tumor cells. *Int J Cancer* 88: 115–120.

Hombach, A., Heuser, C., Marquardt, T., Wieczarkowiecz, A., Groneck, V., Pohl, C., Abken, H. (2001a) Cd4(+) T cells engrafted with a recombinant immunoreceptor efficiently lyse target cells in a mhc antigen- and fas-independent fashion. *J Immunol* 167: 1090–1096.

Hombach, A., Muche, J.M., Gerken, M., Gellrich, S., Heuser, C., Pohl, C., Sterry, W., Abken, H. (2001b) T cells engrafted with a recombinant anti-CD30 receptor target autologous CD30(+) cutaneous lymphoma cells. *Gene Ther* 8: 891–895.

Hombach, A., Sent, D., Schneider, C., Heuser, C., Koch, D., Pohl, C., Seliger, B., Abken, H. (2001c) T-cell activation by recombinant receptors: CD28 costimulation is required for interleukin 2 secretion and receptor-mediated T-cell proliferation but does not affect receptor-mediated target cell lysis. *Cancer Res* 61: 1976–1982.

Hwu, P., Shafer, G.E., Treisman, J., Schindler, D.G., Gross, G., Cowherd, R., Rosenberg, S.A., Eshhar, Z. (1993a) Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor gamma chain. *J Exp Med* 178: 361–366.

Hwu, P., Yannelli, J., Kriegler, M., Anderson, W.F., Perez, C., Chiang, Y., Schwarz, S., Cowherd, R., Delgado, C., Mule, J. et al. (1993b) Functional and molecular characterization of tumor-infiltrating lymphocytes transduced with tumor necrosis factor-alpha cDNA for the gene therapy of cancer in humans. *J Immunol* 150: 4104–4115.

Hwu, P., Yang, J.C., Cowherd, R., Treisman, J., Shafer, G.E., Eshhar, Z., Rosenberg, S.A. (1995) In vivo antitumor activity of T cells redirected with chimeric antibody/T-cell receptor genes. *Cancer Res* 55: 3369–3373.

Jain, R.K. (1990) Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors. *Cancer Res* 50: 814s–819s.

Jensen, M.C., Clarke, P., Tan, G., Wright, C., Chung-Chang, W., Clark, T.N., Zhang, F., Slovak, M.L., Wu, A.M., Forman, S.J., Raubitschek, A. (2000) Human T lymphocyte genetic modification with naked DNA. *Mol Ther* 1: 49–55.

Jensen, M.C., Cooper, L.J., Wu, A.M., Forman, S.J., Raubitschek, A.(2003) Engineered CD20-specific primary human cytotoxic T lymphocytes for targeting B-cell malignancy. *Cytotherapy* 5: 131–138.

Jewett, A. (2001) Activation of c-Jun N-terminal kinase in the absence of NFkappaB function prior to induction of NK cell death triggered by a combination of anti-class I and anti-CD16 antibodies. *Hum Immunol* 62: 320–331.

Jiang, K., Zhong, B., Gilvary, D.L., Corliss, B.C., Hong-Geller, E., Wei, S., Djeu, J.Y. (2000) Pivotal role of phosphoinositide-3 kinase in regulation of cytotoxicity in natural killer cells. *Nat Immunol* 1: 419–425.

Kawata, A., Hosokawa, M., Sawamura, Y., Ito, K., Une, Y., Shibata, T., Uchino, J., Kobayashi, H. (1990) Modification of lymphokine-activated killer cell accumulation into tumor sites by chemotherapy, local irradiation, or splenectomy. *Mol Biother* 2: 221–227.

Kawata, A., Une, Y., Hosokawa, M., Wakizaka, Y., Namieno, T., Uchino, J., Kobayashi, H. (1995) Adjuvant chemoimmunotherapy for hepatocellular carcinoma patients. Adriamycin, interleukin-2, and lymphokine-activated killer cells versus adriamycin alone. *Am J Clin Oncol* 18: 257–262.

Kershaw, M.H., Westwood, J.A., Zhu, Z., Witte, L., Libutti, S.K., Hwu, P. (2000) Generation of gene-modified T cells reactive against the angiogenic kinase insert domain-containing receptor (KDR) found on tumor vasculature. *Hum Gene Ther* 11: 2445–2452.

Kershaw, M.H., Westwood, J.A., Hwu, P. (2002) Dual-specific T cells combine proliferation and antitumor activity. *Nat Biotechnol* 20: 1221–1227.

Kershaw, M.H., Jackson, J.T., Haynes, N.M., Teng, M.W., Moeller, M., Hayakawa, Y., Street, S.E., Cameron, R., Tanner, J.E., Trapani, J.A., Smyth, M.J., Darcy, P.K. (2004) Gene-engineered T cells as a superior adjuvant therapy for metastatic cancer. *J Immunol* 173: 2143–2150.

Kipriyanov, S.M., Cochlovius, B., Schafer, H.J., Moldenhauer, G., Bahre, A., Le Gall, F., Knackmuss, S., Little, M. (2002) Synergistic antitumor effect of bispecific CD19 × CD3 and CD19 × CD16 diabodies in a preclinical model of non-Hodgkin's lymphoma. *J Immunol* 169: 137–144.

Kogure, K., Urabe, M., Mizukami, H., Kume, A., Sato, Y., Monahan, J., Ozawa, K. (2001) Targeted integration of foreign DNA into a defined locus on chromosome 19 in K562 cells using AAV-derived components. *Int J Hematol* 73: 469–475.

Kolanus, W., Romeo, C., Seed, B. (1993) T cell activation by clustered tyrosine kinases. *Cell* 74: 171–183.

Kornacker, M., Moldenhauer, G., Herbst, M., Weilguni, E., Tita-Nwa, F., Harter, C., Hensel, M., Ho, A.D. (2006) Cytokine-induced killer cells against autologous CLL: Direct cytotoxic effects and induction of immune accessory molecules by interferon-gamma. *Int J Cancer* 119: 1377–1382

Kuwana, Y., Asakura, Y., Utsunomiya, N., Nakanishi, M., Arata, Y., Itoh, S., Nagase, F., Kurosawa, Y. (1987) Expression of chimeric receptor composed of immunoglobulin-derived V regions and T-cell receptor-derived C regions. *Biochem Biophys Res Commun* 149: 960–968.

Lal, S., Lauer, U.M., Niethammer, D., Beck, J.F., Schlegel, P.G. (2000) Suicide genes: past, present and future perspectives. *Immunol Today* 21: 48–54.

Lamers, C.H., Gratama, J.W., Pouw, N.M., Langeveld, S.C., Krimpen, B.A., Kraan, J., Stoter, G., Debets, R. (2005) Parallel detection of transduced T lymphocytes after immunogene therapy of renal cell cancer by flow cytometry and real-time polymerase chain reaction: implications for loss of transgene expression. *Hum Gene Ther* 16: 1452–1462.

Lamers, C.H., Sleijfer, S., Vulto, A.G., Kruit, W.H., Kliffen, M., Debets, R., Gratama, J.W., Stoter, G., Oosterwijk, E. (2006) Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 24: e20–22.

Li, J., Baker, M.D. (2000) Mechanisms involved in targeted gene replacement in mammalian cells. *Genetics* 156: 809–821.

Li, Q.S., Tanaka, S., Kisenge, R.R., Toyoda, H., Azuma, E., Komada, Y. (2000) Activation-induced T cell death occurs at G1A phase of the cell cycle. *Eur J Immunol* 30: 3329–3337.

Liberatore, C., Capanni, M., Albi, N., Volpi, I., Urbani, E., Ruggeri, L., Mencarelli, A., Grignani, F., Velardi, A. (1999) Natural

killer cell-mediated lysis of autologous cells modified by gene therapy. *J Exp Med* 189: 1855–1862.

Lipshy, K.A., Kostuchenko, P.J., Hamad, G.G., Bland, C.E., Barrett, S.K., Bear, H.D. (1997) Sensitizing T-lymphocytes for adoptive immunotherapy by vaccination with wild-type or cytokine gene-transduced melanoma. *Ann Surg Oncol* 4: 334–341.

Lister, J., Rybka, W.B., Donnenberg, A.D., deMagalhaes Silverman, M., Pincus, S.M., Bloom, E.J., Elder, E.M., Ball, E.D., Whiteside, T.L. (1995) Autologous peripheral blood stem cell transplantation and adoptive immunotherapy with activated natural killer cells in the immediate posttransplant period. *Clin Cancer Res* 1: 607–614.

Lustgarten, J., Eshhar, Z. (1995) Specific elimination of IgE production using T cell lines expressing chimeric T cell receptor genes. *Eur J Immunol* 25: 2985–2991.

Ma, Q., Gonzalo-Daganzo, R.M., Junghans, R.P. (2002) Genetically engineered T cells as adoptive immunotherapy of cancer. *Cancer Chemother Biol Response Modif* 20: 315–341.

Maasho, K., Marusina, A., Reynolds, N.M., Coligan, J.E., Borrego, F. (2004) Efficient gene transfer into the human natural killer cell line, NKL, using the Amaxa nucleofection system. *J Immunol Methods* 284: 133–140.

Maki, G., Tam, Y.K., Berkahn, L., Klingemann, H.G. (2003) Ex vivo purging with NK-92 prior to autografting for chronic myelogenous leukemia. *Bone Marrow Transplant* 31: 1119–1125.

McGuinness, R.P., Ge, Y., Patel, S.D., Kashmiri, S.V., Lee, H.S., Hand, P.H., Schlom, J., Finer, M.H., McArthur, J.G. (1999) Anti-tumor activity of human T cells expressing the CC49-zeta chimeric immune receptor. *Hum Gene Ther* 10: 165–173.

McKnight, R.A., Wall, R.J., Hennighausen, L. (1995) Expression of genomic and cDNA transgenes after co-integration in transgenic mice. *Transgenic Res* 4: 39–43.

Mellado, M., Rodriguez-Frade, J.M., Manes, S., Martinez, A.C. (2001) Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. *Annu Rev Immunol* 19: 397–421.

Mitsuyasu, R.T., Anton, P.A., Deeks, S.G., Scadden, D.T., Connick, E., Downs, M.T., Bakker, A., Roberts, M.R., June, C.H., Jalali, S., Lin, A.A., Pennathur-Das, R., Hege, K.M. (2000) Prolonged survival and tissue trafficking following adoptive transfer of CD4zeta gene-modified autologous CD4(+) and CD8(+) T cells in human immunodeficiency virus-infected subjects. *Blood* 96: 785–793.

Mizoguchi, H., O'Shea, J.J., Longo, D.L., Loeffler, C.M., McVicar, D.W., Ochoa, A.C. (1992) Alterations in signal transduction molecules in T lymphocytes from tumor-bearing mice. *Science* 258: 1795–1798.

Moeller, M., Haynes, N.M., Trapani, J.A., Teng, M.W., Jackson, J.T., Tanner, J.E., Cerutti, L., Jane, S.M., Kershaw, M.H., Smyth, M.J., Darcy, P.K. (2004) A functional role for CD28 costimulation in tumor recognition by single-chain receptor-modified T cells. *Cancer Gene Ther* 11: 371–379.

Moertel, C.G., O'Fallon, J.R., Go, V.L., O'Connell, M.J., Thynne, G.S. (1986) The preoperative carcinoembryonic antigen test in the diagnosis, staging, and prognosis of colorectal cancer. *Cancer* 58: 603–610.

Moretta, A., Bottino, C., Vitale, M., Pende, D., Cantoni, C., Mingari, M.C., Biassoni, R., Moretta, L. (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol* 19: 197–223.

Moritz, D., Wels, W., Mattern, J., Groner, B. (1994) Cytotoxic T lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells. *Proc Natl Acad Sci USA* 91: 4318–4322.

Moritz, D., Groner, B. (1995) A spacer region between the single chain antibody- and the CD3 zeta-chain domain of chimeric T cell receptor components is required for efficient ligand binding and signaling activity. *Gene Ther* 2: 539–546.

Muhlebach, M.D., Schmitt, I., Steidl, S., Stitz, J., Schweizer, M., Blankenstein, T., Cichutek, K., Uckert, W. (2003) Transduction efficiency of MLV but not of HIV-1 vectors is pseudotype dependent on human primary T lymphocytes. *J Mol Med* 81: 801–810.

Nalesnik, M.A., Rao, A.S., Furukawa, H., Pham, S., Zeevi, A., Fung, J.J., Klein, G., Gritsch, H.A., Elder, E., Whiteside, T.L., Starzl, T.E. (1997) Autologous lymphokine-activated killer cell therapy of Epstein-Barr virus-positive and -negative lymphoproliferative disorders arising in organ transplant recipients. *Transplantation* 63: 1200–1205.

Nolan, K.F., Yun, C.O., Akamatsu, Y., Murphy, J.C., Leung, S.O., Beecham, E.J., Junghans, R.P. (1999) Bypassing immunization: optimized design of "designer T cells" against carcinoembryonic antigen (CEA)-expressing tumors, and lack of suppression by soluble CEA. *Clin Cancer Res* 5: 3928–3941.

North, R.J. (1982) Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. *J Exp Med* 155: 1063–1074.

Ortaldo, J.R., Mason, A.T., O'Shea, J.J. (1995) Receptor-induced death in human natural killer cells: involvement of CD16. *J Exp Med* 181: 339–344.

Patel, S.D., Moskalenko, M., Smith, D., Maske, B., Finer, M.H., McArthur, J.G. (1999) Impact of chimeric immune receptor extracellular protein domains on T cell function. *Gene Ther* 6: 412–419.

Patel, S.D., Moskalenko, M., Tian, T., Smith, D., McGuinness, R., Chen, L., Winslow, G.A., Kashmiri, S., Schlom, J., Stanners, C.P., Finer, M.H., McArthur, J.G. (2000) T-cell killing of heterogenous tumor or viral targets with bispecific chimeric immune receptors. *Cancer Gene Ther* 7: 1127–1134.

Payne, A.S., Cornelius, L.A. (2002) The role of chemokines in melanoma tumor growth and metastasis. *J Invest Dermatol* 118: 915–922.

Perez, P., Hoffman, R.W., Shaw, S., Bluestone, J.A., Segal, D.M. (1985) Specific targeting of cytotoxic T cells by anti-T3 linked to anti-target cell antibody. *Nature* 316: 354–356.

Pule, M.A., Straathof, K.C., Dotti, G., Heslop, H.E., Rooney, C.M., Brenner, M.K. (2005) A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. *Mol Ther* 12: 933–941.

Reinhold, U., Liu, L., Ludtke Handjery, H.C., Heuser, C., Hombach, A., Wang, X., Tilgen, W., Ferrone, S., Abken, H. (1999) Specific lysis of melanoma cells by receptor grafted T cells is enhanced by anti-idiotypic monoclonal antibodies directed to the scFv domain of the receptor. *J Invest Dermatol* 112: 744–750.

Ren Heidenreich, L., Hayman, G.T., Trevor, K.T. (2000) Specific targeting of EGP-2+ tumor cells by primary lymphocytes modified with chimeric T cell receptors. *Hum Gene Ther* 11: 9–19.

Ren-Heidenreich, L., Mordini, R., Hayman, G.T., Siebenlist, R., LeFever, A. (2002) Comparison of the TCR zeta-chain with the FcR gamma-chain in chimeric TCR constructs for T cell activation and apoptosis. *Cancer Immunol Immunother* 51: 417–423.

Riddell, S.R., Elliott, M., Lewinsohn, D.A., Gilbert, M.J., Wilson, L., Manley, S.A., Lupton, S.D., Overell, R.W., Reynolds, T.C., Corey, L., Greenberg, P.D. (1996) T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV-infected patients. *Nat Med* 2: 216–223.

Roberts, M.R., Qin, L., Zhang, D., Smith, D.H., Tran, A.C., Dull, T.J., Groopman, J.E., Capon, D.J., Byrn, R.A., Finer, M.H. (1994) Targeting of human immunodeficiency virus-infected cells by CD8+ T lymphocytes armed with universal T-cell receptors. *Blood* 84: 2878–2889.

Roberts, M.R., Cooke, K.S., Tran, A.C., Smith, K.A., Lin, W.Y., Wang, M., Dull, T.J., Farson, D., Zsebo, K.M., Finer, M.H. (1998) Antigen-specific cytolysis by neutrophils and NK cells expressing chimeric immune receptors bearing zeta or gamma signaling domains. *J Immunol* 161: 375–384.

Roessig, C., Scherer, S.P., Baer, A., Vormoor, J., Rooney, C.M., Brenner, M.K., Juergens, H. (2002) Targeting CD19 with genetically modified EBV-specific human T lymphocytes. *Ann Hematol* 81 (Suppl 2): S42–43.

Romeo, C., Amiot, M., Seed, B. (1992) Sequence requirements for induction of cytolysis by the T cell antigen/Fc receptor zeta chain. *Cell* 68: 889–897.

Rooney, C.M., Smith, C.A., Ng, C.Y., Loftin, S., Li, C., Krance, R.A., Brenner, M.K.,

Heslop, H.E. (1995) Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet* 345: 9–13.

Rooney, C.M., Smith, C.A., Ng, C.Y., Loftin, S.K., Sixbey, J.W., Gan, Y., Srivastava, D.K., Bowman, L.C., Krance, R.A., Brenner, M.K., Heslop, H.E. (1998) Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood* 92: 1549–1555.

Rosenberg, S.A. (1991) Immunotherapy and gene therapy of cancer. *Cancer Res* 51: 5074–5079.

Rosenberg, S.A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R.A., Moen, R., Karson, E.M., Lotze, M.T., Yang, J.C., Topalian, S.L. *et al*. (1990) Gene transfer into humans–immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med* 323: 570–578.

Rosenstein, M., Eberlein, T.J., Rosenberg, S.A. (1984) Adoptive immunotherapy of established syngeneic solid tumors: role of T lymphoid subpopulations. *J Immunol* 132: 2117–2122.

Rossig, C., Bollard, C.M., Nuchtern, J.G., Merchant, D.A., Brenner, M.K. (2001) Targeting of G(D2)-positive tumor cells by human T lymphocytes engineered to express chimeric T-cell receptor genes. *Int J Cancer* 94: 228–236.

Rossig, C., Bollard, C.M., Nuchtern, J.G., Rooney, C.M., Brenner, M.K. (2002) Epstein-Barr virus-specific human T lymphocytes expressing antitumor chimeric T-cell receptors: potential for improved immunotherapy. *Blood* 99: 2009–2016.

Ruggeri, L., Capanni, M., Martelli, M.F., Velardi, A. (2001) Cellular therapy: exploiting NK cell alloreactivity in transplantation. *Curr Opin Hematol* 8: 355–359.

Ruggeri, L., Capanni, M., Urbani, E., Perruccio, K., Shlomchik, W.D., Tosti, A., Posati, S., Rogaia, D., Frassoni, F., Aversa, F., Martelli, M.F., Velardi, A. (2002) Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 295: 2097–2100.

Salvadori, S., Gansbacher, B., Zier, K. (1994) Functional defects are associated with abnormal signal transduction in T cells of mice inoculated with parental but not IL-2 secreting tumor cells. *Cancer Gene Ther* 1: 165–170.

Schirrmann, T., Pecher, G. (2001) Tumor-specific targeting of a cell line with natural killer cell activity by asialoglycoprotein receptor gene transfer. *Cancer Immunol Immunother* 50: 549–556.

Schirrmann, T., Pecher, G. (2002) Human natural killer cell line modified with a chimeric immunoglobulin T-cell receptor gene leads to tumor growth inhibition in vivo. *Cancer Gene Ther* 9: 390–398.

Schirrmann, T., Pecher, G. (2005) Specific targeting of CD33(+) leukemia cells by a natural killer cell line modified with a chimeric receptor. *Leuk Res* 29: 301–306.

Schmidt-Wolf, I.G., Lefterova, P., Mehta, B.A., Fernandez, L.P., Huhn, D., Blume, K.G., Weissman, I.L., Negrin, R.S. (1993) Phenotypic characterization and identification of effector cells involved in tumor cell recognition of cytokine-induced killer cells. *Exp Hematol* 21: 1673–1679.

Schubeler, D., Lorincz, M.C., Cimbora, D.M., Telling, A., Feng, Y.Q., Bouhassira, E.E., Groudine, M. (2000) Genomic targeting of methylated DNA: influence of methylation on transcription, replication, chromatin structure, and histone acetylation. *Mol Cell Biol* 20: 9103–9112.

Shores, E., Flamand, V., Tran, T., Grinberg, A., Kinet, J.P., Love, P.E. (1997) Fc epsilonRI gamma can support T cell development and function in mice lacking endogenous TCR zeta-chain. *J Immunol* 159: 222–230.

Stancovski, I., Schindler, D.G., Waks, T., Yarden, Y., Sela, M., Eshhar, Z. (1993) Targeting of T lymphocytes to Neu/HER2-expressing cells using chimeric single chain Fv receptors. *J Immunol* 151: 6577–6582.

Stitz, J., Buchholz, C.J., Engelstadter, M., Uckert, W., Bloemer, U., Schmitt, I., Cichutek, K. (2000) Lentiviral vectors pseudotyped with envelope glycoproteins derived from gibbon ape leukemia virus and murine leukemia virus 10A1. *Virology* 273: 16–20.

Tam, Y.K., Martinson, J.A., Doligosa, K., Klingemann, H.G. (2003) Ex vivo expansion of the highly cytotoxic human natural killer-92 cell-line under current good manufacturing practice conditions for clinical adoptive cellular immunotherapy. *Cytotherapy* 5: 259–272.

Tonn, T., Becker, S., Esser, R., Schwabe, D., Seifried, E. (2001) Cellular immunotherapy of malignancies using the clonal natural killer cell line NK-92. *J Hematother Stem Cell Res* 10: 535–544.

Tran, A.C., Zhang, D., Byrn, R., Roberts, M.R. (1995) Chimeric zeta-receptors direct human natural killer (NK) effector function to permit killing of NK-resistant tumor cells and HIV-infected T lymphocytes. *J Immunol* 155: 1000–1009.

Uckert, W., Willimsky, G., Pedersen, F.S., Blankenstein, T., Pedersen, L. (1998) RNA levels of human retrovirus receptors Pit1 and Pit2 do not correlate with infectibility by three retroviral vector pseudotypes. *Hum Gene Ther* 9: 2619–2627.

Uckert, W., Becker, C., Gladow, M., Klein, D., Kammertoens, T., Pedersen, L., Blankenstein, T. (2000) Efficient gene transfer into primary human CD8+ T lymphocytes by MuLV-10A1 retrovirus pseudotype. *Hum Gene Ther* 11: 1005–1014.

Uharek, L., Zeis, M., Glass, B., Steinmann, J., Dreger, P., Gassmann, W., Schmitz, N., Muller Ruchholtz, W. (1996) High lytic activity against human leukemia cells after activation of allogeneic NK cells by IL-12 and IL-2. *Leukemia* 10: 1758–1764.

Uherek, C., Tonn, T., Uherek, B., Becker, S., Schnierle, B., Klingemann, H.G., Wels, W. (2002) Retargeting of natural killer-cell cytolytic activity to ErbB2- expressing cancer cells results in efficient and selective tumor cell destruction. *Blood* 100: 1265–1273.

van Oers, N.S., Love, P.E., Shores, E.W., Weiss, A. (1998) Regulation of TCR signal transduction in murine thymocytes by multiple TCR zeta-chain signaling motifs. *J Immunol* 160: 163–170.

Visonneau, S., Cesano, A., Jeglum, K.A., Santoli, D. (1999) Adoptive therapy of canine metastatic mammary carcinoma with the human MHC non-restricted cytotoxic T-cell line TALL-104. *Oncol Rep* 6: 1181–1188.

Visonneau, S., Cesano, A., Porter, D.L., Luger, S.L., Schuchter, L., Kamoun, M., Torosian, M.H., Duffy, K., Sickles, C., Stadtmauer, E.A., Santoli, D. (2000) Phase I trial of TALL-104 cells in patients with refractory metastatic breast cancer. *Clin Cancer Res* 6: 1744–1754.

Vujanovic, N.L., Yasumura, S., Hirabayashi, H., Lin, W.C., Watkins, S., Herberman, R.B., Whiteside, T.L. (1995) Antitumor activities of subsets of human IL-2-activated natural killer cells in solid tissues. *J Immunol* 154: 281–289.

Walker, R.E., Bechtel, C.M., Natarajan, V., Baseler, M., Hege, K.M., Metcalf, J.A., Stevens, R., Hazen, A., Blaese, R.M., Chen, C.C., Leitman, S.F., Palensky, J., Wittes, J., Davey, R.T., Jr., Falloon, J., Polis, M.A., Kovacs, J.A., Broad, D.F., Levine, B.L., Roberts, M.R., Masur, H., Lane, H.C. (2000) Long-term in vivo survival of receptor-modified syngeneic T cells in patients with human immunodeficiency virus infection. *Blood* 96: 467–474.

Weijtens, M.E., Willemsen, R.A., Valerio, D., Stam, K., Bolhuis, R.L. (1996) Single chain Ig/gamma gene-redirected human T lymphocytes produce cytokines, specifically lyse tumor cells, and recycle lytic capacity. *J Immunol* 157: 836–843.

Weijtens, M.E., Willemsen, R.A., Hart, E.H., Bolhuis, R.L. (1998a) A retroviral vector system 'STITCH' in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes. *Gene Ther* 5: 1195–1203.

Weijtens, M.E., Willemsen, R.A., van Krimpen, B.A., Bolhuis, R.L. (1998b) Chimeric scFv/gamma receptor-mediated T-cell lysis of tumor cells is coregulated by adhesion and accessory molecules. *Int J Cancer.* 77: 181–187.

Weijtens, M.E., Hart, E.H., Bolhuis, R.L. (2000) Functional balance between T cell chimeric receptor density and tumor associated antigen density: CTL mediated cytolysis and lymphokine production. *Gene Ther* 7: 35–42.

Westwood, J.A., Smyth, M.J., Teng, M.W., Moeller, M., Trapani, J.A., Scott, A.M.,

Smyth, F.E., Cartwright, G.A., Power, B.E., Honemann, D., Prince, H.M., Darcy, P.K., Kershaw, M.H. (2005) Adoptive transfer of T cells modified with a humanized chimeric receptor gene inhibits growth of Lewis-Y-expressing tumors in mice. *Proc Natl Acad Sci USA* 102: 19051–19056.

Whiteside, T.L. (1999) Signaling defects in T lymphocytes of patients with malignancy. *Cancer Immunol Immunother* 48: 346–352.

Wiedle, G., Johnson-Leger, C., Imhof, B.A. (1999) A chimeric cell adhesion molecule mediates homing of lymphocytes to vascularized tumors. *Cancer Res* 59: 5255–5263.

Willemsen, R.A., Debets, R., Chames, P., Bolhuis, R.L. (2003) Genetic engineering of T cell specificity for immunotherapy of cancer. *Hum Immunol* 64: 56–68.

Williams, D.A., Baum, C. (2003) Medicine. Gene therapy – new challenges ahead. *Science* 302: 400–401.

Yannelli, J.R., Hyatt, C., McConnell, S., Hines, K., Jacknin, L., Parker, L., Sanders, M., Rosenberg, S.A. (1996) Growth of tumor-infiltrating lymphocytes from human solid cancers: summary of a 5-year experience. *Int J Cancer.* 65: 413–421.

Yew, N.S., Wysokenski, D.M., Wang, K.X., Ziegler, R.J., Marshall, J., McNeilly, D., Cherry, M., Osburn, W., Cheng, S.H. (1997) Optimization of plasmid vectors for high-level expression in lung epithelial cells. *Hum Gene Ther* 8: 575–584.

Yun, C.O., Nolan, K.F., Beecham, E.J., Reisfeld, R.A., Junghans, R.P. (2000) Targeting of T lymphocytes to melanoma cells through chimeric anti-GD3 immunoglobulin T-cell receptors. *Neoplasia* 2: 449–459.

Zenner, G., Vorherr, T., Mustelin, T., Burn, P. (1996) Differential and multiple binding of signal transducing molecules to the ITAMs of the TCR-zeta chain. *J Cell Biochem* 63: 94–103.

11
Emerging Therapeutic Concepts IV: Anti-idiotypic Antibodies

Peter Fischer and Martina M. Uttenreuther-Fischer

11.1
Introduction

Over 30 years ago the "network theory of the immune system" was published by Nils K. Jerne (Jerne 1974), for which, together with other "theories concerning the specificity in development and control of the immune system," he was awarded the Nobel Prize in physiology/medicine in 1984. Jerne's Nobel Prize was shared with Georges Köhler and César Milstein (for development of the hybridoma technique). As mentioned in the Nobel Prize announcement (http://nobelprize.org/), Jerne's theory had practical consequences for using anti-antibodies as a tool in medicine. Anti-idiotypic antibodies may be developed as vaccines against infectious diseases or tumors on the one hand and for the induction of tolerance by inhibition of (1) antipollen antibodies in allergy, (2) autoantibodies in autoimmune disease, and (3) graft rejection in transplantation as well. However, anti-idiotypic antibodes may themselves cause disease (e.g. autoimmunity) (Abu-Shakra et al. 1996) or suppression of the immune response (Metlas and Veljkovic 2004).

11.2
Definition of Anti-idiotypic Antibodies

The term "idiotype," which corresponds to the ensemble of idiotopes (i.e. the individual, unique variable regions of an antibody) was first introduced by Oudin, Michel, and Kunkel in 1963 (Abu-Shakra et al. 1996). The principle of Jerne's network theory was the observation that antibodies (Ab1) induce anti-antibodies (Ab2) directed against the idiotype of the first. In addition, those Ab2 will lead to anti-anti-antibodies (Ab3) and so on, resulting in a self-regulation of the antibody repertoire by stimulation and supression of B cells (Fig. 11.1).

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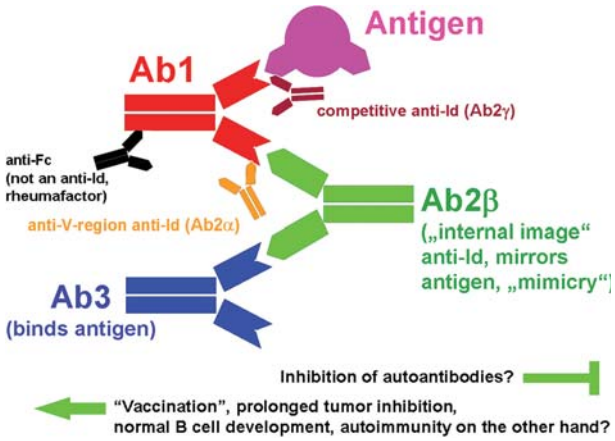

Fig. 11.1 Definition of anti-idiotypic antibodies. Antibodies (Ab1) induce anti-antibodies (Ab2) directed against the idiotype (unique variable region) of the first. In addition, those Ab2 will lead to anti-anti-antibodies (Ab3) and so on, resulting in a self-regulation of the antibody repertoire by stimulation and suppression of B cells. For details see text.

Importantly, some of the Ab2 may mirror the internal image of the original antigen recognized by Ab1. This type of Ab2, designated Ab2β, may mimic the antigen, eliciting Ab3 that bind to the original antigen similar to Ab1 (Abu-Shakra et al. 1996). In this way, Ab2β may be used as a vaccine instead of the antigen itself (Fig. 11.1). This may be advantageous when the antigen is not readily available (e.g. in the case of a dangerous pathogen or toxin, or a nonproteinous antigen such as a carbohydrate). This specific kind of anti-idiotypic interaction with the complementarity determining regions (CDRs) has been confirmed in crystallography and comparison of the complex between antigen (lysozyme) and Ab1 (anti-lysozyme antibody D1.3) to the complex of Ab2 (anti-D1.3 antibody E5.2) and Ab1. D1.3 contacted the antigen lysozyme and the anti-idiotope E5.2 through essentially the same combining-site residues, mimicking the noncovalent binding interactions (Fields et al. 1995).

Another variant of anti-idiotypic antibodies, Ab2γ, also inhibits the binding of Ab1 to its antigen. Ab2γ are also directed against the antigen-binding region (paratope) of Ab1, but they do not mirror the antigen and thus are not useful as a vaccine (Fig. 11.1). In contrast, Ab2α anti-idiotypic antibodies bind outside of the paratope and thus do not interfere with antigen binding. They may recognize parts of both CDR and framework regions (Fig. 11.1). Anti-constant region antibodies (anti-Fc) are known as rheumatoid factors which might enhance the destructive function of autoantibodies (Yang et al. 1999).

11.3
Anti-idiotypic Antibodies as Autoantigens

Anti-idiotypic antibodies against anti-pathogen antibodies (e.g. against mycobacteria or *Klebsiella*) may be the cause or trigger of autoimmune diesease by continuously inducing Ab3 against autoantigens (Shoenfeld 1994; Galeazzi et al. 1998). On the other hand, autoantibodies may be helpful in the treatment of autoimmune disease by blocking autoantigens or downregulation of cytokines (Shoenfeld 2005). Autoimmunity is not the focus of this chapter because there are excellent reviews and books available elsewhere (Peter and Shohat 1996; Shoenfeld et al. 1997; Sherer and Shoenfeld 2000).

11.4
Anti-idiotypes as a Tool for Generating Specific Antibodies

In phage display, the direct biopanning of specific antibody-presenting phage may be difficult or impossible, when the specificity or affinity for the antigen is too low compared with the background phage, resulting in nonspecific adherence of the phage. In many cases, the antigen is not available in a suitable form, because the expression or purification of the biomolecules, such as membrane proteins or carbohydrates, is not possible in sufficient quantities.

In those situations it may be advantageous to use anti-idiotypic antibodies for the panning procedure. These have been generated, for example, by immunization of rabbits with human IgG Fab (idiotype) that were affinity purified with the target antigen (Ishida et al. 1995). Alternatively, anti-idiotypic antibodies mimicking the target antigen were used to isolate the specific phage (Hombach et al. 1998; Fischer et al. 1999). In addition, the cloning of the anti-idiotypic antibodies themselves may be required to isolate antigen-mimicking antibody fragments (e.g. for vaccination, or to derive a tool for targeting clonal myeloma cells) (Willems et al. 1998).

In a different setting, the cloning of unknown antibodies that are the target of regulatory anti-idiotypic antibodies may be the primary goal. This was the case when autoantibodies from patients with autoimmune disease were selected by anti-idiotypic panning using intravenous IgG preparations (IVIG). This technique not only enabled the determination of the genetic origin of those antibodies (Jendreyko et al. 1998) but also allowed platelet-reactive IgG antibodies to be cloned from patients with autoimmune thrombocytopenia (AITP) for the first time (Fischer et al. 1999).

11.5
Intravenous Immunoglobulin Preparations (IVIG)

IVIG prepared by purification of serum IgG from several thousand healthy donors have shown positive effects in a variety of immuneological disorders.

IVIG have been successfully used in the treatment of autoimmune diseases such as autoimmune thrombocytopenia (Imbach et al. 1985; Imholz et al. 1988), acute myocarditis (Drucker et al. 1994), Kawasaki disease (Fischer et al. 1996b), and certain cases of systemic lupus erythematosus (SLE) (Schroeder et al. 1996; Sherer et al. 1999). Variable results and sometimes worsening of disease symptoms in SLE have been partially attributed to the existence of pathogenic anti-DNA idiotype-reactive IgG in IVIG preparations (Silvestris et al. 1994).

The diagnosis of Kawasaki disease, a mucocutaneous lymphadenopathy syndrome (Kawasaki 1967), mainly depends on clinical symptoms such as fever persisting for more than 5 consecutive days, since no specific laboratory criteria are available (Leung 1993; Fischer et al. 1996b). The etiology of Kawasaki disease is still unknown, although epidemiological and clinical data indicate an infectious origin (Kotzin et al. 1993; Burns and Matsubara 1995; Kawasaki 1995). Compiling the information from the literature, we proposed that the disease may be caused by simultaneous or successive dual infection with superantigen-producing and other pathogens (Fischer et al. 1996a,b). Without early diagnosis and treatment, up to 40% of all patients with Kawasaki disease will ultimately develop severe cardiac complications (Dajani et al. 1994). Although a specific therapy for Kawasaki disease is not available, coronary complications can be significantly reduced with high doses of IVIG combined with aspirin (acetylsalicylic acid) (Dajani et al. 1994).

The specific mechanisms of IVIG in autoimmune diseases are not clear. Various studies have suggested the inhibition of effector cells through blockade of Fc receptors (Debre et al. 1993), healing of persisting infections, induction of cytokines (Lacroix-Desmazes et al. 1996), inhibition of autoantibodies by *anti*-idiotypic antibodies (Berchtold et al. 1989; Levy et al. 1999), B cell modulation by *anti*-B cell antigen receptor antibodies and the reconstruction of the "idiotypic" or "V-region" connected (Lacroix-Desmazes et al. 1996) network (Jerne 1974) by IVIG.

However, the documented increase of serum IgM, decrease of certain autoantibodies and long-term therapeutic effects far beyond the half-life of infused IVIG often observed in children treated for AITP (Imholz et al. 1988) cannot be explained by nonspecific mechanisms such as Fc receptor blockade alone. For this effect on antibodies and B cells, the additional anti-idiotypic involvement of variable regions from IVIG molecules is required (Lacroix-Desmazes et al. 1996; Levy et al. 1999). Anti-idiotypic IVIG molecules may fit the antigen-binding site, particularly the complementarity determining regions (CDR) of antibodies, thus mimicking the original antigen (Ab2 beta, "internal image"). Other subsets of IVIG may be directed against idiotypes distinct from the paratope, recognizing mainly the framework regions (Ab2 alpha) (Fig. 1) (Abu-Shakra et al. 1996). The above-mentioned functions of IVIG may act synergistically in the regulation of T cell as well as B cell-dependent immune responses.

11.6
Anti-idiotypic Antibodies as Possible Superantigens?

To elucidate possible specific interactions of IVIG with (auto)antibodies, we recently investigated patient-derived monoclonal IgGs that were bound by IVIG in an anti-idiotypic manner, applying the combinatorial antibody phage display system (Barbas et al. 1991; Breitling et al. 1991; Duchosal et al. 1992; Yang et al. 1997, 1999; Hoet et al. 1998). From three different patients with AITP, a large number of clones specifically reacting with IVIG molecules were enriched. Many IVIG-selected Fab-phage from AITP strongly reacted with platelets in ELISA and fluorescent-activated cell sorting (FACS), in contrast to IVIG-selected Fab-phage derived from a healthy individual (Fischer et al. 1999) or a patient with SLE (Osei et al. 2000).

Sequencing revealed that the most frequently used germline gene loci of all IVIG-bound, platelet-negative Fabs from the three AITP libraries were 3–23 or 3–30/3–30.5 in the case of heavy chains and 3l, 2a2, and 3r for the light chains (Jendreyko et al. 1998; Fischer et al. 1999), while most platelet-reactive Fab were from V_H4 germline origin.

We observed the same favorite selection of 3–23 or 3–30/3–30.5-derived IgG and IgM Fabs by IVIG from libraries of healthy individuals (Hoffmann et al. 2000) and patients with SLE (Osei et al. 2000). Light chains, antigen specificity, and the high variation in mutation rates and CDR3 composition had little influence on this selection by IVIG. The observed interaction of IVIG with Fabs was characteristic for the binding of a B-cell superantigen (SAg).

Several potential B-cell SAgs defined by their ability to bind to the B-cell receptor (BCR) of certain V_H genes (mostly V_H3) outside of the antigen-binding groove have been characterized recently (reviewed in: Silverman 1997). B-cell SAgs include *Staphylococcus*-derived SpA (Sasano et al. 1993; Domiati-Saad and Lipsky 1998; Potter et al. 1998; Graille et al. 2000), mouse monoclonal "superantibody" D12 (Potter et al. 1998), CD5 (Pospisil et al. 1996), HIV gp120 (Karray et al. 1998; Neshat et al. 2000), and a novel clan III-restricted chicken monoclonal antibody (Cary et al. 2000). So far, only one internal human protein with SAg properties has been identified, the 175-kDa gut-associated sialoprotein Fv (pFv) (Silverman et al. 1995). Comparing the sequences, our IVIG-selected Fabs shared most of its critical amino acids with target antibodies of SpA, but comparative ELISAs revealed that at least some of the contact residues on Fabs for IVIG must be different from those for SpA.

The combined results suggested a specific interaction of a subset of IVIGs (normal immunoglobulin repertoires) with B cells that present BCRs derived from these two germline genes. Because 3–23 and 3–30/3–30.5 are the most frequently rearranged V_H germline gene segments among human B cells (Huang et al. 1996; Dewildt et al. 1999), this restricted anti-idiotypic interaction may have an important role for the development and control of the normal B-cell repertoire in health and disease (Fischer et al. 1998; Hoffmann et al. 2000). To investigate

this further, we cloned and selected Fab fragments from a patient with Kawasaki disease before and after IVIG therapy. Again, a favored selection of antibodies derived from both the 3–23 or 3–30/3–30.5 germline gene segments was observed. Importantly, the reactivity with IVIG was significantly higher for clones from the library prepared after the IVIG treatment, providing the first *in vivo* functional evidence that a subset of IVIG may selectively activate B cells of this germline origin. This SAg-like, anti-idiotypic mechanism may add to the therapeutic effect of IVIG in the treatment of Kawasaki disease (Leucht et al. 2001).

11.7 Anti-idiotypic Antibodies in Cancer Therapy

The first report of successful use of a monoclonal antibody for the treatment of human cancer appeared in 1982 (Miller et al. 1982), describing the treatment of B-cell lymphoma with a patient-specific, monoclonal anti-idiotype antibody (reviewed in Waldmann 2003). The principle of anti-idiotypic vaccination and animal models are reviewed in detail elsewere (Veelken 2001; Corthay et al. 2004). A clinical benefit of idiotypic vaccination in patients with follicular lymphoma was proven recently (Inogès et al. 2006).

Numerous clinical trials using Ab2β as an antitumor vaccine have been performed or are currently underway (Hurvitz and Timmerman 2005; Maloney 2005), investigating Ab3 development and clinical remissions (Wettendorff et al. 1989; Mittelman et al. 1995; Reinartz et al. 1999; Foon et al. 2000; Chapman et al. 2004; Reinartz et al. 2004; Pritchard-Jones et al. 2005). For example, when human anti-idiotypic antibody 105AD7, produced by fusion of a human/mouse heteromyeloma and B cells of a patient treated with anti-791T/36, a mouse antibody directed against gp72, was applied to osteosarcoma patients, 11/28 patients had an Ab3 response (Austin et al. 1989; Pritchard-Jones et al. 2005).

Searching the National Institutes of Health (NIH) clinical trials database (www.clinicaltrials.gov/) in December 2005 for "idiotypic or idiotype" reveiled 32 studies, with nine of them still recruiting. However, not all of these directly investigate anti-idiotypic antibodies as a therapy. More specific results were observed from directly searching for cancer trials (www.cancer.gov/clinicaltrials/findtrials), searching for "idio" in the option for specific drugs. This revealed 20 closed and four open trials explicitly with idio- or anti-idiotypic antibodies. The targets in many of these studies were gangliosides, complex glycosphingolipids overexpressed in tumors of neuroectodermal origin, such as neuroblastoma or melanoma.

Despite continuing therapeutic efforts, advanced or relapsed stage IV neuroblastoma has a poor prognosis (Matthay et al. 1994; Matthay et al. 1999; Berthold et al. 2003). Passive immunotherapy with murine or human/mouse chimeric antibodies directed against GD2, a disialoganglioside overexpressed on neuroblastoma cells, in phase I/II trials demonstrated complete remissions and prolonged event-free survival in some neuroblastoma patients (Cheung et al. 1987, 1992, 1998, 2001; Frost et al. 1997; Handgretinger et al. 1992, 1995; Huang et al.

1992; Murray et al. 1992, 1994; Ziegler et al. 1997; Yu et al. 1998). Initially, the clinical efficacy of native anti-GD2 antibody therapy was mainly attributed to their ability to cause tumor cell killing by antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Kushner and Cheung 1989).

Although the infused antibodies were cleared from circulation after six half-lives, clinical remissions were of longer duration, implying that additional anti-tumor defense mechanisms must have been triggered (Uttenreuther et al. 1992b, 1995b). The potential role of a "vaccination-like" effect in long-term neuroblastoma survivors, suggested by Cheung et al. and others, indicated that patients with an immune response benefited from passive immunotherapy (Cheung et al. 1987, 1993, 1994; Uttenreuther et al. 1992a; Handgretinger et al. 1995). Similar results were confirmed for other tumors in the adult patient population (Wagner et al. 1992; Baum et al. 1994; Madiyalakan et al. 1995).

Human anti-mouse antibodies (HAMAs) seemed to preclude repetitive therapeutic use of murine antibodies by diminished tumor targeting, accelerated clearance, and reduction of direct antitumor effects (Handgretinger et al. 1992; Saleh et al. 1992b; Khazaeli et al. 1993; Cheung et al. 1994; Uttenreuther-Fischer et al. 1995a). Some of these problems were addressed by decreasing the size and the xenogenic protein parts of antibodies (Gillies et al. 1989; Winter and Harris 1993). However, even with chimerized or humanized antibodies, (anti-idiotypic) immunogenicity was still observed (Fig. 11.2), although it neither limited treatment nor made patients prone to increased toxicity (Meredith et al. 1992; Saleh et al. 1992a; Uttenreuther-Fischer et al. 1996a; Yu et al. 1998).

In an analysis of a larger patient population of neuroblastoma survivors, Cheung et al. put up the hypothesis that transient levels of HAMAs, which are mainly directed against the constant regions of murine antibodies, were positively correlated with patient survival (Cheung et al. 1998). The immunogenicity of passively applied mAbs was not disadvantageous for the patient, but appeared beneficial by triggering an activation of the idiotypic network (Jerne 1974).

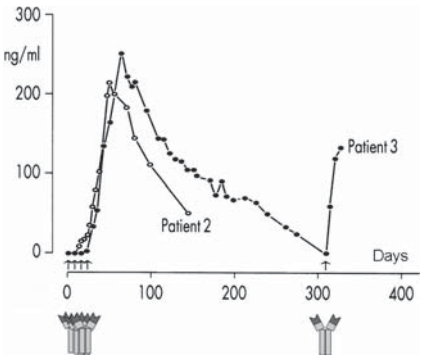

Fig. 11.2 Anti-idiotypic immune response of two ch14.18-treated neuroblastoma patients. Arrows indicate time-points of treatment.

Interestingly, in contrast to some studies (Koprowski et al. 1984; Herlyn et al. 1985; Wettendorff et al. 1989; Baum et al. 1994; Madiyalakan et al. 1995; Fagerberg et al. 1996; Schultes et al. 1998) Cheung et al. found that high HAMA levels prevented Ab3 formation in neuroblastoma patients treated with anti-GD2-MAbs (Cheung et al. 2000b).

Compiling results from previous studies they found that (1) patients with more intensive chemotherapy pretreatment before immunotherapy had lower and usually only transient HAMA/Ab2-levels than others (Cheung et al. 1994); (2) Ab3 production, starting around 6–14 months after MAb-therapy and persisting over years, was positively correlated with improved outcome (i.e. prolonged event-free survival) and more pronounced in patients with lower and only transient HAMA/Ab2-levels (Cheung et al. 2000b); (3) high HAMA concentrations were counterproductive for survival as they limited efficacy of further treatment cycles; (4) heavy chemotherapy eradicated lymphoid structures, reflected by low HAMA/Ab2 responses, and eliminated suppressor T-cell pathways and B cells. Ab2 might also bias the recovering immune repertoire towards the GD2 network (Cheung et al. 1994, 2000a,b; Cheung 2000). Moreover, Cheung et al. assumed that high Ab2 concentrations were not inductive for Ab3. In the murine model only IgM led to Ab3 production, while IgG was rather suppressive. In a healthy immune system dominant B-cell clones (Ab2) may prevent an anti-id response (Ab3) against themselves by early immunoglobulin class switch from immunogenic IgM to suppressive IgG class before anti-id B-cells (anti-Ab2) are activated (Reitan and Hannestad 1995; Cheung et al. 2000b).

11.8
Ab2β Vaccine Trials Mimicking GD_2

Currently two Ab2β vaccines are used for the treatment of GD2-positive malignancies: 1A7, a murine Ab2β against 14G2a, and 4B5 created by murine/human heteromyeloma technology, also directed against 14G2a (Saleh et al. 1993; Foon et al. 1998, 2000; Lutzky et al. 2002; Yu et al. 2002). Proof of principle was provided by all studies in the form of clinical responses and Ab3 serum levels in melanoma and neuroblastoma patients treated with 1A7, and positive Ab3 levels in animals treated with 4B5 (Saleh et al. 1993; Foon et al. 1998; Yu et al. 2002).

However, although Foon et al. did find Ab3-IgM in some of their melanoma patients, in general there was a positive correlation between IgM and IgG levels of Ab3, and isolated high Ab3-IgM levels were accompanied by disease progression (Foon et al. 1998, 2000). As the hypotheses and experimental findings presented above are partially contradictory, Cheung et al. suggested early on that more insight into the activation of the idiotypic network should be obtained by cloning such anti-idiotypic antibodies directly at the B-cell level (Cheung et al. 1994). Most clinical studies instead speculated on an activation of the idiotypic network driven by Ab2 and Ab3 levels detected in sera of patients treated with either Ab1 or Ab2.

Fusion cell lines of human/mouse heteromyelomas, however, frequently are not stable, and they still contain murine glycosylation (Harris et al. 1990; Lewis et al. 1992; Borrebaeck et al. 1993; Uttenreuther-Fischer et al. 1996b; Barnes et al. 2003). Phage display techniques provide an ideal method to clone and analyze the anti-idiotypic repertoire of patients after passive immunotherapy.

11.9 Molecular Characterization of the Anti-idiotypic Immune Response of a Relapse-free Cancer Patient

Assessing anti-ch14.18 levels in 65 serum samples of nine neuroblastoma patients after passive immunotherapy, only three patients showed a significant immune response against the variable region of ch14.18 (Ab2). These three patients are still alive without signs of disease 4.5–7 years later and have been off treatment for 4–5.5 years.

In a recent study, Uttenreuther-Fischer et al. (2004, 2006) reported a set of 40 human anti-idiotypic antibodies against monoclonal antibody ch14.18, cloned directly by antibody phage display technology from B cells of one of the neuroblastoma patients after ch14.18 treatment (Figs 11.3 and 11.4). Upon repetitive selection of lambda and kappa Fab-phage display libraries on target antigens ch14.18 or the murine equivalent 14G2a, positive binders were enriched. Selected Ab2 clones GK2 and GK8 as well as another 38 Fab phage clones demonstrated strong reactivity with both ch14.18 and 14G2a. Specificity and selectivity of binding was confirmed in Western blot analysis. Both anti-idiotypic clones GK2 and GK8 inhibited binding of ch14.18 to tumor-associated antigen GD2. Surprisingly, GK8-Fab alone was able to inhibit binding of the patient's serum to 14G2a by 80%, suggesting that GK8 it is identical to the majority of the patient's "original" anti-idiotypic antibodies. Immunization of rabbits with either purified GK2-

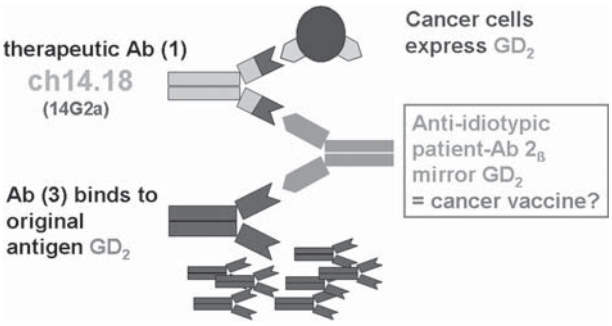

Fig. 11.3 Overview how patients develop anti-idiotypic antibodies against therapeutic ch14.18 which may function as an internal anticancer vaccine, eliciting Ab3 against the tumor-associated antigen GD2.

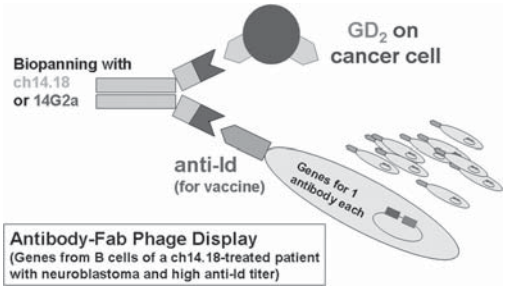

Fig. 11.4 Principle of phage display-cloning of anti-idiotypic antibodies (anti-Id) against ch14.18 as a possible vaccine for GD2-positive tumors. Therapeutic antibodies ch14.18 or the equivalent murine 14G2a are used in biopanning for selection of specific antibody-Fab phages.

and GK8-Fab or the complete Fab phage clearly produced a continuous rise in Ab3 serum levels (Ab3′) in these animals, as indicated by increased GD_2 binding. GK8 (and GK2) may be suitable as a fully human anti-idiotypic vaccine against GD_2-positive tumors.

Sequence analysis revealed at least 10 clones with different immunoglobulin genes. Homologies to putative V_H germline genes ranged between 94.90% and 100%; light chain homologies between 93.90% and 99.60%. An analysis of the R/S-ratio, giving the relation of replacement to silent mutations, suggested an antigen-driven selection of anti-idiotypic antibodies, triggered by ch14.18 treatment of our B-cell donor. Six out of 10 (60%) different clones showed an R/S-ratio above 2.9 for CDRs of their heavy chains and 6/10 (60%) for CDRs of their light chains. Taking into account that some clones appeared several times and giving a numeric relation, 32/36 (89%) clones showed somatic mutations in CDRs 1 and 2 of V_H regions and 11/36 (31%) for CDRs of their V_L regions (Uttenreuther-Fischer et al. 2006).

This was quite interesting, because earlier studies on bone marrow transplant (BMT) recipients had demonstrated that rearrangements in BMT recipients exhibited much less somatic mutations than did rearrangements from healthy subjects (Suzuki et al. 1996; Glas et al. 2000). Although our B-cell donor did not undergo BMT, intensive chemotherapy is also known to destroy lymphoid tissue. As the failure to accumulate somatic mutations in rearranged V_H genes is consistent with a maturational arrest at a very late state of B-cell differentiation, and as somatic mutations and affinity maturation are thought to take place in lymph node germinal centers (GC), it is a popular hypothesis that failure of germinal center processes prevents normal accumulation of somatic mutations following immunization in BMT recipients (Suzuki et al. 1996; Glas et al. 2000).

But unlike studies in BMT recipients, antibody clones picked from our "immunized" library from a heavily pretreated neuroblastoma patient exhibit a proportion of somatic mutations, which is comparable to what we and others found in B-cell libraries of healthy subjects (Suzuki et al. 1996; Hoffmann et al. 2000). In

summary, the immunological capacity of our patient to respond to foreign antigens (i.e. idiotypes) seems comparable to that of a healthy subject.

11.10 Conclusion

Since their description approximately 30 years ago by Jerne, the recognition of the network theory and anti-idiotypic antibodies within the scientific community have had their ups and downs. Almost ignored by immunologists for more than a decade, new techniques such as phage display, DNA-based vaccines, and the recent demand for new tools in generating specific antibodies and effective vaccines against therapy-resistent tumors and old as well as emerging pathogens, for example by "retrovaccinology" (Check 2003), and the problem of breaking tolerance (Saha et al. 2004) has brought anti-idiotypic antibodies back to their attention. This is reflected by an increase of relevant publications (Shoenfeld et al. 1997; Weng et al. 2004; Park et al. 2005) and clinical studies (www.clinicaltrials.gov/) (Hurvitz and Timmerman 2005). Using fully human anti-idiotypic vaccines instead of animal-derived proteins should help to overcome problems of nonspecific immunoreactivity or tolerance (Hernandez et al. 2005).

References

Abu-Shakra, M., Buskila, D., Shoenfeld, Y. (1996) Idiotypes and anti-idiotypic antibodies. In: Peter J.B., Shoenfeld, Y eds. *Autoantibodies*. Amsterdam: Elsevier, pp. 408–416.

Austin, E.B., Robins, R.A., Durrant, L.G., Price, M.R., Baldwin, R.W. (1989) Human monoclonal anti-idiotypic antibody to the tumour-associated antibody 791T/36. *Immunology* 67: 525–530.

Barbas, C.F.I., Kang, A.S., Lerner, R.A., Benkovic, S.J. (1991) Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc Natl Acad Sci USA* 88: 7978–7982.

Barnes, L.M., Bentley, C.M., Dickson, A.J. (2003) Stability of protein production from recombinant mammalian cells, *Biotechnol Bioeng* 81: 631–639.

Baum, R.P., Niesen, A., Hertel, A., Nancy, A., Hess, H., Donnerstag, B., Sykes, T.R., Sykes, C.J., Suresh, M.R., Noujaim, A.A. et al. (1994) Activating anti-idiotypic human anti-mouse antibodies for immunotherapy of ovarian carcinoma. *Cancer* 73: 1121–1125.

Berchtold, P., Dale, G.L., Tani, P., McMillan, R. (1989) Inhibition of autoantibody binding to platelet glycoprotein IIb/IIIa by anti-idiotypic antibodies in intravenous gammaglobulin. *Blood* 74: 2414–2417.

Berthold, F., Hero, B., Kremens, B., Handgretinger, R., Henze, G., Schilling, F.H., Schrappe, M., Simon, T., Spix, C. (2003) Long-term results and risk profiles of patients in five consecutive trials (1979–1997) with stage 4 neuroblastoma over 1 year of age. *Cancer Lett* 197: 11–17.

Borrebaeck, C.K., Malmborg, A.C., Ohlin, M. (1993) Does endogenous glycosylation prevent the use of mouse monoclonal antibodies as cancer therapeutics? *Immunol Today* 14: 477–479.

Breitling, F., Dübel, S., Seehaus, T., Klewinghaus, I., Little, M. (1991) A surface expression vector for antibody screening. *Gene* 104: 147–153.

Burns, J.C., Matsubara, T. (1995) Kawasaki disease: Recent advances. *Trend Cardiovasc Med* 5: 3–7.

Cary, S.P., Lee, J., Wagenknecht, R., Silverman, G.J. (2000) Characterization of superantigen-induced clonal deletion with a novel clan III-restricted avian monoclonal antibody: exploiting evolutionary distance to create antibodies specific for a conserved VH region surface. *J Immunol* 164: 4730–4741.

Chapman, P.B., Williams, L., Salibi, N., Hwu, W.J., Krown, S.E., Livingston, P.O. (2004) A phase II trial comparing five dose levels of BEC2 anti-idiotypic monoclonal antibody vaccine that mimics GD3 ganglioside. *Vaccine* 22: 2904–2909.

Check, E. (2003) AIDS vaccines: back to "plan A", *Nature* 423: 912–914.

Cheung, N.K. (2000) Monoclonal antibody-based therapy for neuroblastoma, *Curr Oncol Rep* 2: 547–553.

Cheung, N.K., Lazarus, H., Miraldi, F.D., Abramowsky, C.R., Kallick, S., Saarinen, U.M., Spitzer, T., Strandjord, S.E., Coccia, P.F., Berger, N.A. (1987) Ganglioside GD2 specific monoclonal antibody 3F8: a phase I study in patients with neuroblastoma and malignant melanoma. *J Clin Oncol* 5: 1430–1440.

Cheung, N.K., Canete, A., Cheung, I.Y., Ye, J.N., Liu, C. (1993) Disialoganglioside GD2 anti-idiotypic monoclonal antibodies. *Int J Cancer* 54: 499–505.

Cheung, N.K., Cheung, I.Y., Canete, A., Yeh, S.J., Kushner, B., Bonilla, M.A., Heller, G., Larson, S.M. (1994) Antibody response to murine anti-GD2 monoclonal antibodies: correlation with patient survival, *Cancer Res* 54: 2228–2233.

Cheung, N.K.V., Kushner, B.H., Cheung, I.Y., Kramer, K., Canete, A., Gerald, W., Bonilla, M.A., Finn, R., Yeh, S.J., Larson, S.M. (1998) Anti-GD(2) antibody treatment of minimal residual stage 4 neuroblastoma diagnosed at more than 1 year of age. *J Clin Oncol* 16: 3053–3060.

Cheung, N.K., Guo, H.F., Cheung, I.Y. (2000a) Correlation of anti-idiotype network with survival following anti-G(D2) monoclonal antibody 3F8 therapy of stage 4 neuroblastoma. *Med Pediatr Oncol* 35: 635–637.

Cheung, N.K., Guo, H.F., Heller, G., Cheung, I.Y. (2000b) Induction of Ab3 and Ab3′ antibody was associated with long-term survival after anti-G(D2) antibody therapy of stage 4 neuroblastoma. *Clin Cancer Res* 6: 2653–2660.

Cheung, N.K., Kushner, B.H., Laquaglia, M., Kramer, K., Gollamudi, S., Heller, G., Gerald, W., Yeh, S., Finn, R., Larson, S.M., Wuest, D., Byrnes, M., Dantis, E., Mora, J., Cheung, I.Y., Rosenfield, N., Abramson, S., O'Reilly, R.J. (2001) N7: a novel multi-modality therapy of high risk neuroblastoma (NB) in children diagnosed over 1 year of age. *Med Pediatr Oncol* 36: 227–230.

Cheung, N.V., Lazarus, H., Miraldi, F.D., Berger, N.A., Abramowsky, C.R., Saarinen, U.M., Spitzer, T., Strandjord, S.E., Coccia, P.F. (1992) Reassessment of patient response to monoclonal antibody 3F8 – letter. *J Clin Oncol* 10: 671–672.

Corthay, A., Lundin, K.U., Munthe, L.A., Froyland, M., Gedde-Dahl, T., Dembic, Z., Bogen, B. (2004) Immunotherapy in multiple myeloma: Id-specific strategies suggested by studies in animal models. *Cancer Immunol Immunother* 53: 759–769.

Dajani, A.S., Taubert, K.A., Takahashi, M., Bierman, F.Z., Freed, M.D., Ferrieri, P., Gerber, M., Shulman, S.T., Karchmer, A.W., Wilson, W. et al. (1994) Guidelines for long-term management of patients with Kawasaki disease. Report from the Committee on Rheumatic Fever, Endocarditis, Kawasaki Disease, Council on Cardiovascular Disease in the Young, American Heart Association. *Circulation* 89: 916–922.

Debre, M., Bonnet, M.C., Fridman, W.H., Carosella, E., Philippe, N., Reinert, P., Vilmer, E., Kaplan, C., Teillaud, J.L., Griscelli, C. (1993) Infusion of Fc gamma fragments for treatment of children with acute immune thrombocytopenic purpura. *Lancet* 342: 945–949.

Dewildt, R.M.T., Hoet, R.M.A., Vanvenrooij, W.J., Tomlinson, I.M., Winter, G. (1999) Analysis of heavy and light chain pairings indicates that receptor editing shapes the human antibody repertoire. *J Mol Biol* 285: 895–901.

Domiati-Saad, R., Lipsky, P.E. (1998) Staphylococcal enterotoxin A induces survival of V(H)3- expressing human B cells by binding to the V-H region with low affinity. *J Immunol* 161: 1257–1266.

Drucker, N.A., Colan, S.D., Lewis, A.B., Beiser, A.S., Wessel, D.L., Takahashi, M., Baker, A.L., Perez Atayde, A.R., Newburger, J.W. (1994) Gamma-globulin treatment of acute myocarditis in the pediatric population. *Circulation* 89: 252–257.

Duchosal, M.A., Eming, S.A., Fischer, P., Leturcq, D., Barbas, C.F.I., McConahey, P.J., Caothien, R.H., Thornton, G.B., Dixon, F.J., Burton, D.R. (1992) Immunization of hu-PBL-SCID mice and the rescue of human monoclonal Fab fragments through combinatorial libraries. *Nature* 355: 258–262.

Fagerberg, J., Ragnhammar, P., Liljefors, M., Hjelm, A.L., Mellstedt, H., Frodin, J.E. (1996) Humoral anti-idiotypic and anti-anti-idiotypic immune response in cancer patients treated with monoclonal antibody 17–1A. *Cancer Immunol Immunother* 42: 81–87.

Fields, B.A., Goldbaum, F.A., Ysern, X., Poljak, R.J., Mariuzza, R.A. (1995) Molecular basis of antigen mimicry by an anti-idiotope. *Nature* 374: 739–742.

Fischer, P., Uttenreuther-Fischer, M.M., Gaedicke, G. (1996a) Superantigens in the aetiology of Kawasaki disease. *Lancet* 348: 202.

Fischer, P., Uttenreuther-Fischer, M.M., Naoe, S., Gaedicke, G. (1996b) Kawasaki disease: update on diagnosis, treatment and a still controversial etiology. *Pediatr Hematol Oncol* 13: 487–501.

Fischer, P., Jendreyko, N., Lerch, H., Uttenreuther-Fischer, M.M., Gaedicke, G. (1998) Genetic origin of IgG antibodies cloned by phage display and anti-idiotypic panning from three patients with autoimmune thrombocytopenia. *Immunobiology* 199: 510–511.

Fischer, P., Jendreyko, N., Hoffmann, M., Lerch, H., Uttenreuther-Fischer, M.M., Chen, P.P., Gaedicke, G. (1999) Platelet reactive IgG antibodies cloned by phage display and panning with IVIG from three patients with autoimmune thrombocytopenia. *Br J Haematol* 105: 626–640.

Foon, K.A., Sen, G., Hutchins, L., Kashala, O.L., Baral, R., Banerjee, M., Chakraborty, M., Garrison, J., Reisfeld, R.A., Bhattacharya-Chatterjee, M. (1998) Antibody responses in melanoma patients immunized with an anti-idiotype antibody mimicking disialoganglioside GD2. *Clin Cancer Res* 4: 1117–1124.

Foon, K.A., Lutzky, J., Baral, R.N., Yannelli, J.R., Hutchins, L., Teitelbaum, A., Kashala, O.L., Das, R., Garrison, J., Reisfeld, R.A., Bhattacharyachatterjee, M. (2000) Clinical and immune responses in advanced melanoma patients immunized with an anti-idiotype antibody mimicking disialoganglioside GD2. *J Clin Oncol* 18: 376–384.

Frost, J.D., Hank, J.A., Reaman, G.H., Frierdich, S., Seeger, R.C., Gan, J., Anderson, P.M., Ettinger, L.J., Cairo, M.S., Blazar, B.R., Krailo, M.D., Matthay, K.K., Reisfeld, R.A., Sondel, P.M. (1997) A phase I/IB trial of murine monoclonal anti-GD2 antibody 14.G2a plus interleukin-2 in children with refractory neuroblastoma: A report of the Children's Cancer Group. *Cancer* 80: 317–333.

Galeazzi, M., Bellisai, F., Sebastiani, G.D., Morozzi, G., Marcolongo, R., Houssiau, F., Cervera, R., Levy, Y., George, J., Sherer, Y., Shoenfeld, Y. (1998) Association of 16/6 and SA1 anti-DNA idiotypes with anticardiolipin antibodies and clinical manifestations in a large cohort of SLE patients, *Clin Exp Rheumatol* 16: 717–720.

Gillies, S.D., Lo, K.M., Wesolowski, J. (1989) High-level expression of chimeric antibodies using adapted cDNA variable region cassettes. *J Immunol Methods* 125: 191–202.

Glas, A.M., van Montfort, E.H., Storek, J., Green, E.G., Drissen, R.P., Bechtold, V.J., Reilly, J.Z., Dawson, M.A., Milner, E.C. (2000) B-cell-autonomous somatic mutation deficit following bone marrow transplant. *Blood* 96: 1064–1069.

Graille, M., Stura, E.A., Corper, A.L., Sutton, B.J., Taussig, M.J., Charbonnier, J.B., Silverman, G.J. (2000) Crystal structure of a Staphylococcus aureus protein A domain complexed with the Fab

fragment of a human IgM antibody: Structural basis for recognition of B-cell receptors and superantigen activity, *Proc Natl Acad Sci USA* 97: 5399–5404.

Handgretinger, R., Baader, P., Dopfer, R., Klingebiel, T., Reuland, P., Treuner, J., Reisfeld, R.A., Niethammer, D. (1992) A phase I study of neuroblastoma with the anti-ganglioside GD2 antibody 14.G2a. *Cancer Immunol Immunother* 35: 199–204.

Handgretinger, R., Anderson, K., Lang, P., Dopfer, R., Klingebiel, T., Schrappe, M., Reuland, P., Gillies, S.D., Reisfeld, R.A., Niethammer, D. (1995) A phase I study of human/mouse chimeric antiganglioside GD2 antibody ch14.18 in patients with neuroblastoma. *Eur J Cancer* 31A: 261–267.

Harris, J.F., Koropatnick, J., Pearson, J. (1990) Spontaneous and radiation-induced genetic instability of heteromyeloma hybridoma cells. *Mol Biol Med* 7: 485–493.

Herlyn, D., Lubeck, M., Sears, H., Koprowski, H. (1985) Specific detection of anti-idiotypic immune responses in cancer patients treated with murine monoclonal antibody. *J Immunol Methods* 85: 27–38.

Hernandez, A.M., Rodriguez, M., Lopez-Requena, A., Beausoleil, I., Perez, R., Vazquez, A.M. (2005) Generation of anti-Neu-glycolyl-ganglioside antibodies by immunization with an anti-idiotype monoclonal antibody: A self versus non-self-matter. *Immunobiology* 210: 11–21.

Hoet, R.M.A., Raats, J.M.H., de Wildt, R., Dumortier, H., Muller, S., Van den Hoogen, F., van Venrooij, W.J. (1998) Human monoclonal autoantibody fragments from combinatorial antibody libraries directed to the U1snRNP associated U1C protein; epitope mapping, immunolocalization and V-gene usage. *Mol Immunol* 35: 1045–1055.

Hoffmann, M., Uttenreuther-Fischer, M.M., Lerch, H., Gaedicke, G., Fischer, P. (2000) IVIG-bound IgG and IgM cloned by phage display from a healthy individual reveal the same restricted germ-line gene origin as in autoimmune thrombocytopenia. *Clin Exp Immunol* 121: 37–46.

Hombach, A., Pohl, C., Heuser, C., Sircar, R., Diehl, V., Abken, H. (1998) Isolation of single chain antibody fragments with specificity for cell surface antigens by phage display utilizing internal image anti-idiotypic antibodies. *J Immunol Methods* 218: 53–61.

Huang, C.S., Uttenreuther, M., Reisfeld, R.A., Yu, A.L. (1992) Immunotherapy of GD2(+) tumors with a murine monoclonal antibody (MAB) 14G2a: A phase I study. *Proc ASCO* 11: 364.

Huang, S.C., Jiang, R.H., Glas, A.M., Milner, E.C.B. (1996) Non-stochastic utilization of Ig V region genes in unselected human peripheral B cells. *Mol Immunol* 33: 553–560.

Hurvitz, S.A., Timmerman, J.M. (2005) Current status of therapeutic vaccines for non-Hodgkin's lymphoma. *Curr Opin Oncol* 17: 432–440.

Imbach, P., Wagner, H.P., Berchtold, W., Gaedicke, G., Hirt, A., Joller, P., Mueller-Eckhardt, C., Muller, B., Rossi, E., Barandun, S. (1985) Intravenous immunoglobulin versus oral corticosteroids in acute immune thrombocytopenic purpura in childhood. *Lancet* 2: 464–468.

Imholz, B., Imbach, P., Baumgartner, C., Berchtold, W., Gaedicke, G., Gugler, E., Hirt, A., Hitzig, W., Mueller-Eckhardt, C., Wagner, H.P. (1988) Intravenous immunoglobulin (i.v. IgG) for previously treated acute or for chronic idiopathic thrombocytopenic purpura (ITP) in childhood: a prospective multicenter study. *Blut* 56: 63–68.

Inogès, S., Rodrìguez-Calvillo, M., Zabalegui, N., Lòpez-Dìaz de Cerio, A., Villanueva, H., Soria, E., Suárez, L., Rodríguez-Caballero, A., Pastor, F., García-Muñóz, R., Panizo, C., Pèrez-Calvo, J., Melero, I., Rocha, E., Orfao, A., Bendandi, M. (2006) Clinical benefit associated with idiotypic vaccination in patients with follicular lymphoma. *J Natl Cancer Inst* 98: 1292–1301.

Ishida, F., Gruel, Y., Brojer, E., Nugent, D.J., Kunicki, T.J. (1995) Repertoire cloning of a human IgG inhibitor of alpha(IIb)beta(3) function. The OG idiotype, *Mol Immunol* 32: 613–622.

Jendreyko, N., Uttenreuther-Fischer, M.M., Lerch, H., Gaedicke, G., Fischer, P. (1998) Genetic origin of IgG antibodies cloned by phage display and anti-idiotypic panning

from three patients with autoimmune thrombocytopenia, *Eur J Immunol* 28: 4236–4247.

Jerne, N.K. (1974) Towards a network theory of the immune system. *Ann Immunol* (*Paris*) 125C: 373–389.

Karray, S., Juompan, L., Maroun, R.C., Isenberg, D., Silverman, G.J., Zouali, M. (1998) Structural basis of the gp120 superantigen-binding site on human immunoglobulins. *J Immunol* 161: 6681–6688.

Kawasaki, T. (1967) Acute febrile mucocutaneous syndrome: Clinical observation of 50 cases. *Jpn J Allergy* 16: 178–222.

Kawasaki, T. (1995) Kawasaki disease. *Acta Paediat* 84: 713–715.

Khazaeli, M.B., Saleh, M., Liu, T., Reisfeld, R.A., LoBuglio, A.F. (1993) Murine and chimeric antibodies to GD2 antigen in melanoma patients: pharmacokinetics and immune response. In: Epenetos, A.A. (ed.) *Monoclonal Antibodies 2. Applications in Clinical Oncology*, 1st edn. London: Chapman and Hall, pp. 413–419.

Koprowski, H., Herlyn, D., Lubeck, M., DeFreitas, E., Sears, H.F. (1984) Human anti-idiotype antibodies in cancer patients: Is the modulation of the immune response beneficial for the patient? *Proc Natl Acad Sci USA* 81: 216–219.

Kotzin, B.L., Leung, D.Y., Kappler, J., Marrack, P. (1993) Superantigens and their potential role in human disease. *Adv Immunol* 54: 99–166.

Kushner, B.H., Cheung, N.K. (1989) GM-CSF enhances 3F8 monoclonal antibody-dependent cellular cytotoxicity against human melanoma and neuroblastoma. *Blood* 73: 1936–1941.

Lacroix-Desmazes, S., Mouthon, L., Spalter, S.H., Kaveri, S., Kazatchkine, M.D. (1996) Immunoglobulins and the regulation of autoimmunity through the immune network. *Clin Exp Rheumatol* 14: S9–S15.

Leucht, S., Uttenreuther-Fischer, M.M., Gaedicke, G., Fischer, P. (2001) The B cell superantigen-like interaction of IVIG with Fab fragments of V_H 3–23 and 3–30/3–30.5 germ-line gene origin cloned from a patient with Kawasaki disease is enhanced after IVIG therapy. *Clin Immunol* 99: 18–29.

Leung, D.Y. (1993) Kawasaki disease. *Curr Opin Rheumatol* 5: 41–50.

Levy, Y., Sherer, Y., George, J., Langevitz, P., Ahmed, A., Bar-Dayan, Y., Fabbrizzi, F., Terryberry, J., Peter, J., Shoenfeld, Y. (1999) Serologic and clinical response to treatment of systemic vasculitis and associated autoimmune disease with intravenous immunoglobulin. *Int Arch Allergy Immunol* 119: 231–238.

Lewis, A.P., Parry, N., Peakman, T.C., Crowe, J.S. (1992) Rescue and expression of human immunoglobulin genes to generate functional human monoclonal antibodies. *Hum Antibodies Hybridomas* 3: 146–152.

Lutzky, J., Gonzalez-Angulo, A.M., Orzano, J.A. (2002) Antibody-based vaccines for the treatment of melanoma. *Semin Oncol* 29: 462–470.

Madiyalakan, R., Sykes, T.R., Dharampaul, S., Sykes, C.J., Baum, R.P., Hor, G., Noujaim, A.A. (1995) Anti-idiotype induction therapy. Evidence for the induction of immune response through the idiotype network in patients with ovarian cancer after administration of anti-CA125 murine monoclonal antibody B43.13. *Hybridoma* 14: 199–203.

Maloney, D.G. (2005) Immunotherapy for non-Hodgkin's lymphoma: monoclonal antibodies and vaccines. *J Clin Oncol* 23: 6421–6428.

Matthay, K.K., Seeger, R.C., Reynolds, C.P., Stram, D.O., Oleary, M.C., Harris, R.E., Selch, M., Atkinson, J.B., Haase, G.M., Ramsay, N.K. (1994) Allogeneic versus autologous purged bone marrow transplantation for neuroblastoma: A report from the Childrens Cancer Group. *J Clin Oncol* 12: 2382–2389.

Matthay, K.K., Villablanca, J.G., Seeger, R.C., Stram, D.O., Harris, R.E., Ramsay, N.K., Swift, P., Shimada, H., Black, C.T., Brodeur, G.M., Gerbing, R.B., Reynolds, C.P. (1999) Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. *N Engl J Med* 341: 1165–1173.

Meredith, R.F., Khazaeli, M.B., Plott, W.E., Saleh, M.N., Liu, T., Allen, L.F., Russell, C.D., Orr, R.A., Colcher, D., Schlom, J. (1992) Phase I trial of iodine-131-chimeric B72.3 (human IgG4) in metastatic colorectal cancer. *J Nucl Med* 33: 23–29.

Metlas, R., Veljkovic, V. (2004) HIV-1 gp120 and immune network. *Int Rev Immunol* 23: 413–422.

Miller, R.A., Maloney, D.G., Warnke, R., Levy, R. (1982) Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. *N Engl J Med* 306: 517–522.

Mittelman, A., Wang, X.H., Matsumoto, K., Ferrone, S. (1995) Antianti-idiotypic response and clinical course of the disease in patients with malignant melanoma immunized with mouse anti-idiotypic monoclonal antibody MK2–23. *Hybridoma* 14: 175–181.

Murray, J.L., Cunningham, J.E., Brewer, H.M., Janus, M.H., Podoloff, D.A., Bhadkamakar, V.P., Kasi, L.P., Shah, R.S., Benjamin, R.S., Legha, S.S., Plager, C., Papadopoulos, N.E., Jaffee, N., Ater, J.L., Mujoo, K., Itoh, K., Ross, M., Bucana, C.D., Rosenblum, M.G. (1992) Phase I trial of murine anti-ganglioside (GD2) monoclonal antibody (Mab) 14G2a in cancer patients. *J Immunother* 11: 135–136.

Murray, J.L., Cunningham, J.E., Brewer, H., Mujoo, K., Zukiwski, A.A., Podoloff, D.A., Kasi, L.P., Bhadkamkar, V., Fritsche, H.A., Benjamin, R.S. et al. (1994) Phase I trial of murine monoclonal antibody 14G2a administered by prolonged intravenous infusion in patients with neuroectodermal tumors. *J Clin Oncol* 12: 184–193.

Neshat, M.N., Goodglick, L., Lim, K., Braun, J. (2000) Mapping the B cell superantigen binding site for HIV-1 gp120 on a V(H)3 Ig. *Int Immunol* 12: 305–312.

Osei, A., Uttenreuther-Fischer, M.M., Lerch, H., Gaedicke, G., Fischer, P. (2000) Restricted V_H3 gene use in phage displayed Fab that are selected by IVIG. *Arthritis Rheum* 43: 2722–2732.

Park, I.H., Youn, J.H., Choi, I.H., Nahm, M. H., Kim, S.J., Shin, J.S. (2005) Anti-idiotypic antibody as a potential candidate vaccine for *Neisseria meningitidis* serogroup B. *Infect Immun* 73: 6399–6406.

Peter, J.B., Shohat, L., eds (1996) *Autoantibodies*. Amsterdam: Elsevier.

Pospisil, R., Fitts, M.G., Mage, R.G. (1996) CD5 is a potential selecting ligand for B cell surface immunoglobulin framework region sequences. *J Exp Med* 184: 1279–1284.

Potter, K.N., Li, Y., Mageed, R.A., Jefferis, R., Capra, J.D. (1998) Anti-idiotypic antibody D12 and superantigen SPA both interact with human V(H)3-encoded antibodies on the external face of the heavy chain involving FR1, CDR2 and FR3. *Mol Immunol* 35: 1179–1187.

Pritchard-Jones, K., Spendlove, I., Wilton, C., Whelan, J., Weeden, S., Lewis, I., Hale, J., Douglas, C., Pagonis, C., Campbell, B., Alvarez, P., Halbert, G., Durrant, L.G. (2005) Immune responses to the 105AD7 human anti-idiotypic vaccine after intensive chemotherapy, for osteosarcoma. *Br J Cancer* 92: 1358–1365.

Reinartz, S., Boerner, H., Koehler, S., Vonruecker, A., Schlebusch, H., Wagner, U. (1999) Evaluation of immunological responses in patients with ovarian cancer treated with the anti-idiotype vaccine ACA125 by determination of intracellular cytokines – a preliminary report. *Hybridoma* 18: 41–45.

Reinartz, S., Kohler, S., Schlebusch, H., Krista, K., Giffels, P., Renke, K., Huober, J., Mobus, V., Kreienberg, R., DuBois, A., Sabbatini, P., Wagner, U. (2004) Vaccination of patients with advanced ovarian carcinoma with the anti-idiotype ACA125: immunological response and survival (phase Ib/II). *Clin Cancer Res*, vol. 10, no. 5: 1580–1587.

Reitan, S.K., Hannestad, K. (1995) A syngeneic idiotype is immunogenic when borne by IgM but tolerogenic when joined to IgG. *Eur J Immunol* 25: 1601–1608.

Saha, A., Chatterjee, S.K., Foon, K.A., Primus, F.J., Sreedharan, S., Mohanty, K., Bhattacharya-Chatterjee, M. (2004) Dendritic cells pulsed with an anti-idiotype antibody mimicking carcinoembryonic antigen (CEA) can reverse immunological tolerance to CEA and induce antitumor immunity in CEA transgenic mice. *Cancer Res* 64: 4995–5003.

Saleh, M.N., Khazaeli, M.B., Wheeler, R.H., Allen, L., Tilden, A.B., Grizzle, W., Reisfeld, R.A., Yu, A.L., Gillies, S.D., LoBuglio, A.F. (1992a) Phase I trial of the chimeric anti-GD2 monoclonal antibody ch14.18 in patients with malignant melanoma. *Hum Antibodies Hybridomas* 3: 19–24.

Saleh, M.N., Khazaeli, M.B., Wheeler, R.H., Dropcho, E., Liu, T., Urist, M., Miller, D.M., Lawson, S., Dixon, P., Russell, C.H. (1992b) Phase I trial of the murine monoclonal anti-GD2 antibody 14G2a in metastatic melanoma. *Cancer Res*52: 4342–4347.

Saleh, M.N., Stapleton, J.D., Khazaeli, M.B., LoBuglio, A.F. (1993) Generation of a human anti-idiotypic antibody that mimics the GD2 antigen. *J Immunol* 151: 3390–3398.

Sasano, M., Burton, D.R., Silverman, G.J. (1993) Molecular selection of human antibodies with an unconventional bacterial B cell antigen. *J Immunol* 151: 5822–5839.

Schroeder, J.O., Zeuner, R.A., Euler, H.H., Loffler, H. (1996) High dose intravenous immunoglobulins in systemic lupus erythematosus: Clinical and serological results of a pilot study. *J Rheumatol* 23: 71–75.

Schultes, B.C., Baum, R.P., Niesen, A., Noujaim, A.A., Madiyalakan, R. (1998) Anti-idiotype induction therapy: anti-CA125 antibodies (Ab(3)) mediated tumor killing in patients treated with Ovarex mAb B43.13 (Ab(1)). *Cancer Immunol Immunother* 46: 201–212.

Sherer, Y., Shoenfeld, Y. (2000) Idiotypic network dysregulation – A common etiopathogenesis of diverse autoimmune diseases. *Appl Biochem Biotechnol* 83: 155–162.

Sherer, Y., Levy, Y., Langevitz, P., Lorber, M., Fabrizzi, F., Shoenfeld, Y. (1999) Successful treatment of systemic lupus erythematosus cerebritis with intravenous immunoglobulin. *Clin Rheumatol* 18: 170–173.

Shoenfeld, Y. (1994) Idiotypic induction of autoimmunity: A new aspect of the idiotypic network. *FASEB J* 8: 1296–1301.

Shoenfeld, Y. (2005) Anti-DNA idiotypes: from induction of disease to novel therapeutical approaches. *Immunol Lett* 100: 73–77.

Shoenfeld, Y., Kennedy, R.C., Ferrone, S. (eds) (1997) *Idiotypes in Medicine: Autoimmunity, Infection and Cancer.* Amsterdam: Elsevier.

Silverman, G.J. (1997) B-cell superantigens. *Immunol Today* 18: 379–386.

Silverman, G.J., Roben, P., Bouvet, J.P., Sasano, M. (1995) Superantigen properties of a human sialoprotein involved in gut-associated immunity. *J Clin Invest* 96: 417–426.

Silvestris, F., Cafforio, P., Dammacco, F. (1994) Pathogenic anti-DNA idiotype-reactive IgG in intravenous immunoglobulin preparations. *Clin Exp Immunol* 97: 19–25.

Suzuki, I., Milner, E.C.B., Glas, A.M., Hufnagle, W.O., Rao, S.P., Pfister, L., Nottenburg, C. (1996) Immunoglobulin heavy chain variable region gene usage in bone marrow transplant recipients: Lack of somatic mutation indicates a maturational arrest. *Blood* 87: 1873–1880.

Uttenreuther, M., Huang, C.S., Tsui, C., Reisfeld, R.A., Gillies, S.D., Yu, A. (1992a) Comparison of biological activity and pharmacokinetics between a mouse and a human-mouse anti-GD2 chimeric monoclonal antibody (MAB). *Proc AACR* 33: 244.

Uttenreuther, M.M., Huang, Ch.S., Reisfeld, R.A., Yu, A.L. (1992b) Pharmacokinetics of chimeric human/mouse antibody ch14.18 in a phase I clinical trial with neuroblastoma patients. *J Cancer Res Clin Oncol* 118(Suppl.): 81.

Uttenreuther-Fischer, M.M., Huang, C.S., Reisfeld, R.A., Yu, A.L. (1995a) Pharmacokinetics of anti-ganglioside GD2 mAb 14G2a in a phase I trial in pediatric cancer patients. *Cancer Immunol Immunother* 41: 29–36.

Uttenreuther-Fischer, M.M., Huang, C.S., Yu, A.L. (1995b) Pharmacokinetics of human-mouse chimeric anti-GD2 mAb ch14.18 in a phase I trial in neuroblastoma patients. *Cancer Immunol Immunother* 41: 331–338.

Uttenreuther-Fischer, M.M., Fischer, P., Reisfeld, R.A., Gaedicke, G., Yu, A.L. (1996a) Monoclonal anti-disialoganglioside (GD2) antibodies in neuroblastoma-therapy: Summary of three phase I/II trials. *Proc ISHage-Meeting*.

Uttenreuther-Fischer, M.M., Yu, A., Gaedicke, G., Fischer, P. (1996b) A human anti-idiotypic monoclonal antibody against anti-GD2 chimeric antibody ch14.18 from a patient treated for neuroblastoma. *Hum Antibodies Hybridomas* 7: 55.

Uttenreuther-Fischer, M.M., Krueger, J.A., Fischer, P. (2004) Anti-idiotypic antibodies against anti-GD2 antibody ch14.18 – a potential tumor vaccine? *Monatsschr Kinderheilkd* 152(Suppl. 1): 229.

Uttenreuther-Fischer, M.M., Krueger, J.A., Fischer, P. (2006) Molecular characterization of the anti-idiotypic immune response of a relapse-free neuroblastoma patient following antibody therapy. A possible vaccine against tumors of neuroectodermal origin? *J Immunol* 176: 7775–7786.

Veelken, H. (2001) Tumor vaccination using monoclonal antibodies. *Internist* (*Berl*) 42: 874–883.

Wagner, U.A., Oehr, P.F., Reinsberg, J., Schmidt, S.C., Schlebusch, H.W., Schultes, B., Werner, A., Prietl, G., Krebs, D. (1992) Immunotherapy of advanced ovarian carcinomas by activation of the idiotypic network. *Biotechnol Ther* 3: 81–89.

Waldmann, T.A. (2003) Immunotherapy: past, present and future. *Nat Med* 9: 269–277.

Weng, W.K., Czerwinski, D., Timmerman, J., Hsu, F.J., Levy, R. (2004) Clinical outcome of lymphoma patients after idiotype vaccination is correlated with humoral immune response and immunoglobulin G Fc receptor genotype. *J Clin Oncol* 22: 4717–4724.

Wettendorff, M., Iliopoulos, D., Tempero, M., Kay, D., DeFreitas, E., Koprowski, H., Herlyn, D. (1989) Idiotypic cascades in cancer patients treated with monoclonal antibody CO17–1A. *Proc Natl Acad Sci USA* 86: 3787–3791.

Willems, P.M.W., Hoet, R.M.A., Huys, E.L.P.G., Raats, J.M.H., Mensink, E.J.B.M., Raymakers, R.A.P. (1998) Specific detection of myeloma plasma cells using anti-idiotypic single chain antibody fragments selected from a phage display library. *Leukemia* 12: 1295–1302.

Winter, G., Harris, W.J. (1993) Humanized antibodies. *Immunol Today* 14: 243–246.

Yang, Y.Y., Fischer, P., Leu, S.J.C., Olee, T., Carson, D.A., Chen, P.P. (1997) IgG rheumatoid factors isolated by surface displaying phage library technique. *Immunogenetics* 45: 301–310.

Yang, Y.Y., Fischer, P., Leu, S.J., Zhu, M., Woods, V.L., Jr., Chen, P.P. (1999) Possible presence of enhancing antibodies in idiopathic thrombocytopenic purpura. *Br J Haematol* 104: 69–80.

Yu, A.L., Uttenreuther-Fischer, M.M., Huang, C.S., Tsui, C.C., Gillies, S.D., Reisfeld, R.A., Kung, F. (1998) Phase I trial of a human-mouse chimeric anti-disialoganglioside monoclonal antibody ch14.18 in patients with refractory neuroblastoma and osteosarcoma. *J Clin Oncol* 16: 2169–2180.

Yu, A.L., Batova, A., Strother, D., Eskenazi, U., Castleberry, R.P., Bash, R., Matthay, K.K., Diller, L. (2002) A pilot trial of a GD-2 directed anti-idiotypic antibody as a vaccine for high risk neuroblastoma. *Proc Adv Neuroblastoma Res* 44: 17–6.

Ziegler, A., Macintosh, S.M., Torrance, L., Simon, W., Slabas, A.R. (1997) Recombinant antibody fragments that detect enoyl acyl carrier protein reductase in *Brassica napus*. *Lipids* 32: 805–809.

Part V
Ongoing Clinical Studies

12
Antibodies in Phase I/II/III: Cancer Therapy

P. Markus Deckert

12.1
Introduction

The promise of Paul Ehrlich's "magic bullet" was soon attached to Köhler and Milstein's – thankfully unpatented – invention of monoclonal antibody technology (Köhler and Milstein 1975) and then went unfulfilled for more than two decades. Only during the past 9 years have antibodies become an established modality of cancer treatment.

Eight antibodies are currently approved in the USA and Europe for the therapy of malignant diseases. Together, they exhibit many of the features to be expected from future antibody therapies. Today, more than 400 antibody varieties are in clinical trials for treatment of malignant diseases. Two hundred and five of these are listed in Tables 12.1–12.15. Although objective criteria have been employed, this implies that the selection will be arbitrary to a certain degree.

In large part, the clinical success of antibodies has been made possible through progress in recombinant technology, as chimerization and humanization helped to overcome the limitations of applying rodent proteins in humans. Furthermore, it has advanced the process of generating a desired binding specificity – from murine hybridoma towards *in vitro* systems such as phage display – and the capacity to design and tailor antigen-binding proteins to specific needs (e.g. by further reducing their immunogenicity or by creating bi- and even trifunctional molecules).

For practical purposes, four generations of monoclonal antibodies may be proposed:

1. Murine monoclonal antibodies generated by vaccination and hybridoma technology (m-mAb).
2. Variable region-grafted chimeric (ch-mAb) and complementarity determining region (CDR)-grafted humanized antibodies (hu-mAb), often collectively referred to as humanized antibodies.

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3. Fully human antibodies generated either by applying the classical vaccination and hybridoma technology on transgenic animals bearing a "human" immune system or by phage display technology (h-mAb).
4. Recombinant antibody constructs such as single-chain variable fragments and diabodies or minibodies as well as recombinant fusion constructs of such fragments with proteins carrying various effector functions, such as enzymes or cytokines.

This outline is certainly not all-encompassing (e.g. it does not include chemical conjugates with drug molecules or radioisotopes, because they have been produced with antibodies of all generations) nor is it generational in a strict chronological sense, but rather marks the distance from the original murine hybridoma.

The future of antibody applications in clinical oncology will comprise members of all four generations. Thus, while most of the antibodies approved for cancer therapy today have been derived from classical hybridomas and improved by humanization, progress in recombinant antibody design is currently moving from bench to bedside as fourth-generation antibodies have entered clinical trials. On the other hand, there are still serious candidates of first-generation murine antibodies being evaluated in clinical studies today.

Along with this development, the discovery of new pathomechanisms in cell signaling and angiogenesis has led to new targets for therapeutic intervention, which are exploited both by antibodies and small molecule pharmaceuticals. Here, too, the focus of antibody research is shifting from the original concept of a cancer-specific surface marker allowing us to label a tumor cell for destruction by the immune system towards employing antibodies to activate or inhibit specific cellular mechanisms.

An up-to-date review of antibodies under clinical investigation has some intrinsic difficulties the reader should be aware of. First, clinical trials may follow years after the initial publication of a new candidate substance, while on the other hand successful trials may be carried out on substances not yet published at all. Second, the inception of a clinical trial is not normally published, nor, necessarily, are its results. Hence, the peer-reviewed literature will not cover the full spectrum of current clinical developments. All other sources, however, are biased in one way or another: the personal insight of a reviewer tends to cover only parts of the field, while company websites and business reviews rarely hold up to the standards of scientific communication. The best source for the start of a comprehensive search would be a central database of clinical trials. With ClinicalTrials.gov, there is such a registry for the United States, and it may be fairly complete for cancer and other diseases deemed life-threatening. However, although the US is certainly a strong motor in this field, there are no comparable registries yet for Europe, Asia, or the Commonwealth. Initiatives to fill this gap are underway, but as of now have not been successful (Haug et al. 2005).

The aim of this chapter is to provide a reasonably complete list of the monoclonal antibodies or antibody-derived constructs currently under clinical investigation (presented mainly in the form of tables) and to review critically the resulting trends and developments.

To this end, the peer-reviewed literature and registered clinical trials were scanned via MedLine and ClinicalTrials.gov, respectively, using various search algorithms, and the retrieved articles and trial protocols reviewed. Antibodies or antibody constructs that appeared in either database, but not in both, were then searched for by various internet search engines to retrieve information from company websites or business reviews. Only where this secondary search yielded reliable, scientifically relevant information were such antibodies included and the sources of information identified.

Following the perspective of this book as a textbook on antibodies, not on clinical oncology, and the author's view of future clinical antibody applications outlined above, this chapter is organized along antibody structure, function, and targets. Thus, bifunctional antibody constructs in clinical trials are presented first, then antibodies with specific targeting or effector mechanisms. The remainder of antibodies in the clinical study phase are grouped by categories of targeted antigen. Only where the antigen is not well described are they finally grouped by the disease studied.

Two important aspects are not mirrored in the outline of this chapter: (1) the original species of an antibody (i.e. whether it is the rodent, humanized, or "fully human" version) – antibody humanization may now be considered standard technology, and where murine antibodies are in clinical trial this is usually not for lack of a humanized version; and (2) the type of therapy (i.e. antibody monotherapy, radioimmunotherapy, combination chemotherapy etc.) – while the integration of antibodies in combination protocols was a major advance in their clinical application, too many antibodies are used in several types of therapy for this criterion to be helpful.

Finally, once an antibody is approved for clinical use in cancer, it leaves the scope of this article.

12.2 Novel Antibody Constructs

Recombinant and bifunctional antibody constructs are not new as such. Almost all currently approved antibodies are recombinantly chimerized or humanized. Early on in the development of antibodies for cancer, radioisotope conjugates have been produced, and two of these, the CD20-specific ^{90}Y-ibritumomab tiuxetan (Zevalin) and ^{131}I-tositumomab (Bexxar) are approved drugs today. The same applies to antibody–drug conjugates such as the CD33-specific chalicheamicin conjugate gemtuzumab-ozogamicin (Mylotarg).

This section, thus, deals with mostly recombinant antibody constructs of a function and structure not predefined by nature, but obtained by adding or subtracting functional domains.

12.2.1 Bispecific Antibodies

The first antibodies combining the binding regions for two different antigens were generated by heterohybridoma technology and were described as early as 1983 (Milstein and Cuello 1983). In cancer therapy, the original idea of bispecific antibodies was to bridge cellular antigens of cancer and immune cells and thus trigger a targeted immune reaction against the tumor (for an overview, see Table 12.1). A newer concept is the use of bispecific antibodies for pretargeting strategies – these are described in Section 12.4.3.

Based on conventional hybridoma technology is the chemically crosslinked dual Fab fragment MDX-210 and its semi-humanized version, MDX-H210 (humanized Fab anti-CD64::Fab anti-HER2/neu): The product of the proto-oncogene Her-2/neu (or erbB-2) is a member of the epithelial growth factor receptor superfamily that is overexpressed in about a third of breast cancers and is also the target of the approved antibody trastuzumab. CD64, synonymous for the Fc γ receptor I (FCγRI) of monocytes, macrophages, dendritic cells, and activated granulocytes, is the central mediator of antibody-dependent cellular cytotoxicity (ADCC). An interesting aspect of the approach chosen here and in other antibodies against effector receptors is the use of an FCγRI-specific idiotope for affinity reasons rather than its natural ligand, the IgG Fc fragment.

Clinical studies have been performed with both bispecific conjugates and have progressed to phase II with MDX-H210, which, interestingly, was first published for Her-2/neu-expressing prostate, not breast cancer (James et al. 2001). In summary, these studies have demonstrated good tolerance of the antibody, and immunological activity defined as binding to circulating monocytes and increases in plasma cytokine levels, saturation of circulating Fc receptors on myeloid cells, and invasion of monocytes into tumor tissue (Repp et al. 2003) was detectable, suggesting functioning of the therapeutic principle. However, no clinical effect was observed when the bispecific antibody was given alone, but only when granulocyte and/or monocyte-activating cytokines such as G-CSF, GM-CSF, or interferon γ (IFNγ), were added to the regimens. The figures generated from these studies are not yet sufficient to estimate response and survival rates, but between one-fifth and one-third of (usually extensively pretreated) patients in these studies showed some degree of response (Valone et al. 1995; James et al. 2001; Repp et al. 2003).

Comparable results were reported earlier in a phase I study of 2B1, an anti-Her-2neu bispecific with FcγRIII as the immunologic effector target (Weiner et al. 1995), whereas H22xKi-4 and MDX-447 are based on the same CD64-binding antibody H22 as MDX-210, but are directed against the CD30 antigen of Hodgkin's lymphoma (Borchmann et al. 2002) and the EGFR (Wallace et al. 2000), respectively. Similarly, with HRS-3/A9, binding FcγRIII and the CD30 antigen, in small phase I studies immunological responses were seen which demonstrated proof of principle, but were not predictive for clinical outcome

Table 12.1 Bispecific antibodies.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
2B1	520C9 + 3G8, murine	p185/HER-2 + CD16	Breast	II	–		National Institutes of Health, Bethesda, MD, USA; Fox Chase Cancer Center, Philadelphia, USA	I	1995/ 1999	
BIS-1	$F(ab')_2$ of RIV-9 + MOC-31, murine	CD3 + EGP-2 (pan-carcinoma associated)	Lung cancer	I	–		Rijksuniversiteit Groningen, Groningen, Netherlands	I	1994/ 2000	Recruits T lymphocytes to tumor
Catumaxomab (Removab)	Trifunctional (trAb), murine/rat	EpCAM + CD3	Ovarian	I/II	NCT00189345	Closed 2005	Fresenius AG, Bad Homburg, Germany; Trion Pharma, München, Germany	–	2005	
H22 × Ki-4	Anti-CD30 + anti-CD64, human/ murine	CD64 + CD30	Reed–Sternberg cells: Hodgkin's lymphoma	I	–		Medarex, Princeton, NJ, USA	I	2002	
HRS-3/A9	HRS-3 + A9, murine	CD30 + FCRIII (CD16)	Reed–Sternberg cells Hodgkin's lymphoma	I/II	–		Universität des Saarlandes, Homburg, Germany; Biotest Pharma, Dreieich, Germany	I/II	2001	

Table 12.1 *(Continued)*

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
MDX-210	Chemically linked chimeric F(ab)′ of M22 + 520C9 murine	CD64 = FcγR1 + p185/HER-2	Leukemia, head-and-neck, breast, skin, pancreatic, renal cell, colon, bladder, and prostatic cancers	III	–		Medarex, Princeton, NJ, USA	I	1997/ 2004	
MDX-H210	Chemically linked humanized F(ab)′, based on H22 + 520C9	CD64 = FcγR1 + p185/HER-2	Head-and-neck, breast, skin, renal cell, ovarian, bladder, and prostatic cancers	II/III	–		Medarex, Princeton, NJ, USA	II	2003/ 2004	
MDX-214	Recombinant bispecific	CD89 (FcαR) + EGFR	Not specified	I/II	–		Medarex, Princeton, NJ, USA	–	–	
MDX-447	Humanized bispecific of H22 + mAb 425	CD64 = FcγR1 + EGFR	Head-and-neck, skin, renal cell, ovarian, bladder, and prostatic cancers	II	NCT00005813	Closed	Medarex, Princeton, NJ, USA; Merck KGaA, Darmstadt, Germany	–	2000	Combination with LAK cells
MT103 (BscCD19 × CD3)	scFv construct, murine	CD19 + CD3	Leukemia, non-Hodgkin's lymphoma	I	–		Micromet AG, München, Germany	–	2005	Derived from mAb HD37 and 145-2C11 (anti-CD3)
OC/TR (OC- TR)	$F(ab')_2$ of Mov18 + anti-CD3, murine	CD3 + folate-binding protein	Ovarian cancer	I	–		Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy; Universiteit Leiden, Leiden, Netherlands	I	1999	Recruits T lymphocytes to tumor

(Renner et al. 2000). Here, too, clinical trials employing costimulation by cytokines were initiated, but the results are not yet available (Hartmann et al. 2001).

A complete recombinant approach has been realized in MT103, a bispecific double scFv construct connecting the CD19 receptor of B lymphocytes as a tumor target of B-cellular non-Hodgkin lymphomas and acute or chronic leukemias with the CD3 part of the T-cell receptor defining the effector cells to be activated. Preclinical observations suggest that this bispecific antibody can induce repeated target cell lysis by activated T cells, while CD19-negative "bystanders" were spared (Hoffmann et al. 2005). *In vivo* T-cell activation and B-cell depletion have been demonstrated in chimpanzees, but data from an ongoing phase I clinical trial are not yet available (Schlereth et al. 2005).

Catumaxomab (Removab) is unique in that it has the structure of a "conventional" quadroma-derived bispecific complete IgG, but acts as a trifunctional agent that binds to tumor cells and harnesses two immunological pathways against the tumor. The trick here is the opposite approach to that introduced above with MDX-210: the natural Fc receptor-binding capacity of the IgG constant region is utilized for ADCC activation, leaving the bispecific variable regions for binding the epithelial cell adhesion molecule (EpCAM) overexpressed in various cancers and the CD3 portion of the T-cell receptor as (second) immunological effector. Thus, both T lymphocytes and antigen-presenting cells are activated, which should lead to a synergistic effect. In addition, the activation of T cells and granulocates/monocytes means the parallel activation of acquired and innnate immunity. Preclinical data demonstrate activation of $CD83^+$ antigen-presenting cells, secretion of IFNγ, and granzyme B-mediated lysis of targeted cells by EpCAM-specific $CD8^+$ T cells after tumor cell opsonization with catumaxomab, and an *in vitro* cytotoxicity comparable to cisplatin (Gronau et al. 2005). As the secretion of cytokines such as TNFα, IFNγ, and IL-2b subsided within 24 h, the authors conclude that this system may be safe without provoking severe adverse events by a "cytokine storm" (Schmitt et al. 2004). Again, data from an ongoing clinical trial are not yet available.

12.2.2 Antibody Fusion Constructs

The idea of attaching an effector function other than its natural Fc fragment to an antibody has arisen early in the development of antibodies for cancer treatment. Again, the advent of recombinant technology has given this concept a boost in helping to overcome the drawbacks of chemical conjugation, namely heterogeneity of products with varying conjugation ratios and molecular attachment sites, problems of stability of the chemical linker, and often a low final yield of the desired product.

There are basically three groups of bifunctional constructs currently in clinical trials: drug conjugates, immunotoxins, and cytokine fusion proteins. They are summarized in Table 12.2.

Table 12.2 Antibody fusion constructs.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
CMB-401	Calicheamycin conjugate of CTM01	RPAP epitope of MUC1	Breast, ovarian cancer	II	–		Wyeth, Madison, NJ, USA	II	2003/ 2005	Drug conjugate
260F9-rRA	260F9::ricin A conjugate, murine	55 kDa breast cancer-associated antigen	Breast, ovarian cancer	I	–		Cetus Corp., Emeryville, CA, USA	I	1989/ 1996	Immunotoxin
Anti-B4-bR	Ricin-A immunotoxin, murine	CD19	B lymphocytes: myeloma, leukemia	II	–		Dana Farber Cancer Institute, Boston, MA, USA	II	2001	Immunotoxin
RFB4(dsFv)-PE38 (BL22)	RFB4 scFv–*Pseudomonas* exotoxin PE38, disulfide stabilized, murine	CD22	B-cell lymphoma	I/II	NCT00071318 NCT00126646 NCT00075309 NCT00114751	Recruiting	Royal Free Hospital, London, UK; National Institutes of Health, Bethesda, MD, USA	I	2005	Immunotoxin
RFB4-dgA	RFB4–ricin A–conjugate, murine	CD22	B-cell lymphoma	I	NCT00006423 NCT00001271	Closed 2003	National Cancer Institute, Rockville, MD, USA	I	2000	Immunotoxin
RFT5(scFv)-ETA′	Recombinant humanized scFv–*Pseudomonas* exotoxin A (ETA)	CD25	Reed– Sternberg cells: Hodgkin's lymphoma	?	–		Medizinische Klinik I, Universität Köln, Köln, Germany	–	2002	Immunotoxin

RFT5-Dga RFT5-SMTP-dGA	Ricin-A immunotoxin, murine	CD25	Reed–Sternberg cells: Hodgkin's lymphoma	I/II	–		Royal Free Hospital, London, UK	I/II	1998	Immunotoxin
LMB-2 (anti-Tac(Fv)-PE38)	Recombinant *Pseudomonas* exotoxin PE38 construct, murine	CD25 (IL-2R)	T lymphocytes leukemia	II	NCT00080535 NCT00077922 NCT00263510 NCT00082004 NCT00080821 NCT00104975	Recruiting	National Institutes of Health, Bethesda, MD, USA	–	2000	Immunotoxin
HuM195/rGel	Chemical conjugate of chimeric huM195 and recombinant gelonin plant toxin	CD33	Myeloic malignancies	I	NCT00038051	Recruiting	University of Texas M.D. Anderson Cancer Center, Houston, TX, USA	–	2003	Immunotoxin
4D5MOCB-ETA	Recombinant humanized scFv–*Pseudomonas* exotoxin A (ETA)	EpCAM	Head-and-neck cancer	I	–		Universität Zurich, Zurich. Switzerland	–	2003	Immunotoxin
LMB-1 (B3-LysPE38)	B3–*Pseudomonas* exotoxin PE38, murine	Le-y	Gastric, ovarian, and colon cancers	II	NCT00001805	Closed 2000	National Institutes of Health, Bethesda, MD, USA	II	2004	Immunotoxin
LMB-7 (B3(Fv)-PE38)	B3 scFv–*Pseudomonas* exotoxin PE38 fusion, murine	Le-y	Leptomeningeal metastases	I	NCT00003020	Closed 2000	National Institutes of Health, Bethesda, MD, USA; Duke University, Durham, NC, USA	I	1995/2003	Immunotoxin

Table 12.2 *(Continued)*

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
LMB-9 (B3(dsFv) PE38)	B3 scFv–*Pseudomonas* exotoxin PE38, disulfide stabilized, murine	Le-y	Breast, lung, gastric, ovarian, and colon cancers	I/II	NCT00001691	Closed 2003	National Institutes of Health, Bethesda, MD, USA	–	2004	Immunotoxin
SS1P (SS1(dsFv)-PE38)	SS1 scFv–*Pseudomonas* exotoxin PE38, disulfide stabilized, murine	Mesothelin	Mesothelioma, lung (NSCLC), and ovarian cancers	I	NCT00065481	Recruiting	NeoPharm, Waukegan, IL, USA; National Institutes of Health, Bethesda, MD, USA	I	2004	Immunotoxin
huKS-IL2 (EMD 273066)	Recombinant huKS1/4:IL2 fusion protein	EpCAM	Lung, ovarian, prostate, and colorectal cancers	I	–		Merck KgaA, Darmstadt, Germany; EMD Pharmaceuticals, Durham, NC, USA	I	2004	Cytokine fusion
EMD 273063	Recombinant hu14.18::IL2(2) fusion protein	GD2	Neuroblastoma, melanoma	II	NCT00082758 NCT00109863	Recruiting	Merck KgaA, Darmstadt, Germany; EMD Pharmaceuticals, Durham, NC, USA	II	2004	Cytokine fusion

12.2.2.1 Antibody–Drug Conjugates

Potent drugs that exert intolerable toxicity when applied directly are the most obvious candidates for antibody-targeted delivery. In this respect, two classes of antibiotics have recently attracted attention: calicheamicins and maytansinoids. Calicheamicins are naturally synthesized by a soil microorganism and bind to the minor groove of DNA, causing double-strand breaks and subsequent cell death by apoptosis (Damle and Frost 2003).

Maytansine and its relatives are members of the ansamycin group of natural products, which are produced by higher plants, mosses and microorganisms (Cassady et al. 2004). Their antitumor activity relies on tubulin binding, inhibiting the microtubule assembly and thus mitosis. First described in 1972, their cytotoxic potency raised great interest for chemotherapeutic application, but despite – or rather, because of – their high cytotoxicity the results of clinical studies were disappointing: In the end, 21 out of about 800 treated patients with various tumors showed partial or, in one case, complete responses.

Because of their cytotoxic potency, both substance classes have been further developed for targeted tumor therapy. While a number of antibody–maytansinoid conjugates are in preclinical development, so far only gemtuzumab-ozogamicin (Mylotarg), directed against the CD33 antigen of acute myeloic leukemia cells, has been approved. CMB-401, a calicheamicin conjugate of the anti-MUC1 antibody CTM01, is currently in phase II clinical trials. While it has shown promising preclinical properties (Hamann et al. 2005), clinical data are not yet available.

12.2.2.2 Immunotoxins

The plant toxin ricin A and *Pseudomonas* exotoxin A are two highly poisonous biologic substances that, like the potent antibiotics above, cannot be applied systemically without deleterious toxicity. Again, the idea here is that targeted delivery via antibodies unleashes their potential against tumor cells without harming the rest of the organism.

Ricin, an *N*-glycosidase from the bean of the castor plant (*Ricinus communis*), is regarded as a biological weapon and has recently gained attention as a potential bioterrorism threat. It consists of an A and B chain, the latter of which binds to cell surface lectins, allowing the A chain to enter the cytosol and exert an essentially toxic ribosome blockade. Clinical effects start with various respiratory or gastrointestinal symptoms, depending on the route of ingestion, and progress to multiorgan failure and finally death. Treatment is supportive as no causal therapy is known (for a review see Audi et al. 2005).

Clinical trials with ricin conjugates have focused on lymphomas, not least due to their high density of specific surface antigens.

Initial clinical trials have tended to be disappointing. Several phase II clinical trials of the blocked ricin immunotoxin anti-B4-bR have been attempted, but they demonstrated too low tumor penetration to bring about a clinical effect in various therapeutic settings in non-Hodgkin's lymphoma patients (Multani et al. 1998;

Grossbard et al. 1999). A combination therapy trial finally yielded durable remissions, but only in a minority of patients (Longo et al. 2000).

RFB4-dgA is a chemically linked conjugate of anti-CD22 with ricin A that was well tolerated in B cell non-Hodgkin's lymphoma patients with a large proportion of circulating tumor cells, whereas the clinical course of patients without circulating cells was unpredictable and included two, probably treatment-associated, deaths. As this has been linked with aggregate formation of the pharmaceutical formulation, no further trials were conducted with this conjugate (Messmann et al. 2000).

Out of 15 heavily pretreated Hodgkin's patients treated with RFT5-dgA, an anti-CD25::ricin A conjugate, three experienced some degree of response. The predominant toxicity was a reversible vascular leak syndrome, and grade III toxicity was observed in all patients receiving the highest dose of $20\,mg\,m^{-2}$. While phase II studies at the maximum tolerated dose of $15\,mg\,m^{-2}$ were proposed (Schnell et al. 1998), to date none have been published.

However, both antibodies have also been fused with *Pseudomonas* exotoxin A, a bacterial toxin, or a truncated version of it, PE 38. RFT5(scFv)-ETA and a derived bispecific double scFv, RFT5/Ki-4(ScFv)-ETA, directed against both CD25 and CD30 (see also Section 12.2.1) have been expressed, and RFT5(scFv)-ETA has been announced for clinical studies after demonstrating specific cytotoxicity against disseminated human Hodgkin's lymphoma in severe combined immunodeficiency (SCID) mice (Barth et al. 2000).

The disulfide-stabilized recombinant *Pseudomonas* exotoxin fusion of RFB4 – RFB4(dsFv)-PE38 or BL22 – did not share the galenic problems of the ricin A conjugate in a phase I study for non-Hodgkin's lymphoma, chronic lymphocytic leukemia (B-CLL) and hairy cell leukemia. Here, 61% complete remissions of a mean duration of 36 months and 19% partial responses were seen in hairy cell leukemia. Among patients who received the maximum tolerated dose established in this study, the rate of complete responses was 86%, prompting a current phase II trial of BL22 for this indication. A characteristic adverse event was transient hemolytic uremic syndrome, observed in 5 out of 31 patients with hairy cell leukemia, but in none of the patients with other conditions. The most common toxicities were again symptoms of capillary leak syndrome, elevated liver enzymes, and fatigue (Kreitman et al. 2005).

The same approach has been followed for LMB-2 (anti-Tac(Fv)-PE38), which has also shown promising results in phase I in patients with various hematologic malignancies. Toxicity in this study was moderate, with fever and transient liver enzyme elevations. Remarkably, only 6 out of 35 patients developed neutralizing antibodies against the construct. One complete and seven partial responses were observed in patients with various hematologic malignancies, including hairy cell leukemia, cutaneous T-cell lymphoma, chronic lymphocytic leukemia, Hodgkin's disease, and T-cell leukemia (Kreitman et al. 2000).

An interesting group of PE38 constructs has been generated based on the anti-Lewis-Y antibody B3, termed LMB-1, LMB-7, and LMB-9. While LMB-1 is a chemical B3-PE38 conjugate, the other two are single-chain Fv (scFv)-based recombinant constructs, with the scFv being disulfide-stabilized in LMB-9.

An intriguing aspect of these recombinant constructs is the separate expression of the component proteins, which contain polyionic adapter peptides, allowing their covalent coupling. This technology facilitates a modular assembly of various antibody–toxin combinations and may in addition offer a solution to a common problem in the design and production of heterogeneous fusion proteins – the refusal of their components to be expressed in the same organism (Kleinschmidt et al. 2003).

LMB-7 and LMB-9 are currently in clinical trials of phase I or II. With LMB-1, clinical responses have been reported from a phase I trial for Le^Y-positive solid tumors unresponsive to conventional treatment (Pai et al. 1996). As with ricin A, the predominant toxicity was a reversible vascular leak syndrome. An ensuing phase II trial addressed the question whether the human anti-murine immune response against the construct could be prevented by pretreatment with the anti-CD20 antibody rituximab, but found that, despite total suppression of circulating B lymphocytes, all patients developed neutralizing antibodies against LMB-1 that prevented repeated administration (Hassan et al. 2004).

With the availability of the smaller and more stable recombinant constructs, the clinical development of LMB-1 was discontinued in favor of LMB-7 and LMB-9. LMB-7 was very efficient in animal models, but disappointing in phase I clinical trials due to low stability and aggregation observed at 37°C. Hence, the disulfide-stabilized scFv underlying the LMB-9 construct was generated, which showed a broader therapeutic window and enhanced stability in preclinical studies, thus opening the opportunity for more intensive dosing schedules such as continuous infusion in order to obtain better tumor penetration (Pastan 2003). LMB-9 is in a clinical phase I study for breast, colon, lung, ovarian, and gastric cancer that completed recruitment in 2003.

Disulfide stabilization has now become almost standard in new scFv-based constructs for clinical applications. It has also been applied in two other recombinant fusion constructs of *Pseudomonas* exotoxin currently in phase I, the anti-EpCAM 4D5MOCB-ETA for head and neck cancer (Di Paolo et al. 2003) and SS1P (SSI(dsFv)-PE38) directed against a mesothelioma antigen (Li et al. 2004).

While these results give the appearance of recombinant PE38 constructs as a comparably safe and potentially efficacious concept, the story of Erb38, a disulfide-stabilized recombinant scFv construct based on the e23 antibody against the Her2/neu (erb2) antigen, tells a cautionary tale. Apart from high expression in 30% of breast cancers, this antigen is also expressed at very low levels by hepatocytes, and despite a huge difference in expression, hepatic toxicity at the lowest dose level led to termination of a phase I trial of Erb38 (Pastan 2003).

As a third toxin, the plant protein gelonin, which inhibits ribosome function, has recently entered the clinical stage. Hum195/rGel is a chemical conjugate of a chimerized version of the anti-CD33 antibody M195 and recombinant gelonin. After initial *ex vivo* bone marrow purging studies (Duzkale et al. 2003), it is currently being evaluated in a still recruiting phase I trial for acute myeloid leukemia.

12.2.2.3 Cytokine Fusion Proteins

Like bispecific antibodies, cytokine fusion antibodies are intended to localize an immune effector function to cancer cells. One of the most effective cytokines in this respect is interleukin 2 (IL-2). It activates both cellular and humoral responses against an antigen. However, it is expressed by T lymphocytes only after their activation following recognition of an HLA-restricted antigen. To bypass this autoregulatory protection against the self-attack of host structures, including cancer cells, IL-2 is being applied with varying success in notoriously chemotherapy-refractory cancers such as melanoma (off-label) and renal cell carcinoma. Its adverse effects are numerous and partly severe.

Thus, as in the concept of drug conjugates and immunotoxins, it is desirable to target this cytokine more specifically to tumor cells in order to intensify its local antitumor effects and to minimize adverse events.

Two such cytokine–antibody fusions, both recombinantly generated, are currently in clinical trials: EMD-273063 and huKS-IL2.

The huKS-IL2 construct is derived from a humanized version of the murine anti-EpCAM antibody KS1/4. While clinical studies have been proposed or are ongoing for ovarian, lung, and colorectal cancer, the results of a phase I study with prostate cancer patients have already been published, indicating a favorable toxicity profile compared with the direct application of IL-2. Neutralizing antibodies were detected, but no hypersensitivity reactions were observed. This study showed increased parameters of IL-2-dependent immune activation, demonstrating the principle, although clinical responses were not reported (Ko et al. 2004).

EMD-273063, generated by the same technology from the same manufacturer as huKS-IL2, is based on the humanized antibody 14.18, which recognizes the GD2 ganglioside. It is currently being investigated in phase II in neuroblastoma. A phase I study in melanoma patients found frequent and partly dose-limiting grade 3 adverse events, including metabolic deregulation, hypotension, hypoxia, and elevated liver enzymes or bilirubin as well as opioid-dependent arthralgia or myalgia, but no grade 4 toxicity. As with huKS-IL2, indicators of an immunological response increased during treatment, but also without clinical responses (King et al. 2004).

12.3 Specific Targeting and Effector Mechanisms

The conventional effect of therapeutic antibodies is the initiation of antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) upon binding to their cognate antigen. The previous section has introduced antibody constructs that have been designed and constructed to exert additional molecular functions. Many therapeutic antibodies, however, exert non-immunological effects without a specific molecular design by activating or blocking the function of their target receptor (which may, of course, still be part of an immunological effect).

As the usual way to discover a hitherto unknown cellular antigen is its initial definition by a new monoclonal antibody, it is not surprising that often the function of a receptor, and thus the mechanism of action of a therapeutic antibody, has only been elucidated after the antibody has entered clinical development. Hence a strict line between "conventional" immunologically and "novel" non-immunologically acting antibodies cannot be drawn, and many of the "novel" antibodies still owe a proportion of their efficacy to ADCC or CDC. Similarly, the different effector principles introduced in this section cannot always be strictly separated. Thus, the proliferation signal pathways of tumor cells and neovasculature share many receptors, so that, for example, EGFR targeting could be classified both as inhibition of tumor cell growth and as antiangiogenesis. Nevertheless, from a systematical and didactic point of view it appears sensible to maintain these distinctions, as long as they are not taken for rigid borders.

12.3.1 Antiangiogenesis

The formation of new microvasculature is a prerequisite for tumor growth, and the recent characterization of the signaling and growth factors involved in this process has fostered the development of antiangiogenesis into a new principle of cancer therapy (Folkman 2006).

A broad array of antiangiogenic substances are now being investigated for their clinical antitumor effects. Of these, antibodies form a large proportion, notably those following the two already approved antiangiogenic antibodies, bevacizumab and cetuximab, in targeting vascular endothelial growth factor (VEGF) or epithelial growth factor receptor (EGFR), respectively. Other targets of antiangiogenic antibodies are phosphatidyl serine, IL-8, fibronectins, and integrin $\alpha_v\beta_3$. Antiangiogenic antibodies are summarized in Table 12.3.

12.3.1.1 Vascular Endothelial Growth Factor

Bevacizumab (Avastin) was the first anti-VEGF antibody approved in combination with chemotherapy for the first-line treatment of colorectal cancer. A4.6.1 and MV833 are two newer antibodies entering or already in clinical phase I trials.

A4.6.1 has been humanized from its murine counterpart by phage display optimization of the framework (Baca et al. 1997). Preclinical studies in an orthotopic mouse model of pancreatic cancer demonstrated additive antitumor efficacy of this antibody in combination with the matrix metalloproteinase inhibitor BB-94, a small-molecule antiangiogenic agent (Hotz et al. 2003). Clinical results are not yet available for this antibody, whereas for MV833 the results of a phase I study have been published. Here, one partial response and disease stabilization in another 9 out of 20 patients were reported. Toxicity was mostly limited to WHO grade I and II, predominantly fatigue, dyspnea, and erythema (Jayson et al. 2005).

Table 12.3 Antibodies with antiangiogenic effect.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
A.4.6.1 (A4.6.1)	Phage-display humanized	VEGF	Rhabdomyosarcoma, pancreatic cancer	I	–		Genentech, South San Francisco, CA, USA	–	2000	
MV833	Murine	VEGF	Epithelial solid tumors	I	–		Protein Design Labs, Fremont, CA, US, Tsukuba Res. Lab., Japan; Toagosei Co., Japan	I	2005	
1121B (IMC-1121)		VEGF-2 (KDR)	Epithelial solid tumors	I	–		Imclone Systems, New York, NY, USA	–	2005	
1C11 (IMC-1C11)	Chimeric from murine	VEGFR-2	Colorectal cancer	I	–		Imclone Systems, New York, NY, USA	I	2003	
2C6 (IMC-2C6)	Humanized	VEGFR-2	Acute myeloid leukemia	I/II	–		Imclone Systems, New York, NY, USA	–	2004	
CDP791	Humanized from murine mAb VR165, PEG conjugate	VEGFR	Lung (NSCLC) cancer	I	NCT00152477	Recruiting	UCB S.A., Bruxelles, Belgium	–	–	No peer-reviewed publications
CNTO 95	Transgenic	α_V integrins	Melanoma	II	NCT00246012	Recruiting	Centocor, Horsham, PA, USA; Medarex, Princeton, NJ, USA	–	2005	
Volociximab Eos200-4 (M200)	Chimeric from murine	Integrin $\alpha_5\beta_1$	Renal cell carcinoma, melanoma	I	NCT00100685 NCT00099970 NCT00103077	Recruiting	Protein Design Labs, Fremont, CA, USA	–	2004	No peer-reviewed publications

Vitaxin	Humanized LM 609	Integrin $\alpha_v\beta_3$	Melanoma, sarcoma, prostatic	I/II	–	Scripps Research Institute, La Jolla, CA, USA	–	2002	
3G4	Humanized	Phosphatidylserine	Breast cancer, sarcoma	I	–	University of Texas Southwestern Medical Center,, Dallas, TX USA	–	2005	
ABX-IL8	Transgenic	IL-8	Melanoma	I	–	Abgenix, Fremont, CA, USA	–	2005	Phase II study on COPD closed in 2003
BC1	Humanized	Fibronectin	Glioma, meningeoma, breast, lung	I/II	–	Istituto Nazionale di Ricerca di Cancro, Genova, Italy; Eidgenossische Technische Hochschule, Zürich, Switzerland	–	2001	
HUIV26	Humanized	Collagen type IV	Melanoma	I	–	University of Southern California, Los Angeles, CA, USA	–	2002	
CDP860	Humanized, PEG conjugated	PDGFβ receptor	Ovarian and colorectal cancers	II	–	UCB S.A., Bruxelles, Belgium	II	2005	Principle: reduction of intratumoral pressure

12.3.1.2 VEGF Receptors

As with EGF and EGFR, targeting the cellular VEGF receptor instead of the soluble ligand appears to be an obvious approach. Although no VEGF-R antibodies have so far been approved for therapy, three of them have recently entered phase I or I/II clinical trials. IMC-1121 and IMC-2C6, high-affinity versions of IMC-1C11, are fully human antibodies raised by phage display against the VEGF-R2, also termed kinase insert domain-containing receptor (KDR). With IMC-2C6, it was first demonstrated that VEGF-R2 can also be expressed by acute myeloid leukemia cells, which also produce VEGF, constituting autocrine and, via stimulation of production of IL-6 and GM-CSF, paracrine activation loops (Zhang et al. 2004). In addition to epithelium-derived solid tumors, for which IMC-1121 and IMC-1C11 are now in clinical phase I, all three antibodies have shown efficacy against leukemia cells in a murine *in vivo* model, probably by inhibition of VEGF-induced migration (Zhu et al. 2003). This study found a correlation between survival time of leukemia-inoculated mice and antibody affinity. IMC-2C6 is being investigated in a clinical phase I/II study.

Another anti-VEGF-R antibody, CDP791, has not been published in peer-reviewed journals yet, but is registered at the National Institutes of Health (NIH) in a phase II study for combination chemotherapy (carboplatin and paclitaxel) against non-small cell lung cancer (ClinicalTrials.gov ID: NCT00152477). It is noteworthy here because it is the only PEG-conjugated antibody in clinical trials the author is aware of. PEGylation has been successfully used to increase the circulating half-life and reduce the immunogenicity of foreign proteins for medical applications – mostly enzymes such as asparaginase, whose comparably small substrate can easily reach the catalytic region through the PEG meshwork. Protein–protein interactions such as antibody binding, however, are a different matter; it has been shown that PEGylation can be optimized so that binding activity is maintained, but immunogenicity diminished (Deckert et al. 2000).

12.3.1.3 Integrins

Integrins are cell surface receptors that mediate interactions of the cell with the extracellular matrix, which are responsible for cell attachment and spatial orientation, but also have crucial effects on growth, differentiation, and apoptosis pathways (Maile et al. 2006).

Vitronectin binding to integrin $\alpha_v\beta_3$ is necessary for the reaction of smooth muscle cells to insulin-like growth factor-I (IGF-I). Vitaxin, the humanized version of the LM609 antibody directed against a conformational epitope of integrin $\alpha_v\beta_3$, blocks this interaction and thus IGF-I-induced cell proliferation and angiogenesis. As this molecular target is expressed not only on endothelial cells, but also on breast cancer, Kaposi's sarcoma, melanoma, and other cancer cells, anti-integrin $\alpha_v\beta_3$ antibodies provide a two-sided method of tumor attack (Rader et al. 2002).

The fully human antibody CNTO 95 recognizes a common epitope of the α_v family of integrins. *In vitro*, this antibody inhibited melanoma cell adhesion, migration, and invasion, and substantially reduced human melanoma growth in

a murine *in vivo* xenograft model. Its specificity for human, but not mouse integrins also suggests additional efficacy independent of its antiangiogenic effect (Trikha et al. 2004). CNTO 95 is being investigated in a recently started phase I/II clinical trial for melanoma patients.

M200 (volociximab), an antibody directed against integrin $\alpha_5\beta_1$, has been reported to induce apoptosis in proliferating endothelial cells and to inhibit cancer cell growth *in vitro* (Bhaskar et al. 2004; Ramakrishnan et al. 2004), but no peer-reviewed clinical information on it has been published. Phase II clinical studies on its use in melanoma and renal cell carcinoma are currently recruiting patients.

12.3.1.4 Other Targets

In addition to the VEGF system and integrins, molecular targets exploited for antiangiogenic antibody therapy include the proangiogenic cytokine IL-8 (fully human antibody ABX-IL8) (Huang et al. 2002), the endothelial surface phospholipid phosphatidylserine (humanized antibody 3G4), which probably becomes exposed preferentially in tumor neovasculature due to oxidative stress (Ran et al. 2005), and structural extracellular matrix molecules such as collagen IV (humanized antibody HUIV26), which is exposed as an early event of vascular budding (Hangai et al. 2002), and fibronectin (BC1), the ligand of integrin $\alpha_5\beta_1$ (Ebbinghaus et al. 2004).

12.3.2 Growth and Differentiation Signaling

If neo-angiogenesis is a supporting prerequisite for tumor growth, aberrant signaling of cell cycle, differentiation, or apoptosis lies at its core. Hence, a main focus of cancer research in past decades has been the identification of these molecular signaling pathways and the development of pharmaceutical ways to influence them – mostly by blocking proliferation signaling. In this effort, ground-breaking small-molecule drugs such as imatinib and other tyrosine kinase inhibitors (-inibs) and monoclonal antibodies stand on a par (for a summary, see Table 12.4).

12.3.2.1 Epithelial Growth Factor Receptor

Many tumors overexpress EGFR (Her1), which enhances both tumor cell proliferation and neoangiogenesis and thus is associated with an unfavorable prognosis. The prototype of anti-EGFR antibodies is cetuximab, which has now been approved for the treatment of colorectal cancer in combination with irinotecan chemotherapy in the US, Europe, and many Asian countries. Its mechanism of action is complex, comprising immunologic activation and target-specific non-immunologic effects. Competing with both EGF and TGFα, thanks to its higher affinity it blocks both ligands from the receptor. This inhibits EGFR tyrosine kinase signaling, which in turn reduces cell proliferation as well as production of angiogenic factors, and sensitizes the cell to chemotherapy and radiation (Petit et al. 1997; Grunwald and Hidalgo 2003).

Table 12.4 Antibodies acting on targets of growth and differentiation signaling.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
225	Murine	EGFR	Melanoma, lung cancer (NSCLC)	I	–		Bristol Myers Squibb, New York, NY, USA	–	2002	Cetuximab predecessor, probably no longer pursued
425	Murine	EGFR	Head-and-neck, laryngeal, and papillary thyroid cancers	II	–		Fraunhofer IME, Aachen, Germany; Merck KGaA, Darmstadt, Germany	–	2004	
528	Murine	EGFR	Glioma, lung, skin, and vulvar cancers	I	–		Oncogene Science, Cambridge, MA, USA	–	2000	
806	Murine	EGFR v III	Glioma, head-and-neck, breast, lung, ovarian, colorectal, and prostatic cancers	I	–		Ludwig Institute for Cancer Research – New York, NY, USA and Melbourne, Australia	–	2005	
11F8 (IMC-11F8)	Recombinant Fab	EGFR	Epithelial cancers	I	–		Imclone Systems, New York, NY, USA	–	2005	
EMD72000 (matuzumab)	Humanized mAb 425	EGFR	Glioma, head-and-neck, laryngeal, and papillary thyroid cancers	II	NCT00215644 NCT00113581 NCT00111839	Recruiting	Merck KGaA, Darmstadt, Germany; EMD Pharmaceuticals, Durham, NC, USA	II	2005	

h-R3 (nimotuzumab)	Humanized version of murine IOR-EGF/R3	EGFR	Glioma, head-and-neck, breast,lung, and colon cancers	II	–		Centro Immunologia Molecular, Habana, Cuba	–	2005	
Humax-EGFR 2F8	Transgenic	EGFR	Head-and-neck cancer	I/II	NCT00093041	Closed 2005	Genmab, København, Denmark; Medarex, Princeton, NJ, USA	–	2005	
CR62	Rat	EGFR	Head-and-neck cancer	I	–		Institute of Cancer Research, Sutton, UK	I	1996/ 2005	
rHuMAb-EGFr (panitumumab, ABX-EGF)	Transgenic	EGFR	Nonsmall cell lung, renal, prostatic, colon, and other cancers	III	NCT00115765 NCT00101894 NCT00101907 . . .	Recruiting	Abgenix, Fremont, CA, USA	II	2004/ 2005	
rhuMab-2C4 (pertuzumab, Omnitarg)	Humanized	p185/HER-2	Breast, lung, ovarian, prostate	II	NCT00096993 NCT00263224	Recruiting	Genentech, South San Francisco, CA, USA	I	2005	
HGS-ETR1 (TRM-1)	Agonist	TRAIL receptor-1 peptides (= TRIAL-R1 or DR4)	Lymphoma (NHL), colon, breast, lung, myeloma	II	NCT00092924 NCT00094848	Closed 2004	Cambridge Antibody Technology, Cambridge, UK	–	2005	
HGS-ETR2 (TRM-2)	High-affinity agonist	TRAIL receptor-2 peptides (= TRIAL-R2 or DR5)	Non-Hodgkin's lymphoma	I	–		Cambridge Antibody Technology, Cambridge, UK	–	2005	
AMG-162 (denosumab)	Recombinant human	RANK ligand	Bone metastases	I/II	NCT00104650	Recruiting	Amgen, Thousand Oaks, CA, USA	–	2005	Clinical study published for osteoporosis

In addition, cetuximab triggers ADCC via its Fc fragment, which contributes significantly to, but is not a prerequisite for its antitumor efficacy, as $F(ab')_2$ fragments also inhibited tumor growth in mice (Aboud-Pirak et al. 1988).

As can be seen so far, these features are characteristic of most if not all anti-EGFR antibodies currently investigated.

Panitumumab (ABX-EGF, rHuMAb-EGF) is a fully human IgG2 antibody. Its efficacy depends on the EGFR density on tumor cells, so that it shows sizable effects with EGFR-overexpressing tumors such as colon cancer. In other aspects, too, it is very similar to cetuximab: it appears to have moderate efficacy as monotherapy in phase II with pretreated and refractory patients, leading to about 10% partial responses and 36% stabilized disease, and it is well tolerated, with reversible acneiform (90% of patients) and other skin symptoms as the most common adverse effects. Whether it also emulates cetuximab in the resensitization of the cancer to irinotecan therapy remains to be seen, but initial clinical data suggest a similar effect (Foon et al. 2004; Rowinsky et al. 2004).

The humanized IgG1 antibody matuzumab (EMD72000) also acts both via immunological and non-immunological mechanisms: while EGFR blockade is regarded its main mechanism of action, it is capable of initiating ADCC. Two clinical phase II trials were reported at the 2005 ASCO meeting, one including 37 heavily pretreated patients with platinum-resistant ovarian or primary peritoneal (Müllerian) malignancies, the other 38 patients with recurrent, platinum-resistent cervical cancer. The spectrum and intensity of adverse effects in both cases was similar to that known for the other anti-EGFR antibodies, except for apparently more marked hepatotoxicity, and one case each of potentially therapy-related pancreatitis. While 21% and 23% of patients in these progression-prone cohorts experienced stable disease, zero and two objective responses were observed in the ovarian cancer study (Seiden et al. 2005) and the cervical cancer study (Blohmer et al. 2005), respectively.

With comparable preclinical data demonstrating the same complex mechanisms of action, the fully human antibody Humax-EGFR (also, but not uniquely, termed 2F8) has entered phase I/II trials (Bleeker et al. 2004).

Another humanized IgG1 anti-EGFR antibody, h-R3 has been radiolabeled with 99m-technetium (^{99m}Tc) and examined in a diagnostic clinical phase I/II trial on 25 patients with suspected epithelial-derived tumours. The highest antibody deposition was seen in the liver (53% of injected dose), followed by the other parenchymous and then hollow abdominal organs. No relevant adverse effects were observed. Tumor localizations were detected with a reported sensitivity of 76.5% and a specificity of 100%.

12.3.2.2 **ErbB-2**

Signal transduction of ErbB-2 (Her-2) is blocked by the humanized version of monoclonal antibody 2C4, pertuzumab (rhuMab-2C4, Omnitarg), exploiting a different mechanism than the approved antibody trastuzumab (Herceptin).

ErbB receptors require dimerization to activate their tyrosine kinase. The ErbB-2 (Her-2) receptor, which does not have a ligand, can be activated either by over-

expression, leading to spontaneous homodimerization, or by heterodimerization with one of the three other erbB receptors upon their binding of ligand. While trastuzumab, besides other effects such as receptor downregulation and shedding inhibition, selectively inhibits ErbB-2 homodimer-induced tyrosine kinase activation, pertuzumab blocks ErbB-2 dimerization with any ErbB receptor (Badache and Hynes 2004). This recently described mechanism makes an obvious case for synergism of both antibodies, or pertuzumab with EGFR receptors, which has indeed been demonstrated *in vitro* (Nahta et al. 2004) and *in vivo*, respectively (Friess et al. 2005). A phase I study of pertuzumab found a favorable toxicity profile and pharmacokinetics allowing a 3-week dosage schedule. Out of 21 patients with progressive tumor disease resistant to chemotherapy or radiation, two experienced partial response and six stable disease for more than 2.5 months (Agus et al. 2005). A phase II double-blind randomized trial of gemcitabine with or without pertuzumab in patients with advanced ovarian, primary peritoneal, or fallopian tube cancer resistant to platinum-based chemotherapy is currently recruiting patients (ClinicalTrials.gov ID: NCT00096993).

12.3.2.3 **TRAIL Receptors**

An interesting approach to inducing proapoptotic rather than suppressing proliferation signals is followed by the fully human antibodies HGS-ETR1 (TRM-1) and HGS-ETR2 (TRM-2), which mimick the TNF-related apoptosis-inducing ligand (TRAIL) in binding the two apoptosis-inducing of its four receptors, TRAIL-R1 and TRAIL-R2, respectively (Younes et al. 2006). *In vitro* studies have shown that cell death is induced by either antibody as seen both in reduced cell survival and the activation of the intrinsic and extrinsic apoptosis pathways in a number of cell lines from different cancer entities, and *in vivo* efficacy against established tumors was demonstrated with colon, renal, and non-small cell lung cancer xenografts. Synergism of HGS-ETR1 with chemotherapy was demonstrated by increased chemosensitivity of cell lines resistant to the antibody alone, when treated with camptothecin, cisplatin, carboplatin, or 5-fluorouracil plus HGS-ETR1, and by increased sensitivity of colon cancer xenograft models treated with both modalities rather than either one alone (Pukac et al. 2005). Similar results have been obtained for both antibodies with primary lymphoma cells and lymphoma cell lines (Georgakis et al. 2005). Both antibodies are in clinical investigation, with a phase II study of HGS-ETR1 in non-small cell lung cancer only recently closed (ClinicalTrials.gov ID: NCT00092924), so that no clinical results are yet available.

12.3.2.4 **RANK-Ligand**

The recombinant fully human antibody denosumab (AMG 162) inhibits bone resorption by shifting the regulatory balance between the ligand of receptor activator of nuclear factor-κB (RANKL), a member of the TNF family, and its opponent, osteoprotegerin (OPG). In this balance, RANKL acts pro-osteoclastic by a number of mechanisms, while osteoprotegerin inhibits osteoclast proliferation and function by blocking RANKL. Denosumab mimicks the function of

osteoprotegerin, but with a longer half-life (Kostenuik 2005). So far, its main clinically investigated application has been the prevention or remediation of osteoporosis in postmenopausal women. Here, while clinical end-points or bone densitometry have not been evaluated, long-lasting reduction in bone turnover could be demonstrated by biochemical surrogate parameters, and the treatment was well tolerated with only temporary and mostly mild effects on calcium metabolism (Bekker et al. 2004). Given that osteoclast activation through cytokines plays a major role in the formation of bone metastases and osteolyses, this concept is of obvious interest for the treatment of many cancers. Hence, in the spring of 2005 a phase II study of denosumab has opened for patients treated with bisphosphonates for bone manifestations of cancer or myeloma (ClinicalTrials.gov ID: NCT00104650).

12.3.3 Pretargeting Strategies

Pretargeting strategies are two-step procedures using antibodies as antigen-specific "anchors," to which cytotoxic effectors bind in a second step, thus localizing their effect to tumor cells. This approach allows the combination of the specific, but slow localization of antibodies with the efficacy of faster to distribute, but more toxic effectors. In addition, it may allow multiple applications of the effector for the duration of antibody localization in the tumor tissue. To improve the desired pharmacokinetic differential, often a clearing agent removing unbound antibody – mostly another monoclonal antibody against the first one – needs to be applied to take full advantage of this approach. Two kinds of cytotoxic effectors are currently in clinical trials: radioisotopes and prodrugs (see Table 12.5).

12.3.3.1 Radioimmunotherapy

In radioimmunotherapeutic pretargeting, the radioisotope is usually bound to a protein or peptide. The peptide binds either to a peptide-specific idiotope of a bispecific antibody, or in biotin–avidin systems to avidin conjugated to the antibody. The radioactive isotope can thus be concentrated in the tumor without prolonged circulation (and thus exposure) of the rest of the body. A preclinical study illustrating the charm of this concept has been performed on a conjugate of IMMU-106 (anti-CD20, see below) with a murine antihistamine–succinyl–glycine (HSG) antibody and ^{111}In- or ^{90}Y-labeled HSG. Here, 10 times higher tumor-to-blood ratios were found, and the same maximum dose of HSG-radionuclide was reached at a much earlier time point compared with directly radiolabeled IMMU-106 (Sharkey et al. 2005b).

A streptavidin–biotin system was clinically tested with NR-LU-10/SA. The antibody NR-LU-10 recognizes a colon cancer-associated antigen. In a phase II study, the streptavidin conjugate was followed 48 h later by biotin–galactose–human serum albumin as a clearing agent. The radioisotope ^{90}Y bound to biotin via the linker tetra-azacyclododecanetetra-acetic acid (DOTA) was administered another 24 h later in a dose of 110 mCi m^{-2}. While there was considerable toxicity

Table 12.5 Pretargeting strategies.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
A5PC	Conjugate of murine A5B7 with carboxypeptidase G2	CEA	CEA-expressing epithelial tumors	I	–		Cancer Research UK Targeting and Imaging Group, UK	I	2002	
hMN-14 × m734	Colorectal, ovarian	CEA	CEA-expressing epithelial tumors	I	–		Nuclear Medicine Department, Rene Gauducheau Cancer Center, France	I	2003	
NR-LU-10/SA	Streptavidin conjugate, murine	EpCAM	Colon cancer	II	–		Stanford University, Stanford, CA, USA; University of Nebraska, Lincoln, NE, USA	II	2000	
96.5 (P96.5, 96.5-hCE2)	Irinotecan-activating ADEPT approach with carboxylesterase 2, murine	p97 melanotransferrin	Melanoma, glioma	I	–		Bristol Myers Squibb, New York, NY, USA	I	1985/ 2001	Past trials with 96.5 alone, current preclinical investigation into ADEPT

with grade 3 or 4 diarrhea experienced by about a third of patients and grade 3 or 4 hematologic toxicities by a cumulative 40%, and objective responses were seen in less than 10%, the feasibility of pretargeting radioimmunotherapy was demonstrated (Knox et al. 2000). A similar pretargeting system for yttrium-90, however, based on BC2, an anti-tenascin antibody in phase I/II clinical studies for glioblastoma, found a substantial survival advantage for treated over untreated patients (Grana et al. 2002). Another biotin-based approach to pretargeted radioimmunotherapy has been reported using an anti-TAG72 antibody fusion protein based on the CC49 antibody (Shen et al. 2005).

The bispecific antibody approach has been realized with hMN-14 × m734 and ^{131}I-labeled di-diethylenetriaminepentaacetic acid-indium. A clinical dose-optimization study highlights the difficulty of the exact dosing and timing necessary to apply such complex therapy regimens successfully. Radioactively trace-labeled antibody was first administered in dose escalation and repeated dose adjustments to measure its tumor uptake, then the uptake of a fixed dose of the therapeutic radioconjugate was monitored. The antibody dose generating the highest uptake also led to considerable toxicity. Reducing the antibody dose by about half, however, optimized the blood clearance of the radioconjugate so that good tumor accretion was achieved with more acceptable toxicity. (Kraeber-Bodere et al. 2003).

12.3.3.2 **Antibody-directed Enzyme Prodrug Therapy**

In antibody-directed enzyme prodrug therapy (ADEPT), the targeting construct is an antibody–enzyme fusion protein or conjugate. After its tumor localization and clearance from the bloodstream, an inert prodrug is administered which is then selectively cleaved by the enzyme component to deliver a toxic chemotherapeutic agent. A number of clinical studies have been performed which in aggregate demonstrate the feasibility of clinical ADEPT (Martin et al. 1997; Francis et al. 2002). While the general problem of antibody immunogenicity preventing repeated therapy cycles is shared by this approach, recombinant humanization could so far not solve, but only alleviate it for ADEPT: the enzyme must be essentially non-human, otherwise enzymatic prodrug activation would occur "wild" and no longer be controlled by the antibody construct.

Nevertheless, the elucidation of the catalytic regions of enzymes and their three-dimensional structures on the one hand and the identification of particularly immunogenic protein motifs on the other has spurned a similar deimmunization development for enzymes as seen before with the chimerization and then humanization of antibodies (Mayer et al. 2004).

Of the clinical studies published so far, a recent one focused on the need for a clearing agent. A5CP, a galactosylated conjugate of a murine F(ab′)$_2$ fragment of A5B7 (an anti-CEA antibody) and carboxypeptidase G2 had been previously tested in a clinical trial, but showed low efficacy due to prolonged antibody conjugate circulation. Thus, a new prodrug bis-iodo phenol mustard (ZD2767P) was designed to avoid the necessity of a clearing antibody by means of its high potency and short half-life. However, although the regimen was comparably well tolerated, no clinical or radiological responses were seen, and a closer look at antibody–

enzyme conjugate localization revealed inadequate tumor localization (Francis et al. 2002), stressing once more the need not only for more potent drug–prodrug systems, but in particular for antibody–enzyme constructs with better tumor penetration and affinity. One current approach to this is the design of single-chain fragment-based recombinant antibody–enzyme fusion proteins (Svensson et al. 1992; Siemers et al. 1997; Deckert et al. 2003).

12.3.4 Immune Signaling

Strictly speaking, the classical immunological effect of antibody therapy via Fc-mediated ADCC and CDC is in itself a form of targeted immune signaling. For the purposes of this section, however, this term addresses antibodies whose cognate antigens are receptors or ligands of the immune system (see Table 12.6). As is so often the case, a strict line cannot be drawn without force, as the first example will show.

12.3.4.1 Cytokines and Cytokine Receptors

Interleukin-2 receptor α The high-affinity receptor for IL-2, IL-2 receptor α, also termed CD25 or Tac, is the target of anti-Tac-H or daclizumab (Zenapax). Activation of the IL-2 receptor plays a central role in the activation of specific or acquired immunity in general and in several forms of autoimmune reactions. Hence, as a functional antibody that blocks immune signaling, daclizumab is applied in autoimmune conditions and for immunosuppression (it was originally approved in 1997 for use in kidney transplantation). Its role in oncology, however, appears to be more that of a conventional tumor-targeting antibody, which it plays on the predominantly CD25-positive cells of adult T-cell leukemia/lymphoma (ATL), hairy cell leukemia (HCL), cutaneous T-cell lymphoma (CTCL), chronic lymphocytic leukemia (CLL), Hodgkin's disease, non-Hodgkin's lymphoma (NHL), and other lymphoid leukemias or lymphomas. This is not true without exception, though: in their very early stages, malignant cells may grow IL-2-dependent, and thus this principle may be important e.g. in minimal residual disease (Staak et al. 2004).

In murine models, the efficacy of daclizumab in the treatment of hematologic malignancies has been demonstrated. Currently, this humanized antibody is in clinical studies for patients with bone marrow failure, Hodgkin's disease, non-Hodgkin's lymphoma, and lymphoid leukemia (NCT00001962, NCT00001575, NCT00001941).

Interleukin 6 Interleukin 6 (IL-6) is an important growth and survival factor in several cancers, contributing to drug resistance, cachexia and formation of bone metastases. A disease in which its role has been well characterized is multiple myeloma. Elsilimomab (B-E8) is a murine anti-IL-6 antibody and has been given in parallel with chemotherapy and autologous stem cell rescue in this disease.

Table 12.6 Antibodies acting on immune signaling.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
Anti-Tac-H (daclizumab, Zenapax)	Humanized version of murine 1H4	CD25, IL-2 receptor	T-cell lymphoma, leukemia	I/II	NCT00001962 NCT00001575 NCT00001941	Recruiting	Protein Design Labs, Fremont, CA, USA; Stanford University, Stanford, CA, USA	I	2003/ 2005	Approved for transplant therapy
B-E8 (elsilimomab)	Murine	IL-6	Lymphoma (NHL), myeloma	I/II	–		Diaclone, Besançon, France	I/II	2005	
CNTO 328 (cCLB8)	Chimeric from murine, blocker of amyloid A	IL-6	Cancer cachexia, renal	I/II	NCT00265135	Recruiting	Centocor, Horsham, PA, USA	I/II	2005	Clinical study: abstract
Humax-Lymphoma	Transgenic	IL-15 receptor	Lymphoma, myeloma	II	–		Amgen, Thousand Oaks, CA, USA; Genmab, København, Denmark	–	–	No peer-reviewed publ.
PM-1 (rsHu-PM1, atlizumab, tocilizumab)	Humanized version of murine PM-1	IL-6 receptor	Multiple myeloma	II	–		Chugai Pharmaceuticals Co. Ltd, Tokyo, Japan	–	2003	
G5/44	Human	CD22	B-cell lymphoma (NHL)	I	–		Wyeth, Madison, NJ, USA	–	2005	
HD37	Murine	CD19	B-cell lymphoma (NHL)	I	–		Deutsches Krebsforschungszentrum, Heidelberg, Germany	I	2000	

hLL2 (epratuzumab, LymphoCide)	Humanized version of LL2	CD22	B-lymphocytic non-Hodgkin's lymphoma (NHL)	III	NCT00113802 NCT00113971 NCT00098839 NCT00111306	Recruiting	Immunomedics, Morris Plains, NJ, USA	I	2005	
Humax-CD20	Transgenic	–	Lymphoma (NHL)	I/II	–		Genmab, København, Denmark	–	–	
IDEC-152 (lumiliximab)	Primatized chimeric	CD23	Leukemia (CLL)	I	NCT00103558	Recruiting	Biogen IDEC, Cambridge, MA, USA; Seikagaku Corp., Tokyo, Japan	I	2004	IgE Fc receptor
IMMU-106	Humanized, identical backbone with hLL2	CD20	Lymphoma	I/II	–		Immunomedics, Morris Plains, NJ, USA	–	2005	
LL2 bectumomab	$F(ab')_2$, murine	CD22	B-cell lymphoma (NHL)	III	–		Immunomedics, Morris Plains, NJ, USA	I	2002/ 2003	
Lym-1	Murine	HLA-DR	B-cell lymphoma (NHL), leukemia	II/III	NCT00008021 NCT00009776 NCT00028613	Closed	University of Southern California, Los Angeles, CA, USA; Schering AG, Berlin, Germany	I/II	1999	RIT, CIT
MB-1	Murine	CD37	B-cell lymphoma (NHL)	I	–		Stanford University, Stanford, CA, USA	I	1997	
TACI-Ig	Recombinant fusion protein targeting TNF receptors (BlyS, APRIL)	–	B-cell leukemia (B-cellular autoimmune diseases)	I	–		Zymogenetics, Seattle, WA, USA	–	2005	Soluble TACI receptor

Table 12.6 *(Continued)*

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
Ber-Act8	Murine	CD103	T lymphocytes: hairy cell leukemia	I	–		Freie Universität Berlin, Berlin, Germany	–	1997	
CP-675,206	Transgenic	CTLA-4	T lymphocytes various cancers	I	NCT00090896 NCT00075192	Recruiting	Abgenix, Fremont, CA, USA	I	2005	
Humax-CD4 (zanolimumab)	Transgenic	CD4	Lymphoma	II	NCT00127881	Recruiting	Medarex, Princeton, NJ, USA; Genmab, København, Denmark	II	2004/ 2005	
IDEC-114 galiximab	Primatized chimeric	CD80	T-cell lymphoma (NHL)	II	NCT00117975	Recruiting	Biogen IDEC, Cambridge, MA, USA	I/II	2005	
MDX-010	Transgenic	CTLA-4	T lymphocytes: melanoma, prostatic cancer	III	NCT00094653 NCT00108888	Recruiting	Medarex, Princeton, NJ, USA	I	2005	(>10 studies)
MEDI-507 (siplizumab)	Humanized from rat	CD2	Lymphoma	II	NCT00071825 NCT00105313 NCT00123942 NCT00063817 NCT00113646	Recruiting	Medimmune, Gaithersburg, MD, USA	I	2005	
OKT3	Murine	CD3	Colorectal, melanoma, ovarian, renal, leukemia	I	NCT00091611 NCT00001832 NCT00080353	Recruiting	Ortho Biotech, Bridgewater, NJ, USA	I	2005	Approved
T101	Fab, murine	CD5	T-cell leukemia (CLL), lymphoma	I/II	–		Scripps Research Institute, La Jolla, CA, USA	I	1998/ 1999	RIT

Visilizumab (HuM291, Nuvion)	Humanized, competes with OKT3	CD3	T-cell leukemia, T-cellular lymphoma, myelodysplastic syndrome, transplant rejection	II	–		Protein Design Labs, Fremont, CA, USA	I/II	2005	Immunosuppressant, studied in GvHD, not leukemia itself
1D10 (apolizumab, Remitogen)	Humanized from murine 1D10 (IgG2a)	28/32 (HLA-DR)	B- and T-cell lymphoma (NHL), leukemia	II	NCT00089154 NCT00022971	Recruiting	Protein Design Labs, Fremont, CA, USA	II	2001	Phase II: abstract only
BC8	Murine	CD45	Leukemia	I	–		Fred Hutchinson Cancer Center, Seattle, WA, USA	I	1999/ 2004	
M195	Murine	CD33	leukemia (AML)	I/II	NCT00014495 NCT00016159	Recruiting	Memorial Sloan-Kettering Cancer Center, New York, NY, USA	I	1995	RIT
MDX-33 (H22)	Humanized M22	CD64 (Fc gamma receptor I)	Various cancers, idiopathic thrombocytopenic purpura	II	–		Medarex, Princeton, NJ, USA	II	2000/ 2003	Clinical study: abstract
MN-3 (lamelesomab, sulesomab)	Fab′ fragment, murine	NCA-90 (CD66)	Granulocytes: osteomyelitis, bone metastasis	II	–		Immunomedics, Morris Plains, NJ, USA	–	2005	
SGN-40 PRO64553	Humanized from murine S2C6	CD40	Leukemia, multiple myeloma	I	NCT00079716 NCT00103779	Recruiting	Genentech, South San Francisco, CA, USA	I	2005	
YTH54.12	Rat	CD45	Leukemia	I	–		Addenbrooks Hospital and Cantab Research Ltd.,	–	2003	
791T/36	Murine	CD55 (gp72)	Osteosarcoma, colorectal cancer	I	–		Cambridge, UK Cancer Research UK, London, UK	–	2001	
SC-1	Heterohybridoma	CD55/SC-1	Gastric	I/II	–		Universität Würzburg, Würzburg, Germany	I	2000/ 2004	

RIT, radioimmunotherapy; CIT, chemoimmunotherapy; GvHD, graft versus host disease.

Evaluation of C-reactive protein production showed neutralization of IL-6 activity, and mucositis and fever were significantly reduced. Moreover, a median event-free survival of 35 months and an overall survival of 68.2% at 5 years were observed in this uncontrolled trial (Rossi et al. 2005). Another study focused on cachexia in multiple myeloma patients investigating CNTO 328, a chimeric anti-IL-6 antibody. A phase I study demonstrated good tolerability and a half-life of approximately 17 days, making it applicable in prolonged dosage intervals. Preclinical data indicated that IL-6 blockade may counteract cachexia (Zaki et al. 2004; Mulders et al. 2005).

12.3.4.2 **B-cell Signaling**

Judging from preclinical data, the humanized anti-CD20 antibody IMMU-106 appears to resemble the approved chimeric anti-CD20 antibody rituximab in almost every respect. Due to its completely human framework, it is expected to reveal improved pharmacokinetics, tolerability, and efficacy when applied to humans. Clinical trials have reportedly commenced, but no results are published as of this writing (Stein et al. 2004).

In response to anti-CD20 treatment, non-Hodgkin's lymphoma cells have been shown to increase their expression of CD22. Hence it has been suggested that IMMU-106 treatment should be combined with the humanized anti-CD22 antibody epratuzumab (hLL2). Epratuzumab has been examined in a phase I radioimmunotherapy trial of Re-186–epratuzumab with 15 patients, of whom five reached objective responses with only mild or moderate toxicity (Postema et al. 2003), and a phase I/II immunotherapy trial in non-Hodgkin's lymphoma patients revealed objective responses in about 10% of patients, one of whom was refractory to rituximab, without dose-limiting toxicity. This antibody is now in phase I or II studies for Waldenström's disease (NCT00113802), relapsed acute lymphoblastic leukemia (NCT00098839), and systemic lupus erythematodes (NCT00113971, NCT00111306).

12.3.4.3 **T-cell Signaling**

The anti-CD2 antibody siplizumab (MEDI-507) is designed to achieve T cell-depletion in various clinical settings (Dey et al. 2005). It is currently in clinical phase I for T-cellular lymphoproliferative disease (NCT00071825), for CD2-positive lymphoma/leukemia (NCT00105313, NCT00123942), as an immunosupressant in kidney and bone marrow transplantation (NCT00063817), and for *ex vivo* T-cell depletion (NCT00113646).

Humax CD4 or zanolimumab is a transgenic fully human antibody from transgenic mice, expressed in CHO cells. It prevents T-cell activation by blocking CD4 interaction with MHC-II (Skov et al. 2003). Mainly developed for psoriasis vulgaris and rheumatoid arthritis, it has been tested clinically in cutaneous T-cell lymphoma (Kim et al. 2004) with encouraging results, and a case report has been published on angioimmunoblastic T-cell lymphoma (Hagberg et al. 2005).

CD80, also named B7 or BB1, a member of the immunoglobulin superfamily that is involved in the alloactivation of T cells and plays a critical role in autoim-

mune, humoral, and transplant responses, is the cognate antigen of galiximab (IDEC-114). Apart from autoimmune diseases such as psoriasis (Gottlieb et al. 2002), it has been investigated clinically in patients with relapsed or refractory follicular lymphoma, most of stage III or IV disease, by weekly infusions. The therapy was well tolerated without dose-limiting toxicities. A response rate of 11% was reported, interestingly with a time to best response of up to 12 months, which makes a causal assignment difficult, but two responders were reported progression-free at 22 and 24.4 months (Czuczman et al. 2005).

12.3.4.4 **Other Lymphocyte Signals**

The anti-CD40 antibody SGN-40 is a humanized IgG1 antibody in phase I for the treatment of multiple myeloma that has also shown antilymphoma activity against other B-lineage lymphomas preclinically (Law et al. 2005). Further elucidating its mechanism of action, blockade of sCD40L-mediated phosphatidylinositol 3′-kinase and nuclear factor kappa B activation was demonstrated in myeloma cells, and proliferation triggered by IL-6 was inhibited (Tai et al. 2004). Clinical phase I trials on its use for multiple myeloma (NCT00079716) and non-Hodgkin's lymphoma (NCT00103779) are under way.

CTLA-4 has been implicated in peripheral immunologic tolerance, so that its blocking may lead to effective anticancer responses. The fully human MDX-010 has already been investigated in renal cancer (Yang et al. 2005) and in high-risk resected stage III and IV melanoma in combination with gp100, MART-1, and tyrosinase vaccines. Apparently, development of autoimmune responses correlated with a better clinical outcome (Sanderson et al. 2005). Similar results were found with CP675,206, also a fully human anti-CTLA4 monoclonal antibody (Ribas et al. 2005).

SC-1 is a human monoclonal antibody found to bind a stomach carcinoma-associated isoform of CD55 or decay-accelerating factor B. In a clinical phase I trial, a significant induction of apoptotic activity was reported in 90% of the cases, and a significant regression of tumor mass in 50% of the patients. The authors suggest this antibody for adjuvant therapy (Vollmers et al. 2000).

12.3.4.5 **The Cautionary Tale of TGN1412**

TGN1412, a T cell-activating antibody, made sad headlines in early 2006 because of an utterly failed phase I clinical trial. Since then "the strange case of TGN1412" has been thoroughly examined and reviewed (Farzaneh et al. 2006; Hansen and Leslie 2006; Schneider et al. 2006; Bhogal and Combes 2006a,b). Briefly, six healthy volunteers who received the antibody rather than placebo experienced a cytokine storm disease within minutes to hours after application, leading to multiorgan failure and prolonged intensive care in all of them. Although as of the writing of this chapter, all six have survived and left the hospital, albeit with serious sequelae, this event has raised general questions about the conduct of clinical studies and the secrecy in which the generated data are kept. Thus, although no regulations had been broken, with hindsight it appears absurd to apply the experimental drug to all six volunteers almost simultaneously, and the

future development of similar drugs trying to avoid the risk of TGN1412 will be hindered by the fact that the data continue to be kept confidential.

While these aspects are independent of antibodies and T-cell stimulatory drugs, the more specific lessons from this trial are more complicated. The target of TGN1412, CD28, acts as a costimulatory signal in T cell activation: upon binding of the antigen-laden MHC-II complex to the T-cell receptor, the intracellular signal cascades for cytokine production and release as well as proliferation are actually released by binding of B7 to CD28. However, both receptors can be "superstimulated," thus bypassing the need for the other signal to activate the T cell. TGN1412 acts as such a superagonist on CD28.

At least two mechanisms normally act to counterbalance and regulate T-cell activation by CD28. First, there is a dose-dependent activation of regulatory T cells, which are preferentially activated at lower anti-CD28 doses in experimental models, thus suppressing an autoreactive T-cell response. In addition, the natural CD28 ligand B7 has a regulatory function in that it is also a ligand for CTLA-4, which is expressed by activated T lymphocytes and promotes apoptosis or anergy. Both mechanisms were outrun by the nonlocalized, systemic superactivation of CD28.

In addition, this cautionary tale highlights species specificity as another problem of drug development in general, exacerbated in antibody therapeutics. The distribution and quantitative reaction patterns of a human receptor molecule and its, say, avian homolog may very well differ substantially. This is also true for the targets of small molecule drugs. In antibodies, however, their species-specific sequence and structure add another twist to this conundrum: even with identical target function, a human (or humanized) antibody administered in a macaque may not reveal all its beneficial or adverse effects because of its immunogenicity in the animal. The other way round, using a nonhumanized version for preclinical tests may obscure molecular effects unique to the human sequence.

Thus, preclinical animal testing will continue to have its intrinsic limitations, and the first application of any new drug in a human being will always carry a risk that can at best be minimized, but never avoided. Which speaks against having six first human beings at once.

12.3.5 Anti-Idiotype Vaccines

If the tumor vaccines are still regarded as highly experimental by many, anti-idiotype vaccine concepts may at first glance appear to be outright esoteric. They relate to Niels K. Jerne's concept of idiotype networks and the observation that the idiotope of an antibody may itself be another antibody's cognate antigen. Thus, antibody 1 may mimick a tumor antigen and thus induce an anti-idiotypic antibody 2 that not only binds antibody 1, but also cross-reacts with the tumor antigen. Taken one step further, another antibody 1 may bind to a tumor antigen, leading to induction of a tumor-mimicking anti-idiotypic antibody 2 – a so-called internal image antibody of the tumor antigen – which in turn induces an anti-

anti-idiotypic antibody 3, that recognizes again antibody 2 and the tumor antigen. The advantages of this approach are the potentially precise definition of the antigen used as vaccine, the possibility of vaccinating against antigens not yet fully characterized or sequenced, and the general advantage of active vaccination: a potentially sustained response of the host.

105AD7 is an example of both approaches characterized above, divided between an original recipient of antibody 1 who generated antibody 2, and the prospective recipients of this antibody 2, who may generate the antitumor antibody 3: the heterohybridoma antibody 105AD7 was originally isolated from a colorectal cancer patient receiving the antitumor antibody 791T/36. This antibody's binding site has been identified as a defined part of the complement regulatory protein CD55. Thus, 105AD7 is a spontaneous CD55-mimicking anti-idiotype to 791T/36. Indeed, amino acid and structural homology between the CDRs of 105AD7 and the "mimicked" regions of CD55 has been demonstrated (Spendlove et al. 2000). This vaccine has been tested in several clinical trials. Phase I studies confirmed that the antibody is well tolerated and that it generated T-cell responses. That result encouraged a prospective, randomized, double-blind, placebo-controlled phase II survival study of 162 patients with advanced colorectal cancer. The result was a nonsignificant survival disadvantage for the vaccine patients, which the authors attribute to high tumor burden or poor patient compliance (only 50% of patients completed at least three immunizations) (Maxwell-Armstrong et al. 2001).

Another study looked at myelosuppressively pretreated young patients with osteosarcoma, finding a mixed picture as to immune responses, but, remarkably, two patients with possible clinical responses who were allowed to continue immunization for 2 years had remained disease free for around 6 years at the time of reporting (Pritchard-Jones et al. 2005).

Clinical anti-idiotype vaccination trials have been reported on with several antibodies against mucins (see also Section 12.4.4), such as pemtumomab (HMFG1), an antibody against mucin 1 (Nicholson et al. 2004), and the anti-mucin 16 antibodies ACA125 (Reinartz et al. 2004) and oregovomab (B43.13) (Schultes et al. 1998). All have also been used in nonvaccination approaches, and the difference between the two kinds of approaches lies in the repeated administration of the antibody with an adjuvant that enhances the specific immune response. With all three, the induction of anti-idiotypic networks has been observed and partly described in great detail, the therapies were well tolerated, but clinical responses were rare at best. Thus, some authors suggest alternative delivery systems that may improve specific immunogenicity (Hann et al. 2005). Anti-idiotype vaccine strategies are summarized in Table 12.7.

12.4 Antigens Without Known Effector Function

Regardless of the advances in functional tumor targeting outlined so far, antigens selected only for their cancer specificity as "dumb" targets continue to play an

Table 12.7 Anti-idiotype vaccines.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
105AD7 (Onyvax-105)	Heterohybridoma, p72/CD55 image	mAb 791T/36	Osteosarcoma, colorectal carcinoma	I/II	NCT00007826	Closed 2003	Cancer Research UK, London, UK	I/II	2005	
11D10 (TriAb)	HMFG image, murine	mAb BrE1	Breast, ovarian cancers	II	NCT00006470 NCT00033748 NCT00045617	Closed 2004	Titan Pharmaceuticals, South San Francisco, CA, USA	I	2000	Abstract only
1A7 (TriGem)	Image of ganglioside GD2, murine	mAb 14.G2a	Melanoma, neuroblastoma, sarcoma, small cell lung cancers	II	NCT00045617	Closed 2005	Titan Pharmaceuticals, South San Francisco, CA, USA	I/II	1998	
1E10	Image of ganglioside GM3, murine	mAb P3	Melanoma	I/II	–		Centro Immunologia Molecular, Habana, Cuba	–	2003	
3H1 (CeaVac)	CEA image, murine	mAb 8019	Colon cancer	II/III	NCT00006470 NCT00033748	Closed 2004	Roswell Park Memorial Institute, Buffalo, USA	–	2004	Combination vaccine with 11D10
ACA125	CA125 image, murine	mAb OC125, CA125 anti-id	Ovarian, breast cancers	I/II	NCT00103545 NCT00058435	Closed	Cell Control Biomedical, Martinsried, Germany	II	2005	

Mitumomab (BEC-2)	Image of ganglioside GD3, murine	mAb R24	Melanoma, small cell lung cancer	III	NCT00037713 NCT00003279 NCT00006352	Closed 2001	Memorial Sloan-Kettering Cancer Center, New York, NY, USA	–	2004	
BR3E4	Image of mAb CO17-1A, rat	mAb 17-1A	Colon cancer	I	–		Wistar Institute, Philadelphia, PA, USA	I	2003	
I-Mel(pg)-2 MEL-2	Murine	mAb MEM-136	Melanoma	I/II	–		Biogen IDEC, Cambridge, MA,	I	1998/ 2004	
IGN101	Murine mAb 17-1A adsorbed on aluminium hydroxide as adjuvant	EpCAM	Nonsmall cell lung cancer	II/III	–	Closed 2005	Igeneon, Vienna, Austria	–	–	Anti-anti-idiotype vaccine. Company information only.
MF11-30	Murine	mAb 225.28	Melanoma	I/II	–		Biogen IDEC, Cambridge, MA, USA	I	1990/ 2004	
MK2-23	Image of HMW-MAA, murine	mAb 763.74	Melanoma	I	–		New York Medical College, Valhalla, NY, USA	–	2005	

important role in the development of new antibody-based cancer therapies. They are introduced here in an attempt to categorize them. The author is aware of two caveats: this categorization is not possible without inconsistencies, and today's "dumb" target may be tomorrow's functional trigger, as happened in the past and will be pointed out where appropriate.

12.4.1 Adhesion Molecules

Adhesion molecules comprise a heterogeneous group of proteins involved in mechanical cell adhesion, but also in cell–cell interaction and signaling. Antibodies against adhesion molecules are listed in Table 12.8.

12.4.1.1 EpCAM

The epithlial cell adhesion molecule (EpCAM, also termed GA733-2, KSA, 17-1A antigen) is a cell surface glycoprotein expressed by the majority of epithelial malignancies. The antigen had been implicated in mechanisms allowing tumors to escape immune surveillance (Armstrong and Eck 2003), but the suggested underlying ligand interaction, intriguing though it was, turned out to be an artifact (Nechansky and Kircheis 2005). Recently, EpCAM has been found to colocalize with tight-junction proteins and to form complexes with CD44v and tetraspanins, which may play a role in apoptosis resistance (Ladwein et al. 2005). This finding may finally give a clue to its function and overexpression in carcinomas.

Antibodies directed against EpCAM are supposed to act by ADCC and CDC, and anti-idiotype network activity has been hypothesized.

Edrecolomab (17-1A, Panorex) was the first anti-EpCAM antibody, defining the antigen. It was also the first antibody approved for treatment of colorectal cancer (in Germany only), and probably the first approved antibody in clinical use to be withdrawn from the market. Its story is emblematic for the pitfalls on the way from an anticancer antibody to an approved drug (and back).

A chimeric version of the murine IgG2a antibody has been produced, and two pharmacokinetic clinical studies were published in the early 1990s (Trang et al. 1990; Meredith et al. 1991).

With the murine original, a number of clinical studies have been conducted, proving good tolerability, with anaphylactic reactions to the murine protein as the most critical ones. Adjuvant therapy in patients with resected Dukes' stage C colorectal cancer and minimal residual disease was found to improve survival and reduce tumor recurrence significantly. According to the pivotal study by Riethmueller et al., conducted on 189 patients, adjuvant edrecolomab treatment reduced the risk of distant metastases by 32% over a follow-up period of 7 years (Riethmuller et al. 1998). While the differences seen in this study were significant, their power was actually small, with broad overlap of the 95% confidence intervals.

Since then, a number of clinical trials in advanced stages of colorectal or pancreatic cancer found limited efficacy at best for antibody monotherapy or

Table 12.8 Antibodies targeting adhesion molecules.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
17-1A (CO17-1A edrecolomab Panorex)	Murine	EpCAM	Breast, lung, gastric, colorectal cancers	II/III	NCT00002968 NCT00002664	Closed 2001	Wistar Institute, Philadelphia, PA, USA	III	2005	
17-1A chimeric	Chimeric from murine	EpCAM	Gastric, colorectal cancers	I	–		Centocor, Horsham, PA, USA	I	1991/ 1996	
323/A3	Murine	EpCAM	Breast, ovarian, and prostatic cancers	I/II	–		Centocor, Horsham, PA, USA	–	2003	
3622W94	Humanized	EpCAM	Prostatic cancer	I/II	–		Glaxo-SmithKline, Brentford, UK	–	1999	
GA73.3 (GA733-2)	Murine	EpCAM	Gastric, colorectal cancers	I	–		Wistar Institute, Philadelphia, PA, USA	–	2002	
ING-1	Recombinant chimeric	EpCAM	Breast cancer	II	NCT00051675	Closed 2003	Xoma LLC, Berkeley, CA, USA	I	2005	
MT201 adecatumumab	Recombinant ("human engineered")	EpCAM	Breast, prostatic cancers	II	–		Micromet AG, München, Germany	I	2004/ 2005	
A33	Murine	gpA33	Pancreatic and colorectal cancers	I/II	–		Ludwig Institute for Cancer Research, New York, NY, USA; Memorial Sloan-Kettering Cancer Center, New York, NY, USA	I/II	1996/ 2005	

Table 12.8 *(Continued)*

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
hu-A33	Humanized	gpA33	Gastric, colorectal cancers	I	NCT00199797 NCT00003360 NCT00003543 NCT00199862	Recruiting	Ludwig Institute for Cancer Research, New York, NY, USA; Memorial Sloan-Kettering Cancer Center, New York, NY, USA	I	2005	RIT, CIT
BIWA-1	Murine	CD44v6	Head-and-neck, lung, breast cancers, multiple myeloma	I	–		Boehringer Ingelheim, Ingelheim, Germany	I	2000/2002	
BIWA-4 (bivatuzumab)	Humanized	CD44v6	Head-and-neck, lung, breast cancers, multiple myeloma	I	–		Boehringer Ingelheim, Ingelheim, Germany	I	2004/2005	
E48	$F(ab')_2$, murine	E48 (GPI-linked Ly-6 antigen)	Head and neck, vulvar cancers	I/II	–		Vrije Universiteit Medisch Centrum, Amsterdam, Netherlands	I	1997/2000	
ERIC-1	Murine	CD56 (NCAM)	NK cells: retinoblastoma, glioma	I	–		University of Bristol, Bristol, UK	–	2001	

FC-2.15	Murine	CD15 (Le-x), Ag2.15	Leukemia (AML), Hodgkin's lymphoma, breast and colorectal cancers	I	–	Instituto de Investigaciones Bioquimicas, Fundación Campomar, Buenos Aires, Argentina	I	1995/ 1999	
G-22	Murine	CD44 spliced form	Glioma, melanoma, lung cancer	I	–	Nagoya University, Nagoya, Japan	–	1997	
Leu-M1	Murine	CD15 (Le-x)	Reed–Sternberg cells: Hodgkin's lymphoma, renal cell carcinoma	I	–	BD Biosciences, San Jose, CA, USA	–	1998	
MOC-1	Murine	NCAM	Neuroectodermal mesothelioma	I	–	Rijksuniversiteit Groningen, Groningen, Netherlands	–	1999	Diagnostic clinical studies
hu-N901	Humanized	CD56 (NCAM)	NK-cells: neuroblastoma, lung cancer (SCLC)	II	–	Biogen IDEC, Cambridge, MA, USA	I	1997/ 2005	
RS7-3G11	Humanized	EGP-1 (GA733-1)	Lung (NSCLC), breast, gastric, ovarian, prostatic, bladder cancer, etc.	I	–	Immunomedics, Morris Plains, NJ, USA	–	2001	

combination regimens (Adkins and Spencer 1998). Combining the antibody with the cytokines GM-CSF and IL-2 showed no objective responses (Fiedler et al. 2001); another study combining edrecolomab with the oral fluoropyrimidine capecitabine (Xeloda) saw one complete and two partial remissions among 27 patients treated (Makower et al. 2003).

The relative clinical ineffectiveness of edrecolomab had become a given with the advent of new small-molecule drugs such as irinotecan and oxaliplatin, when one study found an increase in tumor-protective cytokines and immune reactivity in patients treated with edrecolomab compared with standard therapy only, leading the authors to conclude that edrecolomab could be helpful in postoperative restoration of immune function (Tsavaris et al. 2004).

A recent phase III study looked again at the adjuvant setting in advanced nonmetastasized (UICC stage II or Dukes' stage B) colon cancer. A total of 377 patients were stratified according to tumor extension (T3 or T4) after resection and randomly assigned either to edrecolomab or observation. No significant difference in overall and disease-free survival was found after a median follow-up of 42 months (Hartung et al. 2005). This study, together with the body of existing evidence, overturned the very rationale on which edrecolomab had been approved in Germany, albeit to no regulatory consequences: this study itself had to be terminated in 2000 because edrecolomab was withdrawn from the market and is no longer produced.

In its time, however, the pivotal study by Riethmüller et al. was state of the art, and in a way, edrecolomab was a pioneer that paved the way for the successful modern antibodies bevacizumab and cetuximab now approved for colon cancer therapy.

A number of other anti-EpCAM antibodies are or have been in clinical studies, many of which appear to have gone "silent" in the literature during the past 5 years. Of two recombinant human antibodies, ING-1 and MT201, early clinical results have recently been presented at meetings. For both of these, good tolerability with only mild toxicity, preliminary evidence of tumor localization, comparably low or absent human anti-human antibody (HAHA) immunogenicity, and no objective responses were reported (Naundorf et al. 2002; Peters et al. 2004; de Bono et al. 2004; Kiner-Strachan et al. 2005). Whether these antibodies will open new clinical potential for EpCAM targeting remains to be seen.

12.4.1.2 **gpA33**

The history of antibodies against the A33 antigen (now termed gpA33) is another – and not yet decided – example of the complications encountered on the way from a tumor-selective antibody to controlled studies and a working drug. A33 antigen has been sequenced and identified as a member of the immunglobulin superfamily (Heath et al. 1997). It is now accepted as an adhesion molecule, although details of its function and possible natural ligand are still unclear. The antigen, recently termed gpA33, is expressed by gastrointestinal epithelia including pancreas and by more than 95% of colon cancers. The first clinical phase I/II radioimmunotherapy trials commenced in the early 1990s. Interestingly, in a trial

with ^{131}I-A33, there was little or no bowel toxicity despite expression of the antigen in normal gastrointestinal tissue. Radioimmunoscintigrams confirmed the localization of the radioisotope to almost all known primary or metastatic tumor sites. Furthermore, while there was initial uptake in normal bowel, time-dependent specificity became visible with a maximum at about 2 weeks after injection, when normal tissue had almost completely cleared the antibody, while tumor sites still showed intensive activity (Welt et al. 1994). Another study with ^{125}I-labeled A33 found modest antitumor activity with improved tolerability in the absence of gastrointestinal or hematological toxicity (Welt et al. 1996). The controlled studies encouraged by these results had to wait for the CDR-grafted humanized version. Even with this huA33, however, immunogenicity remained a problem as HAHA developed in 8 out of 11 patients, going along with strong infusion reactions. Only patients with high HAHA titers developed significant toxicity in four cases, and the only radiographic objective response was observed in one of the HAHA-negative patients (Welt et al. 2003a).

Very similar results were observed in a parallel combination chemotherapy study with BCNU, vincristin, fluorouracil, streptozocin (BOF-Step) in a fixed dose and huA33 dose escalation. Apart from higher toxicity because of the chemotherapy component, the results were encouraging, with 3 of 12 patients reaching radiographic partial responses, but were almost identical with respect to HAHA reactions (7 of 12 patients) (Welt et al. 2003b).

More recent studies found a somewhat more favorable HAHA incidence (Chong et al. 2005). In a neoadjuvant setting, one study demonstrated selective and rapid localization of radiolabeled huA33 to colorectal carcinoma sites and penetration into the centers of large necrotic tumors by postsurgical analysis of tumors resected after radioimmunotherapy (Scott et al. 2005).

To overcome the problem of HAHA reactions, an scFv against gpA33 has been developed in a phage display system (Rader et al. 2000). Based on this A33scFv, recombinant fusion proteins for pretargeting enzymes that activate inert prodrugs in ADEPT are currently in preclinical development (Deckert et al. 2003).

12.4.1.3 **CD44v6**

A transmembrane glycoprotein expressed by cells of almost all tissues, CD44 is a receptor for hyaluronan which mediates interactions with other cells and with the extracellular matrix. It helps anchoring cells to basal membranes and thus to achieve and maintain a polar orientation. It is also involved in leukocyte aggregation and their adhesion to endothelia and in mechanisms of cancer proliferation and dissemination. The human CD44 gene in fact encodes a variety of proteins which differ in glycosylation, but essentially are splice variants of the 19 human exons. CD44 variants containing variant domain 6 (CD44v6), in particular, seem to play a role in tumor cell invasion into tissues and metastasis of carcinomas (Heider et al. 2004).

Clinical trials have been reported on for bivatuzumab (BIWA-4). Apart from safety of administration and low immunogenicity, antitumor effects in incurable

head-and-neck cancer patients with bulky disease were reported from a radioimmunotherapy study (Borjesson et al. 2003). The same approach tested in early stage breast cancer, however, revealed disappointing tumor-to-blood and tumor-to-normal tissue ratios, which could not be correlated with CD44v6 expression or the other tumor characteristics investigated (Koppe et al. 2004).

12.4.2 Stromal Antigens and Molecules Interacting with Extracellular Matrix

Tumor stroma and the extracellular matrix form essential support structures of a tumor, but the cells involved are not part of the malignant process and are not transformed themselves. Like neo-angiogenesis, this makes them attractive targets for tumor therapy as they are less prone to develop escape mechanisms than tumor cells themselves. Antibodies of this category currently in clinical trials are listed in Table 12.9.

12.4.2.1 FAP Antigen

Fibroblast activation protein is a stromal antigen involved in normal growth during childhood, wound healing, and tumorigenesis. Sibrotuzumab (BIBH-1) is the CDR-grafted humanized version of F-19, an anti-FAP antibody that has shown good tolerability in early-phase clinical trials and in particular did not interfere significantly with wound healing. A phase I radioimmunotherapy trial of ^{131}I-sibrotuzumab in patients with colorectal and non-small cell lung cancer found no objective responses, whereas the toxicity profile again was very mild (Scott et al. 2003). Another study of the unconjugated antibody in patients with metastatic colorectal cancer receiving weekly intravenous infusions did not reveal any objective responses. In contrast, the disease was progressive during the study in all but two patients, who progressed post study (Hofheinz et al. 2003).

12.4.2.2 Extracellular Matrix

The interactions of cells with extracellular matrix (ECM) proteins play a crucial role in the establishment and proliferation of solid tumors. The underlying mechanisms and the extent of their contribution in various cancer entities is still under investigation, but a couple of interesting ECM proteins or their cellular ligands such as tenascin C and annexin II have emerged, and some anti-ECM antibodies have reached clinical trials. 81C6, an anti-tenascin antibody whose murine and chimeric versions have been published under the same name, has shown prolonged and thereby specific tumor uptake in tumor compared to visceral organs, and objective responses in 2 of 9 patients in a phase I study of ^{131}I-radioimmunotherapy for lymphoma (Rizzieri et al. 2004). Phase II results of intracavity radioimmunotherapy after tumor resection in patients with recurrent malignant glioma indicate prolonged median survival over historical controls, encouraging modified phase II studies and a multicenter phase III trial (Akabani et al. 2005; Reardon et al. 2006).

Table 12.9 Antibodies against stromal and extracellular matrix antigens.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
BIBH-1 (sibrotuzumab)	Humanized F19	FAP (fibroblast activation protein)	Colorectal cancer	II	NCT00004042 NCT00005616	Closed 2000	Boehringer Ingelheim, Ingelheim, Germany	II	2004	
Po66	Murine	Po66-CBP	Lung cancer (NSCLC)	I	–		Centres de Lutte contre le Cancer, Rennes, France	–	2003	
81C6	Chimeric of murine G2b/k	Tenascin	Glioma, lymphoma	I/II	NCT00002752 NCT00002753 NCT00003461 NCT00003478 NCT00003484	Closed 2000	Duke University, Durham, NC, USA	II	2005	RIT
BC2 (BC2-biotin)	Conjugate for pretargeting approach, murine	Tenascin	Glioma	I/II	–		Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy	–	2003	

12.4.3 Gangliosides

Gangliosides are cytoplasmic membrane glycolipids characteristic of the myelin sheaths of neurons. Their oligosaccharide chains extend from the cell surface into the extracellular space and thus play an important role in cell identification and molecular recognition. They are overexpressed in many tumors of neuroectodermal origin. Gangliosides D2 (GD2), D3 (GD3), and M2 (GM2), in particular, have been investigated as targets for immunotherapy (see Table 12.10) because antibodies directed against them are retained for a longer time than those recognizing other gangliosides (Hanai et al. 2000).

12.4.3.1 Gangliosides D2 and D3

Of phase I and II studies of the murine anti-GD2/3 antibody IgG 3 antibody 3F8 in patients with stage 4 neuroblastoma, objective responses, including a sizable proportion of complete responses, and, more importantly, long-term survival of a subset of patients were reported with tolerable acute adverse effects and no delayed neurological toxicity (Cheung et al. 1998). Stratified for resistance to induction therapy, recurrent disease and progressive disease, the majority of patients in the first group achieved complete remission, whereas the effect in patients with progressive disease was small. (Kushner et al. 2001). In a larger controlled study of stage 4 neuroblastoma, the chimeric antibody ch14.18 was studied in 334 patients older than 1 year who had completed initial treatment without event, 166 of whom received the antibody. The tolerability again was good with reversible acute symptoms. While no significant differences in event-free survival were seen between treatment and control groups, the result for overall survival was unclear (Simon et al. 2004). These results were confirmed in a study with infants younger than 1 year (Simon et al. 2005).

Combination therapy of ch14.18 with antibody R24 and IL-2 in patients with melanoma or sarcoma lowered the maximum tolerated dose of each antibody, but despite objective responses and induction of immunological reactions, a therapeutic advantage could not be established (Choi et al. 2005).

12.4.3.2 Gangliosides M2 and M3

The GM2-recognizing human IgG1 kappa chimeric antibody chL6 has been tested in phase I trials for non-small cell lung, colon, and breast cancer. Apart from fever, chills, nausea, and drops in blood cell counts, the antibody was well tolerated, and good tumor localization was demonstrated in biopsies. While human anti-mouse antibody (HAMA) responses in the vast majority of patients hindered the announced phase II trials after encouraging phase I results with the murine monoclonal antibody, antibodies against the chimeric version were detected in only 4 of 18 patients. However, as with the murine version, no objective responses were seen (Goodman et al. 1993).

Chimeric L6-based radioimmunotherapy of patients with chemotherapy-refractory metastatic breast cancer then led to objective tumor responses in about

Table 12.10 Antibodies against ganglioside antigens.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
ch14.18	Humanized mAb 14.18 (derived IL-2 fusion protein: EMD-273063)	GD2	Neuroblastoma	II/III	NCT00026312 NCT00030719	Recruiting	EMD Pharmaceuticals, Durham, NC, USA	I	2005	
14.G2a	Isotype variant of mAb 14.18, murine	GD2	Neuroblastoma	I	–		Scripps Research Institute, La Jolla, CA, USA	I	1997/ 1999	
3F8	Murine	GD2	Neuroblastoma, melanoma, osteosarcoma	II	NCT00072358 NCT00089258 NCT00058370	Recruiting	Case Western Reserve University, Cleveland, OH, USA	II	2004	Clinical abstract
KM871 (ecromeximab, KW-2871, KW-2971)	Chimeric of mAb KM641	GD3	Melanoma	I	–		Kyowa Hakko, Tokyo, Japan	I	2001	
ME36.1	Murine	GD2 and GD3	Melanoma	I	–		Wistar Institute, Philadelphia, PA, USA	–	2000	
R24	Murine	GD3	Leukemia, lymphoma, melanoma, soft tissue sarcoma	I/II	–		Memorial Sloan-Kettering Cancer Center, New York, NY, USA	I	1998/ 2003	

Table 12.10 *(Continued)*

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
L55	EBV-transformed line	GM2 (OFA-I-1)	Breast, ovarian, prostatic, colon cancer, melanoma	I	–		John Wayne Cancer Institute, Santa Monica, CA, USA	I	1995/ 1999	
L6	Murine	GM2	Breast, lung (NSCLC), ovarian, prostatic, and colon cancers	I	–		Scripps Research Institute, La Jolla, CA, USA	I	1995/ 2005	
chL6	Chimeric	GM2	Breast, lung (SCLC), ovarian, and colon cancers	I/II	–		Bristol Myers Squibb, New York, NY, USA	I	1995/ 2005	
L612	Recombinant human	GM3, GM4	Melanoma	I	–		John Wayne Cancer Institute, Santa Monica, CA, USA	I	2004	

50%. Myelosuppression was the limiting toxicity. Hence, transfusions of G-CSF-mobilized peripheral blood progenitor cells were tested in three patients. This reduced hematologic toxicity, but antibodies against chL6 emerged as a problem in the first two patients, so that ciclosporin was administered in the third patient. She did not develop specific antibodies or significant toxicity and enjoyed decreased tumor parameters and improved performance status for a follow-up of 9 months (Richman et al. 1995). Another strategy to overcome the problem of emerging antibodies against the therapeutic antibody is the administration of deoxyspergualin, which was shown to suppress to some extent HAMA responses to L6 (Dhingra et al. 1995). While no other clinical studies following these have been published yet, the preclinical development of new therapeutic approaches with this antibody appears to continue (DeNardo et al. 2005a).

A fully human IgM antibody against ganglioside M3, L612 has been tested in melanoma patients. Two of nine patients with resected tumors had been followed up for more than 5 years without evidence of tumor (Irie et al. 2004).

12.4.4 Mucins and Mucin-like Proteins

Mucins are large *O*-glycoproteins with clustered oligosaccharides linked to threonine, serine, and proline-rich tandem repeats. They fall into two distinct classes: gel-forming mucins are the main component of the secretions produced by mucous glands and goblet cells, whereas transmembrane mucins are cell surface proteins that appear to act rather as adhesion molecules. The latter comprise mucins 1, 3a and b, 4, 12, and 17. Mucin 1 (MUC1) expression is increased in many colon cancers and is correlated with a worse prognosis. MUC16 is one of a number of mucins that do not fit into either class. It has a transmembrane domain, but the structure of its tandem repeats differs from that in other transmembrane mucins. Termed CA125, it has long been known as a tumor marker for ovarian cancer (Porchet and Aubert 2004; Byrd and Bresalier 2004).

As it shares many characteristics with mucins without belonging to the MUC gene family, the tumor-associated glycoprotein 72 (TAG72) has been termed a mucin-like protein (Pavlinkova et al. 1999). Antibodies of this category are compiled in Table 12.11.

12.4.4.1 Mucin 1

A considerable number of antibodies against MUC1 antigens have been in clinical studies. Recently, trials have been reported on for pintumomab and pemtumomab. Pintumomab (170H.82, m170), a murine antibody, has been investigated for hormone-refractory prostate and breast cancer looking at two aspects: combination chemo-radioimmunotherapy with paclitaxel and the prevention of anti-mouse antibodies by immunosuppression rather than deimmunization of the antibody. Targeting of bone and soft tissue metastases was documented in

Table 12.11 Antibodies against mucins and mucin-like antigens.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
170H.82 (m170 pintumomab)	Murine	MUC1	Breast, pancreatic, ovarian cancers	III	–		Cross Cancer Institute, Edmonton, AL, Canada	I/II	2005	
AR20.5 (BrevaRex)	Murine	MUC1	Myeloma, breast, pancreatic cancers	I/II	–		AltaRex, Edmonton, AL, Canada	–	2001	Epithelial cell surface mucin, function see comment
BrE-3	Murine	MUC1 HFMG	Breast	I	NCT00007891	Closed 2002	Cancer Research Fund of Contra Costa, Walnut Creek, CA, USA	I	1998	
h-CTM0	Humanized	MUC1 (RPAP epitope)	Breast, lung, ovarian, endometrial, and colorectal cancers	II	–		UCB S.A., Bruxelles, Belgium	I	1998/ 2005	
HMFG1 (pemtumomab)	Murine	MUC1	Breast, lung (NSCLC), gastric, ovarian, and colorectal cancers	III	NCT00004115	Closed	Cancer Research UK, London, UK	I	2005	RIT. Also used in anti-idiotype approach
hu-HMFG1	Humanized from HMFG1	MUC1	Breast, lung (NSCLC), gastric, ovarian, and colorectal cancers	I	–		Cancer Research UK, London, UK	–	2005	RIT. Idiotypic vaccination

HMFG2	Murine	MUC1	Breast and ovarian cancers	II	–		Cancer Research UK, London, UK; Scripps Research Institute, La Jolla, CA, USA	–	2004	
MA5	Murine	MUC1	Breast and colorectal cancers	I	–		McGill University, Montral, QC, Canada	–	2002	
Nd2	Murine	MUC1-associated antigen	Pancreatic cancer	I	–		Osaka City Univiversity, Osaka, Japan	I	1997/ 2002	Diagnostic study 1999
PAM-4	Murine	MUC1	Pancreatic and colorectal cancers	I/II	–		Garden State Cancer Center, Newark,NJ, USA	–	1997	
SM3	Murine	MUC1	Breast and ovarian cancers	I	–		Cancer Research UK, London, UK	–	1996	
145-9	Murine	CA125, CA130	Ovarian cancer	I	–		Kyoto University, Kyoto, Japan	I	1997	
B43.13 (oregovomab)	Murine	CA125	Ovarian cancer	III	NCT00086632 NCT00050375	Recruiting	Altarex, Waltham, MA, USA	III	2004	
Mu-9	Murine	CSA-p	Colorectal cancer	I	–		Garden State Cancer Center, Newark, NJ, USA	I	1994/ 2005	CSA-p is related to and possibly identical with CA125
B72.3 chimeric	Chimeric	TAG-72 (CA72-4)	Adenocarcinoma of various origin, mesothelioma	I	–		National Institutes of Health, Bethesda, MD, USA	I	2005	
CC49 (HUcc49v10)	Humanized	TAG-72 (CA72-4)	Adenocarcinoma: colorectal, gastric, ovarian	II	–		National Institutes of Health, Bethesda, MD, USA	–	2003	
CC49 (minretumomab, 90Y-CC49)	Murine	TAG-72 (CA72-4)	Adenocarcinoma: colorectal, gastric	III	NCT00002734 NCT00025532 NCT00023933	Closed	National Institutes of Health, Bethesda, MD, USA	I	2005	RIT: phase I

Table 12.11 *(Continued)*

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
scFvHu-CC49	scFv humanized	TAG-72 (CA72-4)	Adenocarcinoma: colorectal, gastric	I/II	–		National Institutes of Health, Bethesda, MD, USA	–	2001	
hu-CC49	Humanized	TAG-72 (CA72-4)	Adenocarcinoma: colorectal, gastric	I	–		National Institutes of Health, Bethesda, MD, USA	–	2003	
hu-CC49 delta CH2	Humanized, constant heavy 2 domain-deleted	TAG-72 (CA72-4)	Adenocarcinoma: colorectal, gastric	I	NCT00025532	Closed	Ohio University, Athens, OH, USA	I	2005	RIT
MDX-220	Bispecific humanized	TAG-72	Breast, prostate cancers	I/II	–		Medarex, Princeton, NJ, USA	–	–	
3E1.2	Murine	MSA (mammary serum antigen)	Lung, breast, and colon cancers	I	–		Melbourne University, Melbourne, Australia	–	1998	
ABX-MA1 (c3.19.1)	Transgenic	MUC18	melanoma	I	–		Abgenix, Fremont, CA, USA	–	2003	
MLS102	Murine	sialyl-Tn	Pancreatic and colorectal cancers	I	–		Kyoto University, Kyoto, Japan	–	1997	

almost all patients, and ciclosporin cotreatment limited human anti-mouse antibodies to one of 16 patients (compared with 12 of 17 patients in a previous trial without ciclosporin), encouraging the authors to test multidose fractionated radioimmunotherapy in future trials (Richman et al. 2005).

As the limiting toxicity was grade 4 neutropenia, requiring autologous peripheral blood stem cell support in the combination therapy cohort, another trial looked at increasing the tumor-to-normal tissue ratio by a cathepsin-cleavable linker between antibody and radioisotope and an scFv-based pretargeting approach. While targeting remained successful and clinical responses were observed in a subset of patients, myelosuppression still limited applicable dose and patient eligibility (DeNardo et al. 2005b).

Several radioimmunotherapy approaches for ovarian cancer have been evaluated with pemtumomab (HMFG1), which binds to an epitope of MUC1 also termed human milk fat globulin 1 (hence the antibody acronym). Peritoneal single administration of ^{90}Y-pemtumomab was tested in 52 patients with ovarian cancer at stage I C through IV who had completed platinum-containing standard chemotherapy, 21 of whom were in complete remission, while 31 had residual disease. For those patients in complete remission, this study found long-term survival with a 10-year rate of close to 80%, while no profit was seen for patients with residual disease (Epenetos et al. 2000).

Combining external radiotherapy and radioimmunotherapy as a means of intensifying radiotherapy but not adverse effects was evaluated in another trial including 23 patients with nonsmall cell lung cancer. It showed low tumor uptake, low tumor-to-normal tissue ratios, and a short residence time despite good radioimaging of tumor localizations (Garkavij et al. 2005).

An anti-idiotypic vaccination approach with this antibody has been mentioned above.

12.4.4.2 **Mucin 16 or CA125**

The murine monoclonal anti-MUC16 antibody oregovomab (B43.13) had initially been applied for diagnostic immunoscintigraphy, and the observation of a survival benefit for the patients involved led to the hypothesis of an anti-idiotypic network generating CA125-neutralizing antibodies as the therapeutic principle. Further scrutiny then revealed that the observed induction of anti-CA125 responses correlated with the concentration of CA125 circulating in the blood at the time of antibody injection. The epitopes recognized by the induced antibodies were not restricted to that bound by oregovomab. These observations speak against anti-idiotypic immunization and in favor of direct immunization by CA125, probably induced by rapid formation of antigen–antibody complexes after oregovomab injection. Detection of oregovomab-induced CA125-specific immune responses correlated with improved survival (Noujaim et al. 2001). A retrospective analysis of immune responses and survival of patients with recurrent ovarian carcinoma who had received ^{99m}Tc-labeled oregovomab confirmed the initial observation of a survival benefit conferred by oregovomab. According to this study, 56.8% of these heavily pretreated patients survived for longer than 12

months, 34.1% for more than 24 months, and 6 out of 44 patients were still alive 4–7.5 years after the initial antibody dose. The detection of HAMA and anti-CA125 responses was associated with 2 to 3 times prolonged average survival (Mobus et al. 2003). Based on these results, a randomized study of consolidation therapy for patients with stage III or IV ovarian cancer in complete response after primary treatment compared oregovomab against placebo. While only a marginal and nonsignificant survival benefit was reported for the oregovomab group in total (13.3 months for oregovomab versus 10.3 months for placebo), patients who did mount an anti-CA125 immune response tended to have a prolonged time until the disease relapsed. Subgroup analyses revealed prognostic factors for improvement of the time to relapse, which was more than doubled to 24 months in one group (Berek et al. 2004). A phase III study to assess this effect prospectively has been announced.

12.4.4.3 **Tumor-associated Glycoprotein 72**

The mucin-like TAG72 has also been classified as an oncofetal antigen. It is expressed in adenocarcinomas, but except for secretory endometrium, no appreciable presence in normal tissues has been found.

The murine original antibody CC49 (minretumomab) is parent to chimerized, humanized, single-chain and C_H2 domain-deleted versions (see Table 12.11) which have been investigated at several different institutions in numerous clinical phase I and II trials including patients with lung, colorectal, breast, ovarian, and prostate cancers. The predominant treatment modality has been radioimmunotherapy, with ^{131}I, ^{90}Y, and ^{177}Lu as therapeutic radioisotopes and ^{111}In as trace label. A thorough and recent review of the clinical trials performed with CC49 and its derivatives has been compiled by Meredith et al. (2003b). This review finds consistent pharmacokinetic and biodistribution characteristics for the individual antibody entities, independent of the tumor diagnosis or bone versus soft tissue metastases. Unsurprisingly, the longest biologic half-life was seen with the chimeric complete IgG antibody cB72.3, and the shortest with the heavy-chain constant region-deleted version HuCC49dCH2, with the "conventional" CC49 in the middle. The longest half-life was about twice as long as the shortest. Myelosuppression was the dose-limiting toxicity in radioimmunotherapy. Hence, localized application such as interaperitoneal administration increased the tolerated dose. Combination therapy with interferon increased expression of TAG72 and possibly tumor uptake of ^{131}I-CC49. A methodologic problem highlighted by this review was the lack of standardization in study design, monitoring, and reporting, so that dosimetry and other data are difficult to compare between studies and to translate into predictions of safe dose levels (Meredith et al. 2003a).

Recently, ^{90}Y-mCC49 was evaluated in patients with non-small cell lung cancer in combination with interferon α for TAG72 upregulation and paclitaxel. In addition, the chelators EDTA and DTPA were tested for their ability to reduce myelosuppression. The latter was found true to a modest extent for DTPA.

No objective tumor responses were observed (Forero et al. 2005), fitting this study into the pattern previously described.

Another recent study evaluated the suitability of the domain-deleted Hu-CC49DeltaC_H2 for a diagnostic rather than therapeutic role in radioimmunoguided surgery. This is the intraoperative identification of tumor localizations by gamma-detecting probes after systemic application of a radiolabeled antibody. Exploratory laparotomy was performed between days 3 and 20 after antibody injection, and pharmacokinetic data monitored in the meantime. The results fitted a two-compartment pharmacokinetic model. After day 3, the antibody was preferentially localized in tumor tissue, especially in intestinal and metastatic liver lesions (Xiao et al. 2005).

12.4.5 Lineage- or Tissue-specific Antigens

Of many antigens targeted for cancer therapy, immunologic identity and expression specificity for a certain differentiation line or stage have been established although the molecule itself and its function have not yet been defined or are themselves lineage- or tissue-specific. For an overview of such antibodies in clinical trials see also Table 12.12.

12.4.5.1 CD30

The type 1 glycoprotein CD30 was originally identified as the Ki-1 antigen on Reed–Sternberg cells in Hodgkin's disease and non-Hodgkin's lymphomas such as diffuse large cell, anaplastic large cell, and immunoblastic lymphomas. It is a member of the TNF receptor family and activates nuclear factor κB (NFκB) via TNF receptor-associated factors (TRAF) 2 and 5, leading to pleiotropic effects *in vitro* ranging from cell proliferation to cell death. It is also expressed on lymphocytes infected with HIV, HTLV-1 or EBV, and crosslinking of HIV-infected T cells is involved in the activation of HIV production. Its selective expression in lymphomas gave rise to hopes of repeating the success of antibody therapy seen with B-cell non-Hodgkin's lymphomas.

So far, this expectation has yet to be met, but interesting interactions were observed that are encouraging for clinical trials. For example, 5F11 (MDX-060), which was well tolerated and had some clinical activity in a recently closed phase I/II trial, was shown to activate NFκB and the antiapoptotic protein c-flip in Hodgkin's lymphoma-derived cell lines. This limitation of the clinical use of 5F11 has been overcome *in vitro* and *in vivo* by combination with the NFκB-suppressing proteasome inhibitor bortezomib. The combination had a synergistic cytotoxic effect if 5F11 is followed by bortezomib, indicating a bortezomib-sensitizing function of the antibody (Boll et al. 2005). Similarly, a drug-sensitizing effect of the chimeric antibody SGN-30, currently in clinical phase II trials, was demonstrated for a number of chemotherapeutic drugs in Hodgkin's and anaplastic large cell lymphoma cell lines *in vitro* and partly *in vivo* (Cerveny et al. 2005).

Table 12.12 Antibodies against lineage- or tissue-specific antigens.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
5F11 (MDX-060)	Transgenic, fully human	CD30	Lymphoma	I/II	NCT00059995	Closed 2005	Medarex, Princeton, NJ, USA	I/II	2004/ 2005	T and B cell activation, M. Hodgkin, embryonal tumors
Ber-H2	Murine	CD30	Reed–Sternberg cells: Hodgkin's lymphoma, testicular cancer	I/II	–		Freie Universität Berlin, Berlin,Germany	–	1999	T and B cell activation, M. Hodgkin, embryonal tumors
HeFi-1	Murine	CD30	Reed–Sternberg cells: Hodgkin's lymphoma	I	NCT00048880	Recruiting	National Institutes of Health, Bethesda, MD, USA	–	2004	T and B cell activation, M. Hodgkin, embryonal tumors
HRS-3	Murine	CD30	Reed–Sternberg cells: Hodgkin's lymphoma	I	–		Cancer Research UK, London, UK; Biotest AG, Dreieich, Germany	I/II	2001	T and B cell activation, M. Hodgkin, embryonal tumors
SGN-30	Chimeric of murine AC10	CD30	Hodgkin's lymphoma	II	–		Seattle Genetics, Bothell, WA, USA	–	2005	T and B cell activation, M. Hodgkin, embryonal tumors

1F5	Murine	CD20	B lymphocytes: leukemia, lymphoma	I	–		Bristol Myers Squibb, New York, NY, USA	I	2000/ 2003	RIT. Intracellular accumulation
CAMPATH-1M YTH66.9	Studied for *ex vivo* applications, rat	CD52	leukemia	I	–		Cambridge University, Cambridge, UK	I	1998/ 2002	Campath history: –1 M → 1 G → 1 H alemtuzumab
Lintuzumab (HuM195, Zamyl)	Humanized immunotoxin see Hum-195/ rGel	CD33	Myelodysplastic syndrome, acute myeloid leukemia	III	–		Memorial Sloan-Kettering Cancer Center, New York, NY, USA	I	2003	Studied for *ex vivo* purging
G250	Murine	G250 (MN/ CA9 or MN/CA IX)	Renal cell carcinoma	II	–		Universiteit Leiden, Leiden, Netherlands; Wilex, München, Germany	I/II	1998/ 2005	RIT
G250, c-WX-G250	Chimeric	G250 (MN/ CA9 or MN/CA IX)	Renal cell carcinoma	II/III	NCT00199875 NCT00087022 NCT00199888	Recruiting	Universiteit Leiden, Leiden, Netherlands; Wilex, München, Germany	II	2004/ 2005	RIT
J591	Murine	PSMA	Prostatic cancer	II	NCT00040586 NCT00195039 NCT00081172	Recruiting	Ludwig Institute for Cancer Research, New York, NY, USA; Millenium Pharmaceuticals, Cambridge, MA, USA	II	2006	
huJ591	Humanized J591	PSMA	Prostatic cancer	II	NCT00195039	Recruiting	Millenium Pharmaceuticals, Cambridge, MA, USA			
3H11	Murine scFv	3H11Ag	Gastric cancer	I	–		Beijing Institute for Cancer Research, Beijing, China	–	2004	T-cell antigen?
C242	Murine	CA242 (CanAg)	Lung, pancreatic, and colorectal cancers	I/II	–		Gøteborgs Universitet, Gøteborg, Sweden; Glaxo-SmithKline, Brentford, UK	–	2003	

Table 12.12 *(Continued)*

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
H11	Humanized	C antigen	Breast, pancreatic, liver, and gastric cancers, lymphoma	II	NCT00058292	Recruiting	Shizuoka University, Shizuoka, Japan; Viventia Biotech, Toronto, ON, Canada	–	2003	RIT
HM1.24	Chimeric	HM1.24	Lymphoma	I	–		Chugai Pharmaceuticals Co. Ltd, Tokyo, Japan	I	1999/ 2005	B-cell differentiation. Phase I trial halted.
MBr1	Murine	CaMBr-1 (GL-6)	Breast and lung (SCLC) cancers	I	–		Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy; Memorial Sloan-Kettering Cancer Center, New York, NY, USA	–	–	
Mel-14	Murine	Chondroitin sulfate (proteoglycan antigen of gliomas, melanomas	Glioma, melanoma, medulloblastoma	I	NCT00002751 NCT00002754	Closed 1999	Ludwig Institute for Cancer Research, Lausanne, Switzerland; Duke University, Durham, NC, USA	I	2001	Phase I study reported in case report. Ab also quoted as Me1-14
RAV12	High-affinity variant of mAb KID3, chimeric	RAAG12	Gastric, pancreatic, and colorectal cancers	I	NCT00101972	Recruiting	Raven Biotechnologies, South San Francisco, CA, USA	–	–	Glycoprotein antigen – ion pump-related?

12.4.5.2 G250 or Carbonic Anhydrase IX

Carbonic anhydrase IX (CA IX) is a membrane-associated enzyme implicated in cell proliferation under hypoxia and thus in oncogenesis and tumor progression. The antigen-defining antibody G250 was raised by immunization of mice with human renal cell carcinoma. CA IX is expressed in various malignancies, in particular renal cell carcinoma (RCC), but is absent in normal tissues except for gastric mucosa. In RCC patients, high expression of CA IX has been determined as a positive prognostic factor for response to IL-2 therapy and survival (Atkins et al. 2005). Selective uptake of G250 antibody by antigen-positive cells has been demonstrated immunohistochemically, and comparably low protein doses were required for effective tumor targeting (Lam et al. 2005).

These findings led to clinical studies with murine G250 in the mid 1990s, but as so often, anti-murine responses abrogated repeated treatment and thus, despite good tumor targeting and tolerable adverse effects, the clinical potential could not be evaluated until a chimeric version, cG250, was available (Divgi et al. 1998). A series of clinical trials have been conducted since then.

The first phase I study with ^{131}I-cG250 observed encouraging clinical effects. Myelosuppression was determined as the dose-limiting toxicity (Steffens et al. 1999). A subsequent escalation trial of whole-body absorbed dose tried to reduce myelotoxicity by fractionated administration. Concomitant monitoring of whole-body and serum clearance allowed rational treatment planning and dynamic dose adaptation, but there was no evidence for fractionation-induced sparing of the hematopoietic system (Divgi et al. 2004).

The unlabeled antibody was investigated in a multicenter phase II study of metastasized RCC. Here, no drug-related grade III or IV toxicity was observed, and only a small proportion of patients experienced grade II toxicity. Among the 36 patients entered, one complete response and one partial response were noted, and five patients with initially progressive disease experienced stabilization for more than 6 months. The overall median survival was 15 months (Bleumer et al. 2004). Given the limited therapeutic options and bleak prognosis of this disease, cG250 may thus be of clinical benefit in renal cell carcinoma.

12.4.5.3 Prostate-specific Membrane Antigen

The transmembrane glycoprotein prostate-specific membrane antigen (PSMA) is primarily expressed on prostatic epithelial cells, including those of prostate cancer, and has been successfully employed for immunoscintigraphy of prostate cancer metastases. The murine IgG1 monoclonal antibody J591 directed against the extracellular domain of PSMA has been epitope deimmunized (Milowsky et al. 2004). It could therefore be administered repeatedly with good tolerability and induced dose-correlated ADCC in a phase I trial of patients with androgen-independent prostate cancer (Morris et al. 2005). A humanized version has also been developed and is also currently in a phase II clinical trial. For radioimmunotherapy with repeated ^{177}Lu-J591, a phase I trial including 35 patients with progressive androgen-independent prostate cancer found dose-limiting myelosuppression after more than three doses of 30 mCi m^{-2}, but no serious nonhema-

tologic toxicity. All known tumor sites in a subset of patients with positive preceding imaging studies were detected by the antibody, and biologic activity as determined by reduction of serum prostate-specific antigen to less than half the initial value was seen in four patients (11.4%) (Bander et al. 2005). Another study found prolonged tumor responses and reduced myelotoxicity as a result of dose fractionation (Vallabhajosula et al. 2005).

12.4.6 Oncofetal Antigens

Oncofetal antigens are a very heterogeneous group whose members are expressed physiologically only during embryonal or fetal development and – obviously associated with cell proliferation – again as a pathological feature of various malignancies (see Table 12.13).

12.4.6.1 Lewis Y

The Lewis Y (Le^Y) antigen was first described as a blood group-related antigen expressed on activated granulocytes. Its association with proteins of the CD66 family of oncofetal antigens led to its classification in this category, while its interaction with the ErbB family of EGF receptors might well justify listing it in Section 12.3.2.2 as interfering with growth signaling (Klinger et al. 2004). Le^Y is expressed in a large proportion of epithelial cancers such as lung, breast, ovarian, and colon carcinoma.

3S193 is a murine anti-Le^Y antibody that has been humanized by linking the murine variable region and human framework cDNA in various combinations and selecting for Le^Y binding. The avidity of the resultant hu3S193 matched that of the murine version, while its capacity to induce ADCC and CDC exceeded that of the parent antibody. *In vivo*, no tumor reduction could be seen in mice bearing established xenograft tumors of the MCF-7 breast cancer cell line against which 3S193 was originally raised, but tumor growth was decelerated in a preventive model compared with controls (Scott et al. 2000). In the same murine xenograft model, ^{131}I-labeled hu3S193 combined with taxol in subtherapeutic doses of each agent significantly inhibited tumor growth in 80% of mice as opposed to either substance alone (Clarke et al. 2000). A clinical phase I study is recruiting patients (NCT00084799), but no clinical results are available yet. Blocking of ErbB1 and ErbB2 signaling has been investigated with IgN311. This humanized anti-Le^Y antibody immunoprecipitated both EGF receptors from detergent lysates of human tumor cells and blocked EGF-stimulated downstream signals with an efficacy similar to that of trastuzumab (Klinger et al. 2004).

12.4.6.2 Carcinoembryonal Antigen

Carcinoembryonal antigen (CEA) is the prototypical oncofetal antigen and has long been used as a diagnostic tumor marker to monitor the course of adenocarcinomas, in particular colon cancer. Hence, despite the problem of circulating soluble CEA – which is the very rationale of its diagnostic use but limits tumor

Table 12.13 Antibodies against oncofetal antigens.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
hu3S193	Humanized	Le-y	Breast, ovarian, and colon cancers	I	NCT00084799	Recruiting	Ludwig Institute for Cancer Research, New York, NY, USA; Biovation Ltd., Aberdeen, Scotland, UK	–	2001	
ABL364 (BR55-2)	Murine	Le-y	Breast, lung, gastric, and colorectal cancers	I	–		Wistar Institute, Philadelphia, PA, USA; Igeneon AG, Vienna, Austria	–	2004	
cBR96	Chimeric	Le-y	Lung, gastric, colon, and prostatic cancers	II	NCT00028483	Closed 2004	Bristol Myers Squibb, New York, NY, USA	I	2000/ 2001	
IGN311	Humanized ABL364	Le-y	Epithelial: carcinomas	I	–		Igeneon AG, Vienna, Austria; Protein Design Labs, Fremont, CA, USA	I	2004/ 2005	
mAb 35	Murine	CEA	Colorectal cancer	I/II	–		Ludwig Institute for Cancer Research, Lausanne, Switzerland	I	2001	
38S1	Murine	CEA	colorectal cancer	I	–		Wenner Gren Institute, Stockholms Universitet, Stockholm, Sweden	–	2000	
F6	Murine	CEA	Thyroid and colorectal cancers	I/II	–		Université de Lausanne, Lausanne, Switzerland; Intervet Danmark AS, Skovlund, Denmark	I/II	1998/ 2005	

Table 12.13 *(Continued)*

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
hMN14 (hMN-14, labetuzumab)	Humanized. Bispecific MN-14 × DTIn-1 in preclinical development publ 2004	CEA	Lung, pancreatic, ovarian, and colorectal cancers	I/II	NCT00040599 NCT00041639 NCT00041652 NCT00041691	Closed 2004	Immunomedics, Morris Plains, NJ, USA	II	2005	
IOR-CEA1	Murine	CEA	Colorectal cancer	II	–		Instituto Nacional di Oncologia y Radiobiologia, Habana, Cuba	II	2000	
MN14 (MN-14)	$F(ab)_2$ fragment, murine	CEA	Ovarian and colorectal cancers	I/II	NCT00004048 NCT00004085 ...	Closed 2004	Immunomedics, Morris Plains, NJ, USA	I/II	2000/ 2004	
PR1A3	Murine	CEA	Colorectal cancer	I	–		Cancer Research UK, London, UK	I	2005	
T84.66	Murine	CEA	Colorectal cancer	I	–		City of Hope Medical Center, Duarte, USA	–	2005	
cT84.66	Chimeric from murine	CEA	Colorectal cancer	I	–		City of Hope Medical Center, Duarte, USA	I	2004/ 2005	
5T4	Fab fragment, murine	5T4	Trophoblast cells: breast, lung, gastric, ovarian, and colorectal cancers	I	–		Cancer Research UK Immunology Group, Manchester, UK	I	2004/ 2005	trophoblast surface cell marker
A6H	Murine	CD26 (dipeptidyl peptidase IV)	T lymphocytes: renal cell cancer	I/II	–		University of Minnesota, Minneapolis, MN, USA; University of Washington, Seattle, WA, USA	I/II	1987/ 2001	
H17E2	Murine	Placental alkaline phosphatase	Lung, breast, ovarian, uterine, and testicular cancers	II	–		Cancer Research UK and Hammersmith Hospital, London, UK	–	2000	

targeting – it is not surprising that numerous approaches to its therapeutic exploitation are being investigated (see Table 12.13).

Among these, the humanized antibody labetuzumab (hMN-14) has been tested in a series of recently completed clinical studies, two of which, on radioimmunotherapy with ^{90}Y- and ^{131}I-labeled conjugates, have already been published. In a combination therapy protocol with doxorubicin and peripheral blood stem cell support, high-dose ^{90}Y-labetuzumab was employed in patients with advanced medullary thyroid cancer. The majority of known tumor lesions were detected, but 20% went insufficiently targeted. Most targeted lesions received more than 20 Gy with an administered dose of less than 1.5 MBq m^{-2}. The average tumor-to-marrow ratio appeared safe at 15.0, albeit with a confidence interval from 4.0 to 26, but a tumor-to-lung ratio of 6.9 ± 6.1 was mirrored in dose-limiting cardiopulmonary toxicity. One patient showed an objective response, two more had minor responses (Sharkey et al. 2005a). Salvage therapy after complete resection of liver metastases of colorectal cancer was the objective of a phase II trial including 23 patients. Doses of 40–60 mCi m^{-2} of ^{131}I-labetuzumab were administered. A median overall survival time of 68.0 months and a median disease-free survival time of 18.0 months with a 5-year survival rate of 51% were reported, compared with historical data giving a 5-year survival of about one-third. Based on these data demonstrating a real clinical benefit, a multicenter, randomized trial has been projected (Liersch et al. 2005).

Another anti-CEA antibody, PR1A3, is remarkable in that its target initially had not been identified as CEA for lack of reaction with circulating serum antigen. Its binding site has then been identified as a conformational epitope involving the glycosyl-phosphatidylinositol (GPI) anchor and the B3 domain at the site of membrane attachment. Putative conformation changes associated with antigen release from the cell surface apparently masked soluble CEA from this antibody (Durbin et al. 1994). A humanized version without a separate name has been constructed and shown to retain the affinity and cell surface specificity of the original (Stewart et al. 1999). Unfortunately, its unique binding pattern could not be translated into clinical benefit. While a couple of clinical imaging studies have been published, the only peer-reviewed therapy trial investigated an anti-idiotype vaccine approach. Despite interesting immunological findings, its result is best summarized by quoting the authors: "Progressive disease was observed in 14 of the 15 patients with minimal toxicity" (Zbar et al. 2005).

A more optimistic outlook can be drawn from reports on a chimeric high-affinity anti-CEA antibody, T84.66. Pretherapy imaging trials demonstrated little toxicity, little immunogenicity, and good tumor targeting, but rapid liver clearance in a subset of patients (Wong et al. 1995). A more recent study combined ^{90}Y-cT84.66 with continuous infusion 5-fluorouracil for patients with chemotherapy-refractory metastatic colorectal cancer. Of 21 heavily pretreated patients, none developed an objective response, but one mixed response was observed and 11 patients with progressive disease at study entry experienced disease stabilization for 3–8 months. An additional effect was reduced incidence of immune reactions against the chimeric antibody (Wong et al. 2003). A new cT84.66-based, ^{123}I-labeled minibody construct demonstrated successful tumor imaging in 7 of 8

patients, maintained tumor residence, and reduced circulation half-life without drug-related adverse reactions (Wong et al. 2004).

12.4.7 Individual or Noncategorized Antigens

A couple of antigens targeted by clinically investigated antibodies are defined and characterized, but do not fit into any of the above categories. These include detrimental products of malignant conditions such as amyloid (11–1F4), or parts of the cytoskeleton and other intracellular antigens that may be exposed as a result of malignant transformation and changes in cell morphology such as annexins (28A32) or cytokeratins (COU-1) or a cytokeratin-associated protein (174H.64). Finally, qualitative or quantitative metabolic markers of tumors such as parathyroid hormone-related protein (CAL) or the alpha folate receptor (MOv18) are falling into this category, which is summarized in Table 12.14.

12.5 Disease-specific Concepts of Unknown Antigen Function and Structure

There are a few antibodies in clinical investigation whose target antigens have not been characterized at all so far apart from their presumed specificity for a certain type of cancer (see Table 12.15). Little published information is available about most of these. An exception is BDI-1, which has so far only been characterized as an antibody against bladder cancer. Studies of this antibody have been published on radioimmunoimaging by intravesical administration, demonstrating immunoreactivity and tumor-specific localization (Zhang et al. 1998). Intravesical administration of BDI-1-RT immunotoxin as adjuvant therapy after tumor resection in 31 bladder cancer patients compared to mitomycin C in a control group of 36 patients revealed about half the recurrence rate in the immunotoxin group, albeit without statistical significance, and significantly lower toxicity. Interestingly, the immunoreactivity of BDI-1-RT was found to correlate with tumor grade (Zang et al. 2000). Recently, the production and *in vitro* characterization of a novel immunotoxin consisting of arsenic trioxide-loaded nanospheres linked to BDI-1 was reported (Zhou et al. 2005). Although the article is at times difficult to follow, the authors go to great lengths to demonstrate specific binding activity and cytotoxic effect, and this may be an interesting approach.

12.6 Summary

Antibodies as anticancer agents have come of age and are now a standard modality of both established and experimental therapies. Originally conceived as a strategy for the specific targeting of "passive" markers of malignant cells, their immediate effects have in fact evolved into a wide array of biological functions.

Table 12.14 Antibodies against individual antigens not otherwise categorized.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
28A32	Heterohybridoma	CTAA 28A32-32K, annexin-related	Colorectal cancer	I/II	–		Intracel, Frederick, MD, USA	I/II	1990/1998	Intracellular
MOv18	Murine	Alpha folate receptor	Epithelial: ovarian cancer	I	–		Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy	I	1995/2005	RIT
11-1F4	Chimeric	Amyloid	Primary amyloidosis	I/II	–		University of Tennessee, Knoxville, TN, USA	–	2003	
174H.64 (174H.64 × antibiotin)	Murine antibody (bispecific conjugate for pretargeting, not in clinical study)	Cytokeratin	Lung, breast, uterine, skin and other cancers	I/II	–		University of Alberta, Edmonton, AL, Canada; Abbott Labs, Abbott Pk, IL, USA	–	2000	
COU-1	Human	Cytokeratin 8 and 18	Lung, breast, ovarian, pancreatic, and colorectal cancers	I/II	–		Bristol Myers Squibb, New York, NY, USA	–	2002	
CAL (anti-PTHrP)	Humanized	Parathyroid hormone related protein (PTHrP)	Breast cancer, bone metastases	I/II	NCT00060138 NCT00051779	Closed 2004	Chugai Pharmaceuticals Co. Ltd, Tokyo, Japan	–	–	

Table 12.15 Antibodies in disease-specific use with undefined antigen.

Antibody name	*Characteristics*	*Target antigen*	*Target cancer*	*Phase*	*NCI study ID*	*Status*	*Source*	*Publ. phase*	*Publ. year*	*Comment*
1A3	Murine	–	Colorectal cancer	I/II	–		Washington University, St. Louis, MO, USA	I	1993/ 2001	Clinical study: abstract
chA7 (chA7Fab-NCS)	Chimeric A7	–	Gastric, pancreatic, and colorectal cancers, leukemia, lymphoma	I	–		Kyoto Prefectural University of Medicine, Kyoto, Japan	I	2003	RIT. Neocarcinostatin conjugate
BDI-1 (BDI-1-RT immunotoxin)	Murine	–	Bladder cancer	I	–		Beijing University, Beijing, China	I	2000/ 2005	Clinical study on immunotoxin
chSF-25	Chimeric	–	Head-and-neck, liver, and colon cancers	I/II	–		Massachusetts General Hospital, Boston, MA, USA; Centocor, Horsham, PA, USA	I/II	1994/ 2002	Radioimmuno-scintigraphy trial
30.6 (c30.6)	Murine (c30.6: chimeric)	–	Colorectal cancer	I	–		Melbourne University, Melbourne, Australia	I	2000	
GAH	Heterohybridoma, Fab fragment	–	Gastrointestinal cancers	I	–		Mitsubishi Pharma, Osaka, Japan	I	2004	Internalized. rGAH-conjugated, doxorubicin encapsulated immunoliposomes
Hepama-1	Murine	–	Liver cancer	I/II	–		Hipple Cancer Research Center, Dayton, OH, USA	I	2004	

In reviewing which antibodies have made it from bench to bedside in terms of clinical trials, two trends become visible regarding antibody structure and therapeutic principle: on the one hand, sophisticated recombinant antibody constructs succeed "plain old" immunoglobulins, on the other, antibodies become increasingly important either as targeting vehicles for the delivery of secondary effector molecules or as specific interactors with cell signaling pathways. While they compete with small molecule drugs in the latter, their unique potential lies in the complex bi- and multifunctional approaches made possible by the former.

In interpreting results of clinical trials, however, one caveat must be kept in mind: It is tempting to judge the potential of the tested therapy by clinical response and survival rates, but this is not what early phase, and particular phase I trials, are designed for. Facing life-threatening diseases, for ethical reasons most investigational new drugs can only be applied in patients who have exhausted all established treatment options. At this point, these patients have been selected for a bad prognosis. Thus, while a significant clinical response in these trials is certainly a good sign, the lack thereof has little predictive value. Especially in solid tumors, the natural strength of antibody-based therapies must be expected from specific treatment of disseminated systemic disease of small total volume, as in early stage or minimal residual disease (Koppe et al. 2005). Successful antibody-based treatment of bulky disease with large tumor mass will probably remain the exception. This does not diminuish the role of antibodies in future cancer therapy, as sufficient therapeutic options are often available to remove large tumors, but disseminated micrometastatic disease or minimal residual disease is the most ominous threat to the patient's survival in most malignancies.

References

Aboud-Pirak, E., Hurwitz, E., Pirak, M.E., Bellot, F., Schlessinger, J., Sela, M. 1988. Efficacy of antibodies to epidermal growth factor receptor against KB carcinoma in vitro and in nude mice. *J Natl Cancer Inst* 80: 1605–1611.

Adkins, J.C., Spencer, C.M. (1998) Edrecolomab (monoclonal antibody 17-1A) *Drugs* 56: 619–626.

Agus, D.B., Gordon, M.S., Taylor, C., Natale, R.B., Karlan, B., Mendelson, D.S., Press, M.F., Allison, D.E., Sliwkowski, M.X., Lieberman, G., Kelsey, S.M., Fyfe, G. (2005) Phase I clinical study of pertuzumab, a novel HER dimerization inhibitor, in patients with advanced cancer. *J Clin Oncol* 23: 2534–2543.

Akabani, G., Reardon, D.A., Coleman, R.E., Wong, T.Z., Metzler, S.D., Bowsher, J.E., Barboriak, D.P., Provenzale, J.M., Greer, K.L., DeLong, D., Friedman, H.S., Friedman, A.H., Zhao, X.G., Pegram, C. N., McLendon, R.E., Bigner, D.D., Zalutsky, M.R. (2005) Dosimetry and radiographic analysis of I-131-labeled anti-tenascin 81C6 murine monoclonal antibody in newly diagnosed patients with malignant gliomas: A phase II study. *J Nucl Med* 46: 1042–1051.

Armstrong, A., Eck, S.L. (2003) EpCAM – A new therapeutic target for an old cancer antigen. *Cancer Biol Ther* 2: 320–325.

Atkins, M., Regan, M., McDermott, D., Mier, J., Stanbridge, E., Youmans, A., Febbo, P., Upton, M., Lechpammer, M., Signoretti, S. (2005) Carbonic anhydrase IX expression predicts outcome of interleukin 2 therapy for renal cancer. *Clin Cancer Res* 11: 3714–3721.

Audi, J., Belson, M., Patel, M., Schier, J., Osterloh, J. (2005) Ricin poisoning: a

comprehensive review. *JAMA* 294: 2342–2351.

Baca, M., Presta, L.G., O'Connor, S.J., Wells, J.A. (1997) Antibody humanization using monovalent phage display. *J Biol Chem* 272: 10678–10684.

Badache, A., Hynes, N.E. (2004) A new therapeutic antibody masks ErbB2 to its partners. *Cancer Cell* 5: 299–301.

Bander, N.H., Milowsky, M.I., Nanus, D.M., Kostakoglu, L., Vallabhajosula, S., Goldsmith, S.J. (2005) Phase I trial of (177)lutetium-labeled J591, a monoclonal antibody to prostate-specific membrane antigen, in patients with androgen-independent prostate cancer. *J Clin Oncol* 23: 4591–4601.

Barth, S., Huhn, M., Matthey, B., Schnell, R., Tawadros, S., Schinkothe, T., Lorenzen, J., Diehl, V., Engert, A. (2000) Recombinant anti-CD25 immunotoxin RFT5(scFv)-ETA′ demonstrates successful elimination of disseminated human Hodgkin lymphoma in SCID mice. *Int J Cancer* 86: 718–724.

Bekker, P.J., Holloway, D.L., Rasmussen, A.S., Murphy, R., Martin, S.W., Leese, P.T., Holmes, G.B., Dunstan, C.R., DePaoli, A.M. (2004) A single-dose placebo-controlled study of AMG 162, a fully human monoclonal antibody to RANKL, in postmenopausal women. *J Bone Miner Res* 19: 1059–1066.

Berek, J.S., Taylor, P.T., Gordon, A., Cunningham, M.J., Finkler, N., Orr, J., Jr., Rivkin, S., Schultes, B.C., Whiteside, T.L., Nicodemus, C.F. (2004) Randomized, placebo-controlled study of oregovomab for consolidation of clinical remission in patients with advanced ovarian cancer. *J Clin Oncol* 22: 3507–3516.

Bhaskar, V., Wales, P., Bremberg, D., Fox, M., Ho, S. (2004) M200, a chimeric antibody against integrin alpha5 betal, inhibits cancer cell growth in vitro. *J Immunother* 27: S9.

Bhogal, N., Combes, R. (2006a) An Update on TGN1412. *Altern Lab Anim* 34: 351–356.

Bhogal, N., Combes, R. (2006b) TGN1412: time to change the paradigm for the testing of new pharmaceuticals. *Altern Lab Anim* 34: 225–239.

Bleeker, W.K., van Bueren, J.J.L., van Ojik, H.H., Gerritsen, A.F., Pluyter, M., Houtkamp, M., Halk, E., Goldstein, J., Schuurman, J., van Dijk, M.A., van de Winkel, J.G.J., Parren, P.W.H.I. (2004) Dual mode of action of a human anti-epidermal growth factor receptor monoclonal antibody for cancer therapy. *J Immunol* 173: 4699–4707.

Bleumer, I., Knuth, A., Oosterwijk, E., Hofmann, R., Varga, Z., Lamers, C., Kruit, W., Melchior, S., Mala, C., Ullrich, S., De Mulder, P., Mulders, P.F., Beck, J. (2004) A phase II trial of chimeric monoclonal antibody G250 for advanced renal cell carcinoma patients. *Br J Cancer* 90: 985–990.

Blohmer, J., Gore, M., Kuemmel, S., Verheijen, R.H., Kimmig, R., Massuger, L. F.A.G., Du Bois, A., Smit, W.M., Kaye, S., Deubelbeiss, C. (2005) Phase II study to determine response rate, pharmacokinetics (PK), pharmacodynamics (PD), safety, and tolerability of treatment with the humanized anti-epidermal growth factor receptor (EGFR) monoclonal antibody EMD 72000 (matuzumab) in patients with recurrent cervical cancer. *J Clin Oncol* 23: 174S.

Boll, B., Hansen, H., Heuck, F., Reiners, K., Borchmann, P., Rothe, A., Engert, A., von Strandmann, E.P. (2005) The fully human anti-CD30 antibody 5F11 activates NF-kappa B and sensitizes lymphoma cells to bortezomib-induced apoptosis. *Blood* 106: 1839–1842.

Borchmann, P., Schnell, R., Fuss, I., Manzke, O., Davis, T., Lewis, L.D., Behnke, D., Wickenhauser, C., Schiller, P., Diehl, V., Engert, A. (2002) Phase 1 trial of the novel bispecific molecule H22xKi-4 in patients with refractory Hodgkin lymphoma. *Blood* 100: 3101–3107.

Borjesson, P.K.E., Postema, E.J., Roos, J.C., Colnot, D.R., Marres, H.A.M., van Schie, M.H., Stehle, G., de Bree, R., Snow, G.B., Oyen, W.J.G., van Dongen, G.A.M.S. (2003) Phase I therapy study with Re-186-labeled humanized monoclonal antibody BIWA 4 (bivatuzumab) in patients with head and neck squamous cell carcinoma. *Clin Cancer Res* 9: 3961S–3972S.

Byrd, J.C., Bresalier, R.S. (2004) Mucins and mucin binding proteins in colorectal cancer. *Cancer Metastasis Rev* 23: 77–99.

Cassady, J.M., Chan, K.K., Floss, H.G., Leistner E. (2004) Recent developments in the maytansinoid antitumor agents. *Chem Pharm Bull (Tokyo)* 52: 1–26.

Cerveny, C.G., Law, C.L., McCormick, R.S., Lenox, J.S., Hamblett, K.J., Westendorf, L.E., Yamane, A.K., Petroziello, J.M., Francisco, J.A., Wahl, A.F. (2005) Signaling via the anti-CD30 mAb SGN-30 sensitizes Hodgkin's disease cells to conventional chemotherapeutics. *Leukemia* 19: 1648–1655.

Cheung, N.K.V., Kushner, B.H., Yeh, S.D.J., Larson, S.M. (1998) 3F8 monoclonal antibody treatment of patients with stage 4 neuroblastoma: a phase II study. *Int J Oncol* 12: 1299–1306.

Choi, B.S., Sondel, P.M., Hank, J.A., Schalch, H., Gan, J., King, D.M., Kendra, K., Mahvi, D., Lee, L.Y., Kim, K., Albertini, M.R. (2005) Phase I trial of combined treatment with ch14.18 and R24 monoclonal antibodies and interleukin-2 for patients with melanoma or sarcoma. *Cancer Immunol Immunother* 1–14.

Chong, G., Lee, F.T., Hopkins, W., Tebbutt, N., Cebon, J.S., Mountain, A.J., Chappell, B., Papenfuss, A., Schleyer, P., Murphy, R., Wirth, V., Smyth, F.E., Potasz, N., Poon, A., Davis, I.D., Saunder, T., O'keefe, G.J., Burgess, A.W., Hoffman, E.W., Old, L.J., Scott, A.M. (2005) Phase I trial of 131I-huA33 in patients with advanced colorectal carcinoma. *Clin Cancer Res* 11: 4818–4826.

Clarke, K., Lee, F.T., Brechbiel, M.W., Smyth, F.E., Old, L.J., Scott, A.M. (2000) Therapeutic efficacy of anti-Lewis(y) humanized 3S193 radioimmunotherapy in a breast cancer model: Enhanced activity when combined with taxol chemotherapy. *Clin Cancer Res* 6: 3621–3628.

Czuczman, M.S., Thall, A., Witzig, T.E., Vose, J.M., Younes, A., Emmanouilides, C., Miller, T.P., Moore, J.O., Leonard, J.P., Gordon, L.I., Sweetenham, J., Alkuzweny, B., Finucane, D.M., Leigh, B.R. (2005) Phase I/II study of galiximab, an anti-CD80 antibody, for relapsed or refractory follicular lymphoma. *J Clin Oncol* 23: 4390–4398.

Damle, N.K., Frost, P. (2003) Antibody-targeted chemotherapy with immunoconjugates of calicheamicin. *Curr Opin Pharmacol* 3: 386–390.

de Bono, J.S., Tolcher, A.W., Forero, A., Vanhove, G.F.A., Takimoto, C., Bauer, R.J., Hammond, L.A., Patnaik, A., White, M.L., Shen, S., Khazaeli, M.B., Rowinsky, E.K., LoBuglio, A.F. (2004) ING-1, a monoclonal antibody targeting Ep-CAM in patients with advanced adenocarcinomas. *Clin Cancer Res* 10: 7555–7565.

Deckert, P.M., Jungbluth, A., Montalto, N., Clark, M.A., Finn, R.D., Williams, C. Jr, Richards, E.C., Panageas, K.S., Old, L.J., Welt, S. 2000. Pharmacokinetics and microdistribution of polyethylene glycol-modified humanized A33 antibody targeting colon cancer xenografts. *Int J Cancer* 87: 382–390.

Deckert, P.M., Renner, C., Cohen, L.S., Jungbluth, A., Ritter, G., Bertino, J.R., Old, L.J., Welt, S. (2003) A33scFv-cytosine deaminase: a recombinant protein construct for antibody-directed enzyme-prodrug therapy. *Br J Cancer* 88: 937–939.

DeNardo, S.J., DeNardo, G.L., Miers, L.A., Natarajan, A., Foreman, A.R., Gruettner, C., Adamson, G.N., Ivkov, R. (2005a) Development of tumor targeting bioprobes ((111)In-chimeric L6 monoclonal antibody nanoparticles) for alternating magnetic field cancer therapy. *Clin Cancer Res* 11: 7087s–7092s.

DeNardo, S.J., Richman, C.M., Albrecht, H., Burke, P.A., Natarajan, A., Yuan, A., Gregg, J.P., O'Donnel, R.T., DeNardo, G.L. (2005b) Enhancement of the therapeutic index: From nonmyeloablative and myeloablative toward pretargeted radioimmunotherapy for metastatic prostate cancer. *Clin Cancer Res* 11: 7187S–7194S.

Dey, B.R., McAfee, S., Colby, C., Cieply, K., Caron, M., Saidman, S., Preffer, F., Shaffer, J., Tarbell, N., Sackstein, R., Sachs, D., Sykes, M., Spitzer, T.R. (2005) Anti-tumour response despite loss of donor chimaerism in patients treated with non-myeloablative conditioning and allogeneic stem cell transplantation. *Br J Haematol* 128: 351–359.

Dhingra, K., Fritsche, H., Murray, J.L., LoBuglio, A.F., Khazaeli, M.B., Kelley, S., Tepper, M.A., Grasela, D., Buzdar, A., Valero, V. (1995) Phase I clinical and pharmacological study of suppression of human antimouse antibody response to

monoclonal antibody L6 by deoxyspergualin. *Cancer Res* 55: 3060–3067.

Di Paolo, C., Willuda, J., Kubetzko, S., Lauffer, I., Tschudi, D., Waibel, R., Pluckthun, A., Stahel, R.A., Zangemeister-Wittke, U. (2003) A recombinant immunotoxin derived from a humanized epithelial cell adhesion molecule-specific single-chain antibody fragment has potent and selective antitumor activity. *Clin Cancer Res* 9: 2837–2848.

Divgi, C.R., Bander, N.H., Scott, A.M., O'Donoghue, J.A., Sgouros, G., Welt, S., Finn, R.D., Morrissey, F., Capitelli, P., Williams, J.M., Deland, D., Nakhre, A., Oosterwijk, E., Gulec, S., Graham, M.C., Larson, S.M., Old, L.J. (1998) Phase I/II radioimmunotherapy trial with iodine-131-labeled monoclonal antibody G250 in metastatic renal cell carcinoma. *Clin Cancer Res* 4: 2729–2739.

Divgi, C.R., O'Donoghue, J.A., Welt, S., O'Neel, J., Finn, R., Motzer, R.J., Jungbluth, A., Hoffman, E., Ritter, G., Larson, S.M., Old, L.J. (2004) Phase I clinical trial with fractionated radioimmunotherapy using I-13-labeled chimeric G250 in metastatic renal cancer. *J Nucl Med* 45: 1412–1421.

Durbin, H., Young, S., Stewart, L.M., Wrba, F., Rowan, A.J., Snary, D., Bodmer, W.F. (1994) An epitope an carcinoembryonic antigen defined by the clinically relevant antibody Pr1A3. *Proc Natl Acad Sci USA* 91: 4313–4317.

Duzkale, H., Pagliaro, L.C., Rosenblum, M.G., Varan, A., Liu, B.S., Reuben, J., Wierda, W.G., Korbling, M., McMannis, J.D., Glassman, A.B., Scheinberg, D.A., Freireich, E.J. (2003) Bone marrow purging studies in acute myelogenous leukemia using the recombinant anti-CD33 immunotoxin HuM195/rGel. *Biol Blood Marrow Transplant* 9: 364–372.

Ebbinghaus, C., Scheuermann, J., Neri, D., Elia, G. (2004) Diagnostic and therapeutic applications of recombinant antibodies: targeting the extra-domain B of fibronectin, a marker of tumor angiogenesis. *Curr Pharm Des* 10: 1537–1549.

Epenetos, A.A., Hird, V., Lambert, H., Mason, P., Coulter, C. (2000) Long term survival of patients with advanced ovarian cancer treated with intraperitoneal radioimmunotherapy. *Int J Gynecol Cancer* 10(S1): 44–46.

Farzaneh, L., Kasahara, N., Farzaneh, F. (2006) The strange case of TGN1412. *Cancer Immunol Immunother* (in press).

Fiedler, W., Kruger, W., Laack, E., Mende, T., Vohwinkel, G., Hossfeld DK. (2001) A clinical trial of edrecolomab, interleukin-2 and GM-CSF in patients with advanced colorectal cancer. *Oncol Rep* 8: 225–231.

Folkman, J. (2006) Angiogenesis. *Annu Rev Med* 57: 1–18.

Foon, K.A., Yang, X.D., Weiner, L.M., Belldegrun, A.S., Figlin, R.A., Crawford, J., Rowinsky, E.K., Dutcher, J.P., Vogelzang, N.J., Gollub, J., Thompson, J.A., Schwartz, G., Bukowski, R.M., Roskos, L.K., Schwab, G.M. (2004) Preclinical and clinical evaluations of ABX-EGF, a fully human anti-epidermal growth factor receptor antibody. *Int J Radiat Oncol Biol Phys* 58: 984–990.

Forero, A., Meredith, R.F., Khazaeli, M.B., Shen, S., Grizzle, W.E., Carey, D., Busby, E., LoBuglio, A.F., Robert, F. (2005) Phase I study of 90Y-CC49 monoclonal antibody therapy in patients with advanced non-small cell lung cancer: effect of chelating agents and paclitaxel co-administration. *Cancer Biother Radiopharm* 20: 467–478.

Francis, R.J., Sharma, S.K., Springer, C., Green, A.J., Hope-Stone, L.D., Sena, L., Martin, J., Adamson, K.L., Robbins, A., Gumbrell, L., O'Malley, D., Tsiompanou, E., Shahbakhti, H., Webley, S., Hochhauser, D., Hilson, A.J., Blakey, D., Begent, R.H. (2002) A phase I trial of antibody directed enzyme prodrug therapy (ADEPT) in patients with advanced colorectal carcinoma or other CEA producing tumours. *Br J Cancer* 87: 600–607.

Friess, T., Scheuer, W., Hasmann, M. (2005) Combination treatment with erlotinib and pertuzumab against human tumor xenografts is superior to monotherapy. *Clin Cancer Res* 11: 5300–5309.

Garkavij, M., Samarzija, M., Ewers, S.B., Jakopovic, M., Tezak, S., Tennvall, J. (2005) Concurrent radiotherapy and tumor targeting with 111In-HMFG1-F(ab')$_2$ in

patients with MUC1-positive non-small cell lung cancer. *Anticancer Res* 25: 4663–4671.
Georgakis, G.V., Li, Y., Humphreys, R., Andreeff, M., O'Brien, S., Younes, M., Carbone, A., Albert, V., Younes, A. (2005) Activity of selective fully human agonistic antibodies to the TRAIL death receptors TRAIL-R1 and TRAIL-R2 in primary and cultured lymphoma cells: induction of apoptosis and enhancement of doxorubicin- and bortezomib-induced cell death. *Br J Haematol* 130: 501–510.
Goodman, G.E., Hellstrom, I., Yelton, D.E., Murray, J.L., O'Hara, S., Meaker, E., Zeigler, L., Palazollo, P., Nicaise, C., Usakewicz J, . (1993) Phase I trial of chimeric (human-mouse) monoclonal antibody L6 in patients with non-small-cell lung, colon, and breast cancer. *Cancer Immunol Immunother* 36: 267–273.
Gottlieb, A.B., Lebwohl, M., Totoritis, M.C., Abdulghani, A.A., Shuey, S.R., Romano, P., Chaudhari, U., Allen, R.S., Lizambri, R.G. (2002) Clinical and histologic response to single-dose treatment of moderate to severe psoriasis with an anti-CD80 monoclonal antibody. *J Am Acad Dermatol* 47: 692–700.
Grana, C., Chinol, M., Robertson, C., Mazzetta, C., Bartolomei, M., De Cicco, C., Fiorenza, M., Gatti, M., Caliceti, P., Paganelli, G. (2002) Pretargeted adjuvant radioimmunotherapy with yttrium-90-biotin in malignant glioma patients: a pilot study. *Br J Cancer* 86: 207–212.
Gronau, S.S., Schmitt, M., Thess, B., Reinhardt, P., Wiesneth, M., Schmitt, A., Riechelmann, H. (2005) Trifunctional bispecific antibody-induced tumor cell lysis of squamous cell carcinomas of the upper aerodigestive tract. *Head Neck* 27: 376–382.
Grossbard, M.L., Multani, P.S., Freedman, A.S., O'Day, S., Gribben, J.G., Rhuda, C., Neuberg, D., Nadler, L.M. (1999) A Phase II study of adjuvant therapy with anti-B4-blocked ricin after autologous bone marrow transplantation for patients with relapsed B-cell non-Hodgkin's lymphoma. *Clin Cancer Res* 5: 2392–2398.
Grunwald, V., Hidalgo, M. (2003) Developing inhibitors of the epidermal growth factor receptor for cancer treatment. *J Natl Cancer Inst* 95: 851–867.
Hagberg, H., Pettersson, M., Bjerner, T., Enblad, G. (2005) Treatment of a patient with a nodal peripheral T-cell lymphoma (angioimmunoblastic T-cell lymphoma) with a human monoclonal antibody against the CD4 antigen (HuMax-CD4). *Med Oncol* 22: 191–194.
Hamann, P.R., Hinman, L.M., Beyer, C.F., Lindh, D., Upeslacis, J., Shochat, D., Mountain, A. (2005) A calicheamicin conjugate with a fully humanized anti-MUC1 antibody shows potent antitumor effects in breast and ovarian tumor xenografts. *Bioconjug Chem* 16: 354–360.
Hanai, N., Nakamura, K., Shitara, K. (2000) Recombinant antibodies against ganglioside expressed on tumor cells. *Cancer Chemother Pharmacol* 46: S13–S17.
Hangai, M., Kitaya, N., Xu, J.S., Chan, C.K., Kim, J.J., Werb, Z., Ryan, S.J., Brooks, P.C. (2002) Matrix metalloproteinase-9-dependent exposure of a cryptic migratory control site in collagen is required before retinal angiogenesis. *Am J Pathol* 161: 1429–1437.
Hann, E., Reinartz, S., Clare, S.E., Passow, S., Kissel, T., Wagner, U. (2005) Development of a delivery system for the continuous endogenous release of an anti-idiotypic antibody against ovarian carcinoma. *Hybridoma* 24: 133–140.
Hansen, S., Leslie, R.G. (2006) TGN1412: scrutinizing preclinical trials of antibody-based medicines. *Nature* 441: 282.
Hartmann, F., Renner, C., Jung, W., da Costa, L., Tembrink, S., Held, G., Sek, A., Konig, J., Bauer, S., Kloft, M., Pfreundschuh, M. (2001) Anti-CD16/CD30 bispecific antibody treatment for Hodgkin's disease: Role of infusion schedule and costimulation with cytokines. *Clin Cancer Res* 7: 1873–1881.
Hartung, G., Hofheinz, R.D., Dencausse, Y., Sturm, J., Kopp-Schneider, A., Dietrich, G., Fackler-Schwalbe, I., Bornbusch, D., Gonnermann, M., Wojatschek, C., Lindemann, W., Eschenburg, H., Jost, K., Edler, L., Hochhaus, A., Queisser, W. (2005) Adjuvant therapy with edrecolomab versus observation in stage II colon cancer: A multicenter randomized phase III study. *Onkologie* 28: 347–350.
Hassan, R., Williams-Gould, J., Watson, T., Pai-Scherf, L., Pastan, I. (2004)

Pretreatment with rituximab does not inhibit the human immune response against the immunogenic protein LMB-1. *Clin Cancer Res* 10(1 Pt 1): 16–18.

Haug, C., Gotzsche, P.C., Schroeder, T.V. (2005) Registries and registration of clinical trials. *N Engl J Med* 353: 2811–2812.

Heath, J.K., White, S.J., Johnstone, C.N., Catimel, B., Simpson, R.J., Moritz, R.L., Tu, G.F., Ji, H., Whitehead, R.H., Groenen, L.C., Scott, A.M., Ritter, G., Cohen, L., Welt, S., Old, L.J., Nice, E.C., Burgess, A.W. (1997) The human A33 antigen is a transmembrane glycoprotein and a novel member of the immunoglobulin superfamily. *Proc Natl Acad Sci USA* 94: 469–474.

Heider, K.H., Kuthan, H., Stehle, G., Munzert, G. (2004) CD44v6: a target for antibody-based cancer therapy. *Cancer Immunol Immunother* 53: 567–579.

Hoffmann, P., Hofmeister, R., Brischwein, K., Brandl, C., Crommer, S., Bargou, R., Itin, C., Prang, N., Baeuerle, P.A. (2005) Serial killing of tumor cells by cytotoxic T cells redirected with a CD19-/CD3-bispecific single-chain antibody construct. *Int J Cancer* 115: 98–104.

Hofheinz, R.D., al Batran, S.E., Hartmann, F., Hartung, G., Jager, D., Renner, C., Tanswell, P., Kunz, U., Amelsberg, A., Kuthan, H., Stehle, G. (2003) Stromal antigen targeting by a humanised monoclonal antibody: An early phase II trial of sibrotuzamab in patients with metastatic colorectal cancer. *Onkologie* 26: 44–48.

Hotz, H.G., Hines, O.J., Hotz, B., Foitzik, T., Buhr, H.J., Reber, H.A. 2003. Evaluation of vascular endothelial growth factor blockade and matrix metalloproteinase inhibition as a combination therapy for experimental human pancreatic cancer. *J Gastrointest Surg* 7: 220–227.

Huang, S.Y., Mills, L., Mian, B., Tellez, C., McCarty, M., Yang, X.D., Gudas, J.M., Bar-Eli, M. (2002) Fully humanized neutralizing antibodies to interleukin-8 (ABX-IL8) inhibit angiogenesis, tumor growth, and metastasis of human melanoma. *Am J Pathol* 161: 125–134.

Irie, R.F., Ollila, D.W., O'Day, S., Morton, D.L. (2004) Phase I pilot clinical trial of human IgM monoclonal antibody to ganglioside GM3 in patients with metastatic melanoma. *Cancer Immunol Immunother* 53: 110–117.

James, N.D., Atherton, P.J., Jones, J., Howie, A.J., Tchekmedyian, S., Curnow, R.T. (2001) A phase II study of the bispecific antibody MDX-H210 (anti-HER2 x CD64) with GM-CSF in HER2+advanced prostate cancer. *Br J Cancer* 85: 152–156.

Jayson, G.C., Mulatero, C., Ranson, M., Zweit, J., Jackson, A., Broughton, L., Wagstaff, J., Hakansson, L., Groenewegen, G., Lawrance, J., Tang, M., Wauk, L., Levitt, D., Marreaud, S., Lehmann, F.F., Herold, M., Zwierzina, H. (2005) Phase I investigation of recombinant anti-human vascular endothelial growth factor antibody in patients with advanced cancer. *Eur J Cancer* 41: 555–563.

Kim, Y.H., Obitz, E., Iversen, L., Oesterborg, A., Whittaker, S., Illidge, T.M., Schwarz, T., Kaufmann, R., Gniadecki, R., Duvic, M., Cooper, K., Jensen, P., Baadsgaard, O., Knox, S.J. (2004) HuMax-CD4, fully human monoclonal antibody: Phase II trial in cutaneous T cell lymphoma. *J Invest Dermatol* 122: A57.

Kiner-Strachan, B., Goel, S., Vanhove, G., Bauer, R.J., Verdier-Pinard, D., Karri, S., Desai, K., Bulgaru, A., Macapinlac, M., Mani, S. (2005) Phase I and pharmacokinetic study of a subcutaneously administered human-engineered monoclonal antibody ING-1 in patients with advanced adenocarcinomas. *J Clin Oncol* 23: 180S.

King, D.M., Albertini, M.R., Schalch, H., Hank, J.A., Gan, J., Surfus, J., Mahvi, D., Schiller, J.H., Warner, T., Kim, K., Eickhoff, J., Kendra, K., Reisfeld, R., Gillies, S.D., Sondel, P. (2004) Phase I clinical trial of the immunocytokine EMD 273063 in melanoma patients. *J Clin Oncol* 22: 4463–4473.

Kleinschmidt, M., Rudolph, R., Lilie, H. (2003) Design of a modular immunotoxin connected by polyionic adapter peptides. *J Mol Biol* 327: 445–452.

Klinger, M., Farhan, H., Just, H., Drobny, H., Himmler, G., Loibner, H., Mudde, G.C., Freissmuth, M., Sexl, V. (2004) Antibodies directed against Lewis-Y antigen inhibit signaling of Lewis-Y

modified ErbB receptors. *Cancer Res* 64: 1087–1093.

Knox, S.J., Goris, M.L., Tempero, M., Weiden, P.L., Gentner, L., Breitz, H., Adams, G.P., Axworthy, D., Gaffigan, S., Bryan, K., Fisher, D.R., Colcher, D., Horak, I.D., Weiner, L.M. (2000) Phase II trial of yttrium-90-DOTA-biotin pretargeted by NR-LU-10 antibody/streptavidin in patients with metastatic colon cancer. *Clin Cancer Res* 6: 406–414.

Ko, Y.J., Bubley, G.J., Weber, R., Redfern, C., Gold, D.P., Finke, L., Kovar, A., Dahl, T., Gillies, S.D. (2004) Safety, pharmacokinetics, and biological pharmacodynamics of the immunocytokine EMD 273066 (huKS-IL2) – Results of a phase 1 trial in patients with prostate cancer. *J Immunother* 27: 232–239.

Köhler, G., Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256: 495–497.

Koppe, M., van Schaijk, F., Roos, J., van Leeuwen, P., Heider, K.H., Kuthan, H., Bleichrodt, R. (2004) Safety, pharmacokinetics, immunogenicity, and biodistribution of Re-186-labeled humanized monoclonal antibody BIWA 4 (bivatuzumab) in patients with early-stage breast cancer. *Cancer Biother Radiopharm* 19: 720–729.

Koppe, M.J., Bleichrodt, R.P., Oyen, W.J., Boerman, O.C. (2005) Radioimmunotherapy and colorectal cancer. *Br J Surg* 92: 264–276.

Kostenuik, P.J. (2005) Osteoprotegerin and RANKL regulate bone resorption, density, geometry and strength. *Curr Opin Pharmacol* 5: 618–625.

Kraeber-Bodere, F., Faivre-Chauvet, A., Ferrer, L., Vuillez, J.P., Brard, P.Y., Rousseau, C., Resche, I., Devillers, A., Laffont, S., Bardies, M., Chang, K., Sharkey, R.M., Goldenberg, D.M., Chatal, J.F., Barbet, J. (2003) Pharmacokinetics and dosimetry studies for optimization of anti-carcinoembryonic antigen x anti-hapten bispecific antibody-mediated pretargeting of Iodine-131-labeled hapten in a phase I radioimmunotherapy trial. *Clin Cancer Res* 9: 3973S–3981S.

Kreitman, R.J., Wilson, W.H., White, J.D., Stetler-Stevenson, M., Jaffe, E.S., Giardina, S., Waldmann, T.A., Pastan, I. (2000) Phase I trial of recombinant immunotoxin anti-Tac(Fv)PE38 (LMB-2) in patients with hematologic malignancies. *J Clin Oncol* 18: 1622–1636.

Kreitman, R.J., Squires, D.R., Stetler-Stevenson, M., Noel, P., Fitzgerald, D.J.P., Wilson, W.H., Pastan, I. (2005) Phase I trial of recombinant immunotoxin RFB4(dsFv)-PE38 (BL22) in patients with B-cell malignancies. *J Clin Oncol* 23: 6719–6729.

Kushner, B.H., Kramer, K., Cheung, N.K.V. (2001) Phase II trial of the anti-G(D2) monoclonal antibody 3F8 and granulocyte-macrophage colony-stimulating factor for neuroblastoma. *J Clin Oncol* 19: 4189–4194.

Ladwein, M., Pape, U.F., Schmidt, D.S., Schnolzer, M., Fiedler, S., Langbein, L., Franke, W.W., Moldenhauer, G., Zoller, M. (2005) The cell-cell adhesion molecule EpCAM interacts directly with the tight junction protein claudin-7. *Exp Cell Res* 309: 345–357.

Lam, J.S., Pantuck, A.J., Belldegrun, A.S., Figlin, R.A. (2005) G250: a carbonic anhydrase IX monoclonal antibody. *Curr Oncol Rep* 7: 109–115.

Law, C.L., Gordon, K.A., Collier, J., Klussman, K., McEarchern, J.A., Cerveny, C.G., Mixan, B.J., Lee, W.P., Lin, Z.H., Valdez, P., Wahl, A.F., Grewal, I.S. (2005) Preclinical antilymphoma activity of a humanized anti-CD40 monoclonal antibody, SGN-40. *Cancer Res* 65: 8331–8338.

Li, Q., Verschraegen, C.F., Mendoza, J., Hassan, R. (2004) Cytotoxic activity of the recombinant anti-mesothelin immunotoxin, SS1(dsFv)PE38, towards tumor cell lines established from ascites of patients with peritoneal mesotheliomas. *AntiCancer Res* 24: 1327–1335.

Liersch, T., Meller, J., Kulle, B., Behr, T.M., Markus, P., Langer, C., Ghadimi, B.M., Wegener, W.A., Kovacs, J., Horak, I.D., Becker, H., Goldenberg, D.M. (2005) Phase II trial of carcinoembryonic antigen radioimmunotherapy with ^{131}I-labetuzumab after salvage resection of colorectal metastases in the liver: five-year safety and efficacy results. *J Clin Oncol* 23: 6763–6770.

Longo, D.L., Duffey, P.L., Gribben, J.G., Jaffe, E.S., Curti, B.D., Gause, B.L., Janik, J.E., Braman, V.M., Esseltine, D., Wilson, W.H., Kaufman, D., Wittes, R.E., Nadler, L.M., Urba, W.J. (2000) Combination chemotherapy followed by an immunotoxin (anti-B4-blocked ricin) in patients with indolent lymphoma: results of a phase II study. *Cancer J* 6: 146–150.

Maile, L.A., Busby, W.H., Sitko, K., Capps, B.E., Sergent, T., Badley-Clarke, J., Clemmons, D.R. (2006) Insulin-like growth factor-i signaling in smooth muscle cells is regulated by ligand binding to the 177CYDMKTTC184 Sequence of the β_3-subunit of $\alpha_v\beta_3$. *Mol Endocrinol* 20: 405–413.

Makower, D., Sparano, J.A., Wadler, S., Fehn, K., Landau, L., Wissel, P., Versola, M., Mani, S. (2003) A pilot study of edrecolomab (Panorex, 17-1A antibody) and capecitabine in patients with advanced or metastatic adenocarcinoma. *Cancer Invest* 21: 177–184.

Martin, J., Stribbling, S.M., Poon, G.K., Begent, R.H., Napier, M., Sharma, S.K., Springer, C.J. (1997) Antibody-directed enzyme prodrug therapy: pharmacokinetics and plasma levels of prodrug and drug in a phase I clinical trial. *Cancer Chemother Pharmacol* 40: 189–201.

Maxwell-Armstrong, C.A., Durrant, L.G., Buckley, T.J.D., Scholefield, J.H., Robins, R.A., Fielding, K., Monson, J.R.T., Guillou, P., Calvert, H., Carmichael, J., Hardcastle, J.D. (2001) Randomized double-blind phase II survival study comparing immunization with the anti idiotypic monoclonal antibody 105AD7 against placebo in advanced colorectal cancer. *Br J Cancer* 84: 1443–1446.

Mayer, A., Sharma, S.K., Tolner, B., Minton, N.P., Purdy, D., Amlot, P., Tharakan, G., Begent, R.H., Chester, K.A. (2004) Modifying an immunogenic epitope on a therapeutic protein: a step towards an improved system for antibody-directed enzyme prodrug therapy (ADEPT). *Br J Cancer* 90: 2402–2410.

Meredith, R.F., LoBuglio, A.F., Plott, W.E., Orr, R.A., Brezovich, I.A., Russell, C.D., Harvey, E.B., Yester, M.V., Wagner, A.J., Spencer, S.A., Wheeler, R.H., Saleh, M.N., Rogers, K.J., Polansky, A., Salter, M.M., Khazaeli, M.B. (1991) Pharmacokinetics, immune-response, and biodistribution of iodine-131-labeled chimeric mouse human Igg1,K 17-1A monoclonal-antibody. *J Nucl Med* 32: 1162–1168.

Meredith, R., Shen, S., Macey, D., Khazaeli, M.B., Carey, D., Robert, F., LoBuglio, A. (2003a) Comparison of biodistribution, dosimetry, and outcome from clinical trials of radionuclide-CC49 antibody therapy. *Cancer Biother Radiopharm* 18: 393–404.

Meredith, R., Shen, S., Macey, D., Khazaeli, M.B., Carey, D., Robert, F., LoBuglio, A. (2003b) Comparison of biodistribution, dosimetry, and outcome from clinical trials of radionuclide-CC49 antibody therapy. *Cancer Biother Radiopharm* 18: 393–404.

Messmann, R.A., Vitetta, E.S., Headlee, D., Senderowicz, A.M., Figg, W.D., Schindler, J., Michiel, D.F., Creekmore, S., Steinberg, S.M., Kohler, D., Jaffe, E.S., Stetler-Stevenson, M., Chen, H.C., Ghetie, V., Sausville, E.A. (2000) A phase I study of combination therapy with immunotoxins IgG-HD37-deglycosylated ricin A chain (dgA) and IgG-RFB4-dgA (Combotox) in patients with refractory CD19(+), CD22(+) B cell lymphoma. *Clin Cancer Res* 6: 1302–1313.

Milowsky, M.I., Nanus, D.M., Kostakoglu, L., Vallabhajosula, S., Goldsmith, S.J., Bander, N.H. (2004) Phase I trial of yttrium-90-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 for androgen-independent prostate cancer. *J Clin Oncol* 22: 2522–2531.

Milstein, C., Cuello, A.C. (1983) Hybrid hybridomas and their use in immunohistochemistry. *Nature* 305: 537–540.

Mobus, V.J., Baum, R.P., Bolle, M., Kreienberg, R., Noujaim, A.A., Schultes, B.C., Nicodemus, C.F. (2003) Immune responses to murine monoclonal antibody-B43.13 correlate with prolonged survival of women with recurrent ovarian cancer. *Am J Obstet Gynecol* 189: 28–36.

Morris, M.J., Divgi, C.R., Pandit-Taskar, N., Batraki, M., Warren, N., Nacca, A., Smith-Jones, P., Schwartz, L., Kelly, W.K., Slovin, S., Solit, D., Halpern, J., Delacruz, A.,

Curley, T., Finn, R., O'Donoghue, J.A., Livingston, P., Larson, S., Scher, H.I. (2005) Pilot trial of unlabeled and indium-111-labeled anti-prostate-specific membrane antigen antibody J591 for castrate metastatic prostate cancer. *Clin Cancer Res* 11: 7454–7461.

Mulders, P.F., Debruyne, F.M.J., de Weijer, K., Rossi, J.F., Patil, S., Cooper, J., Prabhakar, U., Garay, C., Corringham, R. (2005) A phase I/II study of a chimeric antibody against interleukin-6 (CNTO 328) in subjects with metastatic renal cell carcinoma. *J Urol* 173: 380.

Multani, P.S., O'Day, S., Nadler, L.M., Grossbard, M.L. (1998) Phase II clinical trial of bolus infusion anti-B4 blocked ricin immunoconjugate in patients with relapsed B-cell non-Hodgkin's lymphoma. *Clin Cancer Res* 4: 2599–2604.

Nahta, R., Hung, M.C., Esteva, F.J. (2004) The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. *Cancer Res* 64: 2343–2346.

Naundorf, S., Preithner, S., Mayer, P., Lippold, S., Wolf, A., Hanakam, F., Fichtner, I., Kufer, P., Raum, T., Riethmuller, G., Baeuerle, P.A., Dreier, T. (2002) In vitro and in vivo activity of MT201, a fully human monoclonal antibody for pancarcinoma treatment. *Int J Cancer* 100: 101–110.

Nechansky, A., Kircheis, R. (2005) EpCAM is not a LAIR-1 ligand! *Cancer Biol Ther* 4: 357.

Nicholson, S., Bomphray, C.C., Thomas, H., McIndoe, A., Barton, D., Gore, M., George, A.J. (2004) A phase I trial of idiotypic vaccination with HMFG1 in ovarian cancer. *Cancer Immunol Immunother* 53: 809–816.

Noujaim, A.A., Schultes, B.C., Baum, R.P., Madiyalakan, R. (2001) Induction of CA125-specific B and T cell responses in patients injected with MAb-B43.13–evidence for antibody-mediated antigen-processing and presentation of CA125 in vivo. *Cancer Biother Radiopharm* 16: 187–203.

Pai, L.H., Wittes, R., Setser, A., Willingham, M.C., Pastan, I. (1996) Treatment of advanced solid tumors with immunotoxin LMB-1: an antibody linked to *Pseudomonas* exotoxin. *Nat Med* 2: 350–353.

Pastan, I. (2003) Immunotoxins containing *Pseudomonas* exotoxin A: a short history. *Cancer Immunol Immunother* 52: 338–341.

Pavlinkova, G., Beresford, G.W., Booth, B.J., Batra, S.K., Colcher, D. (1999) Pharmacokinetics and biodistribution of engineered single-chain antibody constructs of MAb CC49 in colon carcinoma xenografts. *J Nucl Med* 40: 1536–1546.

Peters, M., Dasilva, A., Weckermann, D., Oberneder, R., Ebner, B., Kirchinger, P., Fetter, A., Kohne-Volland, R., Baeuerle, P., Gjorstrup, P. (2004) Phase I study of the novel fully human monoclonal antibody MT201, directed against epithelial cellular adhesion molecule (Ep-CAM), in patients with hormone-refractory prostate cancer (HRPC). *J Clin Oncol* 22: 188S.

Petit, A.M., Rak, J., Hung, M.C., Rockwell, P., Goldstein, N., Fendly, B., Kerbel, R.S. (1997) Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 151: 1523–1530.

Porchet, N., Aubert, J.P. (2004) [MUC genes: mucin or not mucin? That is the question]. *Med Sci (Paris)* 20: 569–574.

Postema, E.J., Raemaekers, J.M.M., Oyen, W.J.G., Boerman, O.C., Mandigers, C.M.P.W., Goldenberg, D.M., van Dongen, G.A.M.S., Corstens, F.H.M. (2003) Final results of a phase I radioimmunotherapy trial using Re-186-eprat uzumab for the treatment of patients with non-Hodgkin's lymphoma. *Clin Cancer Res* 9: 3995S–4002S.

Pritchard-Jones, K., Spendlove, I., Wilton, C., Whelan, J., Weeden, S., Lewis, I., Hale, J., Douglas, C., Pagonis, C., Campbell, B., Alvarez, P., Halbert, G., Durrant, L.G. (2005) Immune responses to the 105AD7 human anti-idiotypic vaccine after intensive chemotherapy, for osteosarcoma. *Br J Cancer* 92: 1358–1365.

Pukac, L., Kanakaraj, P., Humphreys, R., Alderson, R., Bloom, M., Sung, C.,

Riccobene, T., Johnson, R., Fiscella, M., Mahoney, A., Carrell, J., Boyd, E., Yao, X.T., Zhang, L., Zhong, L., von Kerczek, A., Shepard, L., Vaughan, T., Edwards, B., Dobson, C., Salcedo, T., Albert, V. (2005) HGS-ETR1, a fully human TRAIL-receptor 1 monoclonal antibody, induces cell death in multiple tumour types in vitro and in vivo. *Br J Cancer* 92: 1430–1441.

Rader, C., Ritter, G., Nathan, S., Elia, M., Gout, I., Jungbluth, A.A., Cohen, L.S., Welt, S., Old, L.J., Barbas, C.F., III. (2000) The rabbit antibody repertoire as a novel source for the generation of therapeutic human antibodies. *J Biol Chem* 275: 13668–13676.

Rader, C., Popkov, M., Neves, J.A., Barbas, C.F. (2002) Integrin alpha v beta 3-targeted therapy for Kaposi's sarcoma with an in vitro-evolved antibody. *FASEB J* 16.

Ramakrishnan, V., Bhaskar, V., Law, D.A., Wong, M.H.L., Green, J., Tso, J., Jeffry, U., Finck, B., Murray, R. (2004) M200, an integrin alpha 5 beta 1 antibody, promotes apoptosis in proliferating endothelial cells. *J Clin Oncol* 22: 240S.

Ran, S., He, J., Huang, X., Soares, M., Scothorn, D., Thorpe, P.E. (2005) Antitumor effects of a monoclonal antibody that binds anionic phospholipids on the surface of tumor blood vessels in mice. *Clin Cancer Res* 11: 1551–1562.

Reardon, D.A., Akabani, G., Coleman, R.E., Friedman, A.H., Friedman, H.S., Herndon, J.E., McLendon, R.E., Pegram, C.N., Provenzale, J.M., Quinn, J.A., Rich, J.N., Vredenburgh, J.J., Desjardins, A., Guruangan, S., Badruddoja, M., Dowell, J.M., Wong, T.Z., Zhao, X.G., Zalutsky, M.R., Bigner, D.D. (2006) Salvage radioimmunotherapy with murine iodine-131-labeled antitenascin monoclonal antibody 81C6 for patients with recurrent primary and metastatic malignant brain tumors: phase II study results. *J Clin Oncol* 24: 115–122.

Reinartz, S., Kohler, S., Schlebusch, H., Krista, K., Giffels, P., Renke, K., Huober, J., Mobus, V., Kreienberg, R., duBois, A., Sabbatini, P., Wagner, U. (2004) Vaccination of patients with advanced ovarian carcinoma with the anti-idiotype ACA125: Immunological response and survival (Phase Ib/II) *Clin Cancer Res* 10: 1580–1587.

Renner, C., Hartmann, F., Jung, W., Deisting, C., Juwana, M., Pfreundschuh, M. (2000) Initiation of humoral and cellular immune responses in patients with refractory Hodgkin's disease by treatment with an anti-CD16/CD30 bispecific antibody. *Cancer Immunol Immunother* 49: 173–180.

Repp, R., van Ojik, H.H., Valerius, T., Groenewegen, G., Wieland, G., Oetzel, C., Stockmeyer, B., Becker, W., Eisenhut, M., Steininger, H., Deo, Y.M., Blijham, G.H., Kalden, J.R., van de Winkel, J.G., Gramatzki, M. (2003) Phase I clinical trial of the bispecific antibody MDX-H210 (anti-FcgammaRI × anti-HER-2/neu) in combination with Filgrastim (G-CSF) for treatment of advanced breast cancer. *Br J Cancer* 89: 2234–2243.

Ribas, A., Camacho, L.H., Lopez-Berestein, G., Pavlov, D., Bulanhagui, C.A., Millham, R., Comin-Anduix, B., Reuben, J.M., Seja, E., Parker, C.A., Sharma, A., Glaspy, J.A., Gomez-Navarro, J. (2005) Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206. *J Clin Oncol* 23: 8968–8977.

Richman, C.M., DeNardo, S.J., O'Grady, L.F., DeNardo, G.L. (1995) Radioimmunotherapy for breast cancer using escalating fractionated doses of ^{131}I-labeled chimeric L6 antibody with peripheral blood progenitor cell transfusions. *Cancer Res* 55(23 Suppl): 5916s–5920s.

Richman, C.M., DeNardo, S.J., O'Donnell, R.T., Yuan, A., Shen, S., Goldstein, D.S., Tuscano, J.M., Wun, T., Chew, H.K., Lara, P.N., Kukis, D.L., Natarajan, A., Meares, C.F., Lamborn, K.R., DeNardo, G.L. (2005) High-dose radioimmunotherapy combined with fixed, low-dose paclitaxel in metastatic prostate and breast cancer by using a MUC-1 monoclonal antibody, m170, linked to indium-111/yttrium-90 via a cathepsin cleavable linker with cyclosporine to prevent human anti-mouse antibody. *Clin Cancer Res* 11: 5920–6927.

Riethmuller, G., Holz, E., Schlimok, G., Schmiegel, W., Raab, R., Hoffken, K., Gruber, R., Funke, I., Pichlmaier, H., Hirche, H., Buggisch, P., Witte, J., Pichlmayr, R. (1998) Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial. *J Clin Oncol* 16: 1788–1794.

Rizzieri, D.A., Akabani, G., Zalutsky, M.R., Coleman, R.E., Metzler, S.D., Bowsher, J.E., Toaso, B., Anderson, E., Lagoo, A., Clayton, S., Pegram, C.N., Moore, J.O., Gockerman, J.P., DeCastro, C., Gasparetto, C., Chao, N.J., Bigner, D.D. (2004) Phase 1 trial study of I-131-labeled chimeric 81C6 monoclonal antibody for the treatment of patients with non-Hodgkin lymphoma. *Blood* 104: 642–648.

Rossi, J.F., Fegueux, N., Lu, Z.Y., Legouffe, E., Exbrayat, C., Bozonnat, M.C., Navarro, R., Lopez, E., Quittet, P., Daures, J.P., Rouille, V., Kanouni, T., Widjenes, J., Klein, B. (2005) Optimizing the use of anti-interleukin-6 monoclonal antibody with dexamethasone and 140 mg/m^2 of melphalan in multiple myeloma: results of a pilot study including biological aspects. *Bone Marrow Transplant* 36: 771–779.

Rowinsky, E.K., Schwartz, G.H., Gollob, J.A., Thompson, J.A., Vogelzang, N.J., Figlin, R., Bukowski, R., Haas, N., Lockbaum, P., Li, Y.P., Arends, R., Foon, K.A., Schwab, G., Dutcher, J. (2004) Safety, pharmacokinetics, and activity of ABX-EGF, a fully human anti-epidermal growth factor receptor monoclonal antibody in patients with metastatic renal cell cancer. *J Clin Oncol* 22: 3003–3015.

Sanderson, K., Scotland, R., Lee, P., Liu, D. X., Groshen, S., Snively, J., Sian, S., Nichol, G., Davis, T., Keler, T., Yellin, M., Weber, J. (2005) Autoimmunity in a phase I trial of a fully human anti-cytotoxic T-lymphocyte antigen-4 monoclonal antibody with multiple melanoma peptides and montanide ISA 51 for patients with resected stages III and IV melanoma. *J Clin Oncol* 23: 741–750.

Schlereth, B., Quadt, C., Dreier, T., Kufer, P., Lorenczewski, G., Prang, N., Brandl, C., Lippold, S., Cobb, K., Brasky, K., Leo, E., Bargou, R., Murthy, K., Baeuerle, P.A. (2005) T-cell activation and B-cell depletion in chimpanzees treated with a bispecific anti-CD19/anti-CD3 single-chain antibody construct. *Cancer Immunol Immunother* 1–12.

Schmitt, M., Schmitt, A., Reinhardt, P., Thess, B., Manfras, B., Lindhofer, H., Riechelmann, H., Wiesneth, M., Gronau, S. (2004) Opsonization with a trifunctional bispecific (alphaCD3 x alphaEpCAM) antibody results in efficient lysis in vitro and in vivo of EpCAM positive tumor cells by cytotoxic T lymphocytes. *Int J Oncol* 25: 841–848.

Schneider, C.K., Kalinke, U., Lower, J. (2006) TGN1412 – a regulator's perspective. *Nat Biotechnol* 24: 493–496.

Schnell, R., Vitetta, E., Schindler, J., Barth, S., Winkler, U., Borchmann, P., Hansmann, M.L., Diehl, V., Ghetie, V., Engert, A. (1998) Clinical trials with an anti-CD25 ricin A-chain experimental and immunotoxin (RFT5-SMPT-dgA) in Hodgkin's lymphoma. *Leukemia Lymphoma* 30: 525.

Schultes, B.C., Baum, R.P., Niesen, A., Noujaim, A.A., Madiyalakan, R. (1998) Anti-idiotype induction therapy: anti-CA125 antibodies (Ab3) mediated tumor killing in patients treated with Ovarex mAb B43.13 (Ab1). *Cancer Immunol Immunother* 46: 201–212.

Scott, A.M., Geleick, D., Rubira, M., Clarke, K., Nice, E.C., Smyth, F.E., Stockert, E., Richards, E.C., Carr, F.J., Harris, W.J., Armour, K.L., Rood, J., Kypridis, A., Kronina, V., Murphy, R., Lee, F.T., Liu, Z.Q., Kitamura, K., Ritter, G., Laughton, K., Hoffman, E., Burgess, A.W., Old, L.J. (2000) Construction, production, and characterization of humanized anti-Lewis Y monoclonal antibody 3S193 for targeted immunotherapy of solid tumors. *Cancer Res* 60: 3254–3261.

Scott, A.M., Wiseman, G., Welt, S., Adjei, A., Lee, F.T., Hopkins, W., Divgi, C.R., Hanson, L.H., Mitchell, P., Gansen, D.N., Larson, S.M., Ingle, J.N., Hoffman, E.W., Tanswell, P., Ritter, G., Cohen, L.S., Bette, P., Arvay, L., Amelsberg, A., Vlock, D., Rettig, W.J., Old, L.J. (2003) A phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic

fibroblast activation protein-positive cancer. *Clin Cancer Res* 9: 1639–1647.

Scott, A.M., Lee, F.T., Jones, R., Hopkins, W., MacGregor, D., Cebon, J.S., Hannah, A., Chong, G., U, P., Papenfuss, A., Rigopoulos, A., Sturrock, S., Murphy, R., Wirth, V., Murone, C., Smyth, F.E., Knight, S., Welt, S., Ritter, G., Richards, E., Nice, E.C., Burgess, A.W., Old, L.J. (2005) A phase I trial of humanized monoclonal antibody A33 in patients with colorectal carcinoma: biodistribution, pharmacokinetics, and quantitative tumor uptake. *Clin Cancer Res* 11: 4810–4817.

Seiden, M., Burris, H.A., Matulonis, U., Hall, J., Armstrong, D., Speyer, J., Tillner, J., Weber, D., Muggia, F. (2005) A phase II trial of EMD7(2000) (matuzumab), a humanized anti-EGFR monoclonal antibody in subjects with heavily treated and platinum-resistant advanced Mullerian malignancies. *J Clin Oncol* 23: 229S.

Sharkey, R.M., Hajjar, G., Yeldell, D., Brenner, A., Burton, J., Rubin, A., Goldenberg, D.M. (2005a) A phase I trial combining high-dose 90Y-labeled humanized anti-CEA monoclonal antibody with doxorubicin and peripheral blood stem cell rescue in advanced medullary thyroid cancer. *J Nucl Med* 46: 620–633.

Sharkey, R.M., Karacay, H., Chang, C.H., McBride, W.J., Horak, I.D., Goldenberg, D.M. (2005b) Improved therapy of non-Hodgkin's lymphoma xenografts using radionuclides pretargeted with a new anti-CD20 bispecific antibody. *Leukemia* 19: 1064–1069.

Shen, S., Forero, A., LoBuglio, A.F., Breitz, H., Khazaeli, M.B., Fisher, D.R., Wang, W., Meredith, R.F. (2005) Patient-specific dosimetry of pretargeted radioimmunotherapy using CC49 fusion protein in patients with gastrointestinal malignancies. *J Nucl Med* 46: 642–651.

Siemers, N.O., Kerr, D.E., Yarnold, S., Stebbins, M.R., Vrudhula, V.M., Hellstrom, I., Hellstrom, K.E., Senter, P.D. (1997) Construction, expression, and activities of L49-sFv-beta-lactamase, a single-chain antibody fusion protein for anticancer prodrug activation. *Bioconjug Chem* 8: 510–519.

Simon, T., Hero, B., Faldum, A., Handgretinger, R., Schrappe, M., Niethammer, D., Berthold, F. (2004) Consolidation treatment with chimeric anti-GD2-antibody ch14.18 in children older than 1 year with metastatic neuroblastoma. *J Clin Oncol* 22: 3549–3557.

Simon, T., Hero, B., Faldum, A., Handgretinger, R., Schrappe, M., Niethammer, D., Berthold, F. (2005) Infants with stage 4 neuroblastoma: the impact of the chimeric anti-GD2-antibody ch14.18 consolidation therapy. *Klin Padiatr* 217: 147–152.

Skov, L., Kragballe, K., Zachariae, C., Obitz, E.R., Holm, E.A., Jemec, G.B.E., Solvsten, H., Ibsen, H.H., Knudsen, L., Jensen, P., Petersen, J.H., Menne, T., Baadsgaard, O. (2003) HuMax-CD4 – A fully human monoclonal anti-CD4 antibody for the treatment of psoriasis vulgaris. *Arch Dermatol* 139: 1433–1439.

Spendlove, I., Li, L., Potter, V., Christiansen, D., Loveland, B.E., Durrant, L.G. (2000) A therapeutic human anti-idiotypic antibody mimics CD55 in three distinct regions. *Eur J Immunol* 30: 2944–2953.

Staak, J.O., Colcher, D., Wang, J.Y., Engert, A., Raubitschek, A.A. (2004) Radioimmunotherapy utilizing the humanized anti-CD25 MAb daclizumab is highly effective in mice bearing human Hodgkin's lymphoma xenografts. *Blood* 104: 241B–242B.

Steffens, M.G., Boerman, O.C., De Mulder, P.H., Oyen, W.J., Buijs, W.C., Witjes, J.A., van den Broek, W.J., Oosterwijk-Wakka, J.C., Debruyne, F.M., Corstens, F.H., Oosterwijk, E. (1999) Phase I radioimmunotherapy of metastatic renal cell carcinoma with ^{131}I-labeled chimeric monoclonal antibody G250. *Clin Cancer Res* 5(10 Suppl): 3268s–3274s.

Stein, R., Qu, Z.X., Chen, S., Rosario, A., Shi, V., Hayes, M., Horak, I.D., Hansen, H.J., Goldenberg, D.M. (2004) Characterization of a new humanized anti-CD20 monoclonal antibody, IMMU-106, and its use in combination with the humanized anti-CD22 antibody, epratuzumab, for the therapy of non-Hodgkin's lymphoma. *Clin Cancer Res* 10: 2868–2878.

Stewart, L.M., Young, S., Watson, G., Mather, S.J., Bates, P.A., Band, H.A.,

Wilkinson, R.W., Ross, E.L., Snary, D. (1999) Humanisation and characterisation of PR1A3, a monoclonal antibody specific for cell-bound carcinoembryonic antigen. *Cancer Immunol Immunother* 47: 299–306.

Svensson, H.P., Kadow, J.F., Vrudhula, V.M., Wallace, P.M., Senter, P.D. (1992) Monoclonal antibody-beta-lactamase conjugates for the activation of a cephalosporin mustard prodrug. *Bioconjug Chem* 3: 176–181.

Tai, Y.T., Catley, L.P., Mitsiades, C.S., Burger, R., Podar, K., Shringpaure, R., Hideshima, T., Chauhan, D., Hamasaki, M., Ishitsuka, K., Richardson, P., Treon, S.P., Munshi, N.C., Anderson, K.C. (2004) Mechanisms by which SGN-40, a humanized anti-CD40 antibody, induces cytotoxicity in human multiple myeloma cells: Clinical implications. *Cancer Res* 64: 2846–2852.

Trang, J.M., LoBuglio, A.F., Wheeler, R.H., Harvey, E.B., Sun, L., Ghrayeb, J., Khazaeli, M.B. (1990) Pharmacokinetics of a mouse/human chimeric monoclonal antibody (C-17-1A) in metastatic adenocarcinoma patients. *Pharm Res* 7: 587–592.

Trikha, M., Zhou, Z., Nemeth, J.A., Chen, Q., Sharp, C., Emmell, E., Giles-Komar, J., Nakada, M.T. (2004) CNTO 95, a fully human monoclonal antibody that inhibits alphav integrins, has antitumor and antiangiogenic activity in vivo. *Int J Cancer* 110: 326–335.

Tsavaris, N.B., Katsoulas, H.L., Kosmas, C., Papalambros, E., Gouveris, P., Papantoniou, N., Rokana, S., Kosmas, N., Skopeliti, M., Tsitsilonis, O.E. (2004) The effect of Edrecolomab (Mo17-1A) or fluorouracil-based chemotherapy on specific immune parameters in patients with colorectal cancer – A comparative study. *Oncology* 67: 403–410.

Vallabhajosula, S., Goldsmith, S.J., Kostakoglu, L., Milowsky, M.I., Nanus, D.M., Bander, N.H. (2005) Radioimmunotherapy of prostate cancer using Y-90- and Lu-177-labeled J591 monoclonal antibodies: Effect of multiple treatments on myelotoxicity. *Clin Cancer Res* 11: 7195S–7200S.

Valone, F.H., Kaufman, P.A., Guyre, P.M., Lewis, L.D., Memoli, V., Deo, Y., Graziano, R., Fisher, J.L., Meyer, L., Mrozekorlowski, M., Wardwell, K., Guyre, V., Morley, T.L., Arvizu, C., Fanger, M.W. (1995) Phase Ia/Ib trial of bispecific antibody Mdx-210 in patients with advanced breast or ovarian-cancer that overexpresses the protooncogene Her-2/Neu. *J Clin Oncol* 13: 2281–2292.

Vollmers, H.P., Timmermann, W., Hensel, F., Illert, B., Thiede, A., Muller-Hermelink, H.K. (2000) Adjuvant immunotherapy for stomach carcinoma with the apoptosis-inducing human monoclonal antibody SC-1. *Zentralbl Chir* 125: 37–40.

Wallace, P.K., Romet-Lemonne, J.L., Chokri, M., Kasper, L.H., Fanger, M.W., Fadul, C.E. (2000) Production of macrophage-activated killer cells for targeting of glioblastoma cells with bispecific antibody to Fc gamma RI and the epidermal growth factor receptor. *Cancer Immunol Immunother* 49: 493–503.

Weiner, L.M., Clark, J.I., Davey, M., Li, W.S., Garcia, dP., I., Ring, D.B., Alpaugh, R.K. (1995) Phase I trial of 2B1, a bispecific monoclonal antibody targeting c-erbB-2 and Fc gamma RIII. *Cancer Res* 55: 4586–4593.

Welt, S., Divgi, C.R., Kemeny, N., Finn, R.D., Scott, A.M., Graham, M., Germain, J.S., Richards, E.C., Larson, S.M., Oettgen, H.F. (1994) Phase I/II study of iodine 131-labeled monoclonal antibody A33 in patients with advanced colon cancer. *J Clin Oncol* 12: 1561–1571.

Welt, S., Scott, A.M., Divgi, C.R., Kemeny, N.E., Finn, R.D., Daghighian, F., Germain, J.S., Richards, E.C., Larson, S.M., Old, L.J. (1996) Phase I/II study of iodine 125-labeled monoclonal antibody A33 in patients with advanced colon cancer. *J Clin Oncol* 14: 1787–1797.

Welt, S., Ritter, G., Williams, C., Jr., Cohen, L.S., John, M., Jungbluth, A., Richards, E.A., Old, L.J., Kemeny, N.E. (2003a) Phase I study of anticolon cancer humanized antibody A33. *Clin Cancer Res* 9: 1338–1346.

Welt, S., Ritter, G., Williams, C., Jr., Cohen, L.S., Jungbluth, A., Richards, E.A., Old, L.J., Kemeny, N.E. (2003b) Preliminary report of a phase I study of combination chemotherapy and humanized A33 antibody immunotherapy in patients with

advanced colorectal cancer. *Clin Cancer Res* 9: 1347–1353.

Wong, J.Y.C., Williams, L.E., Yamauchi, D.M., OdomMaryon, T., Esteban, J.M., Neumaier, M., Wu, A.M., Johnson, D.K., Primus, F.J., Shively, J.E., Raubitschek, A.A. (1995) Initial experience evaluating (90)yttrium-radiolabeled anticarcinoembryonic antigen chimeric T84.66 in A phase-I radioimmunotherapy trial. *Cancer Res* 55: S5929–S5934.

Wong, J.Y.C., Shibata, S., Williams, L.E., Kwok, C.S., Liu, A., Chu, D.Z., Yamauchi, D.M., Wilczynski, S., Ikle, D.N., Wu, A.M., Yazaki, P.J., Shively, J.E., Doroshow, J.H., Raubitschek, A.A. (2003) A phase I trial of Y-90-anti-carcinoembryonic antigen chimeric T84.66 radioimmunotherapy with 5-fluorouracil in patients with metastatic colorectal cancer. *Clin Cancer Res* 9: 5842–5852.

Wong, J.Y.C., Chu, D.Z., Williams, L.E., Yamauchi, D.M., Ikle, D.N., Kwok, C.S., Liu, A., Wilczynski, S., Colcher, D., Yazaki, P.J., Shively, J.E., Wu, A.M., Raubitschek, A.A. (2004) Pilot trial evaluating an I-123-labeled 80-kilodalton engineered anticarcinoembryonic antigen antibody fragment (cT84.66 minibody) in patients with colorectal cancer. *Clin Cancer Res* 10: 5014–5021.

Xiao, J., Horst, S., Hinkle, G., Cao, X., Kocak, E., Fang, J., Young, D., Khazaeli, M., Agnese, D., Sun, D., Martin, E. Jr. (2005) Pharmacokinetics and clinical evaluation of 125I-radiolabeled humanized CC49 monoclonal antibody (HuCC49deltaC(H)2) in recurrent and metastatic colorectal cancer patients. *Cancer Biother Radiopharm* 20: 16–26.

Yang, J.C., Beck, K.E., Blansfield, J.A., Tran, K.Q., Lowy, I., Rosenberg, S.A. (2005) Tumor regression in patients with metastatic renal cancer treated with a monoclonal antibody to CTLA4 (MDX-010) *J Clin Oncol* 23: 166S.

Younes, M., Georgakis, G.V., Rahmani, M., Beer, D., Younes, A. (2006) Functional expression of TRAIL receptors TRAIL-R1 and TRAIL-R2 in esophageal adenocarcinoma. *Eur J Cancer* 42: 542–547.

Zaki, M.H., Nemeth, J.A., Trikha, M. (2004) CNTO 328, a monoclonal antibody to IL-6, inhibits human tumor-induced cachexia in nude mice. *Int J Cancer* 111: 592–595.

Zang, Z., Xu, H., Yu, L., Yang, D., Xie, S., Shi, Y., Li, Z., Li, J., Wang, J., Li, M., Guo, Y., Gu, F. (2000) Intravesical immunotoxin as adjuvant therapy to prevent the recurrence of bladder cancer. *Chin Med J (Engl)* 113: 1002–1006.

Zbar, A.P., Thomas, H., Wilkinson, R.W., Wadhwa, M., Syrigos, K.N., Ross, E.L., Dilger, P., Allen-Mersh, T.G., Kmiot, W.A., Epenetos, A.A., Snary, D., Bodmer, W.F. (2005) Immune responses in advanced colorectal cancer following repeated intradermal vaccination with the anti-CEA murine monoclonal antibody, PR1A3: results of a phase I study. *Int J Colorectal Dis* 20: 403–414.

Zhang, C.L., Yu, L.Z., Gu, F.L., Buka, S.D., Zu, S.L., Xie, S.S., Pan, Z.Y. (1998) Targeted diagnosis of bladder and ureteral carcinoma using radiolabelled BDI-1. *Urol Res* 26: 343–348.

Zhang, H.F., Li, Y.W., Li, H.L., Bassi, R., Jimenez, X., Witte, L., Bohlen, P., Hicklin, D.J., Zhu, Z.P. (2004) Inhibition of both the autocrine and the paracrine growth of human leukemia with a fully human antibody directed against vascular endothelial growth factor receptor 2. *Leuk Lymphoma* 45: 1887–1897.

Zhou, J., Zeng, F.Q., Li, C., Tong, Q.S., Gao, X., Xie, S.S., Yu, L.Z. (2005) Preparation of arsenic trioxide-loaded albuminutes immuno-nanospheres and its specific killing effect on bladder cancer cell in vitro. *Chin Med J (Engl)* 118: 50–55.

Zhu, Z., Hattori, K., Zhang, H., Jimenez, X., Ludwig, D.L., Dias, S., Kussie, P., Koo, H., Kim, H.J., Lu, D., Liu, M., Tejada, R., Friedrich, M., Bohlen, P., Witte, L., Rafii, S. (2003) Inhibition of human leukemia in an animal model with human antibodies directed against vascular endothelial growth factor receptor 2. Correlation between antibody affinity and biological activity. *Leukemia* 17: 604–611.

13
Antibodies in Phase I/II/III: Targeting TNF

Martin H. Holtmann and Markus F. Neurath

13.1
Introduction

The past decades have dramatically increased our understanding of the pathophysiology of chronic inflammatory bowel disease (IBD). This has mainly been facilitated by the advent of molecular biological and recombinant techniques. The focus on immunological aspects, in particular on T-cell immunology with the analysis of regulatory cytokine signaling pathways, has provided the basis for innovative treatment strategies. Many of these novel strategies are still experimental and subject to clinical studies, while others have already been incorporated into the treatment armamentarium of routine clinical management in IBD. The availability of an increasing number of recombinant proteins in the treatment of IBD is a unique example of translational research from the bedside to the bench and back to the bedside.

Interestingly, while the elucidation of the immunoregulatory pathomechanisms provided the basis for the development of these novel strategies, ongoing studies on the mechanisms of action of these substances in the clinical setting further increase our pathophysiological understanding considerably. This will further promote the development of more effective and more specific treatment strategies.

It is the purpose of this chapter to outline our current pathoimmunological understanding of IBD with special focus on the characterization of those proinflammatory players that have become targets for the rational development of recombinant antagonistic proteins. In particular, in trying to understand the mechanisms of action of the various different antitumor necrosis factor (anti-TNF) strategies, important insights in TNF signaling pathways in IBD and other diseases have been made.

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13.2
Inflammatory Bowel Disease

Crohn's disease and ulcerative colitis are the two most common forms of IBD and have gained increasing medical and health economic importance during recent decades, especially in Western countries due to their early manifestion in life between 15 and 35 years of age and their chronic character. The etiology of IBD is still unclear and should be considered as multifactorial according to recent studies (Podolsky 2002). Genetic factors seem to play a pathogenetic role as well as environmental, infectious, and immunological factors (Fig. 13.1) (Fiocchi 1998).

Mucosal surfaces such as the intestinal mucosa are special interfaces for the interaction between the organism and the environment and possess an especially adapted immune system (gut-associated lymphoid tissue – GALT) (Nagler-Anderson 2000). Due to its large surface the gut can be regarded as the biggest immune organ of the human body. In the gut the organism is physiologically exposed to a large amount of antigens from the natural flora and the food. Whereas a systemic immune response to these foreign antigens should be prevented in order not to damage the organism, potentially pathogenic antigens have to be recognized and eliminated at a local level. The intestinal epithelium with the mucus represents a mechanical barrier, but it is mainly the role of the GALT to keep this fragile immunological balance between hyporesponsiveness and efficient immune defense.

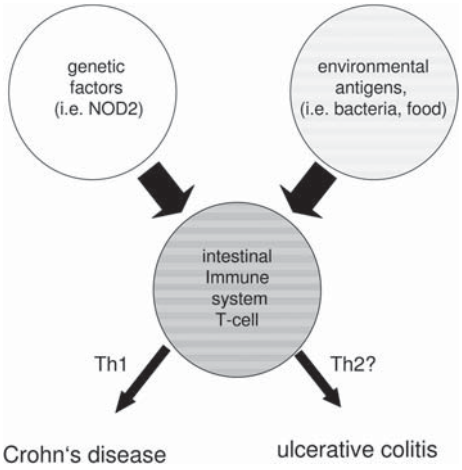

Fig. 13.1 Multifactorial pathogenesis of inflammatory bowel disease (IBD). According to the current paradigm the etiology of IBD is multifactorial. It evolves in genetically susceptibel patients (i.e. NOD2/CARD15), who are exposed to yet unknown environmental antigens (i.e. bacterial or nutritional antigens). This leads to an activation of the intestinal immune system with a pivotal role of T cell-mediated immune responses. While Crohn's disease is prototypic of a Th1-mediated disease, data supporting a Th2-driven pathogenesis of ulcerative colitis are less convincing.

Substantial progress has been made in our understanding of the pathogenesis of IBD in recent years, pursuing the view that IBD could result from disturbances of the intestinal barrier and a pathologic activation of the intestinal immune response towards luminal bacterial and nutritional antigens in a genetically susceptible individual. The main focus of immunologic research in IBD has been on the role and behavior of the T cell and its interaction with other cell populations (Neurath et al. 2002).

13.3 Pathophysiologic Role of T Cells

The acute and chronic inflammation of the bowel goes along with an activation of lamina propria T cells, leading to increased cytokine production (Strober et al. 1998). The cytokine patterns in Crohn's disease and ulcerative colitis show significant differences. In Crohn's disease, $CD4^+$ T cells produce increased amounts of interleukin 12 (IL-12), TNF-α, and interferon gamma (IFN-γ), which would fit to a Th1 cytokine profile. The production of the Th2 cytokines IL-4 and IL-5 in Crohn's disease is decreased. In ulcerative colitis, IL-5 production is increased while IFN-γ production by anti-CD2/CD28-stimulated lamina propria T cells is unchanged compared with control patients (Fuss et al. 1996). IL-13 has recently been identified as a key mediator in experimental ulcerative colitis-like disease (Heller et al. 2005). However, the cytokine profile in ulcerative colitis cannot be classified as a Th2 phenotype without restrictions, since the Th2 cytokine IL-4 is decreased in ulcerative colitis.

The relevance of the Th1–Th2 paradigm has been questioned additionally by the emergence of a third type of $CD4^+$ helper cells termed Th3 cells, mainly producing transforming growth factor beta (TGF-β). In an experimental model, blockade of endogenous TGF-β aggravates colitis, suggesting an anti-inflammatory, protective role of TGF-β (Fuss et al. 2002). $CD4^+CD25^+$ regulatory T cells have also recently been identified as a distinct T-cell population characterized by the production of the anti-inflammatory cytokine IL-10 (Maul et al. 2005).

Numerous experimental animal models, especially knockout or transgenic models, have documented the pathophysiological relevance of distinct cytokine dysregulations (i.e. TNF-α, IL-2, IL-6, IL-10, IL-12, and INF-γ) (Wirtz and Neurath 2000).

13.4 Tumor Necrosis Factor-α

TNF-α is one of the best characterized cytokines in IBD (Holtmann et al. 2002a). The primary translational product of the human TNF-α is the membrane-bound TNF-α (mTNF-α) of 233 amino acids length (26 kDA). Metalloproteinases such as TACE and ADAM 10 cleave the extracellular domain of mTNF-α and thus

release soluble TNF-α (sTNF-α) of 157 amino acids (17 kDa). A probably crucial role of mTNF-α signaling via TNF receptor 2 in chronic inflammatory disease states was not recognized until recently, as will be discussed below. Both mTNF-α and sTNF-α form noncovalently linked homotrimers, which occur intracellularly. Membrane-bound TNF-α is capable of inducing signaling in a receptor-independent manner (reverse signaling) (Eissner et al. 2000).

The pathogenic role of TNF-α in inflammatory bowel disease is well established by clinical and experimental studies (Holtmann et al. 2002a). Although serum levels of TNF-α are not elevated significantly in patients with IBD (Nielsen et al. 2000), lamina propria mononuclear cells from colon biopsies of untreated patients with Crohn's disease and ulcerative colitis spontanously produce more TNF-α than cells from controls when cultured *in vitro* (Reinecker et al. 1993).

An important pathogenic mechanism of TNF-α in the mucosa seems to be the stimulation of a Th1 T-cell response. Lamina propria T lymphocytes from colon biopsies of patients with Crohn's disease incubated with TNF-α, produce increased amounts of the Th1 cytokines IL-2, IFNγ, and TNF-α itself (Plevy et al. 1997). The stimulation of TNF-α secretion by TNF-α itself suggests a possible positive feedback mechanism, which could potentially contribute to the perpetuation of inflammation.

Yet another possible mechanism of TNF-α action could be the activation of endogenous matrix metalloproteinases (MMP), which results in damage of the extracellular matrix of the mucosa (Pender et al. 1997).

The critical role of TNF-α for the development of colitis has been reproduced in various established experimental animal models of colitis including the TNBS model (2,4,6-trinitrobenzene sulfonic acid), the DSS model (dextrane sulfate sodium), the IL-10 knockout mouse and the $CD4^+CD45RB^{high}$ respectively $CD4^+CD62L^+$ adoptive transfer model (Kuhn et al. 1993; Atreva et al. 2000; Neurath et al. 2000; Singh et al. 2001).

13.5 TNF Receptors and Signaling

TNF-α exerts its effects via two TNF-specific membrane-bound receptors with a molecular weight of approximately 55–60 kDA (TNF-R1, p55) and 75–80 kDA (TNF-R2, p75) respectively, which belong to the TNF/nerve growth factor (NGF) receptor family (Fig. 13.2) (Loetscher et al. 1991). Both TNF-R1 and TNF-R2 are composed of two identical subunits and are glycosylated. While TNF-R1 and TNF-R2 are quite similar in their extracellular regions (both receptors possess multiple cysteine-rich motifs), their intracellular domains exhibit striking structural differences, most likely reflecting different signaling pathways.

Extracellular ligand binding elicits complex intracellular signaling cascades. Important signaling cascades of TNF-R1 include the activation of kinases, the induction of apoptosis, and the activation of the proinflammatory transcription factor nuclear factor kappa B (NFκB). NFκB is found in the cytosol of nonacti-

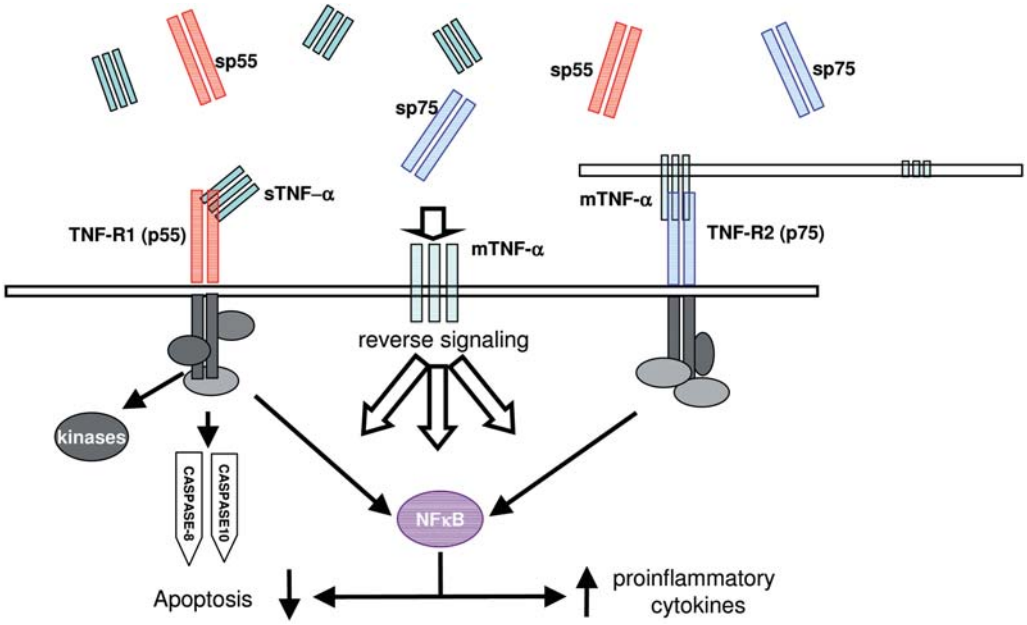

Fig. 13.2 Tumor necrosis factor signaling. TNF-α mediates its effects via two specific cell surface receptors, TNF-R1 (p55) and TNF-R2 (p75). TNF-R1 is mainly activated via soluble TNF-α (sTNF-α), while membrane-bound TNF-α (mTNF-α) is the principal ligand of TNF-R2. Recent data suggest that signaling via mTNF-α/TNF-R2 plays a critical role in the pathogenesis of Crohn's disease. Signaling via TNF-R2 leads to activation of NFκB, a strong proinflammatory transcription factor. Concurrent inhibition of proapoptotic factor may futher promote inflammation. TNF receptors exist in a soluble form generated by shedding of the extracellular portions. These soluble receptor have preserved ligand binding capacity and might thus contribute to the regulation of ligand availability. The finding that mTNF-α is capable of eliciting intracellular signaling without receptor interaction (reverse signaling) adds yet another level of complexity to TNF signaling. Novel recombinant protein-based anti-TNF strategies target different factors of this pathway, some by imitating the mechanism of action of endogenous components, such as soluble receptor p55.

vated monocytes and T cells as an inactive heterodimer of a p50 and p65 subunit, bound to the inhibitory IκB protein. Upon stimulation of the cell IκB is phosphorylated by IκB kinase and consecutively releases NFκB (Auphan et al. 1995). The active subunit of NFκB, p65, moves into the nucleus and directly interacts with the promoter region of several proinflammatory genes such as TNF-α, IL-1, IL-6, and IL-12 (Neurath et al. 2005). Inhibition of NFκB is the underlying mechanism of many established anti-inflammatory treatment strategies. While salicylic acids inhibit IκB kinase (IKK), corticosteroids stimulate IκB synthesis and additionally inhibit p65 in the nucleus by direct complexation (Yin et al. 1998).

For a long time TNF-R1 was considered the principal mediator of TNF signal transduction. This view was mainly based on the fact that TNF-R2 binds sTNF-α

with a 20-fold lower binding affinity than TNF-R1 (Grell et al. 1995). However, it was then found that TNF-R2 is preferentially activated by mTNF-α with high affinity in a paracrine fashion and probably in an autocrine loop, too (Haas et al. 1999). The main cellular response of TNF-R2 is the activation of NFκB. These observations make it possible that the mTNF-α/TNF-R2 system could play an important immunoregulatory role at the local level.

In order to assess the net cellular response of TNF signaling it is important to consider that the two principal effects of TNF-α signal transduction, activation of NFκB and induction of apoptosis, can be antagonistic to each other. NFκB inhibits apoptosis through upregulation of antiapoptotic signaling factors (Roth et al. 1995; Wang et al. 1998). It is conceivable that concurrent inhibition of apoptosis in addition to the upregulation of proinflammatory cytokines is an important proinflammatory mechanism of NFκB.

The hypothesis that TNF-α signaling via TNF-R2 may play an independent pathogenic role can be demonstrated impressively in a transgenic mouse model overexpressing two alleles of the human TNF-R2, which can be activated by murine TNF-α just as efficiently as the mouse receptor (Douni and Kollias 1998), These mice spontanously developed a severe general inflammatory syndrome involving pancreas, liver, kidneys, and lungs. NFκB was constitutively increased in mononuclear cells of the peripheral blood.

Strong direct evidence for a crucial role of TNF-R2 signaling in the pathogenesis of Crohn's disease has been provided by recent experimental studies (Holtmann et al. 2002b). In this work, it could be shown that TNF-R2 expression is significantly increased on mononuclear cells in peripheral blood and in the lamina propria of patients with active Crohn's disease. In a murine model system of experimental colitis overexpression of TNF-R2 led to severe aggravation of colitis. This was mediated by induction of a Th1-like cytokine profile of mononuclear cells and by inhibition of apoptosis in the lamina propria. Both pathomechanisms are relevant in Crohn's disease, too. These data contribute to our understanding of the differential clinical efficacy of different anti-TNF strategies (see below).

Elevated levels of soluble TNF-R1 and TNF-R2 can be detected in the urine of patients with Crohn's disease and ulcerative colitis and correlate with high disease indices (Hadziselimovic et al. 1995). Elevated levels of soluble TNF-R2 can also be detected in various severe inflammatory or autoimmune disease states (i.e. sepsis, chronic viral hepatitis, acute pancreatitis, systemic lupus erythematosus, rheumatoid arthritis, and AIDS (Godfried et al. 1993; Schroder et al. 1995; Marinos et al. 1995; de Beaux et al. 1996; Gabay et al. 1997). It is unclear, however, how these elevated levels of soluble TNF-R2 can be explained. They could represent a regulatory mechanism to bind and inactivate soluble TNF-α ligand.

TNF signaling thus represents a complex network of ligands in a membrane-bound and soluble form, two receptors with different ligand affinities and intracellular signaling cascades, soluble forms of receptors that modify the availability of ligand and reverse signaling of the membrane-bound ligand without receptor

involvement. These different components of TNF signaling are targeted by the anti-TNF strategies available or are mimicked in order to block TNF signaling.

13.6 Anti-TNF Antibodies and Fusion Proteins in Clinical Testing

Currently there are four recombinant anti-TNF antibodies and two recombinant fusion proteins available or under clinical testing in IBD, respectively: infliximab, adalimumab, certolizumab, CDP571, etanercept, and onercept. Although they share TNF-antagonizing properties, these drugs display striking differences in clinical efficacy in distinct inflammatory disorders. The elucidation of their different mechanisms of action in distinct clinical settings helps us to learn more about the details and peculiarities of TNF signal transduction in distinct inflammatory disorders.

The different features of TNF-α antagonists are summarized in Table 13.1 and Fig. 13.3.

Table 13.1 Mechanisms of action of anti-TNF strategies.

Substance	*Construct*	*Binding properties*	*Complement fixation*	*Antibody-dependent cytotoxicity*	*Induction of apoptosis*	*Efficacy in IBD*	*Efficacy in rheumatological disorders*
Infliximab	Chimeric mouse-human IgG1 mAb	sTNF, mTNF	Yes	Yes	Yes	Yes	Yes
Adalimumab	Human IgG1 mAb	sTNF, mTNF	Yes	Yes	Yes	Yes	Yes
Certolizumab (CDP870)	Humanized Fab fragment linked to PEG	sTNF, mTNF	No	No	?	(Yes)	(Yes)
CDP571	Humanized IgG4 mAb	sTNF, mTNF	No	No	?	No	No
Etanercept	IgG1 Fc fragment linked to 2 p75	sTNF	No	No	No	No	Yes
Onercept	Soluble p55	sTNF, mTNF	No	No	?	?	?

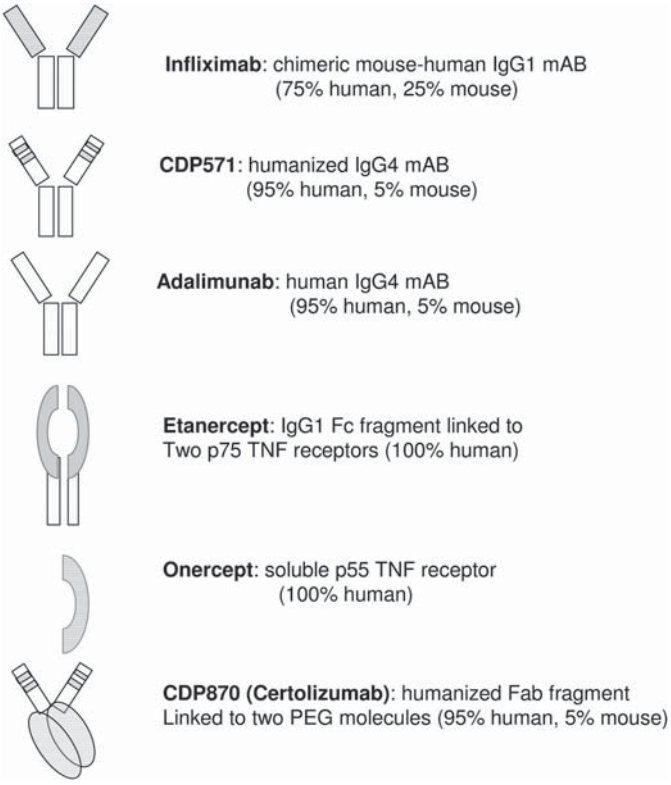

Fig. 13.3 Recombinant anti-TNF strategies. Infliximab was the first recombinant anti-TNF antibody. The residual murine proportions have been considered a major drawback in clinical practise because the mouse epitopes were thought to be responsible for the development of human antichimeric antibodies (HACAs), causing allergic side effects. In an attempt to reduce immunogenicity, humanized antibodies were generated, further reducing the proportions of murine origin (CDP571). Adalimumab is a fully human monoclonal antibody. However, antibodies to CDP571 and adalimumab can still be detected (human anti-human antibodies). Etanercept and onercept represent extracellular portions of p75 and p55 with preserved ligand-binding capacity, thus probably imitating the regulatory role of endogenous soluble receptor. CDP870 distinguished itself by the attachment of two polyethylene glycol molecules, ensuring prolonged continuous release after subcutanous application.

13.6.1 Infliximab

Most clinical experience exists for infliximab (cA2). This is a genetically engineered IgG1 murine–human monoclonal antibody with a constant region of human IgG1κ-immunoglobulin representing 75% of the molecule and a variable region of a monoclonal mouse anti-human antibody representing 25%. Infliximab binds both sTNF-α and mTNF-α and most likely blocks the interaction of TNF-α with the TNF receptors this way (Siegel et al. 1995; Agnholt et al. 2003).

Clinical studies were first performed in rheumatoid arthritis, where good efficacy could be shown (Maini 2004). Other rheumatological disorders followed, including psoriatic arthritis (Antoni et al. 2005), juvenile chronic arthritis (Gerloni et al. 2005), psoriasis (Reich et al. 2005), and ankylosing spondylitis (Marzo-Ortega et al. 2005).

The efficacy of infliximab in the induction and maintenance of remission in patients with both luminal and fistulizing Crohn's disease has been established in several controlled studies (Rutgeerts et al. 1999; Hanauer et al. 2002; Sands et al. 2004). Recent data support the utilization of infliximab in severe refractory ulcerative colitis, too (Rutgeerts et al. 2005; Sandborn et al. 2005a).

The general tolerability of infliximab is good. Most adverse side effects are probably related to the immunogenicity of infliximab that leads to the formation of antibodies to infliximab (ATI) (human antichimeric antibodies, HACA) in 3–17% of the cases (Hanauer et al. 2004) and include infusion reactions (in 4–16%), delayed hypersensitivity-like reactions (in 1%), and autoimmune phenomena. A rare, but severe side effect is the exacerbation of latent infections, especially tuberculosis (Keane et al. 2001; Myers et al. 2002). This reflects the important role of TNF-α in the antimicrobial defense of the immune system. Available long-term safety data provide no evidence for an increased risk for lymphoproliferative disorders or other malignancies (Lichtenstein et al. 2005).

13.6.2 Adalimumab

Adalimumab is a recombinant human IgG1 monoclonal antibody that binds to sTNF-α and mTNF-α and induces apoptosis in monocytes (Shen et al. 2005). A recent, large, phase III study has shown that adalimumab is efficacious and well tolerated in the long-term treatment of rheumatoid arthritis (Weinblatt et al. 2006). Short-term efficacy could also be shown for psoriatic arthritis (Mease et al. 2005). The role of adalimumab in IBD is currently under investigation. Uncontrolled studies showed the clinical efficacy of adalimumab in cases of infliximab intolerability and refractoriness (Sandborn et al. 2004a). This efficacy could be confirmed in a larger controlled study in infliximab-naive patients (Sandborn et al. 2005b,c).

The theoretical advantage of adalimumab with regard to adverse side effects is reduced immunogenicity due to the lack of mouse epitopes. However, development of human anti-human antibodies (anti-adalimumab antibodies, AAAs) is still observed. Side effects include injection-site reactions and infections.

13.6.3 Certolizumab

Certolizumab (CDP870) is a Fab fragment of a humanized monoclonal anti-TNF antibody attached to polyethylene glycol molecules. Although it binds to both

sTNF-α and mTNF-α, certolizumab does not induce apoptosis in peripheral blood lymphocytes and monocytes (Fossati and Nesbitt 2005). The clinical efficacy of certolizumab has not yet been established. In a phase II trial, certolizumab showed clinical response after 12 weeks only in a subgroup of patients with elevated C-reactive protein level as sign of systemic inflammation (Schreiber et al. 2005). Results of larger phase III trials have not been published, yet. Adverse events include injection-site reactions, exacerbations of Crohn's disease, and infections.

13.6.4 CDP571

CDP571 (nerelimomab) is a humanized anti-TNF antibody derived from a mouse anti-human TNF-α monoclonal antibody. The complementarity determining region of this antibody has been linked to a human IgG4 antibody. Binding occurs to both sTNF-α and mTNF-α, but no data have been reported regarding induction of apoptosis. CDP571 has shown only temporary marginal efficacy 2 weeks after treatment and in a subgroup of patients with elevated C-reactive protein (Sandborn et al. 2004b). Tolerability and safety data are limited and are comparable to infliximab.

13.6.5 Etanercept

Etanercept is a fusion protein consisting of two identical chains of recombinant human anti-TNF receptor p75 monomers fused to the Fc domain of human IgG1. Etanercept binds only to sTNF-α, but not to mTNF-α. Unlike infliximab and adulimumab, etanercept fails to induce apoptosis in lamina propria T lymphocytes (Van den Brande et al. 2003). Etanercept has shown good efficacy in rheumatoid arthritis and the indication has been extended to many other rheumatoid disorders, including psoriatic arthritis, juvenile chronic arthritis, plaque psoriasis, and ankylosing spondylitis (Moreland et al. 1999; Bathon et al. 2000; Takei et al. 2001; Leonardi et al. 2003; Mease et al. 2004; Davis et al. 2005; Tyring et al. 2006). Interestingly, in Crohn's disease etanercept has failed to show any efficacy (Sandborn et al. 2001).

13.6.6 Onercept

Onercept is a recombinant form of human soluble TNF-R1 (p55). In an initial pilot study on 12 patients with Crohn's disease, onercept showed clinical response beyond placebo (Rutgeerts et al. 2003). This result could not be confirmed in a phase II study (Rugeerts et al. 2004). More importantly, however, phase III trials on onercept in psoriasis had to be discontinued because of two cases of severe sepsis, one of which was lethal.

13.7 Mechanisms of Action

Soon after clinical efficacy of infliximab was shown, the mechanisms of action *in vivo* were investigated. In preclinical studies using a transfection model system with expression of uncleavable mTNF-α, it had been shown that inflximab has a higher binding affinity to mTNF-α than etanercept, while both agents bind sTNF-α (Scallon et al. 1995). These results could be confirmed on activated lamina propria T lymphocytes (Van den Brande et al. 2003). The potential of infliximab and etanercept to neutralize biologically active sTNF-α was similar (Van den Brande et al. 2005).

Since defective apoptosis of mononuclear cells seems to play a role in the pathogenesis of IBD (Boirivant et al. 1999), it was soon hypothesized that the rapid clinical effect of infliximab might be due to induction of apoptosis. And in fact, in Crohn's disease patients treated with infliximab, dose-dependent induction of peripheral blood monocyte apoptosis by a CD95/CD95L-dependent pathway within a few hours could be shown (Lugering et al. 2001). This effect could be imitated by $F(ab)_2$ fragments of infliximab which lack the Fc domain. The finding that infliximab induces increased apoptosis of lamina propria T cells in patients with Crohn's disease is probably even more relevant (ten Hove et al. 2002).

The ability to bind mTNF-α correlates with the inducibility of apoptosis, since only infliximab, but not etanercept induces apoptosis in lamina propria T lymphocytes. Interestingly, induction of apoptosis in cultured peripheral blood monocytes could also be shown for adalimumab, which shares the isotype class IgG1 with infliximab (Shen et al. 2005). In this experimental setting, adalimumab, infliximab and etanercept reduced the levels of sTNF-α in the culture, but only adalimumab and infliximab reduced the production of IL-10 and IL-12.

For infliximab and adalimumab, additional relevant mechanisms of action may be the ability to fix complement and antibody-dependent cytotoxicity. These are features that may be referred to their common subtype class IgG1.

However, binding to mTNF-α does not always lead to blockade of TNF signaling, but can elicit reverse trans-signaling. In an intriguing experimental approach, wildtype and serin-replaced mutant forms of mTNF-α were stably transfected in human Jurkat T cells. Treatment with infliximab, but not with etanercept led to IL-10 production, apoptosis, and G0/G1 cell cycle arrest. These effects were abolished by substitution of all three cytoplasmic serine residues of mTNF-α by alanine residues (Mitoma et al. 2005). This work does not only reveal an additional mechanism of action of infliximab, but also provides insight into a so far unrecognized role of mTNF-α in chronic inflammation.

13.8 Other Anti-TNF Biologicals

CNTO-148 (golimumab) is another fully human anti-TNF-antibody, that can be administered subcutaneously. Clinical testing has been restricted to rheumatoid

arthritis so far and preliminary unpublished data suggest statistically significant reductions of signs and symptoms of rheumatoid arthritis. Afelimomab is a monoclonal anti-TNF antibody $F(ab')_2$ fragment. Published data exist from controlled clinical trials in patients with severe sepsis syndrome. Afelimomab slightly reduces 28-day all-cause mortality in a subgroup of patients with sepsis with elevated IL-6 levels (Reinhart et al. 2001; Panecek et al. 2004). No data or press releases, however, are available on these agents in IBD.

13.9 Other Cytokine-based and Anti-CD4$^+$ T-Cell Approaches

Many of the proinflammatory cytokines considered as pathogenetically relevant in IBD have become targets of novel recombinant treatment approaches. Fontolizumab is a humanized anti-IFNγ antibody developed by Protein Design Labs and currently under investigation in phase I and II studies (Harmony I and II) in patients with Crohn's disease. The human IgG1 monoclonal anti-IL-12p40 antibody ABT-874 from Abbott Laboratories has been successfully tested in a controlled phase II study in 76 patients with CD and shows efficacy in remission induction (Mannon et al. 2004). The anti-inflammatory IL-10 (from Schering Plough) has been utilized directly as recombinant protein delivered subcutanously in three large phase III trials comprising 800 patients, but has failed to show any effect (van Deventer et al. 1997; Fedorak et al. 2000; Schreiber et al. 2005). Phase II trials with IL-11 (from Genetics Institute) were discontinued, too. MRA is a humanized anti-IL-6 receptor antibody from Roche currently tested in a phase II trial in Crohn's disease.

The pivotal role of the CD4$^+$ T-helper cell in the pathogenesis of IBD is also taken into account in treatment strategies that attempt to downregulate T-cell recruitment to the inflamed focus by blocking adhesion molecules. Early studies blocking the intercellular adhesion molecule 1 (ICAM-1) with an antisense oligonucleotide showed no clinical efficacy (Schreiber et al. 2001).

A monoclonal antibody approach was pursued with natalizumab, a humanized IgG4 antibody to α_4 integrin (Fig. 13.4). While effective in multiple sclerosis, which is T-cell mediated, too, natalizumab failed to show any clinically relevant efficacy in several large studies in active Crohn's disease (Gordon et al. 2001; Ghosh et al. 2003; Miller et al. 2003; Sandborn et al. 2005d). Of note, three cases of JC virus-associated progressive multifocal leukoencephalopathy under natalizumab treatment have been reported (Kleinschmidt-DeMasters and Tyler 2005; Van Assche et al. 2005).

MLN-02 is another humanized IgG1 monoclonal anti-integrin antibody directed against the heterodimeric epitope $\alpha_4\beta_7$ (Fig. 13.4). This antibody showed efficacy in ulcerative colitis (Feagan et al. 2005). Concerns regarding the risk of polymorphonuclear leukocytes under MLN-02 treatment have been allayed by the following considerations. Natalizumab blocks specifically the α_4 subunit of integrins that is incorporated in both the $\alpha_4\beta_1$ heterodimer that interacts with VCAM-1 in

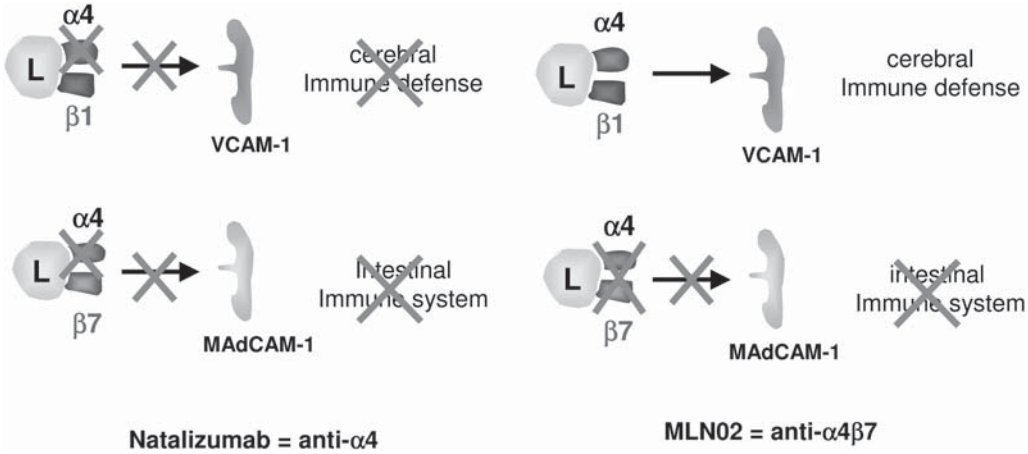

Fig. 13.4 Differential mechanisms of action of anti-integrins. Inhibition of T-cell homing is a therapeutic approach to decrease inflammation. Migration of T cells is mediated by distinct adhesion molecules which interact specifically. T cells can pass the blood–brain barrier through interaction of the $\alpha_4\beta_1$ integrin heterodimer with the adhesion molecule VCAM-1. For homing into the gut, T cells expressing the heterodimer $\alpha_4\beta_7$ interact with the adhesion molecule MAdCAM-1, which is specifically expressed in the gut. The monoclonal anti-integrin antibody natalizumab blocks the α subunit and thus inhibits homing of T cells to both the brain and the gut. The antigenetic epitope of MLN-02 is the heterodimer $\alpha_4\beta_7$ as a whole. MLN-02 thus blocks homing of T cells to the gut, but not to the brain.

the brain and the $\alpha_4\beta_7$ heterodimer that interacts with MAdCAM-1, specifically expressed in the gut. Natalizumab thus interferes with T cell homing in both the brain and the gut. Being directed against the heterodimer $\alpha_4\beta_7$ as a whole, MLN-02 only blocks MAdCAM-1-mediated T-cell homing in the gut, thus preventing the negative impact on immune defense in the brain. It remains to be seen if these theoretical considerations really apply in clinical practice in the long run.

13.10 Perspective

It is striking that only 50–70% of all patients respond clinically to distinct anti-cytokine strategies such as anti-TNF-α or anti-IL-12 antibodies given the pivotal role of these cytokines in murine models of colitis. In fact, there is increasing evidence that the classic Th1-Th2 paradigm derived from the focus on T-cell immunology may be too simplistic to explain IBD.

Two considerations may be relevant. Crohn's disease and ulcerative colitis may represent common final pathophysiological pathways of different etiological triggers. These different pathomechanisms may involve different signaling cascades. In addition, individual cytokines may play different roles in different phases of

the disease. In particular Crohn's disease is characterized by two distinct phases, an initial, inductive phase and a chronic, effector phase.

Increasing evidence suggests that for the inductive phase, cell components of the innate immune system, such as monocytes/macrophages, play a predominant role (Kelsall and Strober 1999; Berrebi et al. 2003). Monocytes/macrophages are important antigen presentation cells and produce proinflammatory cytokines such as IL-1, TNF-α, IL-6, and possibly IL-12 and IL-18. The first Crohn's disease susceptibility gene codes for the intracellular protein NOD2/CARD15, which represents an intracellular receptor for bacterial products, in particular muramyl dipeptide, strongly expressed in monocytes/macrophages and epithelial cells (Hugot et al. 2001; Ogura et al. 2001; Hisamatsu et al. 2003). *In vitro* studies on loss of function mutations of NOD2 revealed that wildtype NOD2/CARD15 activates NFκB.

This apparently paradoxical finding has led to the view that in Crohn's disease the initial response of the innate immune system to antigens of the physiological intestinal flora or the food may be defective, resulting in a detrimental activation of the acquired immune system with chronic inflammation. In this phase dysregulation of T cells and T cell-associated cytokine regulatory networks play the crucial role. And in fact, in a murine model of a loss-of-function mutation of NOD2, elevated NFκB activation in response to muramyl dipeptide could be shown with increased susceptibility to bacterial-induced inflammation (Kobayashi et al. 2005; Maeda et al. 2005).

It is thus conceivable that the blockade of a certain cytokine – although a key proinflammatory cytokine in colitis models – could be beneficial under certain circumstances, but ineffective or even detrimental otherwise. This is likely to apply not only to the targets of anticytokine strategies currently available, but also for all potential future targets such as IL-13, IL-18, IL-23, IL-27, and others. For medical and cost-effectiveness reasons it will be challenging to identify predictive markers for the responsiveness of an individual patient to a distinct cytokine-targeted treatment strategy.

With regard to safety, the serious adverse events following natalizumab treatment and the risk of exacerbation of silent infections and sepsis under anti-TNF-α treatment should alert to the two faces of Janus inherent in T cell and T cell-associated cytokine-targeted strategies.

References

Agnholt, J., Dahlerup, J.F., Kaltoft, K. (2003) The effect of etanercept and infliximab on the production of tumour necrosis factor alpha, interferon-gamma and GM-CSF in in vivo activated intestinal T lymphocyte cultures. *Cytokine* 23: 76–85.

Antoni, C.E., Kavanaugh, A., Kirkham, B., Tutuncu, Z., Burmester, G.R., Schneider, U., Furst, D.E., Molitor, J., Keystone, E., Gladman, D., Manger, B., Wassenberg, S., Weier, R., Wallace, D.J., Weisman, M.H., Kalden, J.R., Smolen, J. (2005) Sustained benefits of infliximab therapy for dermatologic and articular manifestations of psoriatic arthritis: results from the infliximab multinational psoriatic arthritis

controlled trial (IMPACT). *Arthritis Rheum* 52: 1227–1236.

Atreya, R., Mudter, J., Finotto, S., Mullberg, J., Jostock, T., Wirtz, S., Schutz, M., Bartsch, B., Holtmann, M., Becker, C., Strand, D., Czaja, J., Schlaak, J.F., Lehr, H.A., Autschbach, F., Schurmann, G., Nishimoto, N., Yoshizaki, K., Ito, H., Kishimoto, T., Galle, P.R., Rose-John, S., Neurath, M.F. (2000) Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in Crohn disease and experimental colitis in vivo. *Nat Med* 6: 583–588.

Auphan, N., DiDonato, J.A., Rosette, C., Helmberg, A., Karin, M. (1995) Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* 270: 286–290.

Bathon, J.M., Martin, R.W., Fleischmann, R.M., Tesser, J.R., Schiff, M.H., Keystone, E.C., Genovese, M.C., Wasko, M.C., Moreland, L.W., Weaver, A.L., Markenson, J., Finck, B.K. (2000) A comparison of etanercept and methotrexate in patients with early rheumatoid arthritis. *N Engl J Med* 343: 1586–1593.

Berrebi, D., Maudinas, R., Hugot, J.P., Chamaillard, M., Chareyre, F., De Lagausie, P., Yang, C., Desreumaux, P., Giovannini, M., Cezard, J.P., Zouali, H., Emilie, D., Peuchmaur, M. (2003) Card15 gene overexpression in mononuclear and epithelial cells of the inflamed Crohn's disease colon. *Gut* 52: 840–846.

Boirivant, M., Marini, M., Di Felice, G., Pronio, A.M., Montesani, C., Tersigni, R., Strober, W. (1999) Lamina propria T cells in Crohn's disease and other gastrointestinal inflammation show defective CD2 pathway-induced apoptosis. *Gastroenterology* 116: 557–565.

Davis, J.C., van der Heijde, D.M., Braun, J., Dougados, M., Cush, J., Clegg, D., Inman, R.D., Kivitz, A., Zhou, L., Solinger, A., Tsuji, W. (2005) Sustained durability and tolerability of etanercept in ankylosing spondylitis for 96 weeks. *Ann Rheum Dis* 64: 1557–1562.

de Beaux, A.C., Goldie, A.S., Ross, J.A., Carter, D.C., Fearon, K.C. (1996) Serum concentrations of inflammatory mediators related to organ failure in patients with acute pancreatitis. *Br J Surg* 83: 349–353.

Douni, E., Kollias, G. (1998) A critical role of the p75 tumor necrosis factor receptor (p75TNF-R) in organ inflammation independent of TNF, lymphotoxin alpha, or the p55TNF-R. *J Exp Med* 188: 1343–1352.

Eissner, G., Kirchner, S., Lindner, H., Kolch, W., Janosch, P., Grell, M., Scheurich, P., Andreesen, R., Holler, E. (2000) Reverse signaling through transmembrane TNF confers resistance to lipopolysaccharide in human monocytes and macrophages. *J Immunol* 164: 6193–6198.

Feagan, B.G., Greenberg, G.R., Wild, G., Fedorak, R.N., Pare, P., McDonald, J.W., Dube, R., Cohen, A., Steinhart, A.H., Landau, S., Aguzzi, R.A., Fox, I.H., Vandervoort, M.K. (2005) Treatment of ulcerative colitis with a humanized antibody to the alpha4beta7 integrin. *N Engl J Med* 352: 2499–2507.

Fedorak, R.N., Gangl, A., Elson, C.O., Rutgeerts, P., Schreiber, S., Wild, G., Hanauer, S.B., Kilian, A., Cohard, M., LeBeaut, A., Feagan, B. (2000) Recombinant human interleukin 10 in the treatment of patients with mild to moderately active Crohn's disease. The Interleukin 10 Inflammatory Bowel Disease Cooperative Study Group. *Gastroenterology* 119: 1473–1482.

Fiocchi, D. (1998) Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology* 115: 182–205.

Fossati, G., Nesbitt, A.M. (2005) Effect of the anti-TNF agents, adalimumab, etanercept, infliximab, and certolizumab PEGOL (CDP870) on the induction of apoptosis in activated peripheral blood lymphocytes and monocytes (abstract). *Am J Gastroenterol* 100: S298.

Fuss, I.J., Neurath, M., Boirivant, M., Klein, J.S., de la Motte, C., Strong, S.A., Fiocchi, C., Strober, W. (1996) Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* 157: 1261–1270.

Fuss, I.J., Boirivant, M., Lacy, B., Strober, W. (2002) The interrelated roles of TGF-beta

and IL-10 in the regulation of experimental colitis. *J Immunol* 168: 900–908.

Gabay, C., Cakir, N., Moral, F., Roux-Lombard, P., Meyer, O., Dayer, J.M., Vischer, T., Yazici, H., Guerne, P.A. (1997) Circulating levels of tumor necrosis factor soluble receptors in systemic lupus erythematosus are significantly higher than in other rheumatic diseases and correlate with disease activity. *J Rheumatol* 24: 303–308.

Gerloni, V., Pontikaki, I., Gattinara, M., Desiati, F., Lupi, E., Lurati, A., Salmaso, A., Fantini, F. (2005) Efficacy of repeated intravenous infusions of an anti-tumor necrosis factor alpha monoclonal antibody, infliximab, in persistently active, refractory juvenile idiopathic arthritis: results of an open-label prospective study. *Arthritis Rheum* 52: 548–553.

Ghosh, S., Goldin, E., Gordon, F.H., Malchow, H.A., Rask-Madsen, J., Rutgeerts, P., Vyhnalek, P., Zadorova, Z., Palmer, T., Donoghue, S. (2003) Natalizumab for active Crohn's disease. *N Engl J Med* 348: 24–32.

Godfried, M.H., van der Poll, T., Jansen, J., Romijin, J.A., Schattenkerk, J.K., Endert, E., van Deventer, S.J., Sauerwein, H.P. (1993) Soluble receptors for tumour necrosis factor: a putative marker of disease progression in HIV infection. *Aids* 7: 33–36.

Gordon, F.H., Lai, C.W., Hamilton, M.I., Allison, M.C., Srivastava, E.D., Fouweather, M.G., Donoghue, S., Greenlees, C., Subhani, J., Amlot, P.L., Pounder, R.E. (2001) A randomized placebo-controlled trial of a humanized monoclonal antibody to alpha4 integrin in active Crohn's disease. *Gastroenterology* 121: 268–274.

Grell, M., Douni, E., Wajant, H., Lohden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., Scheurich, P. (1995) The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDA tumor necrosis factor receptor. *Cell* 83: 793–802.

Haas, E., Grell, M., Wajant, H., Scheurich, P. (1999) Continuous autotropic signaling by membrane-expressed tumor necrosis factor. *J Biol Chem* 274: 18107–18112.

Hadziselimovic, F., Emmons, L.R., Gallati, H. (1995) Soluble tumour necrosis factor receptors p55 and p75 in the urine monitor disease activity and the efficacy of treatment of inflammatory bowel disease. *Gut* 37: 260–263.

Hanauer, S.B., Feagan, B.G., Lichtenstein, G.R., Mayer, L.F., Schreiber, S., Colombel, J.F., Rachmilewitz, D., Wolf, D.C., Olson, A., Bao, W., Rutgeerts, P. (2002) Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 359: 1541–1549.

Hanauer, S.B., Wagner, C.L., Bala, M., Mayer, L., Travers, S., Diamond, R.H., Olson, A., Bao, W., Rutgeerts, P. (2004) Incidence and importance of antibody responses to infliximab after maintenance or episodic treatment in Crohn's disease. *Clin Gastroenterol Hepatol* 2: 542–553.

Heller, F., Florian, P., Bojarski, C., Richter, J., Christ, M., Hillenbrand, B., Mankertz, J., Gitter, A.H., Burgel, N., Fromm, M., Zeitz, M., Fuss, I., Strober, W., Schulzke, J.D. (2005) Interleukin-13 is the key effector Th2 cytokine in ulcerative colitis that affects epithelial tight junctions, apoptosis, and cell restitution. *Gastroenterology* 129: 550–564.

Hisamatsu, T., Suzuki, M., Reinecker, H.C., Nadeau, W.J., McCormick, B.A., Podolsky, D. (2003) CARD15/NOD2 functions as an anti-bacterial factor i human intestinal epithelial cells. *Gastroenterology* 124: 993–1000.

Holtmann, M.H., Schutz, M., Galle, P.R., Neurath, M.F. (2002a) Functional relevance of soluble TNF-alpha, transmembrane TNF-alpha and TNF-signal transduction in gastrointestinal diseases with special reference to inflammatory bowel diseases. *Z Gastroenterol* 40: 587–600.

Holtmann, M.H., Douni, E., Schutz, M., Zeller, G., Mudter, J., Lehr, H.A., Gerspach, J., Scheurich, P., Galle, P.R., Kollias, G., Neurath, M.F. (2002b) Tumor necrosis factor-receptor 2 is up-regulated on lamina propria T cells in Crohn's disease and promotes experimental colitis in vivo. *Eur J Immunol* 32: 3142–3151.

Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M.,

Binder, V., Finkel, Y., Cortot, A., Modigliani, R., Laurent-Puig, P., Gower-Rousseau, C., Macry, J., Colombel, J. F., Sahbatou, M., Thomas, G. (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 411: 599–603.

Keane, J., Gershon, S., Wise, R.P., Mirabile-Levens, E., Kasznica, J., Schwieterman, W.D., Siegel, J.N., Braun, M.M. (2001) Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med* 345: 1098–1104.

Kelsall, B., Strober, W. (1999) *Gut-Associated Lymphoid Tissue: Antigen Handling and T Lymphocyte Responses*. San Diego: Academic Press, 1999.

Kleinschmidt-DeMasters, B.K., Tyler, K.L. (2005) Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis. *N Engl J Med* 353: 369–374.

Kobayashi, K.S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nunez, G., Flavell, R.A. (2005) Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307: 731–734.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., Muller, W. (1993) Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75: 263–274.

Leonardi, C.L., Powers, J.L., Matheson, R.T., Goffe, B.S., Zitnik, R., Wang, A., Gottlieb, A.B. (2003) Etanercept as monotherapy in patients with psoriasis. *N Engl J Med* 349: 2014–2022.

Lichtenstein, G.R., Feagan, B.G., Cohen, R.D., Salzberg, B.A., Diamond, R.H., Chen, D.M., Pritchard, M.L., Sandborn, W. J. (2005) Safety of infliximab and other Crohn's disease therapies – updated TREAT registry data with over 10,000 patient-years of follow-up (abstract). *Gastroenterology* 128: A-580.

Loetscher, H., Brockhaus, M., Dembic, Z., Gentz, R., Gubler, U., Hohmann, H.P., Lahm, H.W., Van Loon, A.P., Pan, Y.C., Schlaeger, E.J., et al. (1991) Two distinct tumour necrosis factor receptors – members of a new cytokine receptor gene family. *Oxf Surv Eukaryot Genes* 7: 119–142.

Lugering, A., Schmidt, M., Lugering, N., Pauels, H.G., Domschke, W., Kucharzik, T. (2001) Infliximab induces apoptosis in monocytes from patients with chronic active Crohn's disease by using a caspase-dependent pathway. *Gastroenterology* 121: 1145–1157.

Maeda, S., Hsu, L.C., Liu, H., Bankston, L.A., Iimura, M., Kagnoff, M.F., Eckmann, L., Karin, M. (2005) Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science* 307: 734–738.

Maini, S.R. (2004) Infliximab treatment of rheumatoid arthritis. *Rheum Dis Clin North Am* 30: 329–347, vii.

Mannon, P.J., Fuss, I.J., Mayer, L., Elson, C.O., Sandborn, W.J., Present, D., Dolin, B., Goodman, N., Groden, C., Hornung, R.L., Quezado, M., Yang, Z., Neurath, M. F., Salfeld, J., Veldman, G.M., Schwertschlag, U., Strober, W. (2004) Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med* 351: 2069–2079.

Marinos, G., Naoumov, N.V., Rossol, S., Torre, F., Wong, P.Y., Gallati, H., Portmann, B., Williams, R. (1995) Tumor necrosis factor receptors in patients with chronic hepatitis B virus infection. *Gastroenterology* 108: 1453–1463.

Marzo-Ortega, H., McGonagle, D., Jarrett, S., Haugeberg, G., Hensor, E., O'Connor, P., Tan, A.L., Conaghan, P.G., Greenstein, A., Emery, P. (2005) Infliximab in combination with methotrexate in active ankylosing spondylitis: a clinical and imaging study. *Ann Rheum Dis* 64: 1568–1575.

Maul, J., Loddenkemper, C., Mundt, P., Berg, E., Giese, T., Stallmach, A., Zeitz, M., Duchmann, R. (2005) Peripheral and intestinal regulatory $CD4^+$ $CD25^{high}$ T cells in inflammatory bowel disease. *Gastroenterology* 128: 1868–1878.

Mease, P.J., Kivitz, A.J., Burch, F.X., Siegel, E.L., Cohen, S.B., Ory, P., Salonen, D., Rubenstein, J., Sharp, J.T., Tsuji, W. (2004) Etanercept treatment of psoriatic arthritis: safety, efficacy, and effect on disease progression. *Arthritis Rheum* 50: 2264–2272.

Mease, P.J., Gladman, D.D., Ritchlin, C.T., Ruderman, E.M., Steinfeld, S.D., Choy, E.H., Sharp, J.T., Ory, P.A., Perdok, R.J.,

Weinberg, M.A. (2005) Adalimumab for the treatment of patients with moderately to severely active psoriatic arthritis: results of a double-blind, randomized, placebo-controlled trial. *Arthritis Rheum* 52: 3279–3289.

Miller, D.H., Khan, O.A., Sheremata, W.A., Blumhardt, L.D., Rice, G.P., Libonati, M. A., Willmer-Hulme, A.J., Dalton, C.M., Miszkiel, K.A., O'Connor, P.W. (2003) A controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med* 348: 15–23.

Mitoma, H., Horiuchi, T., Hatta, N., Tsukamoto, H., Harashima, S., Kikuchi, Y., Otsuka, J., Okamura, S., Fujita, S., Harada, M. (2005) Infliximab induces potent anti-inflammatory responses by outside-to-inside signals through transmembrane TNF-alpha. *Gastroenterology* 128: 376–392.

Moreland, L.W., Schiff, M.H., Baumgartner, S.W., Tindall, E.A., Fleischmann, R.M., Bulpitt, K.J., Weaver, A.L., Keystone, E.C., Furst, D.E., Mease, P.J., Ruderman, E.M., Horwitz, D.A., Arkfeld, D.G., Garrison, L., Burge, D.J., Blosch, C.M., Lange, M.L., McDonnell, N.D., Weinblatt, M.E. (1999) Etanercept therapy in rheumatoid arthritis. A randomized, controlled trial. *Ann Intern Med* 130: 478–486.

Myers, A., Clark, J., Foster, H. (2002) Tuberculosis and treatment with infliximab. *N Engl J Med* 346: 623–626.

Nagler-Anderson, C. (2000) Tolerance and immunity in the intestinal immune system. *Crit Rev Immunol* 20: 103–120.

Neurath, A.R., Strick, N., Li, Y.Y., Debnath, A.K. (2005) *Punica granatum* (pomegranate) juice provides an HIV-1 entry inhibitor and candidate topical microbicide. *Ann NY Acad Sci* 1056: 311–327.

Neurath, M., Fuss, I., Strober, W. (2000) TNBS-colitis. *Int Rev Immunol* 19: 51–62.

Neurath, M.F., Finotto, S., Glimcher, L.H. (2002) The role of Th1/Th2 polarization in mucosal immunity. *Nat Med* 8: 567–573.

Nielsen, O.H., Vainer, B., Madsen, S.M., Seidelin, J.B., Heegaard, N.H. (2000) Established and emerging biological activity markers of inflammatory bowel disease. *Am J Gastroenterol* 95: 359–367.

Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H., Achkar, J.P., Brant, S.R., Bayless, T.M., Kirschner, B.S., Hanauer, S.B., Nunez, G., Cho, J.H. (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 411: 603–606.

Panacek, E.A., Marshall, J.C., Albertson, T.E., Johnson, D.H., Johnson, S., MacArthur, R.D., Miller, M., Barchuk, W. T., Fischkoff, S., Kaul, M., Teoh, L., Van Meter, L., Daum, L., Lemeshow, S., Hicklin, G., Doig, C. (2004) Efficacy and safety of the monoclonal anti-tumor necrosis factor antibody $F(ab')_2$ fragment afelimomab in patients with severe sepsis and elevated interleukin-6 levels. *Crit Care Med* 32: 2173–2182.

Pender, S.L., Tickle, S.P., Docherty, A.J., Howie, D., Wathen, N.C., MacDonald, T.T. (1997) A major role for matrix metalloproteinases in T cell injury in the gut. *J Immunol* 158: 1582–1590.

Plevy, S.E., Landers, C.J., Prehn, J., Carramanzana, N.M., Deem, R.L., Shealy, D., Targan, S.R. (1997) A role for TNF-alpha and mucosal T helper-1 cytokines in the pathogenesis of Crohn's disease. *J Immunol* 159: 6276–6282.

Podolsky, D. (2002) Inflammatory bowel disease. *N Engl J Med* 347: 417–429.

Reich, K., Nestle, F.O., Papp, K., Ortonne, J.P., Evans, R., Guzzo, C., Li, S., Dooley, L.T., Griffiths, C.E. (2005) Infliximab induction and maintenance therapy for moderate-to-severe psoriasis: a phase III, multicentre, double-blind trial. *Lancet* 366: 1367–1374.

Reinecker, H.C., Steffen, M., Witthoeft, T., Pflueger, I., Schreiber, S., MacDermott, R.P., Raedler, A. (1993) Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Clin Exp Immunol* 94: 174–181.

Reinhart, K., Menges, T., Gardlund, B., Harm Zwaveling, J., Smithes, M., Vincent, J.L., Tellado, J.M., Salgado-Remigio, A., Zimlichman, R., Withington, S., Tschaikowsky, K., Brase, R., Damas, P., Kupper, H., Kempeni, J., Eiselstein, J.,

Kaul, M. (2001) Randomized, placebo-controlled trial of the anti-tumor necrosis factor antibody fragment afelimomab in hyperinflammatory response during severe sepsis: The RAMSES Study. *Crit Care Med* 29: 765–769.

Rothe, M., Sarma, V., Dixit, V.M., Goeddel, D.V. (1995) TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. *Science* 269: 1424–1427.

Rutgeerts, P., D'Haens, G., Targan, S., Vasiliauskas, E., Hanauer, S.B., Present, D.H., Mayer, L., Van Hogezand, R.A., Braakman, T., DeWoody, K.L., Schaible, T.F., Van Deventer, S.J. (1999) Efficacy and safety of retreatment with anti-tumor necrosis factor antibody (infliximab) to maintain remission in Crohn's disease. *Gastroenterology* 117: 761–769.

Rutgeerts, P., Lemmens, L., Van Assche, G., Noman, M., Borghini-Fuhrer, I., Goedkoop, R. (2003) Treatment of active Crohn's disease with onercept (recombinant human soluble p55 tumour necrosis factor receptor): results of a randomized, open-label, pilot study. *Aliment Pharmacol Ther* 17: 185–192.

Rutgeerts, P., Fedorak, R.N., Rachmilewitz, D., Tarabar, D., Gibson, P., Nielsen, O.H., Wild, G., Schreiber, S., Zignani, M. (2004) Onercept (recombinant human soluble p55 tumour necrosis factor receptor) treatment in patients with active Crohn's disease: randomized, placebo-controlled, dose-finding phase II study (abstract). *Gut* 53: A47.

Rutgeerts, P., Feagan, B.G., Olson, A., Johanns, J., Travers, S.B., Present, D.H., Sands, B.E., Sandborn, W.J. (2005) A randomized placebo-controlled trial of infliximab therapy for active ulcerative colitis: act i trial (abstract). *Gastroenterology* 128: A-105.

Sandborn, W.J., Hanauer, S.B., Katz, S., Safdi, M., Wolf, D.G., Baerg, R.D., Tremaine, W.J., Johnson, T., Diehl, N.N., Zinsmeister, A.R. (2001) Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial. *Gastroenterology* 121: 1088–1094.

Sandborn, W.J., Hanauer, S., Loftus, E.V., Jr., Tremaine, W.J., Kane, S., Cohen, R., Hanson, K., Johnson, T., Schmitt, D., Jeche, R. (2004a) An open-label study of the human anti-TNF monoclonal antibody adalimumab in subjects with prior loss of response or intolerance to infliximab for Crohn's disease. *Am J Gastroenterol* 99: 1984–1989.

Sandborn, W.J., Feagan, B.G., Radford-Smith, G., Kovacs, A., Enns, R., Innes, A., Patel, J. (2004b) CDP571, a humanised monoclonal antibody to tumour necrosis factor alpha, for moderate to severe Crohn's disease: a randomised, double blind, placebo controlled trial. *Gut* 53: 1485–1493.

Sandborn, W.J., Rachmilewitz, D., Hanauer, S.B., Lichtenstein, G.R., Villiers, W.J., Olson, A., Johanns, J., Travers, S.B., Colombel, J.F. (2005a) Infliximab induction and maintenance therapy for ulcerative colitis: the act 2 trial (abstract). *Gastroenterology* 128: A-104.

Sandborn, W.J., Hanauer, S.B., Lukas, M., Wolf, D.C., Isaacs, K.L., MacIntosh, D., Panaccione, R. (2005b) Maintenance of remission over 1 year in patients with active Crohn's disease treated with adalimumab: results of a blinded, placebo-controlled study (abstract). *Am J Gastroenterol* 100: S311.

Sandborn, W.J., Hanauer, S.B., Lukas, M., Wolf, D.C., Isaacs, K.L., MacIntosh, D., Panaccione, R., Rutgeerts, P., Pollack, P. (2005c) Remission and clinical response induced and maintained in patients with active Crohn's disease treated for 1-year open-label with adalimumab. *Am J Gastroenterol* 100: S316.

Sandborn, W.J., Colombel, J.F., Enns, R., Feagan, B.G., Hanauer, S.B., Lawrance, I.C., Panaccione, R., Sanders, M., Schreiber, S., Targan, S., van Deventer, S., Goldblum, R., Despain, D., Hogge, G.S., Rutgeerts, P. (2005d) Natalizumab induction and maintenance therapy for Crohn's disease. *N Engl J Med* 353: 1912–1925.

Sands, B.E., Anderson, F.H., Bernstein, C.N., Chey, W.Y., Feagan, B.G., Fedorak, R.N., Kamm, M.A., Korzenik, J.R., Lashner, B.A., Onken, J.E., Rachmilewitz, D., Rutgeerts, P., Wild, G., Wolf, D.C., Marsters, P.A., Travers, S.B., Blank, M.A., van Deventer, S.J. (2004) Infliximab

maintenance therapy for fistulizing Crohn's disease. *N Engl J Med* 350: 876–885.

Scallon, B.J., Moore, M.A., Trinh, H., Knight, D.M., Ghrayeb, J. (1995) Chimeric anti-TNF-alpha monoclonal antibody cA2 binds recombinant transmembrane TNF-alpha and activates immune effector functions. *Cytokine* 7: 251–259.

Schreiber, S., Nikolaus, S., Malchow, H., Kruis, W., Lochs, H., Raedler, A., Hahn, E. G., Krummenerl, T., Steinmann, G. (2001) Absence of efficacy of subcutaneous antisense ICAM-1 treatment of chronic active Crohn's disease. *Gastroenterology* 120: 1339–1346.

Schreiber, S., Rutgeerts, P., Fedorak, R.N., Khaliq-Kareemi, M., Kamm, M.A., Boivin, M., Bernstein, C.N., Staun, M., Thomsen, O.O., Innes, A. (2005) A randomized, placebo-controlled trial of certolizumab pegol (CDP870) for treatment of Crohn's disease. *Gastroenterology* 129: 807–818.

Schroder, J., Stuber, F., Gallati, H., Schade, F.U., Kremer, B. (1995) Pattern of soluble TNF receptors I and II in sepsis. *Infection* 23: 143–148.

Shen, C., Assche, G.V., Colpaert, S., Maerten, P., Geboes, K., Rutgeerts, P., Ceuppens, J.L. (2005) Adalimumab induces apoptosis of human monocytes: a comparative study with infliximab and etanercept. *Aliment Pharmacol Ther* 21: 251–258.

Siegel, S.A., Shealy, D.J., Nakada, M.T., Le, J., Woulfe, D.S., Probert, L., Kollias, G., Ghrayeb, J., Vilcek, J., Daddona, P.E. (1995) The mouse/human chimeric monoclonal antibody cA2 neutralizes TNF in vitro and protects transgenic mice from cachexia and TNF lethality in vivo. *Cytokine* 7: 15–25.

Singh, B., Read, S., Asseman, C., Malmstrom, V., Mottet, C., Stephens, L.A., Stepankova, R., Tlaskalova, H., Powrie, F. (2001) Control of intestinal inflammation by regulatory T cells. *Immunol Rev* 182: 190–200.

Strober, W., Ludviksson, B.R., Fuss, I.J. (1998) The pathogenesis of mucosal inflammation in murine models of inflammatory bowel disease and Crohn disease. *Ann Intern Med* 128: 848–856.

Takei, S., Groh, D., Bernstein, B., Shaham, B., Gallagher, K., Reiff, A. (2001) Safety and efficacy of high dose etanercept in treatment of juvenile rheumatoid arthritis. *J Rheumatol* 28: 1677–1680.

ten Hove, T., van Montfrans, C., Peppelenbosch, M.P., van Deventer, S.J. (2002) Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. *Gut* 50: 206–211.

Tyring, S., Gottlieb, A., Papp, K., Gordon, K., Leonardi, C., Wang, A., Lalla, D., Woolley, M., Jahreis, A., Zitnik, R., Cella, D., Krishnan, R. (2006) Etanercept and clinical outcomes, fatigue, and depression in psoriasis: double-blind placebo-controlled randomised phase III trial. *Lancet* 367: 29–35.

Van Assche, G., Van Ranst, M., Sciot, R., Dubois, B., Vermeire, S., Noman, M., Verbeeck, J., Geboes, K., Robberecht, W., Rutgeerts, P. (2005) Progressive multifocal leukoencephalopathy after natalizumab therapy for Crohn's disease. *N Engl J Med* 353: 362–368.

Van den Brande, J.M., Braat, H., van den Brink, G.R., Versteeg, H.H., Bauer, C.A., Hoedemaeker, I., van Montfrans, C., Hommes, D.W., Peppelenbosch, M.P., van Deventer, S.J. (2003) Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. *Gastroenterology* 124: 1774–1785.

Van den Brande, J., Hommes, D.W., Peppelenbosch, M.P. (2005) Infliximab induced T lymphocyte apoptosis in Crohn's disease. *J Rheumatol Suppl* 74: 26–30.

van Deventer, S.J., Elson, C.O., Fedorak, R.N. (1997) Multiple doses of intravenous interleukin 10 in steroid-refractory Crohn's disease. Crohn's Disease Study Group. *Gastroenterology* 113: 383–389.

Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V., Baldwin, A.S., Jr. (1998) NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* 281: 1680–1683.

Weinblatt, M.E., Keystone, E.C., Furst, D.E., Kavanaugh, A.F., Chartash, E.K., Segurado, O.G. (2006) Long-term efficacy and safety of adalimumab plus methotrexate in patients with rheumatoid

arthritis: ARMADA 4-year extended study. *Ann Rheum Dis* 65: 753-759.

Wirtz, S., Neurath, M.F. (2000) Animal models of intestinal inflammation: new insights into the molecular pathogenesis and immunotherapy of inflammatory bowel disease. *Int J Colorectal Dis* 15: 144–160.

Yin, M.J., Yamamoto, Y., Gaynor, R.B. (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature* 396: 77–80.

Volume III
Approved Therapeutics

1
Adalimumab (Humira)

Hartmut Kupper, Jochen Salfeld, Daniel Tracey, and Joachim R. Kalden

1.1
Introduction

Adalimumab (Humira; Abbott Laboratories, Abbott Park, IL, USA) is the first fully human recombinant immunoglobulin G1 (IgG1) monoclonal antibody designed to inhibit tumor necrosis factor alpha (TNF-α or TNF). Previous anti-TNF monoclonal antibodies were composed of both murine and human components, which increased the potential for immune responses and limited the long-term use. In contrast, because adalimumab is fully human, it is able to elicit a long-term response with a low degree of immunogenicity, with or without concomitant administration of immunosuppressants such as methotrexate (MTX) (Rau 2002; van de Putte et al. 2003).

Adalimumab is composed of human-derived heavy and light chain variable regions and human IgG1:κ constant regions, engineered through phage display technology (US Humira PI 2006). Phage display technology is designed to recapitulate the physiologic antibody generation process in the laboratory. Comparable to the natural selection process for the B-cell displaying the appropriate antibody, phage display allows for the selection of a fully human antibody specific for an antigen, in this case TNF, from a large repertoire of antibodies. If the desired antibody is felt to be rare in the repertoire, a variant of the technology allows for more rapid "guided selection" in a two-stage process. Therefore, the first step in the generation of adalimumab was a guided selection approach using the murine anti-human TNF antibody MAK195 to isolate a human antibody that recognized the same neutralizing epitope as MAK195. MAK195 is a potent neutralizing monoclonal antibody, which has a high affinity and a low off-rate constant for human TNF. The MAK195 VH and VL (variable portion of heavy and light chains) were paired with human cognate repertoires, and these phage antibody libraries underwent antigen binding selection using recombinant human TNF as the antigen. The selected human VH and VL genes were then combined to generate a fully human anti-TNF antibody.

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Early human anti-TNF antibodies were optimized in a process mirroring the natural process for antibody optimization. The final antibody, adalimumab, is a full-length IgG1:κ molecule with optimized heavy and light chains characterized by high specificity, affinity, and potency (van de Putte et al. 2003). Adalimumab contains no nonhuman components or artificially fused human peptide sequences. The resulting molecule is, therefore, indistinguishable in structure and function from a naturally occurring human IgG1, and has a comparable terminal half-life of approximately 2 weeks (Salfeld et al. 1998).

Adalimumab is produced in a Chinese hamster ovary (CHO) host cell that is transfected with a plasmid vector containing the expression cassettes for adalimumab heavy and light chains. Adalimumab is produced by a standard, well-controlled fermentation and purification process (US Humira PI 2006). Each batch of adalimumab is characterized rigorously in a series of biochemical and biophysical assays in order to meet prespecified release criteria.

1.2 Pharmacology

In the body, TNF is protective against infection and injury through multiple biologic mechanisms that also can play a role in inflammation. This cytokine interacts with endothelial cells, synovial fibrobasts, keratinocytes, dendritic cells, and other components of the immune system as part of a complex cascade of response-to-injury events (Mease and Goffe 2005). In this setting, TNF promotes the accumulation of inflammatory cells, activates endothelial adhesion molecules, and promotes the synthesis of other proinflammatory cytokines (e.g., interleukin [IL]-1, IL-6, granulocyte-macrophage colony-stimulating factor) and chemokines (Fig. 1.1) (Lee and Kavanaugh 2005; Maini and Taylor 2000; Mease and Goffe 2005). Despite a critical, protective role in the immune response, chronically elevated levels of TNF have been implicated as a pathogenic component of a number of disease states, including rheumatoid arthritis (RA), psoriasis, psoriatic arthritis (PsA), ankylosing spondylitis (AS), and Crohn's disease.

Three lines of evidence support a role for TNF in these conditions. First, high concentrations of TNF are found in the synovial fluid of patients with RA, psoriatic lesions of patients with psoriasis (Partsch et al. 1997; Ritchlin et al. 1998), sacroiliac joint biopsy specimens from patients with AS (Braun et al. 1995), and in stool, mucosa, and blood of patients with Crohn's disease (Braegger et al. 1992; Murch et al. 1991, 1993). Second, in animal models of RA, TNF has been shown to accelerate disease activity; and third, anti-TNF antibodies have been shown to decrease disease activity in animal models of RA (Cooper et al. 1992; Williams et al. 1992).

Adalimumab binds to soluble TNF with high specificity and high affinity ($K_d = 6 \times 10^{-10}$ M). Adalimumab binding to TNF prevents interaction with TNF-RI/II cell-surface receptors (Lee and Kavanaugh 2005; Salfeld et al. 1998; US Humira PI 2006). Adalimumab neutralizes TNF proinflammatory activity (Fig.

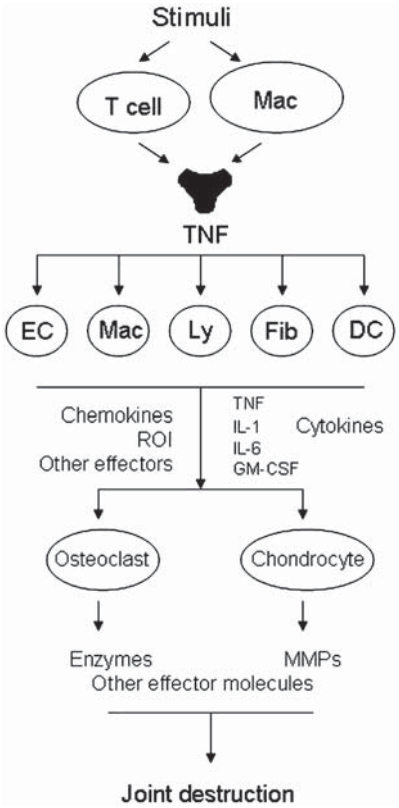


Fig. 1.1 The role of tumor necrosis factor (TNF) in the pathogenesis of rheumatoid arthritis. DC = dendritic cells; EC = endothelial cells; Fib = fibroblasts; GM-CSF = granulocyte-macrophage colony-stimulating factor; IL = interleukin; Ly = lymphocytes; Mac = macrophages; MMPs = matrix metalloproteases; ROI = reactive oxygen intermediates.

1.2) (Lee and Kavanaugh 2005; Salfeld et al. 1998), and also binds to and neutralizes the cell membrane-associated form of TNF, which may play a role in disease (Georgopoulos et al. 1996). Adalimumab does not bind to or inactivate lymphotoxin (LTα, formerly called TNF-β). The effects of adalimumab treatment on parameters of inflammatory disease are consistent with neutralization of TNF as the primary mechanism of action. The effects of adalimumab in patients with RA on various disease-related parameters were initially evaluated in a phase I, single-dose, placebo-controlled trial (den Broeder et al. 2002a). Patients were randomized to receive a single dose of 0.5, 1, 3, 5, or 10 $mg\,kg^{-1}$ adalimumab or placebo. At 1 week after injection of adalimumab, the acute-phase reactant C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) showed an impressive decrease from baseline at all doses (den Broeder et al. 2002a). Levels of IL-6, IL-1 receptor antagonist, and IL-1β messenger RNA also decreased rapidly (Barrera et al. 2001).

In patients receiving adalimumab, levels of matrix metalloproteases (MMP-1 and -3) decreased over 6 months (den Broeder et al. 2002a; Weinblatt et al. 2003). Long-term monotherapy with adalimumab modulated cartilage and synovium turnover, and was associated with improved radiologic outcomes (den Broeder

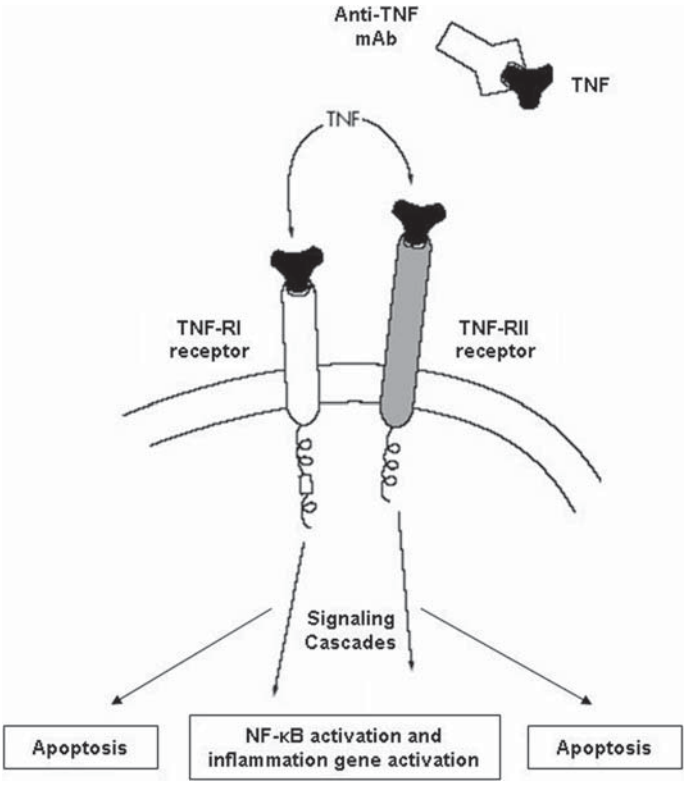


Fig. 1.2 Neutralization of tumor necrosis factor (TNF) by anti-TNF monoclonal antibody (mAb) prevents TNF binding to either the TNF-RI or the TNF-RII receptors. NF-κB = nuclear factor kappa B. (Adapted with permission from Mease 2002.)

et al. 2002b). Treatment with adalimumab also has been shown to alter expression of adhesion molecules responsible for leukocyte migration (endothelial leukocyte adhesion molecule-1, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1) (US Humira PI 2006).

1.3 Pharmacokinetics

Adalimumab follows linear pharmacokinetic properties throughout the clinical dose range in healthy adults. The absolute bioavailability of adalimumab following a single 40-mg subcutaneous (SC) dose is 64%, and maximum plasma concentrations ($4.7 \pm 1.6\,\mu g\,mL^{-1}$) are achieved at 131 ± 56 h. Following the recommended dose of 40 mg SC every other week (EOW), steady-state serum trough adalimumab concentrations are three- to seven-fold higher than the expected

effective dose in 50% of patients (Granneman et al. 2003). Concomitant MTX treatment does not significantly alter adalimumab pharmacokinetics. Neither does the presence of MTX not adversely affect adalimumab serum concentrations. At the recommended dose of 40 mg EOW, mean steady-state serum concentrations are 5 $\mu g\,mL^{-1}$ without MTX and 8–9 $\mu g\,mL^{-1}$ with MTX. After single and multiple dosing, MTX reduced adalimumab apparent clearance by 29% and 44%, respectively. Synovial fluid concentrations of adalimumab in patients with RA are 31% to 96% of those found in serum (US Humira PI 2006). Pharmacokinetic studies have not been conducted in children, or in patients with hepatic or renal impairment (US Humira PI 2006).

1.4 Adalimumab Comparisons with Infliximab and Etanercept

There are some structural and functional differences between adalimumab and the other TNF antagonists: infliximab, a chimeric murine/human monoclonal antibody; and etanercept, a TNF receptor fusion protein (Fig. 1.3). These include specificity, TNF binding affinity, immunogenicity, pharmacokinetics, and efficacy across different disease states. Adalimumab and infliximab are highly specific for TNF (in both soluble and membrane-bound forms), whereas etanercept binds and neutralizes LTα as well as TNF. Adalimumab binds to TNF with very high affinity (K_d ~85 pM) (Kaymakcalan et al. 2002), and potently neutralizes TNF in bioassays (IC_{50} ~130 pM) (Salfeld et al. 1998). The kinetic binding parameters of adalimumab and infliximab are similar; however, etanercept dissociates from TNF much more rapidly than adalimumab or infliximab (Kaymakcalan et al. 2002).

Immunogenicity is directly related to the structure of protein therapeutics, and may result in increased adverse effects and diminished efficacy. Antibodies to adalimumab, a fully human monoclonal IgG1 antibody indistinguishable from naturally occurring IgG1, have been observed in a small proportion of patients (US Humira PI 2006). This low level of immunogenicity is reflective of the natural process of anti-idiotypic antibody formation to endogenous antibodies characteristic of the natural immune network (Jerne 1974). Infliximab, a chimeric antibody developed by recombinant fusion of murine and human antibody components, can be immunogenic in a high proportion of patients, unless immunosuppressive drugs are administered concomitantly (Anderson 2005; Baert et al. 2003; Maini et al. 1998). Low levels of immunogenicity have been reported following administration of etanercept, which is composed of human components but in an artificial construct; however, considerable variability has been seen, depending on the assays used to detect anti-etanercept antibodies (Anderson 2005).

Pharmacokinetic differences between the three TNF antagonists are also apparent. Adalimumab has a longer half-life than etanercept (10–20 days versus 3.5–5 days, respectively), allowing for less-frequent dosing. In contrast, infliximab must

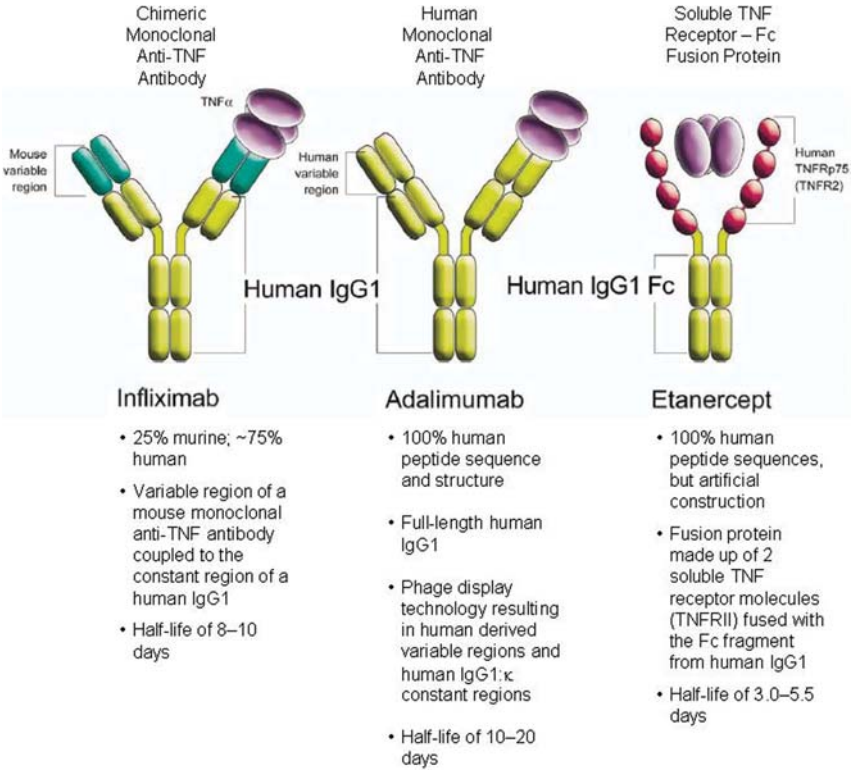


Fig. 1.3 Adalimumab structure in comparison to other tumor necrosis factor (TNF)-antagonists. IgG1 = immunoglobulin G1. (Adapted with permission from Anderson 2005.)

be administered by intravenous infusion, which produces peak concentrations significantly higher and trough concentrations significantly lower than adalimumab and etanercept (Granneman et al. 2003; Maini et al. 1998; Zhou et al. 2004). The clinical implication of these substantial fluctuations in concentrations is unknown.

Finally, there appear to be differences in efficacy among the three TNF antagonists, the efficacy of which has been evaluated in an animal model of RA driven by a human TNF transgene (Kaymakcalan et al. 2002). In this evaluation, adalimumab was more effective than infliximab and etanercept in preventing the development of arthritis, as demonstrated by suppressed histopathologic evidence of synovial inflammation, vascularity, and cartilage and bone erosion. Following administration of etanercept, human TNF was cleared more slowly from serum than after adalimumab or infliximab, suggesting that TNF–etanercept complexes persist longer in the circulation. The results of clinical studies evaluating each agent suggest that the efficacy of adalimumab is comparable to that of infliximab

and etanercept in RA, but that infliximab and adalimumab may be more efficacious than etanercept in Crohn's disease and psoriasis, possibly a result of greater tissue penetration and/or effector function by the antibodies. Although it is accepted that all three agents bind to membrane TNF on transfected cells, *in-vitro* data on binding and initiation of Fc-mediated effector functions such as complement activation or antibody-induced cellular cytotoxicity (ADCC) by normal human cells are inconsistent (Scallon et al. 1995; van den Brande et al. 2003). There is also conflicting *in-vitro* data regarding the induction of apoptosis in T cells, monocytes, or other cells by TNF antagonists (Catrina et al. 2005; Mitoma et al. 2005; Shen et al. 2005; van den Brande et al. 2003); the relevance of these *in-vitro* studies to the efficacy or safety of these agents *in vivo* remains to be determined, however.

1.5 Indications

The currently approved indications for adalimumab as of September 2006 are summarized in Table 1.1. Note that there are subtle differences in the European and United States labeled indications.

1.5.1 Rheumatoid Arthritis

The first indication for adalimumab was for the treatment of RA. In the United States, adalimumab is indicated for reducing signs and symptoms, inducing major clinical response, inhibiting the progression of structural damage, and improving physical function in adult patients with moderately to severely active RA (US Humira PI 2006). The European labeling indicates that adalimumab should be used in the setting of disease-modifying antirheumatic drugs (DMARD) failure. However, in the case of severe, active and progressive RA, European labeling also includes a specific indication for the use of adalimumab in the treatment of adults not previously treated with MTX (EU Humira SPC 2006). US labeling indicates that adalimumab may be used alone or in combination with MTX and other DMARDs; European labeling includes administration in combination with MTX or as monotherapy (EU Humira SPC 2006; US Humira PI 2006).

1.5.2 Psoriatic Arthritis

Adalimumab is also indicated for use in the treatment of PsA. In the United States, the labeling states that adalimumab is indicated for reducing signs and symptoms of active arthritis in patients with PsA (US Humira PI 2006). Again, similar to the labeling in RA, the European labeling for this indication stipulates that adalimumab should be used when the response to previous DMARD therapy

Table 1.1 Approved indications (EU Humira SPC 2006; US Humira PI 2006).

EU Summary of Product Characteristics Therapeutic Indications	*USA Package Insert Indications and Usage*
Rheumatoid arthritis (RA) Humira in combination with MTX, is indicated for • the treatment of moderate to severe, active RA in adult patients when the response to DMARDs including MTX has been inadequate. • the treatment of severe, active and progressive RA in adults not previously treated with MTX. Humira can be given as monotherapy in case of intolerance to MTX, or when continued treatment with MTX is inappropriate. Humira has been shown to reduce the rate of progression of joint damage as measured by radiography, and to improve physical function, when given in combination with MTX.	Humira is indicated for reducing signs and symptoms, inducing major clinical response, inhibiting the progression of structural damage and improving physical function in adult patients with moderately to severely active RA. Humira can be used alone or in combination with MTX or other DMARDs.
Psoriatic arthritis Humira is indicated for the treatment of active and progressive psoriatic arthritis in adults when the response to previous DMARD therapy has been inadequate.	Humira is indicated for reducing signs and symptoms of active arthritis in patients with psoriatic arthritis. Humira can be used alone or in combination with DMARDs.
Ankylosing spondylitis Humira is indicated for reducing signs and symptoms in patients with active ankylosing spondylitis.	Humira is indicated for the treatment of adults with severe active ankylosing spondylitis who have had an inadequate response to conventional therapy.

DMARDs = disease-modifying antirheumatic drugs; MTX = methotrexate.

has been inadequate (EU Humira SPC 2006), The US Food and Drug Administration labeled adalimumab for use alone or in combination with DMARDs; however, the labeling approved by the European Medicines Evaluation Agency for PsA does not mention the use of combination therapy at this time (EU Humira SPC 2006).

1.5.3 Ankylosing Spondylitis

Adalimumab is indicated for reducing signs and symptoms in patients with active ankylosing spondylitis (US Humira PI 2006). In the European Union, adalimumab is indicated for the treatment of adults with severe active ankylosing spondylitis who have had an inadequate response to conventional therapy (EU Humira SPC 2006).

1.5.4 Dosing and Administration

The recommended dose for RA, PSA, and AS is 40 mg SC, EOW (US Humira PI 2006; EU Humira SPC 2006). US labeling states that, in RA, some patients not taking concomitant MTX may derive additional benefit from increasing the dosing frequency to 40 mg weekly (US Humira PI 2006). Weekly dosing also is included in the European labeling for RA patients who are receiving monotherapy and experience a decrease in their response. Further, both labels state that glucocorticoids, salicylates, nonsteroidal anti-inflammatory drugs (NSAIDs), and analgesics may be continued during treatment with adalimumab.

Adalimumab is available as a preservative-free, sterile solution for SC injection, as 40 mg per 0.8 mL in 1-mL prefilled single-dose glass syringes. Under refrigeration (2–8°C), adalimumab has a shelf-life of 18 months. The drug formulation should not be frozen (US Humira PI 2006).

1.6 Clinical Experience

Adalimumab has been studied most extensively in patients with RA (Table 1.2), with more than 10000 patients having been enrolled in studies during the RA clinical development program. A total of 300 of these patients has been followed for at least 5 years, with some patients having been followed into their seventh year of treatment (Schiff et al. 2005b, 2006).

1.6.1 Studies in Rheumatoid Arthritis

The pivotal studies evaluated safety and efficacy of adalimumab treatment in a variety of settings: in combination with MTX in advanced disease (ARMADA [Anti-TNF Research Study Program of the Monoclonal Antibody Adalimumab, DE019), in combination with traditional DMARDs (STAR [The Safety Trial of Adalimumab in Rheumatoid Arthritis]), as monotherapy in patients with severe disease (DE011), and combined with MTX or as monotherapy in early disease (PREMIER). Following the double-blind phases of all of these studies, adalimumab treatment was continued during open-label extension (OLE) trials. In addition to these studies, more than 900 and more than 6600 patients were evaluated in the Act (Access to Therapy) and ReAct (Research in Active Rheumatoid Arthritis) trials, respectively, open-label trials designed to assess prospectively the safety and efficacy of adalimumab in real-life settings (Bombardieri et al. 2004; Burmester et al. 2004, 2005a; Schiff et al. 2006).

1.6.1.1 Adalimumab in Combination with MTX

Two large, randomized, double-blind, placebo-controlled, multicenter studies have evaluated the safety and efficacy of adalimumab in combination with MTX

Table 1.2 Summary of baseline data and 6-month efficacy endpoints for clinical trials evaluating adalimumab in patients with rheumatoid arthritis.* (Adapted with permission from Bansback et al. 2005.)

Trial name	*Treatment*	*Patients [n]*	*Mean age [years]*	*Disease duration [years]*	*Baseline HAQ*	*MTX dose [mg/week]*	*RF+ [%]*	*ACR20*	*ACR50*	*ACR70*
ARMADA (Weinblatt et al. 2003)	Placebo + MTX	62	56	11	1.6	17	79	15	8	10
	Adalimumab + MTX	67	57	12	1.6	17	80	67	55	27
DE019 (Keystone et al. 2004b,c)	Placebo + MTX	200	56	11	1.4	17	82	30	10	3
	Adalimumab + MTX	207	56	11	1.4	17	82	63	39	21
DE011 (van de Putte et al. 2004)	Placebo	110	54	12	1.9	–	90	19	8	2
	Adalimumab	113	53	11	1.8	–	90	46	22	12
STAR (Furst et al. 2003)	Placebo + DMARDs	318	56	12	1.4	NR†	62	35	11	4
	Adalimumab + DMARDs	318	55	9	1.4	NR†	63	53	29	15
PREMIER (Breedveld et al. 2005, 2006	MTX	257	52	1	1.5	20	81	63‡	46‡	28‡
	Adalimumab	274	52	1	1.5	–	83	54‡	42‡	26‡
	Adalimumab + MTX	268	52	1	1.5	20	87	73‡	62‡	46‡

* Note that only licensed indication dosages are shown (adalimumab 40 mg EOW).
† Methotrexate (MTX) used in only 56% of patients; dose not reported.
‡ Results from 12 months.
ACR = American College of Rheumatology; ARMADA = Anti-TNF Research Study Program of the Monoclonal Antibody Adalimumab; DMARDs = disease-modifying antirheumatic drugs; HAQ = Health Assessment Questionnaire; MTX = methotrexate; RF = rheumatoid factor; STAR = Safety Trial of Adalimumab in Rheumatoid Arthritis.

for treatment of long-standing RA in patients with insufficient response to MTX. In ARMADA, 271 patients received either adalimumab 20, 40, or 80 mg or placebo SC EOW in addition to MTX (Weinblatt et al. 2003). A total of 25% to 32% of patients receiving adalimumab achieved at least 20% improvement in American College of Rheumatology responses after 1 week (ACR20). At the end of 24 weeks, significantly more patients treated with adalimumab 20, 40, and 80 mg had achieved an ACR20 response (48%, 67%, and 66% respectively; $P < 0.001$ versus placebo for all comparisons) and an ACR50 response (32%, 55%, 43%, respectively; $P = 0.003$, $P < 0.001$, and $P < 0.001$, versus placebo, respectively) than those who received placebo (ACR20, 15%; ACR50, 8%). Some 27% of patients receiving adalimumab 40 mg ($P < 0.001$ versus placebo), and 19% of those receiving adalimumab 80 mg ($P = 0.02$ versus placebo) achieved an ACR70 response versus only 5% of patients receiving placebo. Scores on the Health Assessment Questionnaire Disability Index (HAQ-DI), fatigue scale, and the Short Form 36 (SF-36) also were improved over baseline, and these improvements were statistically greater than with placebo. Adalimumab-treated patients experienced up to a 40% reduction in the HAQ-DI scores. Clinically meaningful changes (≥10 points) in six domains of the SF-36 were attained by patients receiving adalimumab plus MTX as compared with two domains for those receiving placebo plus MTX. Adalimumab was well tolerated at all doses. Infections occurred at a similar rate in the adalimumab (1.55 per patient-year [PY]) and placebo groups (1.38/PY).

The OLE of this study is ongoing. Among patients completing 5 years of treatment, clinical efficacy was sustained, with 76%, 64%, and 39% of patients achieving ACR20, 50, 70 responses; 52% achieving clinical remission; and 28% having no physical limitations (Weinblatt et al. 2005, 2006). Furthermore, the majority of patients in the OLE were able to reduce corticosteroid and/or MTX dosages without adversely affecting long-term efficacy (Weinblatt et al. 2005). Serious adverse events in the OLE were similar to those observed during the controlled phase. The rate of serious infection was 2.03 events per 100 PY in the OLE phase compared with 2.30 during the blinded phase. The primary reasons for withdrawal from the study over time were lack of efficacy (8%), adverse events (11%), and other reasons (16%).

The long-term efficacy and safety of adalimumab in combination with DMARDs were evaluated in a rollover OLE study (DE020), enrolling 846 RA patients from ARMADA, STAR, and two phase I trials in which all patients received adalimumab and most received concomitant MTX. The significant clinical improvement seen after 6 months of treatment in controlled trials was sustained over 4 years, with patients maintaining significant reductions in Disease Activity Score in 28 joints (DAS28), swollen and tender joint counts, and HAQ-DI scores. ACR20, 50, and 70 response rates achieved at 6 months were also maintained through 4 years of therapy. At the last visit, 49% of patients achieved DAS28 <2.6, 24% had zero tender joints, 21% had zero swollen joints, and 44% achieved HAQ-DI ≤0.5 – parameters indicative of remission. A substantial percentage of patients treated with adalimumab plus MTX were able to reduce their use of steroids and decrease their MTX dose while maintaining control of their disease. Among those patients

achieving remission, those with moderate disease and younger age tended to achieve remission more rapidly and sustain remission for longer periods than older patients and those with severe disease (Emery et al. 2004; Schiff et al. 2004).

DE019 was a randomized, double-blind, placebo-controlled, multicenter study assessing the ability of adalimumab to inhibit radiographic progression and reduce disease activity in 619 RA patients with active disease, despite therapy with MTX. Patients were randomized to receive adalimumab 40 mg EOW, adalimumab 20 mg weekly, or placebo, plus concomitant MTX (Keystone et al. 2004a). At the end of 52 weeks, as was seen in the ARMADA study, significantly more patients treated with adalimumab attained ACR20, 50, and 70 responses (40 mg EOW: 59%, 42%, 23% and 20 mg weekly: 55%, 38%, 21%, respectively) compared with patients receiving placebo (24%, 10%, 5%; $P \leq 0.001$).

Significantly less radiographic progression as measured by total Sharp scores (TSS) was seen after adalimumab treatment. At one year, the mean change in TSS of adalimumab-treated patients was 0.1 units compared with 2.7 units for placebo patients. Of the patients receiving adalimumab 40 mg EOW, 62% had no new erosions at Week 52, as did 58% of those receiving adalimumab 20 mg weekly. A significantly smaller percentage (46%; $P \leq 0.001$, $P \leq 0.05$, respectively) of patients in the placebo group had no new erosions at Week 52. Functional status and quality of life (HAQ-DI and SF-36) also improved significantly in the adalimumab groups, with approximately 40% decreases in HAQ-DI scores in the adalimumab groups versus 17% with placebo, and clinically meaningful improvement in the SF-36 for the patients receiving active treatment versus placebo (Keystone et al. 2004a).

In an OLE of this study, 40 mg adalimumab was administered EOW to 457 of the patients who completed DE019. Of these patients, 79% (n = 363) remained in the study at 3 years. The reasons for discontinuation were loss of efficacy (2%), adverse events (7%), and other reasons (11%). For those in the overall OLE population who remained in the study at 3 years, responses were maintained, with 58% achieving an ACR20 response, 42% achieving an ACR50 response, and 24% achieving an ACR70 response. The long-term effects of adalimumab treatment on disease progression were substantial. At the three-year follow-up, adalimumab plus MTX continued to inhibit structural damage. On radiographic examination of the 129 patients originally randomized to receive adalimumab 40 mg EOW, the mean TSS at the end of 3 years was 0.3. Some 62% of patients had no radiographic progression (defined as ≤0.5 unit increase in TSS from baseline), and 28% showed radiographic improvement (>0.5 unit decrease in TSS from baseline) at 3 years (Keystone et al. 2005). The sustained clinical benefit and inhibition of disease progression were accompanied by improvements in functional status as measured by HAQ (Keystone et al. 2004b,c).

1.6.1.2 **Adalimumab with Traditional DMARDs**

STAR was a randomized, double-blind, placebo-controlled, multicenter study that assessed the safety and efficacy of adalimumab in a heterogeneous group of RA

patients with persistent disease activity despite concomitant DMARDs (Furst et al. 2003). The study was designed to reflect the general patient population and standard treatment regimens typically seen in clinical practice. This 24-week study enrolled 636 patients who had active disease while being treated with standard antirheumatic therapies. Participants received either placebo or adalimumab 40 mg EOW while continuing their standard therapy. MTX, antimalarials, and leflunomide were the most commonly used agents, alone or in combination.

The addition of adalimumab to standard antirheumatic therapy was well tolerated (Furst et al. 2003), with the majority of adverse events being mild or moderate in severity. Adverse event rates and withdrawal rates were similar in both treatment groups, with 91% of each group completing the study. Over the study period, no differences were seen in the incidence of any infection or serious infection between the treatment groups (Furst et al. 2003). Add-on treatment with adalimumab resulted in significantly higher percentages of patients achieving ACR20, 50, 70 responses (53%, 29%, 15%, respectively) compared with placebo plus standard therapy (35%, 11%, 3.5%, respectively; $P \leq 0.001$ for all comparisons) (Furst et al. 2003).

1.6.1.2.1 Additional Experience: Adalimumab with Traditional DMARDs

The Act and ReAct trials were 12-week, open-label studies designed to provide a prospective evaluation of efficacy and safety in real-life settings involving 936 and 6610 patients with active, insufficiently treated RA, respectively (Bombardieri et al. 2004; Burmester et al. 2004, 2005a; Schiff et al. 2006). Participants had various comorbidities, were treated with a broad range of antirheumatic therapies, and were enrolled in varied social care systems. Patients received adalimumab 40 mg EOW as classic add-on therapy (i.e., added to existing, but insufficient antirheumatic therapy). In ReAct, patients were given the option to continue adalimumab for an extension period.

At Week 12 in the ReAct study, 69%, 40%, and 18% of patients achieved ACR20, 50, and 70 responses, respectively. Moderate European League Against Rheumatism (EULAR) responses were achieved in 83% of patients, and 33% achieved a good EULAR response. The mean DAS28 was reduced by 2.1 units, with 20% of patients having a DAS28 <2.6 at Week 12. The mean HAQ scores decreased by 0.54 units. These data demonstrate that adalimumab was effective in more than 6000 difficult-to-treat patients with active RA (Burmester et al. 2005a; also data on file).

1.6.1.3 Adalimumab Monotherapy

The safety and efficacy of adalimumab as monotherapy in severe RA was assessed in 544 difficult-to-treat patients who had failed multiple DMARDs in a phase III randomized, double-blind, placebo-controlled, multicenter study (DE011) (van de Putte et al. 2004). Patients with active disease were randomized to receive adalimumab 20 mg EOW, 20 mg weekly, 40 mg EOW, 40 mg weekly, or placebo for 26 weeks. At 26 weeks, significantly more patients receiving adalimumab achieved

the primary efficacy endpoint ACR20 compared with those in the placebo group ($P \leq 0.01$ for all treatment groups). An ACR20 response was achieved by 36%, 39%, 46%, 53%, and 19% of adalimumab 20 mg EOW, 20 mg weekly, 40 mg EOW, 40 mg weekly, and placebo groups, respectively. Response rates for ACR50 were 19%, 21%, 22%, 35% for adalimumab 20 mg EOW, 20 mg weekly, 40 mg EOW, 40 mg weekly, all of which were significantly higher than the 8% response seen with placebo. Treatment with adalimumab resulted in improved ACR70 rates compared with placebo (van de Putte et al. 2004). Adalimumab 40 mg weekly resulted in slightly higher ACR response than 40 mg EOW. Moderate EULAR response rates were significantly greater with adalimumab than with placebo at 26 weeks (42%, 48%, 56%, 61% versus 26%, $P \leq 0.05$). In addition, clinical improvement was achieved as early as 2 weeks after treatment initiation with adalimumab (van de Putte et al. 2004).

The long-term efficacy and safety of adalimumab as monotherapy were evaluated in a rollover OLE study (DE018), enrolling 794 RA patients from DE011 and three other adalimumab trials in which the majority of patients received monotherapy. The clinical responses achieved at Year 1 were sustained up to 5 years, with 67%, 40%, and 17% of patients maintaining ACR20, 50, and 70 responses, respectively. A total of 81% of these patients had a moderate EULAR response at 5 years, and initial reductions in tender and swollen joint counts were also maintained. High retention rates were achieved, which suggested high tolerability with adalimumab therapy (Burmester et al. 2003).

1.6.1.4 Pivotal Study in Early Rheumatoid Arthritis

Rheumatoid arthritis is characterized by rapid disease progression that is often insidious. By the time joint malalignment and functional disability are evident, substantial irreversible damage has occurred in many patients (Lee and Weinblatt 2001). In 70% of patients, radiographic evidence of joint destruction is present within the first 2 years (Lee and Weinblatt 2001; McQueen et al. 1998). As early as 4 months after disease onset, synovial hypertrophy, bone edema, and early erosion have been detected using magnetic resonance imaging (Lee and Weinblatt 2001; McGonagle et al. 1999). Moreover, active synovitis is detectable in biopsy specimens of symptomless knee joints in patients with early disease (Lee and Weinblatt 2001; Soden et al. 1989). These observations are consistent with data indicating that treatment with combination therapy with DMARDs before irreversible joint destruction has occurred may improve long-term clinical outcomes for patients with RA (Landewé et al. 2002; Möttönen et al. 2002; Tsakonas et al. 2000).

The outcomes of aggressive, early treatment of RA were tested in the PREMIER study, a randomized, double-blind, active comparator, multicenter trial of adalimumab in 799 MTX-naïve patients with very early RA (mean disease duration 0.7 years) (Breedveld et al. 2005, 2006). This trial compared treatment with adalimumab 40 mg EOW plus MTX, adalimumab 40 mg EOW alone, and MTX alone (rapidly escalated to 20 mg per week after study initiation) for 2 years. Combination therapy was consistently more effective than monotherapy with either agent

for all outcomes measured. The ACR50 response rates at one year were 62% for the combination group versus 46% for MTX monotherapy ($P < 0.001$) and 42% for adalimumab monotherapy ($P < 0.001$). Similarly, a greater percentage of patients receiving the combination achieved an ACR70 response (46%) versus those receiving MTX monotherapy (28%; $P < 0.001$) or adalimumab monotherapy (26%; $P < 0.001$). These differences were maintained at 2 years, as shown in Fig. 1.4A.

In this population of MTX-naïve patients with recent-onset RA, inhibition of disease progression as measured radiographically was significantly greater with combination therapy or adalimumab alone than with MTX alone. The change

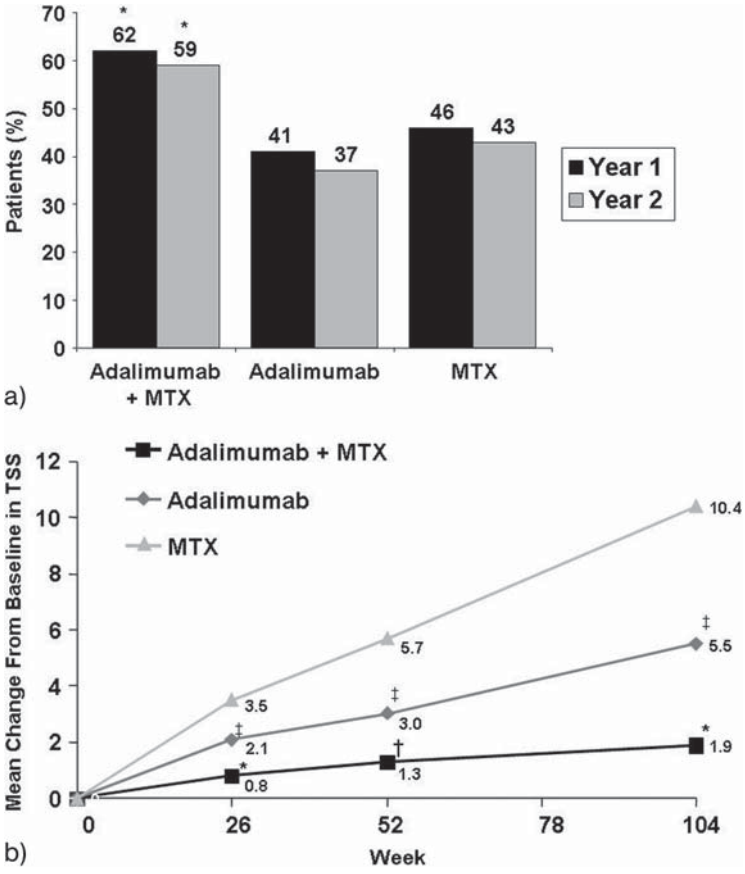


Fig. 1.4 Results of the PREMIER study. (a) American College of Rheumatology 50 response at Years 1 and 2. *$P < 0.001$ for adalimumab + methotrexate (MTX) versus MTX alone and adalimumab + MTX versus adalimumab alone. (b) Mean change from baseline in total Sharp scores (TSS) over time. *$P < 0.001$ for adalimumab + MTX versus MTX alone and adalimumab alone. †$P < 0.001$ for adalimumab + MTX versus MTX alone and $P = 0.002$ for adalimumab + MTX versus adalimumab alone. ‡$P < 0.001$ for adalimumab versus MTX alone. (Reproduced with permission from Breedveld et al. 2006.)

from baseline in mean TSS at one year was significantly lower for adalimumab plus MTX (1.3, $P < 0.001$) compared with MTX alone (5.7) and adalimumab alone (3.0). The differences in progression for patients treated with adalimumab versus MTX alone were even more marked at the end of Year 2, with an accumulated TSS change of 10.4 in the MTX monotherapy arm versus 5.5 in the adalimumab monotherapy arm and 1.9 in the combination arm. Although ACR responses were comparable between the two monotherapy arms, there was statistically less progression in the adalimumab monotherapy arm compared with the MTX monotherapy arm in both Year 1 (3.0 versus 5.7), and Year 2 (5.5 versus 10.4) ($P < 0.001$ for all comparisons; Fig. 1.4B). These findings emphasize that clinical assessment of signs and symptoms of RA may not fully reflect therapeutic benefit (Breedveld et al. 2006).

Interestingly, during Year 2, patients who were treated with MTX monotherapy continued to progress at approximately the same rate seen in Year 1 (5.7 Sharp unit progression in Year 1 and 4.7 Sharp unit progression in Year 2). In contrast, patients who received combination therapy had less than half the progression in Year 2 than they had experienced in Year 1 (1.3 Sharp unit progression in Year 1 and 0.6 Sharp unit progression in Year 2). Moreover, after 2 years, remission as measured by DAS28 <2.6 and major clinical response (continuous ACR70 response for ≥6 months) was achieved by almost half (49%) of the patients receiving combination therapy.

The results of PREMIER provide further evidence that early aggressive combination therapy of progressive RA with MTX and adalimumab can reduce disease progression to a greater extent than treatment of disease with MTX monotherapy. The benefits of early treatment with combination therapy are sustained for at least 2 years, and are associated with improved clinical outcomes.

1.6.1.5 **Adalimumab in Patients Previously Treated with other Anti-TNF Agents**

The ReAct data were analyzed based on use of prior anti-TNF therapy. Of the 6610 enrolled patients, 899 had received prior anti-TNF therapy with etanercept and/or infliximab (median prior treatment of 9.5 months). Despite higher disease activity and use of a greater number of prior DMARDs at baseline, patients with a history of prior anti-TNF therapy demonstrated robust responses with adalimumab. At Week 12, ACR20, 50, and 70 response rates were 60%, 33%, and 13%, respectively, among patients who had received prior anti-TNF therapy, as compared with 70%, 41%, and 19%, respectively, among patients who were naïve to anti-TNF therapy. Response to adalimumab among patients who had discontinued prior anti-TNF therapy due to loss of efficacy or intolerance was similar to that of patients who had never received anti-TNF agents, with 67% achieving ACR20 responses (Bombardieri et al. 2005, 2006; Burmester et al. 2005a).

The results from two additional studies of the use of adalimumab in RA patients who had failed infliximab therapy were consistent with those from ReAct, as described above (Nikas et al. 2006; van der Bijl et al. 2005). In the first study, adalimumab was administered at a dose of 40 mg EOW added to current therapy

in 41 patients with long-standing moderate to severe RA who had discontinued infliximab therapy because of lack of efficacy, loss of efficacy after an initial response, and/or intolerance/adverse side effects (mean infliximab treatment duration, 17 months). After 16 weeks of adalimumab therapy, significant percentages of patients achieved ACR20, ACR50, and moderate EULAR responses (49%, 31%, 65%, respectively). Clinically meaningful decreases in tender and swollen joint counts were apparent by Week 2 of adalimumab therapy. Additionally, patients who switched to adalimumab demonstrated significant reductions in disease activity and disability, as assessed by changes in DAS28 and HAQ scores (Data on file; van der Bijl et al. 2005). In the second study, 24 patients with RA who discontinued therapy with infliximab (mean infliximab treatment duration, 18.5 months) were treated with adalimumab 40 mg EOW for 12 months. The results from these patients were compared with 25 patients receiving adalimumab who had not previously used anti-TNF therapy (controls). After 12 months of treatment, 75% of patients previously receiving infliximab and 76% of control patients achieved ACR20. Mean changes in DAS28 were similar for the two groups (–2.4 versus –2.7). A total of 71% of patients in the infliximab failure group and 72% of control patients achieved a EULAR response (Nikas et al. 2006). In both of these studies, adalimumab was well tolerated, even among patients with previous intolerance to infliximab.

Collectively these results suggest that, even after treatment failure with a TNF antagonist, a significant clinical response can be achieved with a different agent of the same class.

1.6.2 Pivotal Studies in Psoriatic Arthritis

ADEPT (Adalimumab Effectiveness in Psoriatic Arthritis Trial) evaluated adalimumab treatment in patients with moderate to severe PsA and a history of inadequate response to NSAIDs (Mease et al. 2005a–c). In this double-blind, randomized, parallel-group, multicenter, 24-week trial, 315 patients with PsA who had three or more swollen and three or more tender joints and either active psoriatic skin lesions or a documented history of psoriasis were randomized to receive adalimumab 40 mg SC EOW, or placebo (Mease et al. 2005a). Participants were stratified prior to randomization by MTX use (yes or no) and extent of psoriasis (<3% or ≥3%).

At Week 24, the ACR20 response rate was 57% with active treatment, which was significantly higher than with placebo (15%, $P < 0.001$) (Fig. 1.5A) (Mease et al. 2005a). Significantly more patients achieved ACR50 and 70 responses with adalimumab than with placebo. Among the 69 patients in each treatment group who were assessed using the Psoriasis Area and Severity Index (PASI), 59% of those in the adalimumab group achieved a 75% improvement in PASI compared with 1% of those in the placebo group (Fig. 1.5B). Some 42% of patients receiving adalimumab achieved the very marked response of 90% improvement in PASI;

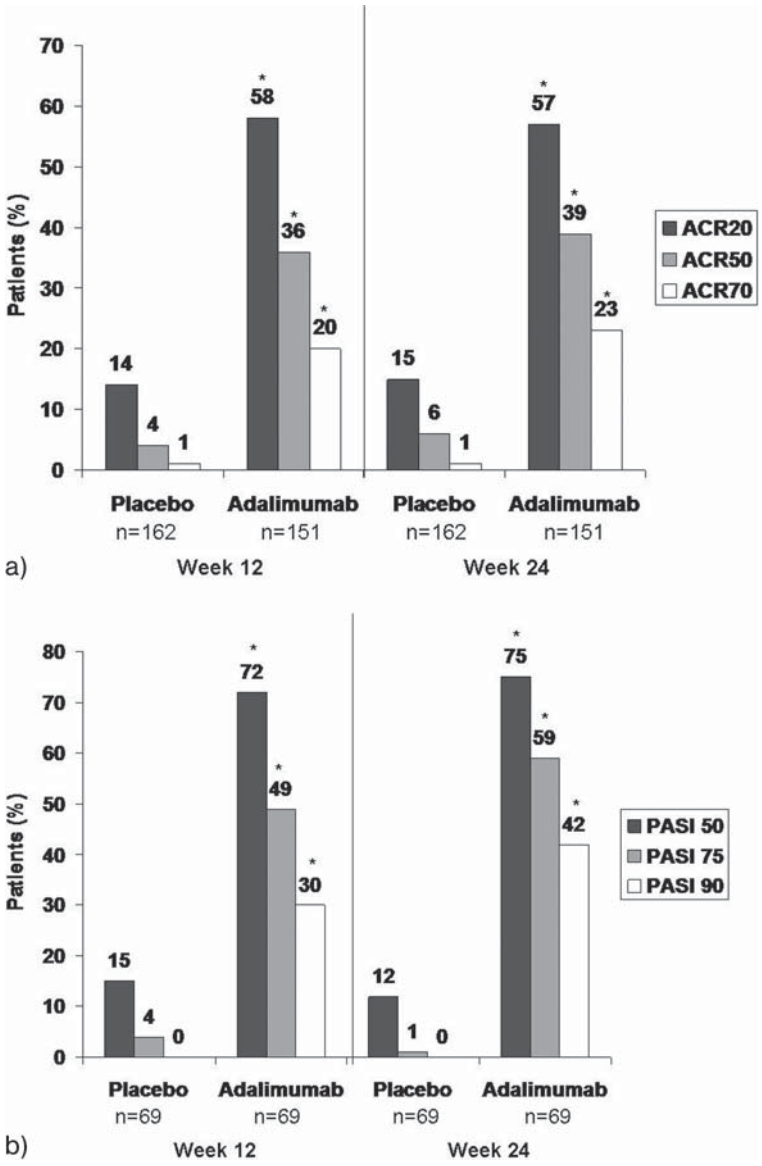


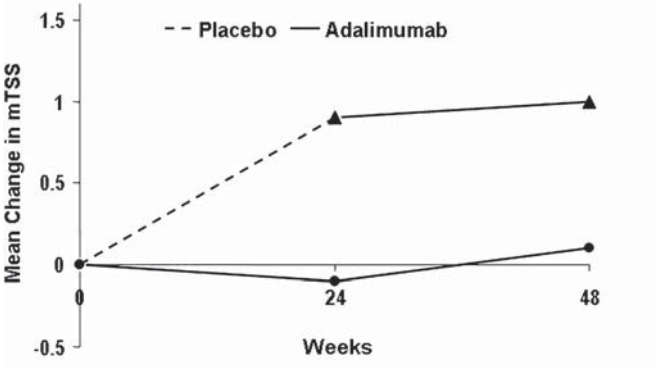
Fig. 1.5 Results from ADEPT (Adalimumab Effectiveness in Psoriatic Arthritis Trial). (a) Percentage of patients with psoriatic arthritis who met American College of Rheumatology (ACR) 20, 50, and 70 response criteria at Week 12 and Week 24. *$P < 0.001$ placebo versus adalimumab, adjusted for baseline methotrexate use and extent of psoriasis at baseline. (b) Percentage of patients with psoriatic arthritis and ≥3% body surface area psoriasis involvement at baseline who met Psoriasis Area and Severity Index (PASI) 50, 75, and 90 response criteria at Week 12 and Week 24. *$P < 0.001$ placebo versus adalimumab, adjusted for baseline methotrexate use. (Reproduced with permission from Mease et al. 2005.)

this level of response was reached by 0% of patients receiving placebo ($P < 0.001$). In addition, 67% of patients receiving active treatment were assessed as "clear" or "almost clear" using the Physician Global Assessment (PGA), compared with 10% of those in the placebo group.

Progression of radiographic damage in these PsA patients was inhibited with adalimumab treatment, yielding a mean change from baseline of the modified TSS equal to −0.2 at 24 weeks (Fig. 1.6) (Mease et al. 2005a). In the placebo group, the mean change in TSS was +1.0 at 24 weeks.

As would be expected, these clinical benefits were associated with improved levels of disability and quality-of-life responses (Mease et al. 2005a). The mean change from baseline of the HAQ-DI was −0.4 in the adalimumab group compared with −0.1 in the placebo group ($P < 0.001$). Physical function, as measured by the Functional Assessment of Chronic Illness Therapy, and quality of life, assessed with the Dermatology Life Quality Index, were similarly improved with active treatment (Mease et al. 2005a).

At the end of the 24-week blinded phase of ADEPT, patients were given the option of continuing in an OLE phase, during which all 285 patients who elected to continue received adalimumab 40 mg SC EOW (Mease et al. 2005b). Among the patients who had initially been randomized to the adalimumab group, the 24-week improvements in ACR, PASI, and HAQ scores were maintained at Week



	N	Baseline	24 Wk Mean Change	48 Wk Mean Change
Placebo	152	21.8	0.9	1.0
Adalimumab	144	23.7	-0.1*	0.1

Fig. 1.6 Results from ADEPT (Adalimumab Effectiveness in Psoriatic Arthritis Trial). Changes in modified total Sharp score (TSS) at Weeks 24 and 48. *$P < 0.001$ placebo versus adalimumab using a ranked analysis of covariance. (From Mease et al. 2005a,c.)

48, as was inhibition of radiographic progression (mean change from baseline in TSS of 0.1 at Week 48) (Mease et al. 2005b,c). For those who had previously been randomized to the placebo group, ACR20, 50, and 70 were achieved at Week 48 of the OLE phase (24 weeks of active treatment) by 54%, 37%, and 21% of patients, respectively. In this group at 48 weeks, PASI50, 75, and 90 responses were attained by 76%, 63%, and 47% of patients respectively, similar to the responses after 24 weeks for patients originally randomized to adalimumab in the blinded phase of the study. Radiographic progression was decreased markedly compared with the 24 weeks on placebo, and the mean change in modified TSS was 0.1 during the first 24 weeks of active treatment with adalimumab (Fig. 1.6). Disability also improved among these patients, with a mean change in the HAQ-DI of −0.4 from baseline (Mease et al. 2005b,c). For patients who failed to demonstrate ≥20% decrease in swollen and total joint count response versus baseline after 12 weeks of treatment in the OLE, an option was given to increase the dose to 40 mg weekly. A total of 12 patients underwent a dose increase, with an ACR20 response resulting in three cases.

1.6.3 Ankylosing Spondylitis

ATLAS (Adalimumab Trial Evaluating Long-term Efficacy and Safety in Ankylosing Spondylitis) was a randomized, double-blind, placebo-controlled, multicenter study comparing adalimumab with placebo in patients with AS. A total of 315 patients was randomized in a 2:1 fashion to receive adalimumab 40 mg EOW versus placebo for 24 weeks. At Week 12, 58% of adalimumab patients versus 21% of placebo patients achieved a 20% improvement in the ASsessment in Ankylosing Spondylitis (ASAS20) International Working Group Improvement Criteria, a difference that was statistically significant (differences of 37.6% [95% CI, 27.4–47.8%], $P < 0.001$). A favorable clinical response was observed as early as 2 weeks after initiation of active treatment (van der Heijde et al. 2006).

Also at Week 12, 45.2% of adalimumab-treated patients achieved at least a 50% improvement in the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) compared with only 15.9% (17/107) of the placebo group (difference of 29.3% [95% CI, 19.6–39.0%], $P < 0.001$) This significant difference was sustained at Week 24 (van der Heijde et al. 2006).

The percentage of patients with at least a 20% improvement in five of the six ASAS assessment domains was significantly greater with adalimumab treatment (44.7%) at Week 24 compared with only 12.1% of the placebo patients (difference of 32.6% [95% CI, 23.4–41.7%], $P < 0.001$). Similar significantly better outcomes were observed with adalimumab for ASAS40 responders and percentage of patients achieving partial remission. These differences were seen at Week 12 and remained significant at Week 24 (Fig. 1.7) (van der Heijde et al. 2006).

The responses of patients with total spinal ankylosis (n = 11) were similar to those without total ankylosis (US Humira PI 2006).

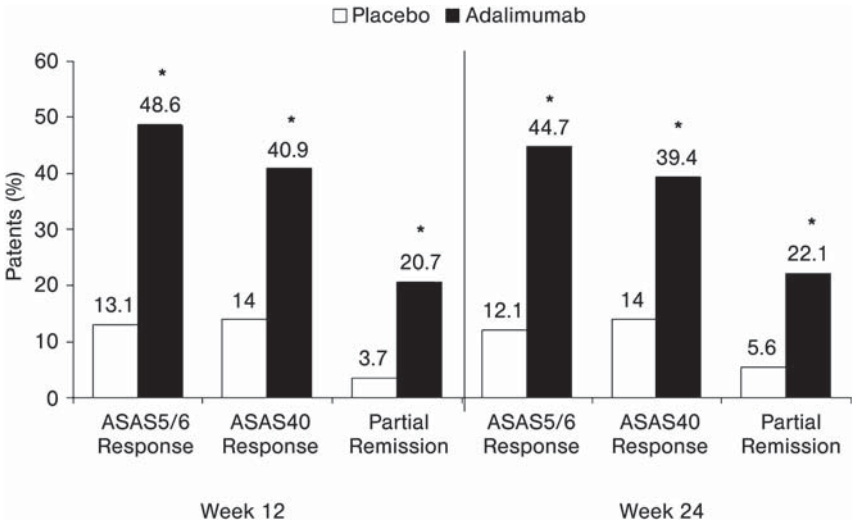


Fig. 1.7 Results from ATLAS (Adalimumab Trial Evaluating Long-term Efficacy and Safety in Ankylosing Spondylitis). ASsessment in Ankylosing Spondylitis (ASAS) 5/6 criteria response, ASAS40 response, and partial remissions. *$P < 0.001$ versus placebo. Values are imputed. (From van der Heijde et al. 2006.)

1.7 Review of Adalimumab Safety

In clinical trials, adalimumab has been generally well tolerated, with less than 10% of patients in clinical trials discontinuing treatment because of adverse events (Lee and Kavanaugh 2005; Furst et al. 2003; Keystone et al. 2004a). Moreover, data from a national registry demonstrate that adalimumab has a high adherence rate (82%) at one-year follow-up, comparable to that for other TNF inhibitors (Kristensen et al. 2004). Primary among the potential safety concerns for inhibitors of TNF are the risk of serious infections, including tuberculosis (TB) and opportunistic infections (Lee and Kavanaugh 2005; Schiff et al. 2006), autoimmune disease (Lee and Kavanaugh 2005), demyelination disorders (Magnano et al. 2004), and malignancies, particularly lymphoma (Lee and Kavanaugh 2005). Assessment of safety with long-term adalimumab suggests that the incidence of these serious adverse events with adalimumab treatment is low.

1.7.1 Safety in Specific Indications

1.7.1.1 Long-Term Safety in Rheumatoid Arthritis

Safety data are available for more than 10 000 patients with moderate to severe RA who have been evaluated in clinical trials; 300 of these patients have had at

least 5 years' exposure to adalimumab, with some followed for up to 7 years. These patients represent a total of 12 506 PY of adalimumab exposure (Schiff et al. 2006). In addition, adalimumab exposure during the US postmarketing period is estimated to be more than 78 500 PY (Schiff et al. 2006). The types of adverse event reported following adalimumab use in RA have been similar throughout the clinical development program and postmarketing surveillance (Schiff et al. 2005a).

Adverse events reported by ≥5% of patients treated with adalimumab during placebo-controlled phases of RA studies are listed in Table 1.3. Injection site reactions (ISR) are the most common adverse events associated with adalimumab, with an incidence of 20% versus 14% for placebo reported in four pivotal trials

Table 1.3 Adverse events reported by ≥5% of patients treated with adalimumab during placebo-controlled periods of rheumatoid arthritis studies (US Humira PI 2006).

Adverse event	*Adalimumab 40 mg EOW [n = 705] (%)*	*Placebo [n = 690] (%)*
Respiratory		
• Upper respiratory tract infection	17	13
• Sinusitis	11	9
• Flu syndrome	7	6
Gastrointestinal		
• Nausea	9	8
• Abdominal pain	7	4
Laboratory tests*		
• Laboratory test abnormal	8	7
• Hypercholesterolemia	6	4
• Hyperlipidemia	7	5
• Hematuria	5	4
• Increased alkaline phosphatase	5	3
Other		
• Injection site pain	12	12
• Headache	12	8
• Rash	12	6
• Accidental injury	10	8
• Injection site reaction†	8	1
• Back pain	6	4
• Urinary tract infection	8	5
• Hypertension	5	3

* Laboratory test abnormalities were reported as adverse events in European trials.
† Does not include erythema and/or itching, hemorrhage, pain, or swelling.
EOW = every other week.

for RA (ARMADA, DE011, DE019, and STAR) (US Humira PI 2006; EU Humira SPC 2006). Most ISRs were mild, were not recurrent, and rarely led to discontinuation from studies (US Humira PI 2006; Wells et al. 2003).

1.7.1.2 Safety Profile in Psoriatic Arthritis and Ankylosing Spondylitis

Although safety evaluations of adalimumab in patients with PsA and AS are less extensive than those for patients with RA, in clinical trials up to one year duration adalimumab has been generally well tolerated. Adverse event profiles are similar to those in RA, and no new safety concerns have arisen (Mease et al. 2005a,b; van der Heijde et al. 2006).

1.7.2 Immune System Function

An immunology substudy of DE019 determined that normal immune function is preserved during adalimumab therapy (Kavanaugh et al. 2002). Adalimumab did not significantly alter the total number of peripheral granulocytes, natural killer cells, monocytes/macrophages, B cells, T cells, or T-cell subsets. The ability of lymphocytes to proliferate, as evidenced by *in-vitro* proliferation to both mitogen and recall antigen, was not altered. There were no clinically relevant differences in delayed-type hypersensitivity reactivity, or in B-cell function, as assessed by total immunoglobulin levels, IgG levels, and antigen-specific antibody response to recall antigen. Phagocytic functions measured in oxidative burst assays and by phagocytosis of fluorescent particles were similar for neutrophils and macrophages from adalimumab- and placebo-treated patients (Kavanaugh et al. 2003).

1.7.3 Infections

In placebo-controlled RA trials, infections rates were approximately one per PY for both adalimumab- and placebo-treated groups. The most frequently reported infectious adverse events among RA trials were upper respiratory tract infections, rhinitis, bronchitis, and urinary tract infections (US Humira PI 2006; EU Humira SPC 2006). Rates of serious infections (5.1/100 PY) did not increase over the duration of treatment, were similar to those found with traditional DMARDs and other TNF antagonists, and were within the range reported among the general RA population (range ~3 to ~9/100 PY) (Schiff et al. 2003a, 2006). In controlled studies, the rate of serious infections among adalimumab-treated patients was not significantly different from placebo in either significance or type; the results of OLEs were similar to those of randomized trials (Lee and Kavanaugh 2005; Schiff et al. 2003a). In the ReAct study of 6610 patients with long-standing RA, the rate of serious infections was 5.5/100 PY, consistent with other data (G.R. Burmester et al., unpublished results).

1.7.3.1 Tuberculosis

Cases of TB have been seen with all three anti-TNF agents. During the initial phase of the development program, no TB screening was performed, but screening prior to treatment initiation was introduced during the phase II studies. Following the introduction of routine screening in European clinical trials, the rate of TB decreased by 75% (0.33/100 PY). In North American clinical RA trials, four cases had been reported after 4914 PY of exposures (0.08/100 PY) (Schiff et al. 2006). In the postmarketing period from December 21, 2002, through June 30, 2005, 17 cases of TB (0.02/100 PY) had been reported in the United States, of which five had extrapulmonary involvement. These data are consistent with that of other TNF inhibitors (Lee and Kavanaugh 2005). However, because of the small risk of reactivation of latent TB, a detailed medical history should be taken, and screening tests (i.e., tuberculin skin test, chest radiography) should be performed in all patients prior to initiating adalimumab therapy in order to rule out active and inactive (latent) TB infection. European labeling includes a contraindication for adalimumab in patients with active TB. Appropriate anti-TB prophylaxis should be initiated for patients with latent TB prior to beginning adalimumab therapy. Moreover, the risks and benefits of adalimumab in these patients should be considered carefully prior to initiating therapy (EU Humira SPC 2006).

1.7.3.2 Opportunistic Infections

Opportunistic infections have been seen with the use of all TNF antagonists. These infections are seen very rarely, and causative agents vary. There have been four cases of histoplasmosis reported in RA trials, all in areas where the condition is endemic (Schiff et al. 2006). Patients should be monitored closely for infections before, during, and after treatment with adalimumab (EU Humira SPC 2006).

1.7.4 Other Conditions

1.7.4.1 Lymphoma

The incidence of lymphoma in the almost 2500 adalimumab-treated patients in the clinical development program for RA was consistent with the rates of lymphoma among patients with moderate to severe RA (Schiff et al. 2003b). After 12 506 PY of adalimumab exposure, 15 lymphomas were seen (0.12/100 PY) The standardized incidence ratio (SIR) for lymphomas in adalimumab clinical trials in comparison with that of the normal RA population in the Surveillance, Epidemiology, and End Results database was 3.19, which is consistent with SIRs reported for RA populations naïve to anti-TNF therapy (Schiff et al. 2006).

1.7.4.2 Demyelinating Conditions

Six cases of multiple sclerosis (MS), two of nonspecific demyelination, and two of Guillain–Barré syndrome have been reported after 12 506 PY of exposure to adalimumab (Schiff et al. 2005a). Patients with MS have a statistically signifi-

cantly higher coexistence of RA, psoriasis, and goiter than matched controls, suggesting that patients with these conditions may innately be at increased risk of MS as compared with the general population (Heinzlef et al. 2000; Magnano et al. 2004). The true impact of TNF inhibitors on the development of this disorder is unknown, however (Lee and Kavanaugh 2005; Magnano et al. 2004).

1.7.4.3 Autoantibodies and Autoimmune Diseases

Between 3% and 12% of patients treated with adalimumab develop autoantibodies to antinuclear antigen and double-stranded DNA (Lee and Kavanaugh 2005). The mechanism by which autoantibodies are generated – and the clinical implications of these antibodies – remain to be defined, because progression to lupus-like illness appears to be uncommon (Lee and Kavanaugh 2005). After 12 506 PY of adalimumab exposure, 13 cases of systemic lupus erythematosus (SLE) and lupus-like syndromes have been reported. However, none of these cases had significant internal organ involvement (Schiff et al. 2006).

1.8 Special Conditions and Populations

1.8.1 Pregnancy and Lactation

The use of adalimumab in pregnant women has not been evaluated. Although animal data have shown no indication of maternal toxicity, embryotoxicity, or teratogenicity, adalimumab should be used during pregnancy only if clearly needed. European labeling recommends that adequate contraception be used for at least 5 months after the last adalimumab treatment (EU Humira SPC 2006).

Whether or not adalimumab is secreted in breast milk or absorbed systemically after ingestion has not been determined. However, because human immunoglobulins are secreted in breast milk, women should not breastfeed for at least 5 months after the last adalimumab treatment (EU Humira SPC 2006).

1.8.2 Elderly

No dose adjustment is required for adalimumab treatment in individuals aged 65 years or more (EU Humira SPC 2006).

1.8.3 Children and Adolescents

Adalimumab is currently not indicated for use in children (US Humira PI 2006; EU Humira SPC 2006). Investigations in juvenile rheumatoid arthritis (JRA) are

currently underway (Lovell et al. 2004); the preliminary results of a study evaluating the use of adalimumab in JRA are presented in Section 1.10.4.

1.8.4
Impaired Renal and/or Hepatic Function

At present, no dosing recommendations are available for patients with impaired renal or hepatic function (EU Humira SPC 2006).

1.8.5
Congestive Heart Failure

In several clinical trials of patients with congestive heart failure (CHF), TNF inhibitors (infliximab, etanercept) failed to benefit patents with this condition (Lee and Kavanaugh 2005). Clinical studies of adalimumab in treating CHF have not been conducted. In some studies in other indications, however, TNF inhibitors (including adalimumab) have been associated with worsening and new-onset CHF (EU Humira SPC 2006). During adalimumab trials, 44 patients reported a medical history of CHF, and three (7%) of these reported CHF events during the trials. Thirty-two cases of CHF were observed among 10 006 (0.3%) patients who did not report any medical history of CHF. Over the 2.5-year period when the rate of CHF was evaluated, the rate at which events occurred appears to have remained stable (Schiff et al. 2006).

A causal association between TNF inhibitors and CHF has not been proven. Nonetheless, because of the potential risk found with other TNF inhibitors, adalimumab is contraindicated in patients with severe heart failure (New York Heart Association class III/IV). Adalimumab should be discontinued in patients who develop new or worsening symptoms of CHF (EU Humira SPC 2006).

1.9
Storage and Administration

Adalimumab must be refrigerated at between 2°C and 8°C (36–46°F), but the formulation should not be frozen. Prefilled syringes should be protected from light (US Humira PI 2006).

Adalimumab is administered via SC injection. Patients may self-inject adalimumab if their physician determines that it is appropriate and with medical follow-up, as necessary, after proper training in injection technique. In order to reduce the incidence of ISRs, the injection sites should be rotated. Injections should not be given in areas of the skin that are tender, bruised, red, or hard (US Humira PI 2006).

1.10 Outlook and New Indications

Adalimumab has been established as a safe and effective treatment for RA, PsA, and AS, and it is currently being evaluated for use in several related indications, including psoriasis (phase III), Crohn's disease (phase III), and JRA (phase III) (Hanauer et al. 2006; Langley et al. 2005; Lovell et al. 2004; Sandborn et al. 2005a).

1.10.1 Psoriasis

In a randomized, double-blind, placebo-controlled, multicenter study to evaluate the efficacy and safety of adalimumab in patients with moderate to severe plaque psoriasis, two adalimumab dosing regimens were evaluated (Langley et al. 2005). A total of 148 patients received either adalimumab 80 mg at Week 0 followed by 40 mg EOW beginning at Week 1 (Group A), adalimumab 80 mg at Weeks 0 and 1 followed by 40 mg weekly beginning at Week 2 (Group B), or placebo for 12 weeks. At Week 12, 53%, 80%, and 4% of patients in Groups A, B, and placebo, respectively, achieved PASI 75. The mean percentage improvements in PASI scores over time are illustrated in Fig. 1.8. The percentage of patients determined by the PGA to be clear or almost clear at Week 12 were 49% and 76% in the two adalimumab treatment groups, compared with 2% with placebo. Adalimumab also produced clinically important and statistically significant improvements in the quality of life of patients with psoriasis, based on Dermatology Life Quality Index, SF-36, and EuroQOL 5D scores (Wallace et al. 2005a–d). Adalimumab was safe and well tolerated, with no significant differences in the incidence of adverse events across the three groups (Langley et al. 2005).

Following the 12 weeks of blinded, placebo-controlled treatment, patients were allowed to continue in an OLE phase. Those who had received placebo during the blinded phase, received adalimumab 80 mg followed by 40 mg EOW beginning at Week 12. Treatment response was sustained throughout the OLE phase for those who had been treated with adalimumab during the blinded phase. After 24 weeks of continuous adalimumab treatment, 67% and 77% of patients in Groups A and B, respectively, achieved PASI 75. Among the patients who converted from placebo to adalimumab at the end of Week 12, 55% achieved PASI 75 at Week 24. Some 69% and 84% of patients in Groups A and B, respectively, and 46% of the placebo/adalimumab group, attained PGA ratings of clear or almost clear at Week 24 (Langley et al. 2005).

1.10.2 Crohn's Disease

The safety and efficacy of adalimumab in patients with Crohn's disease has been evaluated in two randomized, double-blind, placebo-controlled, multicenter trials.

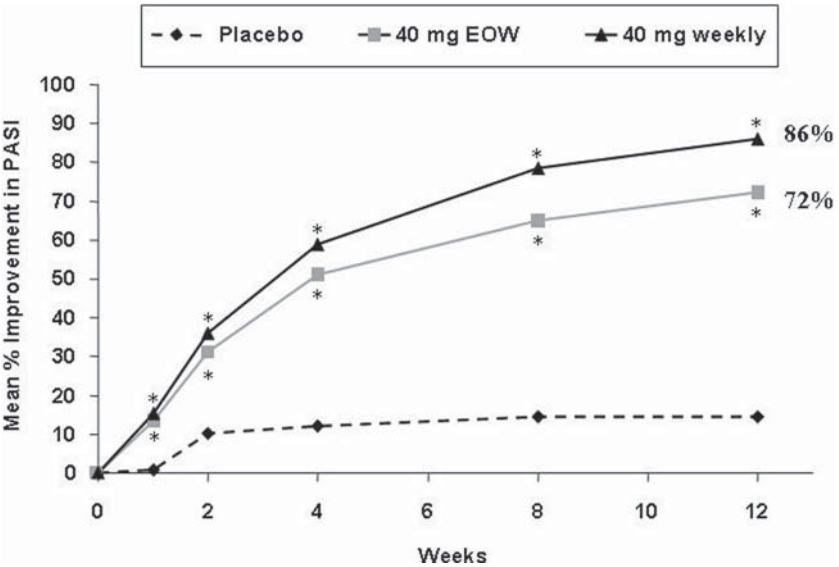


Fig. 1.8 Results from the study evaluating adalimumab in the treatment of moderate to severe chronic plaque psoriasis. Mean percentage improvement in Psoriasis Area and Severity Index (PASI) scores through 12 weeks of treatment using a modified intent-to-treat population. *$P < 0.001$ versus placebo. EOW = every other week. (From Langley et al. 2005.)

CLASSIC-I (Clinical assessment of Adalimumab Safety and efficacy Studied as Induction therapy in Crohn's disease) was a dose-ranging induction trial involving 299 patients with moderate to severe Crohn's disease naïve to anti-TNF therapy (Hanauer et al. 2006). Patients were randomized to one of four induction treatment groups: adalimumab 40 mg at Week 0/20 mg at Week 2 (Group A); 80 mg at Week 0/40 mg at Week 2 (Group B); 160 mg at Week 0/80 mg at Week 2 (Group C); or placebo at Weeks 0 and 2. Patients were followed through Week 4 (Hanauer et al. 2006).

Serum adalimumab concentrations increased proportionately with increasing dose. Adalimumab concentrations were similar at Weeks 2 and 4, indicating that the loading doses achieved stable serum levels by Week 2. Serum adalimumab concentrations achieved with the 160 mg/80 mg and 80 mg/40 mg doses were comparable to those achieved with 40 mg weekly and 40 mg EOW, respectively (Hanauer et al. 2006).

Rates of clinical remission (Crohn's disease activity index [CDAI] <150), a CDAI decrease from baseline of ≥70, and a CDAI decrease from baseline of ≥100 at Week 4 are shown in Fig. 1.9. The optimal induction dosing regimen in this study was adalimumab 160 mg at Week 0 followed by 80 mg at Week 2. The percentages of patients achieving remission at Week 4, the primary efficacy endpoint, in the adalimumab Groups A, B, C, and placebo were 18%, 24%, 36%, and 12%,

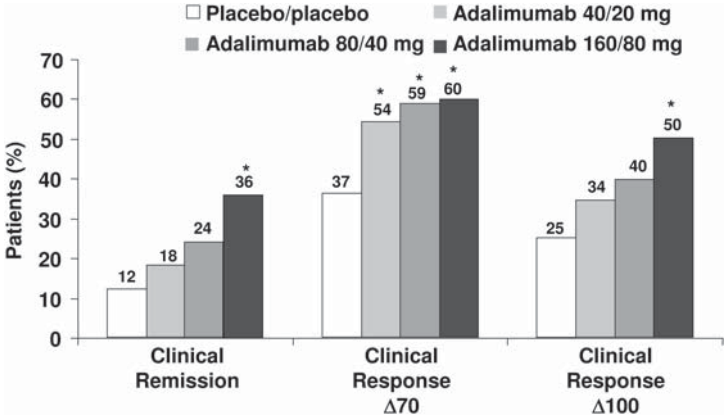


Fig. 1.9 Results from CLASSIC-I (CLinical assessment of Adalimumab Safety and efficacy Studied as Induction therapy in Crohn's disease). Clinical remission and response among adalimumab and placebo-treated patients at Week 4. *$P < 0.05$ versus placebo. Clinical remission = Crohn's disease activity index (CDAI) <150. Clinical response Δ70/Δ100 = CDAI decrease from baseline ≥70 or ≥100. (From Hanauer et al. 2006.)

respectively. Percentages of patients achieving a CDAI decrease from baseline of ≥70 were 54% for Group A, 59% for Group B, 60% for Group C, and 37% for placebo. For a CDAI decrease from baseline of ≥100, the percentages were 34%, 40%, 50%, and 25%, for the respective treatment groups (Hanauer et al. 2006).

Adverse events occurred at similar frequencies in all four treatment groups, except that ISRs were more common among patients treated with adalimumab (26%, 24%, and 38% for Groups A, B, and C, respectively, versus 16% for the placebo group) (Hanauer et al. 2006).

Patients who completed the CLASSIC-I study were eligible to enroll in CLASSIC-II, a two-arm study (open-label and randomized arms) to evaluate adalimumab in the maintenance of clinical remission in subjects with Crohn's disease (Sandborn et al. 2005a). Fifty-five patients who were in remission at Weeks 0 and 4 were randomized to receive adalimumab 40 mg EOW, 40 mg weekly, or placebo for up to one year. The remaining subjects not in remission at both Weeks 0 and 4 received open-label adalimumab 40 mg EOW. The adalimumab dose could be increased to 40 mg weekly for flare or persistent nonresponse. In the open-label arm, the long-term efficacy and safety of adalimumab 40 mg EOW therapy was assessed in 220 patients with moderate to severe Crohn's disease. At 6 months, 71% remained on adalimumab 40 mg EOW (45%, n = 98) or weekly (26%, n = 58). Clinical remission was achieved in 33% of patients. The percentages of patients attaining CDAI decreases of 70 and 100 were 78% and 70%, respectively. Adverse events were mild to moderate in severity, and similar to those observed in previous studies of patients with RA (Sandborn et al. 2005a). Data available after one year supported the maintenance of remission, with clinical remission rates of 43% and clinical (CDAI) response changes of 70 and 100 in 69% and 61%

of patients, respectively (Sandborn et al. 2005b). Among the 55 patients in the randomized arm of the study, 83% of those receiving adalimumab 40 mg weekly, 68% receiving 40 mg EOW, and 39% receiving placebo maintained remission at one year (Sandborn et al. 2005c).

Similar to the data in RA regarding switching from a previous anti-TNF agent to adalimumab, preliminary evidence in patients with Crohn's disease also suggests that adalimumab is clinically beneficial in patients who have lost response or who are intolerant to infliximab (Barthel et al. 2005; Papadakis et al. 2005; Sandborn et al. 2004; Stallmach et al. 2004; Youdim et al. 2004). In one study of seven patients with Crohn's disease who had allergic reactions to infliximab, patients who had active disease and who had a previous response to infliximab (n = 6) also responded to adalimumab, as evidenced by improvement in the Harvey–Bradshaw index (HBI) and inflammatory markers (Youdim et al. 2004). In a second study, 15 patients with Crohn's disease who experienced an attenuated response to infliximab were treated with an adalimumab 80 mg loading dose following by 40 mg EOW for 6 months. Of the 13 patients who completed the study, seven had a complete response (HBI of ≤4 and withdrawal of corticosteroids), four had a partial response (decrease of ≥50% in HBI and reduction in corticosteroid dose), and two were nonresponders. In a third study of patients with Crohn's disease who had lost responsiveness or who had developed intolerance to infliximab and were switched to adalimumab, clinical remission (CDAI ≤150) was achieved by five of 17 patients, and a CDAI decrease of ≥70 was achieved by 10 of 17 patients at 12 weeks. In all of these studies, adalimumab was generally well tolerated, without producing signs or symptoms of allergic reactions (Papadakis et al. 2005; Sandborn et al. 2004; Youdim et al. 2004). These studies, although small in terms of patient numbers, suggest that subjects with Crohn's disease who have lost their response to, or are intolerant of, infliximab may benefit from switching to adalimumab.

1.10.3 Juvenile Rheumatoid Arthritis

Preliminary results from the initial open-label portion of a phase III, randomized, double-blind, placebo-controlled trial in children aged 4 to 17 years with JRA suggest that adalimumab provides rapid and substantial responses in these patients. Patients received 24 mg m^{-2} of adalimumab EOW, and concomitant MTX therapy was allowed. A total of 155 patients (81 receiving adalimumab plus MTX; 74 receiving adalimumab monotherapy) completed the 16-week open-label period. After 16 weeks of therapy, 95% of patients receiving adalimumab plus MTX achieved a pediatric ACR30, as did 88% of those receiving adalimumab monotherapy. ACR50 and ACR70 responses were achieved by 94% and 82%, respectively, of patients receiving the combination, and by 80% and 59%, respectively, of patients receiving adalimumab monotherapy. Adalimumab alone or in combination with MTX was generally safe and well tolerated during the 16-week interim study period (Lovell et al. 2004).

1.11 Conclusions

Despite its protective role in the immune response, TNF has been implicated in a number of chronic inflammatory disease states. Treatment with the fully human anti-TNF antibody, adalimumab, offers improvements in disease symptoms, as well as improvement in quality of life, functional status, and – most importantly – limits disease progression in a number of disease states. Although currently this agent is indicated for the treatment of RA, PsA, and AS, the safety and efficacy of adalimumab for other diseases, including plaque psoriasis, Crohn's disease and JRA, show great promise.

References

Adalimumab [PI; prescribing information] (2006) North Chicago, IL: Abbott Laboratories.

Adalimumab [SPC; summary of product characteristics] (2006) Queenborough, UK: Abbott Laboratories. Available at: http://www.emea.eu.int/humandocs/Humans/EPAR/humira/humira.htm. Accessed September 12, 2006.

Anderson, P.J. (2005) Tumor necrosis factor inhibitors: clinical implications of their different immunogenicity profiles. *Semin Arthritis Rheum* 34 (5 Suppl.1): 19–22.

Baert, F., Noman, M., Vermeire, S., et al. (2003) Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *N Engl J Med* 348: 601–608.

Bansback, N., Brennan, A., and Anis, A.H. (2005) A pharmacoeconomic review of adalimumab in the treatment of rheumatoid arthritis. *Expert Rev Pharmacoeconomics Outcomes Res* 5: 519–529.

Barrera, P., Joosten, L.A., den Broeder, A.A., et al. (2001) Effects of treatment with a fully human anti-tumour necrosis factor α monoclonal antibody on the local and systemic homeostasis of interleukin 1 and TNFα in patients with rheumatoid arthritis. *Ann Rheum Dis* 60: 660–669.

Barthel, H.R., Gille, T., Halbsguth, A., Kramer, M. (2005) Successful treatment with adalimumab in infliximab-resistant Crohn's disease. *J Gastroenterol Hepatol* 20: 1464–1465.

Bombardieri, S., Moutsopoulos, H.M., McKenna, F., Michel, B., Webber, D.G., Kupper, H. (2004) Rapid response to adalimumab after first dose: the ReAct trial [abstract]. *Ann Rheum Dis* 63(Suppl.1): 261.

Bombardieri, S., Tzioufas, A.G., McKenna, F., et al. (2005) Adalimumab (HUMIRA) is effective in treating patients with rheumatoid arthritis who previously failed etanercept and/or infliximab in real-life clinical settings. *Arthritis Rheum* 52(Suppl.): S144.

Bombardieri, S., McKenna, F., Drosos, A., et al. (2006) Efficacy and safety of adalimumab (HUMIRA) in 899 patients with rheumatoid arthritis (RA) who previously failed etanercept and/or infliximab in clinical practice [abstract]. *Ann Rheum Dis* 65(Suppl. II): 178.

Braegger, C.P., Nicholls, S., Murch, S.H., Stephens, S., MacDonald, T.T. (1992) Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet* 339: 89–91.

Braun, J., Bollow, M., Neure, L., et al. (1995) Use of immunohistologic and in situ hybridization techniques in the examination of sacroiliac joint biopsy specimens from patients with ankylosing spondylitis. *Arthritis Rheum* 38: 499–505.

Breedveld, F.C., Weisman, M.H., Kavanaugh, A.F., et al. (2005) The efficacy and safety of adalimumab (Humira) plus methotrexate vs. adalimumab alone or methotrexate alone in the early treatment

of rheumatoid arthritis: 1- and 2-year results of the PREMIER study [abstract]. *Ann Rheum Dis* 64(Suppl.III): 60.

Breedveld, F.C., Weisman, M.H., Kavanaugh, A.F., et al. for the PREMIER investigators (2006) The PREMIER study: a multicenter, randomized, double-blind clinical trial of combination therapy with adalimumab plus methotrexate versus methotrexate alone or adalimumab alone in patients with early, aggressive rheumatoid arthritis who had not had previous methotrexate treatment. *Arthritis Rheum* 54: 26–37.

Burmester, G.R., van de Putte, L.B.A., Rau, R., et al. (2003) Sustained efficacy of adalimumab monotherapy for more than four years in DMARD-refractory RA. *Ann Rheum Dis* 62(Suppl.1): 192–193.

Burmester, G.R., Monteagudo Sáez, I., Malaise, M.G., Canas de Silva, J., Webber, D.G., Kupper, H. (2004) Efficacy and safety of adalimumab (Humira) in European clinical practices: the ReAct trial [abstract]. *Ann Rheum Dis* 63(Suppl.1): 266.

Burmester, G.R., Monteagudo Sáez, I., Malaise, M.G., Kary, S., Kupper, H. (2005a) Adalimumab (Humira) is effective and safe in treating rheumatoid arthritis (RA) in real-life clinical practice: 1-year-results of the ReAct study [abstract]. *Arthritis Rheum* 52(Suppl.): S541–S542.

Burmester, G.R., Monteagudo Sáez, I., Malaise, M.G., et al (2005b) Adalimumab (Humira) is effective in patients who have previously been treated with TNF-antagonists (etanercept and/or infliximab) in widespread clinical practice: 12-week outcomes in the REACT Trial. *Ann Rheum Dis* 64(Suppl.III): 423–424.

Catrina, A.I., Trollmo, C., Klint, E., et al. (2005) Evidence that anti-tumor necrosis factor therapy with both etanercept and infliximab induces apoptosis in macrophages, but not lymphocytes, in rheumatoid arthritis joints: extended report. *Arthritis Rheum* 52: 61–72.

Cooper, W.O., Fava, R.A., Gates, C.A., Cremer, M.A., Townes, A.S. (1992) Acceleration of onset of collagen-induced arthritis by intra-articular injection of tumour necrosis factor or transforming growth factor-beta. *Clin Exp Immunol* 89: 244–250.

den Broeder, A.A., van de Putte, L.B.A., Rau, R., et al. (2002a) A single dose, placebo controlled study of the fully human anti-tumor necrosis factor-α antibody adalimumab (D2E7) in patients with rheumatoid arthritis. *J Rheumatol* 29: 2288–2298.

den Broeder, A.A., Joosten, L.A., Saxne, T., et al. (2002b) Long term anti-tumour necrosis factor alpha monotherapy in rheumatoid arthritis: effect on radiological course and prognostic value of markers of cartilage turnover and endothelial activation. *Ann Rheum Dis* 61: 311–318.

Emery, P., Schiff, M.H., Kalden, J.R., et al. (2004) Adalimumab (HUMIRA) plus methotrexate induces sustained remission in both early and long-standing rheumatoid arthritis. *Arthritis Rheum* 50(Suppl.): S183–S184.

Furst, D.E., Schiff, M.H., Fleischmann, R.M., et al. (2003) Efficacy and safety of the fully human anti-tumour necrosis factor-α monoclonal antibody, and concomitant standard antirheumatic therapy for the treatment of rheumatoid arthritis: results of STAR (Safety Trial of Adalimumab in Rheumatoid Arthritis). *J Rheumatol* 30: 2563–2571.

Georgopoulos, S., Plows, D., Kollias, G. (1996) Transmembrane TNF is sufficient to induce localized tissue toxicity and chronic inflammatory arthritis in transgenic mice. *J Inflamm* 46: 86–97.

Granneman, R.G., Zhang, Y., Noetersheuser, P.A., et al. (2003) Pharmacokinetic/pharmacodynamic (PK/PD) relationships of adalimumab (Humira, Abbott) in rheumatoid arthritis patients during Phase II/III clinical trials [abstract]. *Arthritis Rheum* 48 (Suppl.): S140.

Hanauer, S.B., Sandborn, W.J., Rutgeerts, P., et al. (2006) Human anti-tumor necrosis factor monoclonal antibody (adalimumab) in Crohn's disease: the CLASSIC-I Trial. *Gastroenterology* 130: 323–333.

Heinzlef, O., Alamowitch, S., Sazdovitch, V., et al. (2000) Autoimmune diseases in families of French patients with multiple sclerosis. *Acta Neurol Scand* 101: 36–40.

Jerne, N.K. (1974) Towards a network theory of the immune system. *Ann d'Immunologie* 125C: 373–389.

Kavanaugh, A., Grenwald, M., Zizic, T., et al. (2002) Treatment with adalimumab (D2E7) does not affect normal immune responsiveness [abstract]. *Arthritis Rheum* 45(Suppl.9): S132.

Kavanaugh, A., Cush, J.J., Matteson, E. ACR Hotline (2003) FDA meeting March 2003: update on the safety of new drugs for rheumatoid arthritis. Part II. CHF, infection, and other safety issues. Available at: http://www.rheumatology.org/publications/hotline/0803chf.asp?aud=mem. Accessed September 11, 2006.

Kaymakcalan, Z., Beam, C., Kamen, R., Salfeld, J. (2002) Comparison of adalimumab (D2E7), infliximab, and etanercept in the prevention of polyarthritis in a transgenic murine model of rheumatoid arthritis. *Arthritis Rheum* 46(Suppl.): S304.

Keystone, E.D., Kavanaugh, A.F., Sharp, J.T., et al. (2004a) Radiographic, clinical, and functional outcomes of treatment with adalimumab (a human anti-tumor necrosis factor monoclonal antibody) in patients with active rheumatoid arthritis receiving concomitant methotrexate therapy. *Arthritis Rheum* 50: 1400–1411.

Keystone, E.D., Kavanaugh, A.F., Perez, J.L., Spencer-Green, G.T. (2004b) Adalimumab (Humira) plus methotrexate provides sustained improvements in physical function over 2 years in treatment of patients with rheumatoid arthritis [abstract]. *Ann Rheum Dis* 64(Suppl.1): 278.

Keystone, E., Kavanaugh, A., Perez, J., Spencer-Green, G. (2004c) Adalimumab (Humira) plus methotrexate provides sustained improvements in physical function over 2 years of treatment in patients with rheumatoid arthritis [abstract]. *Ann Rheum Dis* 63(Suppl.1): 278.

Keystone, E.C., Kavanaugh, A.F., Sharp, J.T., et al. (2005) Inhibition of radiographic disease progression in patients with long-standing rheumatoid arthritis following 3 years of treatment with adalimumab (Humira) plus methotrexate [abstract]. *Ann Rheum Dis* 64(Suppl.III): 419.

Kristensen, L., Saxne, T., Geborek, P. (2004) Adherence to therapy of infliximab, etanercept, adalimumab, and anakinra in chronic arthritis patients treated in clinical practice in southern Sweden [abstract]. *Ann Rheum Dis* 64(Suppl.1): 263.

Landewé, R., Boers, M., Verhoeven, A., et al. (2002) COBRA combination therapy in patients with early rheumatoid arthritis: long-term structural benefits of a brief intervention. *Arthritis Rheum* 46: 347–356.

Langley, R., Leonardi, C., Toth, D., et al. (2005) Long-term safety and efficacy of adalimumab in the treatment of moderate to severe chronic plaque psoriasis [abstract]. *J Am Acad Dermatol* 52(3 Suppl.): 203.

Lee, D.M. and Weinblatt, M.E. (2001) Rheumatoid arthritis. *Lancet* 358: 903–911.

Lee, S.J. and Kavanaugh, A. (2005) Adalimumab for the treatment of rheumatoid arthritis. *Therapy* 2: 13–21.

Lovell, D.J., Ruperto, N., Goodman, S., et al. (2004) Preliminary data from the Study of Adalimumab in Children with Juvenile Idiopathic Arthritis (JIA). *Arthritis Rheum* 50(Suppl.): S436–S437.

Magnano, M., Robinson, W.H., Genovese, M.C. (2004) Demyelination and the use of TNF inhibition. *Clin Exp Rheumatol* 22(5 Suppl.35): S134–S140.

Maini, R.N. and Taylor, P.C. (2000) Anticytokine therapy for rheumatoid arthritis. *Annu Rev Med* 51: 207–229.

Maini, R.N., Breedveld, F.C., Kalden, J.R., et al. (1998) Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. *Arthritis Rheum* 41: 1552–1563.

McGonagle, D., Conaghan, P.G., O'Connor, P., et al. (1999) The relationship between synovitis and bone changes in early untreated rheumatoid arthritis: a controlled magnetic resonance imaging study. *Ann Rheum Dis* 42: 1706–1711.

McQueen, F.M., Stewart, N., Crabbe, J., et al. (1998) Magnetic resonance imaging of the wrist in early rheumatoid arthritis reveals a high prevalence of erosions at four months after symptom onset. *Ann Rheum Dis* 57: 350–356.

Mease, P.J. (2002) Tumour necrosis factor (TNF) in psoriatic arthritis:

pathophysiology and treatment with TNF inhibitors. *Ann Rheum Dis* 61: 298–304.
Mease, P. and Goffe, B.S. (2005) Diagnosis and treatment of psoriatic arthritis. *J Am Acad Dermatol* 52: 1–19.
Mease, P.J., Gladman, D.D., Ritchlin, C.T., et al. for the ADEPT Study Group (2005a) Adalimumab for the treatment of patients with moderately to severely active psoriatic arthritis: results of a double-blind, randomized, placebo-controlled trial. ADEPT. *Arthritis Rheum* 52: 3279–3289.
Mease, P.J., Gladman, D.D., Ritchlin, C.T., et al. (2005b) Clinical efficacy and safety of adalimumab for psoriatic arthritis: 48-week results of ADEPT [abstract]. *Arthritis Rheum* 52(Suppl.): S215.
Mease, P., Sharp, J., Ory, P., et al. (2005c) Inhibition of joint destruction in PsA with adalimumab: 48-week results of ADEPT [abstract]. *Arthritis Rheum* 52(Suppl.): S631.
Mitoma, H., Horiuchi, T., Hatta, N., et al. (2005) Infliximab induces potent anti-inflammatory responses by outside-to-inside signals through transmembrane TNF-[945]. *Gastroenterology* 128: 376–392.
Möttönen, T., Hannonen, P., Korpela, M., et al. (2002) Delay to institution of therapy and induction of remission using single-drug or combination-disease-modifying antirheumatic drug therapy in early rheumatoid arthritis. *Arthritis Rheum* 46: 894–898.
Murch, S.H., Lamkin, V.A., Savage, M.O., Walker-Smith, J.A., MacDonald, T.T. (1991) Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. *Gut* 32: 913–917.
Murch, S.H., Braegger, C.P., Walker-Smith, J.A., MacDonald, T.T. (1993) Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. *Gut* 34: 1705–1709.
Nikas, S.N., Voulgari, P.V., Alamanos, Y., et al. (2006) Efficacy and safety of switching from infliximab to adalimumab: a comparative controlled study. *Ann Rheum Dis* 65: 257–260.
Papadakis, K.A., Shaye, O.A., Vasiliauskas, E.A., et al. (2005) Safety and efficacy of adalimumab (D2E7) in Crohn's disease patients with an attenuated response to infliximab. *Am J Gastroenterol* 100: 75–79.
Partsch, G., Steiner, G., Leeb, B.F., Dunky, A., Broll, H., Smolen, J.S. (1997) Highly increased levels of tumor necrosis factor-α and other proinflammatory cytokines in psoriatic arthritis synovial fluid. *J Rheumatol* 24: 518–523.
Rau, R. (2002) Adalimumab (a fully human anti-tumour necrosis factor α monoclonal antibody) in the treatment of active rheumatoid arthritis: the initial results of five trials. *Ann Rheum Dis* 61(Suppl. III): ii70–ii73.
Ritchlin, C., Haas-Smith, S.A., Hicks, D., Cappuccio, J., Osterland, C.K., Looney, R.J. (1998) Patterns of cytokine production in psoriatic synovium. *J Rheumatol* 25: 1544–1552.
Salfeld, J., Kaymakcalan, Z., Tracey, D., Robert, A., Kamen, R. (1998) Generation of fully human anti-TNF antibody D2E7 [abstract]. *Arthritis Rheum* 41(Suppl.): S57.
Sandborn, W.J., Hanauer, S., Loftus, E.V. Jr., et al. (2004) An open-label study of the human anti-TNF monoclonal antibody adalimumab in subjects with prior loss of response or intolerance to infliximab for Crohn's disease. *Am J Gastroenterol* 99: 1984–1989.
Sandborn, W., Hanauer, S., Lukas, M., et al. (2005a) Induction and maintenance of clinical remission and response in subjects with Crohn's disease treated during a 6-month open-label period with fully human anti-TNF-α monoclonal antibody adalimumab (Humira). *Gastroenterology* 128(4 Suppl.2): A-111.
Sandborn, W.J., Hanauer, S., Lukas, M., et al. (2005b) Clinical remission and response in patients with Crohn's disease treated with open-label for 1 year with fully human anti-TNF-α monoclonal antibody adalimumab. *Gut* 54(Suppl.VII): A18.
Sandborn, W.J., Hanauer, S., Enns, R., et al. (2005c) Maintenance of remission over 1 year in patients with active Crohn's disease treated with adalimumab: results of CLASSIC II, a blinded, placebo-controlled study. *Gut* 54(Suppl.VII): A81–A82.
Scallon, B.J., Moore, M.A., Trinh, H., Knigh, D.M., Ghrayeb, J. (1995) Chimeric anti-TNF-alpha monoclonal antibody cA2 binds recombinant transmembrane TNF-alpha

and activates immune effector functions. *Cytokine* 7: 251–259.

Schiff, M., van de Putte, L.B., Breedveld, F.C., et al. (2003a) Rates of infection in adalimumab rheumatoid arthritis clinical trials [abstract]. *Ann Rheum Dis* 62(Suppl. I): 184.

Schiff, M.H., Chartash, E., Spencer-Green, G. (2003b) Malignancies in rheumatoid arthritis (RA) clinical trials with adalimumab (HUMIRA) [abstract]. *Arthritis Rheum* 48(9 Suppl.): S700.

Schiff, M.H., Weisman, M.H., Cohen, S.B., et al. (2004) Significant clinical improvement at 6 months are sustained over 4 years in patients with rheumatoid arthritis treated with adalimumab (Humira) plus methotrexate. *Arthritis Rheum* 50(Suppl.): S182–S183.

Schiff, M.H., Burmester, G.R., Kent, J., et al. (2005a) Safety of adalimumab in long-term treatment of patients with rheumatoid arthritis [abstract]. *J Am Acad Dermatol* 52(3 Suppl.): 203.

Schiff, M.H., Breedveld, F.C., Weisman, M. H., et al. (2005b) Adalimumab (Humira) plus methotrexate is safe and efficacious in patients with rheumatoid arthritis into the seventh year of therapy [abstract]. *Ann Rheum Dis* 64(Suppl. III): 438–439.

Schiff, M.H., Burmester, G.R., Kent, J.D., et al. (2006) Safety analyses of adalimumab (Humira) in global clinical trials with US postmarketing surveillance of patients with rheumatoid arthritis. *Ann Rheum Dis* 65: 889–894.

Shen, C., Assche, G.V., Colpaert, S., et al. (2005) Adalimumab induces apoptosis of human monocytes: a comparative study with infliximab and etanercept. *Aliment Pharmacol Ther* 21: 251–258.

Soden, M., Rooney, M., Cullen, A., Whelan, A., Feighery, C., Bresnihan, B. (1989) Immunohistological features in the synovium obtained from clinically uninvolved knee joints of patients with rheumatoid arthritis. *Br J Rheumatol* 28: 287–292.

Stallmach, A., Giese, T., Schmidt, C., Meuer, S.C., Zeuzem, S.S. (2004) Severe anaphylactic reaction to infliximab: successful treatment with adalimumab – report of a case. *Eur J Gastroenterol Hepatol* 16: 627–630.

Tsakonas, E., Fitzgerald, A.A., Fitzcharles, M.A., et al. (2000) Consequences of delayed therapy with second-line agents in rheumatoid arthritis: a 3 year follow up on the hydroxychloroquine in early rheumatoid arthritis HERA study. *J Rheumatol* 27: 623–629.

van der Bijl, A.E., Breedveld, F.C., Antoni, C.E., et al. (2005) Adalimumab (HUMIRA) is effective in treating patients with rheumatoid arthritis who previously failed infliximab treatment. *Ann Rheum Dis* 64(Suppl.III): 428.

van den Brande, J.M., Braat, H., van den Brink, G.R., et al. (2003) Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. *Gastroenterology* 124: 1774–1785.

van der Heijde, D., Kivitz, A., Schiff, M.H., et al. for the Adalimumab Trial Evaluating Long-term Efficacy and Safety in Ankylosing Spondylitis Study Group (2006) Efficacy and safety of adalimumab in patients with ankylosing spondylitis: results of a randomized, placebo-controlled trial (ATLAS). *Arthritis Rheum* 54: 2136–2146.

van de Putte, L.B.A., Salfeld, J., Kaymakcalan, Z. (2003) Adalimumab. In: Moreland, L.W. Emery, P. (Eds.), *TNF-Inhibition in the Treatment of Rheumatoid Arthritis*. Martin Dunitz, London, pp. 71–93

van de Putte, L.B., Atkins, C., Malaise, M., et al. (2004) Efficacy and safety of adalimumab as monotherapy in patients with rheumatoid arthritis for whom previous disease modifying antirheumatic drug treatment has failed. *Ann Rheum Dis* 63: 508–516.

Wallace, K.L., Langley, R., Bissonnette, R., Melluli, L., Hoffman, R. (2005a) Quality of life in patients with psoriatic arthritis treated with adalimumab: subanalysis of studies in moderate to severe psoriasis [abstract]. *J Am Acad Dermatol* 52(3 Suppl.): 192.

Wallace, K.L., Gordon, K.B., Langley, M., et al. (2005b) Dermatologic quality of life in patients with moderate to severe plaque psoriasis receiving 48 weeks of adalimumab therapy [abstract]. *J Am Acad Dermatol* 52(3 Suppl.): 180.

Wallace, K.L., Bissonnette, R., Leonardi, C., et al. (2005c) Effects of adalimumab on health status as measured by EQ-5D in patients with moderate to severe plaque psoriasis [abstract]. *J Am Acad Dermatol* 52(3 Suppl.): 181.

Wallace, K.L., Leonardi, C., Kempers, S., et al. (2005d) General physical and mental health status in patients with moderate to severe plaque psoriasis receiving 48 weeks of adalimumab therapy [abstract]. *J Am Acad Dermatol* 52(3 Suppl.): 185.

Weinblatt, M.E., Keystone, E.C., Furst, D.E., et al. (2003) Adalimumab, a fully human anti-tumor necrosis factor α monoclonal antibody for the treatment of RA in patients taking concomitant methotrexate. The ARMADA trial. *Arthritis Rheum* 48: 35–45.

Weinblatt, M.E., Keystone, E.C., Furst, D.E., Kavanaugh, A.F., Cartash, E.K., Segurado, O.G. (2005) Long-term efficacy, remission, and safety of adalimumab (HUMIRA) plus methotrexate in patients with rheumatoid arthritis in the ARMADA trial [abstract]. *Arthritis Rheum* 52 (Suppl): 563.

Weinblatt, M.E., Keystone, E.C., Furst, D.E., Kavanaugh, A.F., Chartash, E.K., Segurado, O.G. (2006) Long-term efficacy and safety of adalimumab plus methotrexate in patients with rheumatoid arthritis: ARMADA 4-year extended study. *Ann Rheum Dis* 65: 753–759.

Wells, A.F., Kupper, H., Fischkoff, S., et al. (2003) Injection site reactions in adalimumab rheumatoid arthritis (RA) pivotal clinical trials [abstract]. *Ann Rheum Dis* 62(Suppl.I): 411.

Williams, R.O., Feldmann, M., Miani, R.N. (1992) Anti-tumour necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc Natl Acad Sci USA* 89: 974–978.

Youdim, A., Vasikiauskas, E.A., Targan, S.R., et al. (2004) A pilot study of adalimumab in infliximab-allergic patients. *Inflamm Bowel Dis* 10: 333–338.

Zhou, H., Buckwalter, M., Boni, J., et al. (2004) Population-based pharmacokinetics of the soluble TNFr etanercept: a clinical study in 43 patients with ankylosing spondylitis compared with post hoc data from patients with rheumatoid arthritis. *Int J Clin Pharmacol Ther* 42: 267–276.

2
Alemtuzumab (MabCampath)

Thomas Elter, Andreas Engert, and Michael Hallek

2.1
Introduction

Alemtuzumab (MabCampath) is a member of the Campath-1 family of monoclonal antibodies that recognize the CD52 glycoprotein on human lymphocytes. The development of alemtuzumab evolved from the detailed study of the immunologic properties of various rat monoclonal Campath-1 antibodies. Initially, these anti-CD52 antibodies were generated in the United Kingdom by investigators in the Cambridge University Department of Pathology (hence the name, Campath). At the time, the goal of these investigations was to target lymphocytes for removal from donor bone marrow prior to transplantation, in an effort to prevent graft-versus-host disease (GvHD) in transplant recipients.

The first Campath-1 antibody to be described was a rat monoclonal IgM antibody that could bind to T and B lymphocytes and some monocytes and fix human complement, while leaving stem cells intact (Hale et al. 1983a). In subsequent studies, Campath-1G antibodies of the IgG2b isotype, which also had properties for lymphocyte binding and complement fixation, were shown to have the added feature of inducing antibody-dependent cell-mediated cytotoxicity (ADCC) in human lymphocytes (Hale et al. 1985). This suggested that IgG2b might be an ideal candidate for depleting lymphocytes in patients with lymphoproliferative disorders. Data supporting this hypothesis were obtained from a preliminary study that compared IgM, IgG2a, and IgG2b to determine the variant that depleted lymphocytes most effectively. In patients with chronic lymphocytic leukemia (CLL), the IgM and IgG2a variants induced only a transient depletion of blood lymphocytes, whereas the IgG2b variant, termed Campath-1G, achieved a longlasting depletion of both peripheral blood and bone marrow lymphocytes (Dyer et al. 1989). Consequently, the rat monoclonal Campath-1G antibody was studied for further development.

An important limitation with rat monoclonal antibodies, however, is the development of human anti-murine antibody when administered repeatedly over longer periods of time. In order to minimize the antigenicity of Campath-1G and to optimize its potential as a therapeutic agent, Campath-1G was humanized by

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inserting the hypervariable regions of the rat immunoglobulin into the human IgG1 framework – thus forming Campath-1H (alemtuzumab) (Riechmann et al. 1988). An initial pilot study in patients with non-Hodgkin's lymphoma showed significant responses to Campath-1H, with no detectable antiglobulin responses (Hale et al. 1988).

Alemtuzumab has since been studied in numerous clinical trials in a variety of diseases. It is currently indicated for the treatment of B-cell CLL in patients who have been treated with alkylating agents and who have failed fludarabine therapy (Campath-1H product information 2004).

2.2 Basic Principles

2.2.1 Antibody Features and Production

2.2.1.1 Features of Alemtuzumab (Campath-1H)

The humanization of Campath-1G was carried out by inserting the DNA sequences encoding the hypervariable regions (antigen-binding sites) of the rat IgG2 antibody to the human IgG1 isotype variable framework and constant domains. The human IgG1 isotype was selected based on its superiority to other isotypes in complement fixation, ADCC, and human IgG Fc receptor binding. The structure of the resultant humanized antibody, Campath-1H, is shown in Fig. 2.1 (Rai and Stephenson 2001). Campath-1H was shown to be as effective as Campath-1G at complement fixation, and to be more effective than Campath-1G at ADCC (Riechmann et al. 1988).

2.2.1.2 Antibody Production

The recombinant heavy-chain and light-chain DNAs of the antibody, containing both rat and human sequences as described above, were transfected into Chinese hamster ovary (CHO) cells to obtain clones to be used for the production of large quantities of Campath-1H via cell culture methods. Although the transfected cells are cultured in neomycin-containing medium, no detectable neomycin is present in the final solution of Campath-1H that is used clinically for injection (Campath-1H product information 2004).

2.3 Mechanism of Action

2.3.1 Molecular Target and Target Expression

The CD52 antigen, the target of the Campath-1 antibodies, contains 12 amino acids, with a large complex carbohydrate attached at residue 3 (asparagine) and a

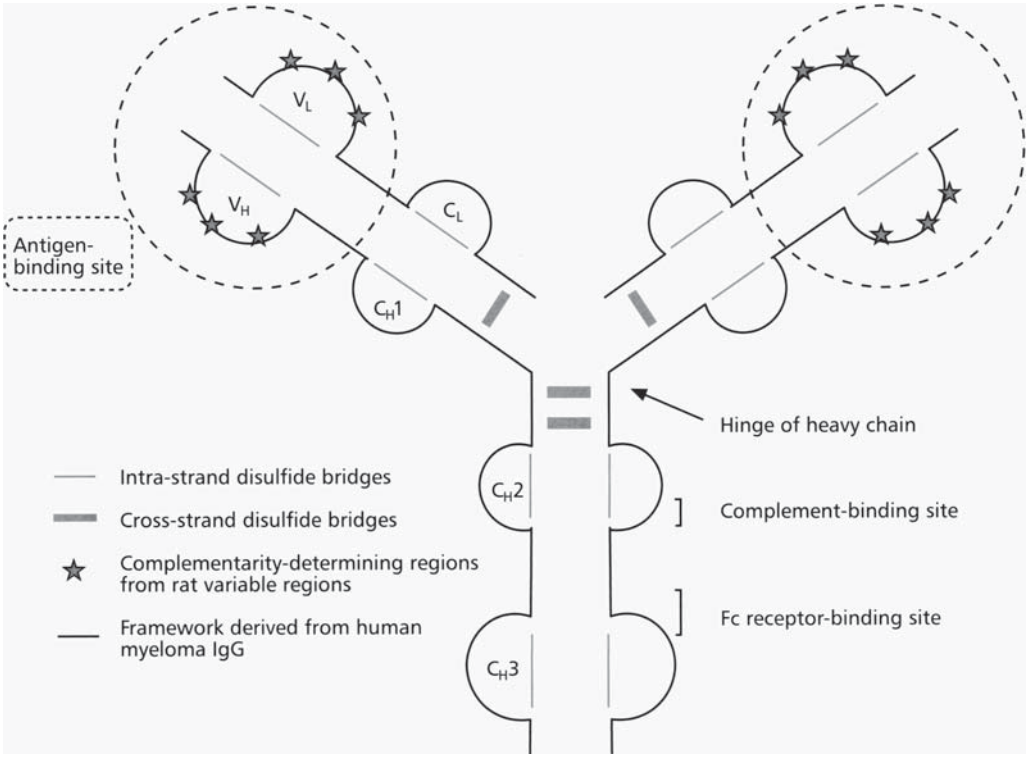

Fig. 2.1 Structure of the Campath-1H (alemtuzumab) monoclonal antibody. (Reproduced with permission from Rai and Stephenson 2001; © The Parthenon Publishing Group)

glycosylphosphatidylinositol (GPI) lipid anchor attached at the carboxy terminus. This anchor serves to maintain CD52 in the cell membrane, and portions of it – along with residues in the carboxy terminus – comprise the epitope recognized by Campath-1H (Fig. 2.2) (Hale 2001). Based upon structural analysis, it is thought that the proximity of this epitope to the cell surface contributes to the efficiency of complement-mediated cell lysis induced by Campath-1 antibodies (Xia et al. 1993).

The function of CD52 remains unknown. It is extensively expressed on lymphocytes, accounting for approximately 5% of the lymphocyte cell surface, and it is expressed at most stages of lymphocyte differentiation (Hale 2001); thus, CD52 may be an ideal target for the treatment of lymphoproliferative disorders. Its expression in the male reproductive tract occurs in the epithelial cells of the epididymis, vas deferens, and seminal vesicles; however, because it is shed from these cells, it is transferred to mature spermatozoa that pass through the genital tract. Nevertheless, no adverse effects of Campath-1H on reproductive function have been observed (Hale 2001; Kirchhoff 1996). Although CD52 is also expressed on monocytes, macrophages, and eosinophils, assessments of the effects of Campath-1H on bone marrow mononuclear cells and CD34(+) hematopoietic

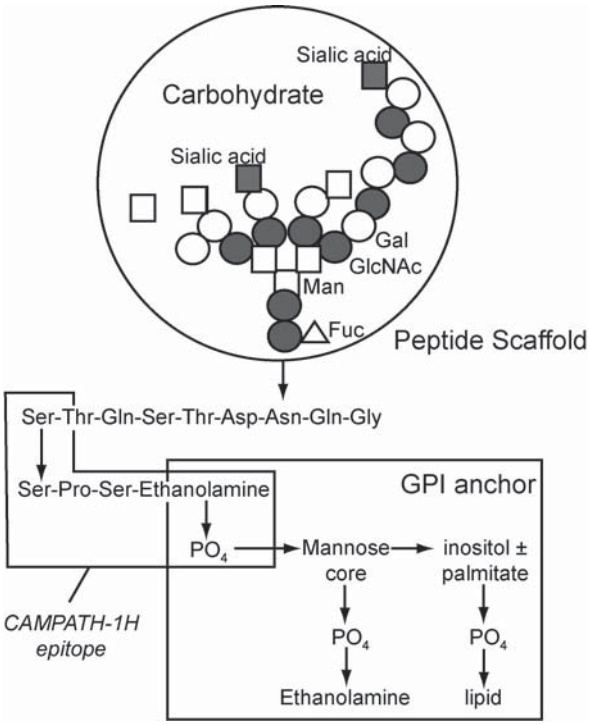

Fig. 2.2 Diagram of the CD52 antigen. CD52 is a glycoprotein comprising 12 amino acids with a complex carbohydrate attached to Asn-3. The protein is held in the outer layer of the cell membrane through a glycosylphosphatidylinositol (GPI)-lipid anchor attached to the C-terminus. (Reproduced with permission from Hale 2001.)

stem cells show that it does not reduce the number of progenitor cells, and importantly, does not affect their ability to proliferate, develop, or establish hematopoiesis (Gilleece and Dexter 1993).

In populations of normal lymphocytes, CD52 expression was shown to be higher on T than on B lymphocytes, and in patients with leukemia, expression was higher in those with T-prolymphocytic leukemia (T-PLL) than in those with B-cell CLL (Ginaldi et al. 1998). These differences in expression may potentially have a role in the variations observed in therapeutic response to Campath-1H among different patients and lymphoid malignancies; patients who responded to treatment with Campath-1H were found to have higher levels of CD52 expression than nonresponders (Ginaldi et al. 1998). The level of therapeutic response may also be influenced by the shedding of CD52 from lymphoid cells. In patients with CLL, high levels of soluble CD52 were found in plasma, and increased level of soluble CD52 correlated with disease severity and decreased survival outcomes. In the plasma of patients treated with alemtuzumab, soluble CD52 was found to

form complexes with alemtuzumab, and results of *ex-vivo* experiments showed that patient plasma containing the soluble form of CD52 blocked the binding of alemtuzumab to CLL cells. Responders to therapy had lower plasma levels of soluble CD52 than nonresponders, prompting the investigators to suggest that levels of soluble CD52 be considered when planning treatment with alemtuzumab (Albitar et al. 2004).

Another consideration in the targeting of the CD52 antigen is the emergence of CD52-subclones. For example, although most cells of a high-CD52-expressing B-cell lymphoma line (Wien 133) incubated with alemtuzumab and crosslinked with anti-human IgG underwent growth inhibition and apoptosis, surviving cells expanded for 2 to 4 weeks were found to have low levels of cell surface CD52 expression. The growth of these cells was not inhibited by subsequent CD52 crosslinking, and analysis suggested that a defect in synthesis or attachment of the GPI anchor conferred resistance (Rowan et al. 1998). Clinically, three of 25 patients with rheumatoid arthritis (RA) treated with Campath-1H 25–80 mg intravenously daily for 5 to 10 days in a phase II clinical trial developed high levels of CD52(−) B and T cells, but the effect on B cells was transient (<3 months), and the persistence of elevated levels of CD52(−) T cells for at least 20 months did not interfere with the therapeutic effect of Campath-1H (Brett et al. 1996). A recent report described the emergence of CD52(−) T-cell subsets in patients with B-CLL who were receiving an 18-week course of alemtuzumab 3 to 30 mg subcutaneously as first-line therapy. These cells comprised 80% of all peripheral T cells at the end of treatment, declined gradually, but persisted 18 months after treatment cessation. Again, no relationship between the level of CD52(−) T cells and response to treatment was apparent (Lundin et al. 2004).

2.3.2 Mechanism of Cell Lysis

Alemtuzumab has been shown to induce cell death in CD52-positive cells by several different mechanisms. As mentioned above, the location of the CD52 alemtuzumab-binding epitope close to the cell surface enables efficient complement-dependent cellular toxicity. Complement consumption by Campath-1M (IgM) was first documented in early studies of monkeys and human patients with lymphoid malignancies (Hale et al. 1983b). More recently, *in-vitro* assays using alemtuzumab and freshly isolated human neoplastic cells demonstrated that, in the presence of human complement, alemtuzumab efficiently induced complement-dependent cytotoxicity in a variety of malignant cells including B-CLL, B-PLL, hairy cell leukemia, Burkitt's lymphoma, mantle cell lymphoma, and follicular lymphoma (Golay et al. 2004).

ADCC also has a role in cell lysis, as Campath-1G (Dyer et al. 1989) and the human IgG1 portion of alemtuzumab have been shown to mediate ADCC (Greenwood et al. 1993). In addition, in a murine model of adult T-cell leukemia (ATL) that did not contain human complement, it was established that the Fc receptor was required for tumor cell killing by alemtuzumab (Zhang et al. 2003).

Moreover, in cultures of CLL patient-derived peripheral blood mononuclear cells, cross-linking of the Fc region of alemtuzumab with an anti-Fc antibody under complement-free conditions enhanced the low levels of apoptosis induced by alemtuzumab alone, produced cell clustering, and stimulated the production of pro-apoptotic proteins (Nückel et al. 2005). These observations suggested that ADCC was involved in the cell death induced by alemtuzumab, in part, via a caspase-dependent apoptotic pathway.

Induction of apoptosis by alemtuzumab appears to be mediated via a caspase-independent pathway, which may provide an advantage in patients resistant to therapeutic agents that induce apoptosis via classic pathways (Nückel et al. 2005; Rowan et al. 1998; Stanglmaier et al. 2004). As described above, alemtuzumab-induced apoptosis *in vitro* is enhanced significantly by the addition of crosslinking antibodies (Nückel et al. 2005; Rowan et al. 1998; Stanglmaier et al. 2004).

2.3.3
Immunogenicity and Antiglobulin Response

Antiglobulin responses, which limit the repeated use of murine monoclonal antibodies in transplant applications, were primarily a concern with the rat-derived Campath-1 antibodies. Given that alemtuzumab is a humanized version of a rat monoclonal antibody, this concern has been greatly reduced. For example, one study in kidney transplant patients found that while 15 of 17 patients had antiglobulin responses to the rat monoclonal antibody Campath-1G, none of 12 patients had detectable antiglobulin responses to alemtuzumab (Rebello et al. 1999). An analysis of antiglobulin responses was performed in two separate clinical trials that evaluated intravenous (IV) or subcutaneous (SC) administration of alemtuzumab in patients with CLL (Hale et al. 2004). In 30 patients who had previously failed therapy with alkylating agents and fludarabine, IV administration of alemtuzumab thrice weekly for up to 12 weeks produced no detectable antigloblulin responses (Hale et al. 2004). In another trial in which an extended dosing regimen of SC alemtuzumab was administered (three times weekly for up to 18 weeks) as first-line treatment, only a limited occurrence of antiglobulin responses to alemtuzumab (in two of 32 patients) was documented (Lundin et al. 2002). Although these responses interfered with treatment efficacy, they were not associated with any serious adverse effects (Hale et al. 2004).

2.4
Clinical Studies with Alemtuzumab

2.4.1
Pharmacokinetic Studies

In the bone marrow transplant (BMT) setting, the evaluation of IV alemtuzumab $10\,mg\,day^{-1}$ administered to patients with chronic myelogenous leukemia on

either a 5-day schedule (from 10 to 5 days prior to BMT) or a 10-day schedule (from 5 days prior to 4 days after BMT) showed that mean peak serum concentrations were 2.5 $\mu g\,mL^{-1}$ after 5 days (5-day schedule) and 6.1 $\mu g\,mL^{-1}$ after 10 days (10-day schedule). The terminal half-lives were 21 and 15 days, respectively (Rebello et al. 2001). In patients with CLL the indicated dosing regimen of alemtuzumab is an initial 2-h IV infusion with 3 mg daily which, when tolerated, should be increased to 10 mg daily. When the 10-mg dose is tolerated, a final increase is made to a maintenance dose of 30 $mg\,day^{-1}$, three times per week on alternate days, for up to 12 weeks. The mean half-life of alemtuzumab obtained in patients with CLL following this schedule was 11 h after the first 30-mg dose, and 6 days after the last 30-mg dose; thus, alemtuzumab displayed nonlinear elimination kinetics (Campath-1H product information 2004). When this dosing schedule was used in CLL patients who had previously failed therapy with alkylating agents and fludarabine, the mean cumulative dose required to reach the therapeutic serum level of 1.0 $\mu g\,mL^{-1}$ was 90 mg (Fig. 2.3). In contrast, for patients in the study of SC alemtuzumab (see Section 2.3.3), the mean cumulative dose required to reach a serum level of 1.0 $\mu g\,mL^{-1}$ was 551 mg (Hale et al. 2004). In patients with relapsed/refractory CLL treated with IV alemtuzumab, the clinical response was positively correlated with the maximum trough concentration of alemtuzumab in patient serum samples (Fig. 2.4A) (Hale et al. 2004). Moreover, throughout 12 weeks of therapy, the mean trough concentration of alemtuzumab was higher among responding patients (defined as those achieving <0.4% CLL cells in the bone marrow) compared to that of nonresponding patients (Fig. 2.4B).

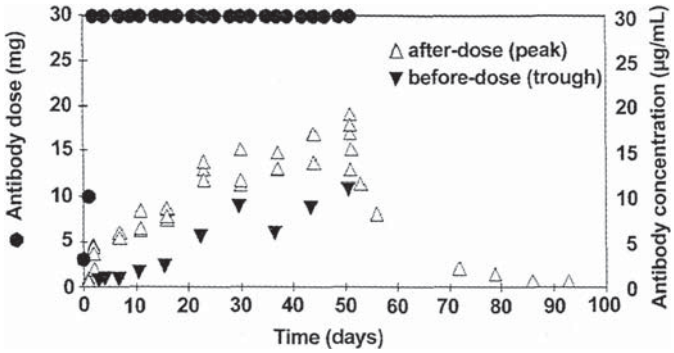

Fig. 2.3 Serum concentrations of alemtuzumab (intravenous administration) in a patient treated for relapsed/refractory chronic lymphocytic leukemia (CLL). Following dose escalation from 3 mg to 30 mg in Week 1, the patient received 30 mg alemtuzumab three times per week for 8 weeks. • indicates the dose of alemtuzumab administered during the course of therapy. (Reproduced from Hale et al. 2004; © American Society of Hematology.)

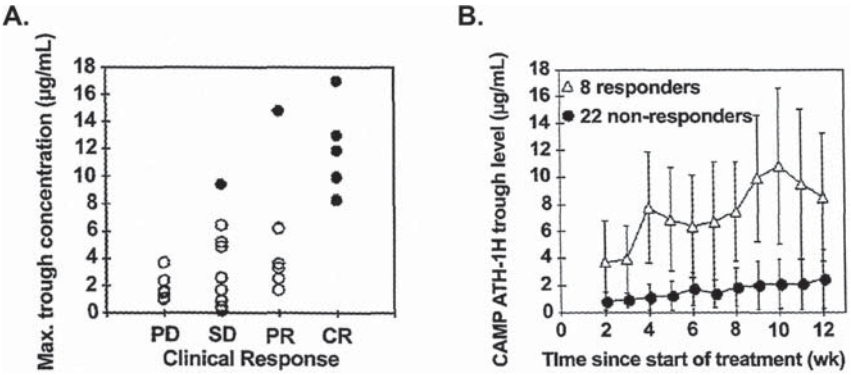

Fig. 2.4 Association between serum concentrations of alemtuzumab and response to therapy in patients with relapsed/refractory chronic lymphocytic leukemia (CLL) treated with intravenous alemtuzumab. (A) Maximum trough concentration of alemtuzumab and clinical response as assessed by NCI-WG criteria. Increase in response correlated with higher trough concentrations ($p = 0.006$; Kruskal-Wallis test). • indicates patients with minimal residual disease (MRD)-negative response defined as <0.1% CLL cells in the bone marrow. Patients with MRD-negative response had significantly higher maximum trough concentrations compared to those with a MRD-positive response ($p < 0.0001$, Kruskal-Wallis test). (B) Mean trough concentrations of alemtuzumab during 12 weeks of intravenous administration. Responders were patients who achieved <0.4% CLL cells in the bone marrow. PD = progressive disease; SD = stable disease; PR = partial response; CR = complete response. (Reproduced from Hale et al. 2004; © American Society of Hematology.)

2.4.2 Chronic Lymphocytic Leukemia (CLL)

2.4.2.1 Relapsed/Refractory CLL

The safety and activity of alemtuzumab were initially evaluated in a phase II study of 29 patients with relapsed (n = 8) or refractory (n = 21) CLL. Most patients (76%) had advanced disease, and 72% had previously received two or more lines of therapy. Alemtuzumab was administered as a 2-h IV infusion, at an initial dose of 3 or 10 mg, based on patient characteristics. The target dose was 30 mg, given three times weekly for up to 12 weeks (Österborg et al. 1997). Responses to therapy were evaluated using the National Cancer Institute Working Group (NCIWG) criteria (Cheson et al. 1988). Alemtuzumab showed significant activity in this patient population, with an overall response rate (ORR) of 42% [4% complete response (CR), 38% partial response (PR)] and a median duration of response of 12 months. Rapid elimination of malignant cells was achieved in the blood (in 1–2 weeks) of 97% of patients, elimination of malignant cells was achieved in the bone marrow (in 6–12 weeks) of 36% of assessable patients, and splenomegaly resolved in 32%. In contrast, activity in the lymph nodes was limited, particularly for patients with bulky lymphadenopathy. Only 17% of patients had progressive disease (PD) after treatment. Of the 12 responders, nine

were refractory to alkylating agents, and three had relapsed after an initial response to prior chemotherapy. All 12 weeks of the treatment period were completed by 12 patients; 10 had withdrawn from therapy due to lack of response, two after 6 weeks of therapy due to stable plateaus after achieving PR, four due to serious infections, and one withdrawal was of informed consent (Österborg et al. 1997). A brief summary of response rates in recent clinical trials of single-agent alemtuzumab in patients with CLL is provided in Table 2.1.

Safety evaluations showed moderate hematologic toxicity. Because some patients had low platelet and/or neutrophil counts at the start of therapy, the overall incidence of grade 4 thrombocytopenia and neutropenia was 24% and 20%, respectively. The incidence of grade 4 myelosuppression that developed during therapy included thrombocytopenia in 7% and neutropenia in 10% of patients. Grade 3/4 anemia developed in 38% of patients while receiving therapy. Nonhematologic toxicities included fever and rigors during the dose-escalation phase, rash, nausea, diarrhea, and hypotension. None of the toxicities was Grade 4, though one case of hypotension was Grade 3. Opportunistic infections were the main nonhematologic toxicity encountered, attributed largely to the longlasting lymphocytopenia induced by alemtuzumab. Infections included herpes simplex virus (HSV) reactivation, oral candidiasis, pneumonia, and septicemia; one case of pneumonia and two cases of septicemia were grade 3, and two cases of septicemia were grade 4. No deaths occurred either during treatment or within 6 months of follow-up (Österborg et al. 1997).

Further evaluation of the safety and efficacy of alemtuzumab was made in a pivotal, multicenter phase II trial of 93 patients with relapsed or refractory CLL. Heavily pretreated patients (median of three prior lines of therapy) who had failed prior therapy with alkylating agents and fludarabine received IV alemtuzumab 30 mg three times weekly for up to 12 weeks, using the same dose-escalation schedule described above, but with a starting dose of 3 mg for all patients (Keating et al. 2002b). Responses to therapy were evaluated using the revised guidelines of the 1996 NCIWG (Cheson et al. 1996). As in the above-described study, treatment with alemtuzumab resulted in a rapid elimination of malignant cells from the bone marrow (Fig. 2.5) (Keating et al. 2002b). The ORR was 33% (2% CR, 31% PR); in those patients who had never responded to fludarabine, the ORR was 29%, versus 38% in those who had previously responded. The median duration of response was 8.7 months. Median overall survival (OS) was 16 months for all patients, and 32 months for those who showed a response (Fig. 2.6) (Keating et al. 2002b). This result is particularly notable given the poor survival outcomes in patients who have become refractory to fludarabine. In a historical series of patients with CLL receiving first salvage therapy (with a variety of agents) for fludarabine-refractory disease, median OS was approximately 9–10 months (Keating et al. 2002c).

Alemtuzumab treatment resulted in the resolution of lymphocytosis (reduced to <30%) in 83% of all patients and in 93% of responders, while normal bone marrow biopsies were obtained in 26% of all patients and 48% of responders. In addition, 47% of patients with lymphadenopathy at baseline demonstrated at least

Table 2.1 Summary of response rates with single-agent alemtuzumab in chronic lymphocytic leukemia (CLL).

No. of patients	*Disease status*	*Route of administration*	*Median no. of prior therapies (range)*	*CR [%]*	*ORR [%]*	*Median response duration, months (range)*	*Reference*
29	Relapsed or refractory	IV	72% received ≥2 prior therapies	4	42	12(6–25+)	Österborg et al. (1997)
24	Primarily relapsed or refractory	IV	3 (1–8)	0	33	15.4(4.6–38+)	Rai et al. (2002)
93	Fludarabine-refractory	IV	3 (2–7)	2	33	8.7 (2.5–22.6+)	Keating et al. (2002b)
42	Advanced or refractory	IV	3 (1–9)	5	31	18 (range NR)* for CR patients	Ferrajoli et al. (2003)
36	Primarily advanced, fludarabine-refractory	IV	3 (1–12)	6	31	10 (3–36)	Lozanski et al. (2004)
91	Relapsed or refractory	IV/SC	3 (1–8)	36	54	TFS not reached for MRD (–) CR; 20 mo. for MRD (+) CR; 13 mo. for PR	Moreton et al. (2005)
41	Previously untreated	SC	None	19	87	TTF 18+ (7–44+)	Lundin et al. (2002)

* Among all responders in this study; the study included patients with various lymphoproliferative disorders (n = 78) including CLL (n = 42), T-cell prolymphocytic leukemia (n = 18), cutaneous T-cell lymphoma (n = 6), and others (n = 12).
CR = complete response; IV = intravenous; NR = not reported; ORR = overall response rate; SC = subcutaneous; TFS = treatment-free survival; TTF = time-to-treatment failure.

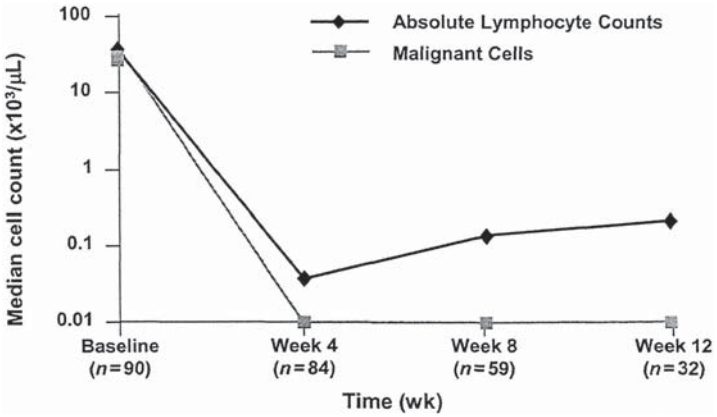

Fig. 2.5 Median number of malignant cells (CD19+/CD5+ cells) in peripheral blood during treatment with alemtuzumab. (Reproduced with permission from Keating et al. 2002b; © American Society of Hematology.)

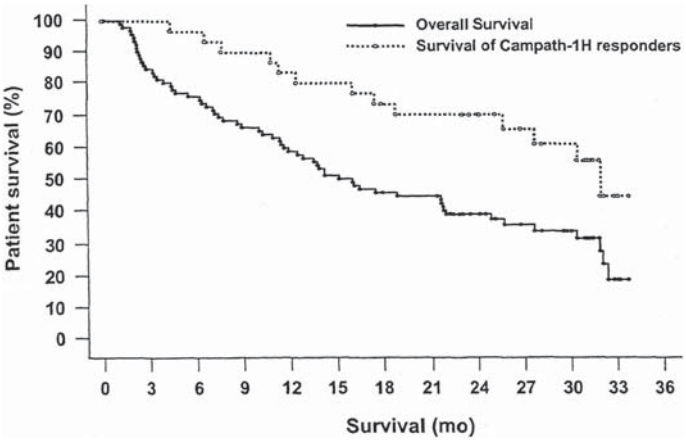

Fig. 2.6 Overall survival in patients with fludarabine-refractory (or fludarabine-failed) chronic lymphocytic leukemia (CLL) treated with alemtuzumab. The median survival was 16 months (95% CI: 11.8–21.9 months) for the intent-to-treat population (n = 93). The median survival for patients responding to treatment (n = 31) was 32 months. (Reproduced with permission from Keating et al. 2002b; © American Society of Hematology.)

a 50% reduction in enlarged nodes after treatment. However, as observed in previous studies, patients with bulky lymphadenopathy (lymph nodes >5 cm) were less likely to achieve resolution of disease in the lymph nodes. Among patients who had hepatomegaly at enrollment, resolution was achieved in 52%, and of those with splenomegaly, resolution was achieved in 54%. In terms of other measures

of clinical benefit assessed at the end of treatment, 76% of all patients experienced resolution of B-CLL symptoms or fatigue, 55% achieved resolution of massive splenomegaly, 49% improved in performance score, and 76% experienced an improvement in anemia. The treatment course was completed according to the protocol by 65 patients, while 28 discontinued prematurely.

The toxicity of alemtuzumab in these severely myelosuppressed patients was acceptable, although some patients required dosing delays until the toxicities were managed. The discontinuation rate due to adverse events related to treatment was 24%. Hematologic toxicities while receiving alemtuzumab included neutropenia and thrombocytopenia. However, 82% of patients had hematologic abnormalities (neutropenia, thrombocytopenia, anemia) at enrollment, and by the 2-month follow-up visit 55% of these patients had improvements in at least one of those abnormalities. No significant shifts of IgG from baseline were noted. Nonhematologic toxicities during dose escalation included fever (17% Grade 3, 3% Grade 4), rigors (14% Grade 3), nausea (all Grade 1 or 2), vomiting (1% Grade 3), and rash (all Grade 1 or 2), which usually decreased in incidence by Week 2 of treatment. Other Grade 3/4 adverse events were dyspnea (28%), hypotension (17%), and hypoxia (3%).

Infections were the most common reason for discontinuation. Grade 3/4 infections, such as septicemia and cytomegalovirus (CMV) reactivation, occurred in 27% of all patients and were significantly more common in alemtuzumab nonresponders than in responders (36% versus 10%, respectively; $P < 0.01$). However, 53% of patients had a history of infection, and 33% had infections during the month prior to alemtuzumab treatment initiation. Nine deaths occurred during the study or within 30 days after treatment (five of these were due to infection and considered related to treatment), 19 occurred between days 30 and 180 after treatment, and 35 occurred more than 180 days after treatment (Keating et al. 2002b). The results of this trial led to the approval of alemtuzumab for the treatment of B-cell CLL in patients who have been treated with alkylating agents and in whom fludarabine therapy had failed.

A smaller phase II multicenter trial of alemtuzumab was conducted in 23 patients with advanced CLL and one patient with T-PLL, all of whom had previously received fludarabine and other regimens (Rai et al. 2002). Alemtuzumab was administered intravenously, starting with an initial dose of 10 mg daily and dose escalation to 30 mg, and a dose of 30 mg thrice weekly was then continued for a maximum of 16 weeks. Treatment response was evaluated using the 1996 NCIWG criteria. Investigators were encouraged to monitor patients for antiglobulin responses every 4 weeks during the treatment period and 28 days after treatment cessation. Patients were assessed monthly for 6 months post treatment and every 3 months thereafter. As in the pivotal phase II trial, the ORR was 33% (all PR). The median duration of response was 15.4 months. Median OS was 27.5 months for all patients, and 35.8 months for responders. Elimination of malignant cells was achieved in the blood of 75% of patients, and in the bone marrow of 37%. In patients with splenomegaly or hepatomegaly at baseline, splenomegaly was resolved in 38% and hepatomegaly in 50%. The platelet counts improved

significantly from baseline to end of treatment, and continued to increase during follow-up (Rai et al. 2002).

The discontinuation rate due to adverse events was 37.5%. Hematologic toxicities were present in several patients at baseline; 20.8% had Grade 3/4 neutropenia and 41.7% had Grade 3 thrombocytopenia. During treatment, the rate of neutropenia increased to 59.1% but returned to near-baseline levels by the 2-month follow-up visit. Grade 3/4 nonhematologic toxicities included fever, rigors, and vomiting (16.7% for each), but these decreased in incidence after the first week of treatment. Grade 3 dyspnea was reported in one patient. Opportunistic infections (any grade) occurred in 41.7% of patients; similar to the pivotal trial, most of these incidences were observed in alemtuzumab nonresponders. Infections led to five treatment discontinuations and two deaths during treatment or within 35 days of treatment cessation. Of 10 patients evaluated for antiglobulin responses (total of 53 samples), one patient had a low-titer response after 7 weeks of treatment (one sample) (Rai et al. 2002).

In a phase II, single-site trial of patients with advanced CLL (n = 42), T-PLL (n = 18), or other chronic lymphoproliferative disorders (n = 18), treatment with alemtuzumab also demonstrated promising activity (Ferrajoli et al. 2003). Most patients were refractory to multiple therapies (median three prior lines of therapy); 55% of patients with CLL were refractory to alkylating agents and 55% to fludarabine, while 40% were refractory to both. The treatment protocol was similar to that used in the pivotal phase II trial, with an initial dose of 3 mg escalated to a final dose of 30 mg three times weekly for a maximum of 12 weeks of treatment. Responses to therapy were again evaluated using the 1996 NCIWG criteria. In those patients with NHL, treatment response was evaluated according to published NCIWG guidelines for NHL (Cheson et al. 1999). The ORR was 35% (13% CR, 22% PR), and the median duration of response was 18 months for patients who achieved a CR and 7 months for those who achieved a PR. The ORR in the subpopulation of patients with CLL (n = 42) was 31% (4% CR, 25% PR, 2% nodular PR). In those refractory to fludarabine, the ORR was 26%, and in those sensitive to fludarabine, the ORR was 37%. Patients with T-PLL showed a high ORR of 55% (44% CR, 11% PR) (Ferrajoli et al. 2003). Elimination of malignant cells was achieved in the blood of 84% of patients and in the bone marrow of 49%. Over half of all patients had a greater than 50% improvement with hepatomegaly and splenomegaly.

Grade 3/4 hematologic toxicities were transient neutropenia (34%) and thrombocytopenia (41%). As in the other trials, infusion-related adverse events (fever, rigors, skin rash, nausea, dyspnea, hypotension, and headache) were common, but decreased in incidence over time (Ferrajoli et al. 2003). Dyspnea of Grade 3/4 was observed in 11%; other toxicities were Grade 3/4 in 1% each (fever, rigors, hypotension, headache) or showed no Grade 3/4 toxicity (rash and nausea). Three patients with T-cell malignancies developed cardiovascular toxicities, and two of these patients discontinued alemtuzumab treatment. Infections and fever of unknown origin were common (experienced by 46% of all patients and 71% of those with CLL), with CMV reactivation being the most common infection

reported (20% of all patients). Pneumonia occurred in 13% of patients, and two died of progressive pneumonia. Septicemia was also responsible for two deaths (Ferrajoli et al. 2003).

Because alemtuzumab demonstrated efficacy in several trials of patients with CLL refractory to conventional treatments, its activity was investigated further in other difficult-to-treat or high-risk populations of CLL patients. Mutations or deletions in the *p53* gene are predictive of a poor response to conventional therapy for CLL. Thus, it was important to determine whether alemtuzumab would provide benefits in patients with CLL who had *p53* abnormalities. A study using cryopreserved cells from previously treated CLL patients (n = 36), a majority (81%) of whom were refractory to fludarabine and who had received a median of three prior therapies, identified 15 patients (42%) who had *p53* mutations or deletions (Lozanski et al. 2004). Alemtuzumab was administered according to the indicated dosing schedule for up to 12 weeks, and responses were assessed using the 1996 NCIWG guidelines. While the ORR was 31% for all patients (6% CR, 25% PR), it was 40% (all PR) for patients with *p53* abnormalities. In all patients, the median duration of response was 10 months, and in those with *p53* abnormalities it was 8 months (Lozanski et al. 2004). Thus, alemtuzumab may be a promising therapeutic option for the subgroup of CLL patients with *p53* aberrations.

2.4.2.2 **Minimal Residual Disease in CLL**

The NCI-WG criteria (Cheson et al. 1996) is the current standard for assessing response to therapy in patients with CLL, and remains an important guideline for both the clinical trials setting and routine clinical practice. However, the NCI-WG definition for CR allows for <30% lymphocytes in the bone marrow, which may still harbor substantial levels of malignant cells, likely leading to disease relapse. Using a highly sensitive four-color flow cytometric assay, almost 25% of CLL patients achieving CR by NCI-WG criteria have been shown to have minimal residual disease (MRD), defined as >0.05% CLL cells among bone marrow leukocytes (Rawstron et al., 2001). MRD levels have been shown to be an important prognostic indicator; MRD-positive status (>0.05% CLL cells) is predictive of significantly decreased event-free survival and overall survival compared with MRD-negative response in patients with CLL (Rawstron et al. 2001).

In light of these findings, investigators in a recent clinical trial with alemtuzumab aimed to eradicate MRD in patients (n = 91) with relapsed/refractory CLL who had received a median of three prior therapies (Moreton et al. 2005). The majority of patients had received prior therapy with purine analogs, and 48% were refractory. After standard dose escalation, patients received alemtuzumab 30 mg (IV, n = 84; SC, n = 7) three times weekly until maximum response. Blood and bone marrow were examined by flow cytometry before, during, and after treatment for evidence of CLL and MRD negativity. Responses were assessed using the 1996 NCIWG criteria. Patients who had a CR by these criteria but were MRD-positive by four-color flow cytometry were designated as MRD-positive CR (Moreton et al. 2005). After a median of 9 weeks of treatment, the ORR was 54% (35% CR, 19% PR). MRD was eradicated in 20% of patients (MRD-negative CR);

15% had an MRD-positive CR. Patients achieving MRD-negative status had a significantly longer median OS compared with patients who achieved an MRD-positive CR, PR, or NR ($P = 0.0007$) (Fig. 2.7) (Moreton et al. 2005). Median treatment-free survival also was significantly longer among patients with MRD-negative CR ($P < 0.0001$), further illustrating the potential survival advantage of eradicating MRD. The results of this study also confirmed the effectiveness of alemtuzumab in patients with fludarabine-refractory disease. In this subgroup of difficult-to-treat patients, the ORR was 50% (27% CR, 23% PR), and MRD was eradicated in 18% (Moreton et al. 2005).

Common hematologic toxicities were Grade 3 and 4 neutropenia (18% and 30%, respectively) and Grade 3/4 thrombocytopenia (46%). Infusion-related adverse events were most frequently Grade 1 or 2 in severity, and they decreased in frequency after Week 3. The most common events were rigors and fever (76% overall, 13% Grade 3/4). Major infections (Grade 3/4), including pulmonary infection (seven patients), febrile neutropenia (four patients), herpesvirus infection (two patients), CMV reactivation (six patients), and fungal infection (three patients) occurred during therapy or within 1 month of completing therapy. CMV reactivation developed in eight patients and was fatal in one case (Moreton et al. 2005).

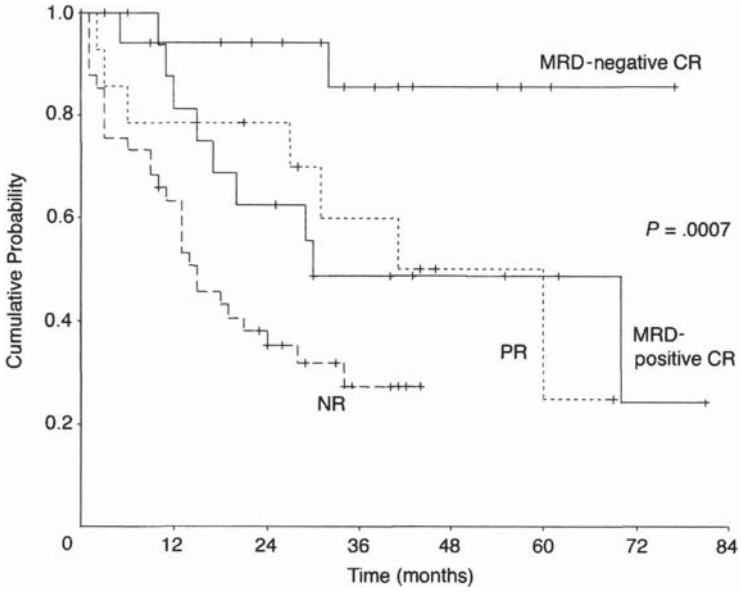

Fig. 2.7 Overall survival by response in patients with relapsed/refractory chronic lymphocytic leukemia (CLL) treated with alemtuzumab. Median survival was not reached for patients achieving MRD-negative complete response (CR), while median survival was 60 months for MRD-positive CR, 70 months for partial response (PR), and 15 months for nonresponders (NR) ($p = 0.0007$). (Reproduced with permission from Moreton et al. 2005.)

The ability of alemtuzumab to eliminate MRD also has been studied in CLL patients who received sequential therapy with alemtuzumab for consolidation following initial response to chemotherapy. These studies are discussed in Section 2.4.2.4.

2.4.2.3 **Treatment-Naïve CLL**

Alemtuzumab has also shown promising activity as first-line therapy for patients with untreated, progressive CLL. In an early pilot study, IV (n = 5) or SC (n = 4) administration of alemtuzumab (dose escalation to a target dose of 30 mg three times weekly for up to 18 weeks) was assessed in nine patients (Österborg et al. 1996). The ORR was 89% (33% CR, 56% PR). The median duration of response had not been reached by the time of publication, but ranged from 8+ to 24+ months. Complete elimination of CLL cells was obtained in 100% of patients, and bone marrow remission in 78% of patients. Hepatomegaly and/or splenomegaly was completely resolved in 67% of patients, and four patients experienced a >50% reduction in enlarged lymph nodes. One patient developed Grade 3 neutropenia, but all other hematologic toxicity was mild. Fever and rigor occurred with initial doses in 89% of patients. One patient developed CMV pneumonitis and oral candidosis (Österborg et al. 1996).

More recently, SC administration of alemtuzumab was evaluated in a phase II multicenter trial as first-line therapy in patients with CLL (n = 41) (Lundin et al. 2002). After dose escalation from 1 mg to 3 mg to the target dose of 30 mg, previously untreated patients received alemtuzumab 30 mg three times weekly for up to 18 weeks as a SC injection in the thigh. After 2 to 3 weeks of treatment, patients self-administered alemtuzumab at home. Responses were assessed using the 1996 NCIWG criteria. The ORR was 87% (19% CR, 68% PR). At the time of publication, the median time to treatment failure had not been reached (18+ months) (see Table 2.1). CLL cells were eliminated in 95% of patients at a median of 21 days, and CR or nodular PR was obtained in the bone marrow in 66% of patients by 18 weeks (Lundin et al. 2002).

Transient injection-site reactions (ISR) were noted in 90% of patients. Grade 1 reactions were erythema/edema, and Grade 2 reactions included pruritus and slight pain. Only 1 Grade 3 ISR was reported, with local pain that led to treatment discontinuation. Infusion-related toxicities frequently observed with the IV administration of alemtuzumab (i.e., rigors, nausea, hypotension) were absent or rare, with no episodes of rash/urticaria, bronchospasm, hypotension, or nausea, and a 17% incidence of transient rigor. Hematologic toxicities included Grade 4 neutropenia, which occurred in 21% of patients (Grade 2/3, 53%), and Grade 4 thrombocytopenia, which occurred in 5% (Grade 2/3, 11%). Although 39% of patients developed Grade 2/3 anemia, no Grade 4 anemia was reported. In addition, no major bacterial infections were observed. One patient who was allergic to prophylactic cotrimoxazole developed *Pneumocystis carinii* pneumonia after receiving treatment for 11 weeks. CMV reactivation occurred in 10% of patients, but did not lead to study withdrawal (Lundin et al. 2002). Importantly, the results of this study also established that single-agent alemtuzumab given subcutane-

ously could achieve response rates similar to those achieved with IV administration, but with an acceptable and more favorable safety profile.

A follow-up to this study was conducted to determine the effects of alemtuzumab on blood lymphocyte subsets at the end of treatment and during the posttreatment period (Lundin et al. 2004). By analyzing blood samples of 23 patients (all responders) with flow cytometry, the investigators found that treatment with alemtuzumab resulted in longlasting immune suppression (e.g., depression of CD4+ and CD8+ cells, as well as natural killer cells, normal B cells, granulocytes, and monocytes). The median end-of-treatment counts were low and remained less than 25% of baseline values for over 9 months after treatment cessation (Lundin et al. 2004). However, these low counts showed no significant association with late-occurring infections or autoimmune phenomena. In addition, no significant relationship between the cumulative dose of alemtuzumab and the severity or duration of lymphopenia was apparent (Lundin et al. 2004).

2.4.2.4 **Chemoimmunotherapy Combinations**

Because alemtuzumab has a mechanism of action which is distinct from those of chemotherapeutic agents used to treat B-CLL, combination therapy with chemotherapeutic agents may lead to synergistic activity and thereby result in improved response compared to either agent alone. Thus, protocols using concurrent and sequential administration of fludarabine and alemtuzumab have been investigated. This combination was evaluated initially in a study of six patients with previously treated CLL (Kennedy et al. 2002). Prior to receiving combination therapy, patients had become refractory to single-agent fludarabine (median eight courses of treatment) and single-agent alemtuzumab. Combination treatment with IV fludarabine (25 mg m^{-2} for 3 days every 28 days) and alemtuzumab (30 mg three times weekly) was given for a minimum of 8 weeks. Responses were assessed using the 1996 NCIWG guidelines. Although a range of responses was obtained, the ORR was 83% (16% CR; 67% PR), with only one patient showing PD. In addition, bone marrow normalized in three patients, with two showing MRD-negative response by flow cytometry, while lymphadenopathy was completely resolved in one patient (Kennedy et al. 2002). Responses in two patients enabled them to undergo subsequent successful autologous peripheral blood stem cell transplantation. Thus, the two agents appeared to act synergistically and were highly active as combination therapy. Acceptable toxicity was seen with this combination approach, with one case of *Pseudomonas* bronchopneumonia during neutropenia, while two patients experienced neutropenia (Kennedy et al. 2002).

In a recent clinical trial, the concurrent administration of fludarabine and alemtuzumab (the FluCam regimen) was evaluated in a phase II, single-center trial conducted in 36 CLL patients with relapsed or refractory disease (Elter et al. 2005). Patients had received a median of two prior therapies; four patients had previously received single-agent alemtuzumab, and 22 had previously received fludarabine as a single agent or in combination with another agent. Some 25%

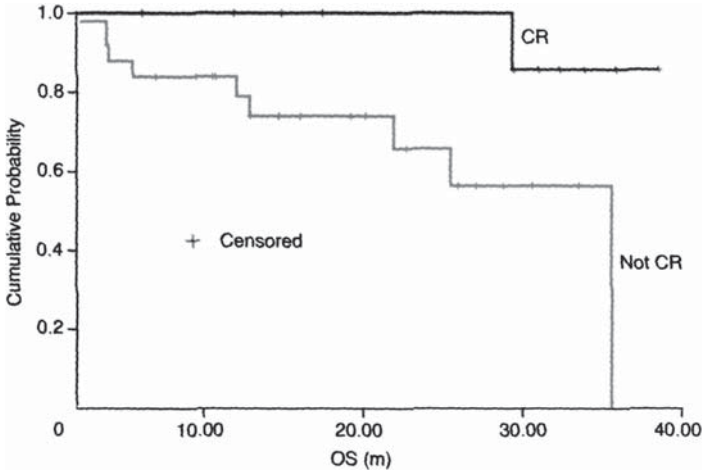

Fig. 2.8 Overall survival by response (CR versus non-CR) in patients with relapsed/refractory chronic lymphocytic leukemia (CLL) treated with combination therapy with alemtuzumab and fludarabine. Median survival was not reached for patients achieving CR, while median survival was approximately 35 months for those not achieving CR. (Reproduced with permission from Elter et al. 2005.)

of patients were refractory to prior fludarabine therapy. The dose of alemtuzumab was escalated on consecutive days from 3 mg to 10 mg to the target dose of 30 mg, after which FluCam (fludarabine 30 mg m^{-2} day for days 1–3 and alemtuzumab 30 mg for days 1–3) was administered every 4 weeks for a maximum of six treatment cycles. Responses were evaluated using the 1996 NCIWG criteria. The ORR was 83% (30% CR, 53% PR), with a median OS of 35.6 months for all patients in this study; the median OS for those who achieved a CR was not reached at the time of publication (Fig. 2.8) (Elter et al. 2005). The median TTP was 12.97 months for all patients, and calculated to be 21.9 months for those achieving a CR. When patients were analyzed by prior response to treatment, eight of the 12 refractory patients responded (4 CR, 4 PR), and 22 of the 24 relapsed patients responded (7 CR, 15 PR). Of those patients who had received fludarabine previously, 18 responded (6 CR, 12 PR), and six of the nine who previously were refractory to fludarabine responded to FluCam (Elter et al. 2005).

Analysis of the 140 treatment cycles assessable for toxicity showed that the major Grade 3/4 toxicities were leukopenia (44%), thrombocytopenia (30%), and neutropenia (26%). Grade 3/4 nonhematologic toxicities were rare (infectious complications in three patients), and most Grade 1/2 toxicities were infusion-related. Thus, the data from this larger trial further demonstrate the synergistic effect of alemtuzumab and fludarabine when given in combination, and indicate that this combination therapy is effective and well tolerated by patients with relapsed/refractory B-CLL (Elter et al. 2005).

Alemtuzumab has also been evaluated as sequential combination therapy (consolidation) in previously treated CLL patients with residual disease after initial

chemotherapy. In a trial of 41 patients who had responded to prior chemotherapy with a PR, a nodular PR, or a CR with bone marrow evidence of residual disease, alemtuzumab 10 mg was administered three times weekly for 4 weeks, and if residual disease persisted, a dose of 30 mg three times weekly for 4 additional weeks was offered (after a 4-week rest period) (O'Brien et al. 2003). The rationale for the lower dose was that patients were likely to have less tumor burden compared with the typical population of relapsed/refractory patients for whom the full dose of alemtuzumab is indicated. However, after the first 24 patients were assessed, the remaining patients who enrolled were given the 30-mg dose to increase response rates. The response criteria in this trial differed from those in the trials described previously; patients were required to convert to a CR or a nodular PR by 1996 NCIWG criteria to be considered responders, and residual disease was allowed only in the bone marrow. For those patients who had a CR with bone marrow involvement at the start of treatment (n = 3), an MRD-negative status was required for them to be considered responders (O'Brien et al. 2003).

The ORR was 46%, comprising a 39% response with the 10-mg dose and a 56% response with the 30-mg dose. Failure to respond was due largely to residual lymphadenopathy. In patients with specific disease site involvement prior to treatment, alemtuzumab consolidation therapy achieved a 48% response in clearance of disease from bone marrow nodules, an 86% response in reducing bone marrow lymphocytes to <30%, and an 86% response by immunophenotyping. Achievement of a MRD-negative response, as assessed by polymerase chain reaction (PCR), was attained by 38% of 29 evaluable patients (O'Brien et al. 2003). At the time of publication, the median overall TTP had not been reached; between 24 and 38 months after therapy, six patients were still in remission. Patients who were PCR-negative had not reached their median TTP, whereas those who were PCR-positive had a median TTP of 15 months. Toxicities included Grade 3/4 neutropenia in 30% and Grade 3/4 thrombocytopenia in 14% of patients, infections (including CMV reactivation) in 15% of patients, and infusion-related events (all Grade 1/2) in most patients (O'Brien et al. 2003).

Alemtuzumab consolidation therapy was also evaluated in 34 patients who had previously responded to fludarabine-based therapy (Montillo et al. 2006). In this study, patients received consolidation therapy with alemtuzumab in an effort to eradicate MRD for peripheral stem cell collection in preparation for autologous transplantation. All patients had previously received first-line treatment with fludarabine-based therapy, including fludarabine monotherapy (n = 31) or fludarabine plus cyclophosphamide (n = 1). Two additional patients initially received fludarabine monotherapy, but were switched to fludarabine plus cyclophosphamide after suboptimal response in the lymph nodes. Responses were evaluated using the 1996 NCI-WG criteria. Patients had achieved a CR (35%), nodular PR (21%), or PR (44%) with their prior fludarabine-based therapy, but all had MRD positivity as measured by consensus primer PCR (Montillo et al. 2006). Alemtuzumab was administered SC with initial dose escalation up to 10 mg, three times weekly for 6 weeks. For stem cell mobilization, patients received granulocyte

colony-stimulating factor (G-CSF) alone or with intermediate-dose ara-C (800 mg m^{-2} every 12 h). The median time from the last dose of fludarabine to initiation of alemtuzumab was 16 weeks (range 12–76 weeks). Following consolidation with alemtuzumab, 53% of patients improved their responses to initial fludarabine-based therapy; overall, 27 patients (79%) achieved a CR with 19 (56%) having a MRD-negative CR. In addition, 24 of 26 patients were successfully able to mobilize peripheral stem cells with adequate stem cell collection in preparation for subsequent transplantation. At the time of publication, 18 patients had undergone stem cell transplantation; at a median follow-up of 28 months after alemtuzumab therapy (range, 11–42 months), 17 of these patients remain in CR (Montillo et al. 2006). The most common adverse events associated with SC alemtuzumab were first-dose reactions such as injection-site reactions (76%) and fever (41%), and were primarily Grade 1 or 2. No hematologic toxicities were associated with alemtuzumab therapy. Although CMV reactivation occurred in 53% of patients (as a result of routine monitoring for CMV antigenemia during the study), the development of active CMV infection was prevented in all cases by pre-emptive therapy with oral ganciclovir (or in three patients, by spontaneous resolution). No bacterial or fungal infections were reported (Montillo et al. 2006). This study demonstrates the activity of alemtuzumab consolidation therapy in eliminating MRD following fludarabine-based induction in patients with CLL, and the feasibility of stem cell mobilization and successful engraftment using this therapeutic approach.

A phase III randomized study compared outcomes with alemtuzumab consolidation therapy versus observation only in patients (n = 21) with residual disease following an initial response to fludarabine-based treatment (Wendtner et al. 2004). Consolidation with alemtuzumab 30 mg three times weekly (for up to 12 weeks) (n = 11) resulted in superior responses compared to observation alone (n = 10) in terms of the proportion of patients achieving MRD negativity (five of six evaluable versus none of three evaluable, respectively; $P = 0.048$) and mean progression-free survival (no progression versus 24.7 months, respectively; $P = 0.036$). In both study arms, patients had previously received six cycles of fludarabine or fludarabine + cyclophosphamide as first-line therapy (Wendtner et al. 2004). Two patients who received alemtuzmab converted from a PR to a CR, and three showed bone marrow clearance. Grade 3/4 hematologic toxicities were common in the alemtuzumab arm, with seven patients with neutropenia, four with thrombocytopenia, and two with anemia. There were eight cases of Grade 3/4 infections (pulmonary aspergillosis, CMV reactivation, HSV, herpes zoster, and pulmonary tuberculosis reactivation). These infectious complications resulted in early termination of the study, such that only two patients completed the planned 12 weeks of therapy; however, all patients completed at least 3 weeks. No Grade 3/4 first-dose reactions were observed (Wendtner et al. 2004). The high incidence of infectious events observed in the alemtuzumab arm of this study was likely due to the relatively short recovery time between the initial therapy and alemtuzumab administration. Further studies are warranted to better establish the optimal rest period between induction and consolidation therapy that would

allow for sufficient recovery of immune function. The benefits of alemtuzumab consolidation were demonstrated further in a recent update of the results of this trial. Thus, studies have shown the feasibility and promising activity of alemtuzumab in combination with chemotherapy, administered either concurrently or sequentially as consolidation therapy to eliminate residual disease following chemotherapy-based treatment.

2.4.2.5 **Immunotherapy Combination**

The feasibility of the combination of alemtuzumab with rituximab, a chimeric murine/human monoclonal antibody targeting the CD20 antigen, was investigated in 48 patients with relapsed/refractory lymphoid malignancies (Faderl et al. 2003). The CD20 antigen is expressed on normal and malignant B lymphocytes, and rituximab is approved for the treatment of patients with relapsed or refractory, low-grade or follicular, CD20-positive, B-cell NHL (Rituxan product information 2005). Patients in this study had poor prognoses and one of the following malignancies coexpressing CD52 and CD20: CLL, CLL/PLL, PLL, mantle cell leukemia/lymphoma, or Richter transformation. The treatment protocol was IV rituximab 375 mg m^{-2} once weekly for 4 weeks, and IV alemtuzumab dose escalation from 3 mg to 10 mg to 30 mg on three consecutive days during Week 1, followed by 30 mg on days 3 and 5 of Weeks 2, 3, and 4. A second cycle was available to patients based on their status after the first cycle. Responses were assessed using the 1996 NCIWG criteria for CLL. The ORR was 52% (8% CR, 4% nodular PR, 40% PR), with 63% of CLL patients and 44% of patients with CLL/PLL responding to this combination regimen (Faderl et al. 2003). The one patient with PLL also responded; however, none of the patients with mantle cell leukemia/lymphoma (n = 4) or Richter transformation (n = 2) responded to therapy. Although the majority of patients had peripheral blood clearance of malignant cells, only 36% of those with CLL, 25% of those with CLL/PLL, and the one patient with PLL had bone marrow responses. At least a 50% decrease in lymphadenopathy and hepatosplenomegaly occurred in 59% of those with CLL and 67% of those with CLL/PLL. The median TTP was 6 months, and the median OS was 11 months (Faderl et al. 2003).

Hematologic toxicities and infusion-related toxicities were common, but most were Grade 2, or less. Over half (52%) of the patients had at least one infectious complication. CMV antigenemia was detected in 27% of patients; 15% of these were symptomatic. Other infections included pneumonia, sinusitis, and fever of unknown origin (Faderl et al. 2003). Overall, the combination was well tolerated, and results supported the feasibility of combining these two monoclonal antibodies.

2.4.2.6 **Safety with Alemtuzumab in CLL**

As can be seen from the toxicities detailed in the individual trials described in Sections 2.4.2.1 to 2.4.2.5, alemtuzumab administration is associated with infusion-related adverse events, hematologic toxicities, and infectious events. Although some of these events may be of Grade 3/4 severity, guidelines for managing these

complications are available and enable patients to derive maximum benefit from alemtuzumab-based therapy.

2.4.2.6.1 Infusion-related Adverse Events

Intravenous administration of alemtuzmab is associated with fever, rigors, nausea, vomiting, skin rash, dyspnea, hypotension, headache, and hypoxia. Most of these adverse events tend to resolve after about 2 weeks from the time of initial dose administration (Keating et al. 2002b; Ferrajoli et al. 2003; Moreton et al. 2005). Recommendations for the prevention and management of infusion-related adverse events were recently published as part of the outcomes of an expert opinion roundtable that generated treatment guidelines for alemtuzumab in CLL (Table 2.2) (Keating et al. 2004). The authors stated that, in their experience,

Table 2.2 Guidelines for managing acute first-dose infusion reactions with intravenous alemtuzumab. (Modified from Keating et al. 2004, with permission.)

Reaction	*Recommendations*
Standard premedications	• Diphenhydramine (50 mg) or chlorpheniramine (≤35 mg) and acetaminophen (650 mg) 30 min before infusion. • Second dose if acetaminophen (4 h later) during the first week of treatment.
Severe infusion-related events	• Add premedication with hydrocortisone (intravenous dose 50–100 mg) to subsequent infusions. • Discontinue steroids as soon as possible, preferably after Week 1
Rigors	• Hold infusion and administer intravenous meperidine or pethidine (25 mg). • Premedicate with anti-emetics if patients experience meperidine/pethidine-induced nausea.
Rash	• Add 25–50 mg diphenhydramine/chlorpheniramine orally every 4 h as needed. • Hydrocortisone may be used if additional diphenhydramine/chlorpheniramine is not effective. • For severe rash, premedicate with H_2 receptor antagonists (e.g., cimetidine, ranitidine).
Hypotension	• Hydrate with normal saline solution unless contraindicated, based on underlying cardiac condition.
Dyspnea	• Hold infusion and treat with inhaled β_2-agonists. • For severe dyspnea, temporary use of steroids may be necessary.
Febrile neutropenia	• Antibiotics or G-CSF may be administered at the discretion of the clinician.

G-CSF = granulocyte colony-stimulating factor.

Table 2.3 Summary of common adverse events and first-dose reactions with intravenous and subcutaneous alemtuzumab. Data expressed as % of patients. (Modified from Keating et al. 2004, with permission.)

	IV administration (n = 93)		***SC administration (n = 41)***	
Events	Grade 1 or 2	Grade 3 or 4	Grade 1 or 2	Grade 3 or 4
Fever	69	13	15	2
Rigors	77	11	15	2
Rash/urticaria	44	4	0	0
Hypotension	14	1	0	0
Dyspnea	11	6	0	0
Nausea/vomiting	78	0	0	0
Diarrhea	12	1	0	0
Headache	13	0	0	0
Fatigue	14	3	5	2
Local injection-site reactions	0	0	88	0

these clinical guidelines have allowed most patients receiving alemtuzumab to continue with therapy. The administration of alemtuzumab via the subcutaneous route may avoid or minimize many of these infusion-related toxicities, as was demonstrated in the phase II trial of previously untreated patients with CLL (Lundin et al. 2002) described in Section 2.4.2.3. Although ISRs were observed in the majority of patients, the infusion-related toxicities described above were rare, or absent (Lundin et al. 2002). While direct comparisons cannot be made between trials due to differences in patient populations, differences in the incidence of infusion-related events in the SC alemtuzumab trial and that of the most common infusion-related events seen in the pivotal trial of IV alemtuzumab suggest that SC administration circumvents many of these toxicities (Table 2.3).

2.4.2.6.2 **Hematologic Toxicities**

Hematologic toxicities are associated with both the IV and SC administration of alemtuzumab, owing to its myelosuppressive properties. In previously treated CLL patients, the major hematologic toxicities reported were transient thrombocytopenia and neutropenia (Ferrajoli et al. 2003; Keating et al. 2002b; Moreton et al. 2005; Rai et al. 2002); these events, which also occur when alemtuzumab is administered to treatment-naïve CLL patients, are typically of higher incidence and greater severity in previously treated patients (Lundin et al. 2002). In addition, the disease itself is often associated with cytopenias, such that patients may frequently present with thrombocytopenia or neutropenia even prior to receiving alemtuzumab therapy.

Hematologic toxicities reported with concurrent chemoimmunotherapy with FluCam were leukopenia, thrombocytopenia, and neutropenia (Elter et al. 2005). With alemtuzumab consolidation therapy, Grade 3/4 hematologic toxicities did not occur in one small study (Montillo et al. 2002), but Grade 3/4 thrombocytopenia and neutropenia were common in a larger trial with a more heavily pretreated patient population (O'Brien et al. 2003), as well as in the randomized trial of alemtuzumab consolidation (Wendtner et al. 2004). The combination of alemtuzumab and rituximab, which was given for only one cycle in most patients in the study, produced myelosuppression in approximately 66% of patients, but events of Grade 3/4 severity were rare (Faderl et al. 2003).

Clinical recommendations for the management of hematologic toxicities are based on the fact that thrombocytopenia appears to be most common during Weeks 2 through 4 of therapy, and neutropenia appears to be most common during Weeks 4 through 8; thus, careful monitoring during these times may help clinicians to monitor and manage these events promptly. Severe events are managed with growth factor support during treatment, and experts do not recommend interrupting therapy (although this is suggested in the alemtuzumab product information), because patients will be vulnerable to infusion-related toxicities when re-starting therapy. The current guidelines for managing thrombocytopenia, neutropenia and anemia (which is not as common in incidence or as severe in presentation as thrombocytopenia and neutropenia) are listed in Table 2.4 (Keating et al. 2004). Experts have found that transient neutropenia at 4 weeks of therapy does not warrant discontinuation of therapy unless the patient has presented with major infections.

2.4.2.6.3 Immunosuppression and Infectious Events

In a retrospective review of the records of 27 fludarabine-refractory CLL/small-cell lymphocytic leukemia (SLL) or B-PLL patients, data analysis showed that, regardless of the salvage therapy administered, 89% of patients developed serious infections, 78.4% of which were bacterial (Perkins et al. 2002). Serious infections were defined as those that required hospital admission and IV antibiotic treatment. The median time to development of the first serious infection was 4 months from the time the patient became refractory to fludarabine, with a median time between hospital admissions of 42 days (Perkins et al. 2002). Thus, chemotherapy-refractory CLL patients are highly vulnerable to serious infections while receiving salvage therapy. Because these patients have poor immune function even before beginning alemtuzumab-based therapy, they may show a high incidence of infectious complications that may not be attributed entirely to the immunosuppressive nature of this agent.

Studies of alemtuzumab in patients with refractory/relapsed CLL have shown that serious infections have developed during therapy, including septicemia, CMV reactivation, pneumonia, and herpes virus infections, with infection-related deaths reported in all trials. There appeared to be a trend toward a lower incidence of infections in patients with the best responses to therapy (Ferrajoli et al. 2003; Keating et al. 2002b; Moreton et al. 2005; Rai et al. 2002). With the FluCam

Table 2.4 Guidelines for managing hematological toxicities.
(Modified from Keating et al. 2004, with permission.)

Condition	*Recommendation(s)*
Anemia	
All patients with anemia	Alemtuzumab may be given with transfusion (with irradiated blood products) or erythropoietin support.
Neutropenia	Dose reduction or discontinuation of alemtuzumab is not recommended unless neutropenia is accompanied by a major infection.
ANC > 500 μL	Support may not be required as cytopenias are likely to resolve spontaneously.
ANC < 500 μL	G-CSF or GM-CSF support may be used at the discretion of the clinician.
ANC < 250 μL	Alemtuzumab treatment should be temporarily interrupted until resolution.
Febrile neutropenia	Alemtuzumab treatment should be temporarily interrupted until resolution.
Thrombocytopenia	
$<25\,000\,\mu L^{-1}$	If no hemorrhage is present, alemtuzumab should be continued at full dose.
$<10\,000\,\mu L^{-1}$	Transfusion support may be given at the discretion of the clinician.
Hemorrhagic event	Alemtuzumab treatment should be temporarily interrupted until resolution.

ANC = absolute neutrophil count; G-CSF = granulocyte colony-stimulating factor; GM-CSF = granulocyte macrophage colony-stimulating factor.

combination, three of 36 patients with relapsed/refractory disease developed infections (two with fungal pneumonia and one with sepsis, which was fatal) (Elter et al. 2005). Again, these patients either had poor performance status at study entry or did not respond to alemtuzumab therapy (i.e., they had progressive disease after treatment). Subclinical CMV reactivation was observed in three patients (Elter et al. 2005). When alemtuzumab was used as consolidation therapy, 37% of patients developed infections, including nine who experienced CMV reactivation. In addition, three patients developed Epstein–Barr virus (EBV) large cell lymphoma (O'Brien et al. 2003). The use of alemtuzumab as first-line therapy in patients with CLL was not associated with any major infections (no Grade >1 incidences) in patients receiving anti-infective prophylaxis, and CMV reactivations were manageable (Lundin et al. 2002). This finding further supports the notion that the immune function of CLL patients may be more intact in the frontline setting or in less-advanced stages of the disease. In the first-line consolidation setting, alemtuzumab was associated with CMV reactivation as well as pulmonary aspergillosis, herpes virus infections, and tuberculosis reactivation (Montillo et al. 2002; Wendtner et al. 2004). While T-cell counts may decrease to

undetectable levels in some patients during treatment, observations in small numbers of patients suggested that no correlations existed between absolute decreases in T-cell counts and the risk of severe infection (Wendtner et al. 2004).

Clinical guidelines for the prevention and management of infectious events in patients receiving alemtuzumab include routine antimicrobial prophylaxis and weekly monitoring for CMV reactivation, which often occurs between Weeks 3 and 6 of therapy (Keating et al. 2004). If CMV reactivation is observed or suspected, then prompt treatment with ganciclovir (or equivalent) is recommended. More detailed recommendations are outlined in Table 2.5 (Keating et al. 2004).

2.4.3 T-Cell Leukemias and Lymphomas

2.4.3.1 T-Cell Lymphomas (Cutaneous/Peripheral T-Cell Lymphoma)

Patients with cutaneous T-cell lymphomas (CTCLs) have few treatment options and poor prognoses after their disease becomes refractory to topical therapies. Because malignant T cells typically have high levels of CD52 expression, studies were conducted to determine whether alemtuzumab had activity in patients with advanced CTCLs.

Promising results were observed when alemtuzumab was evaluated in a phase II, multicenter study of patients with advanced mycosis fungoides or Sézary syndrome (MF/SS), one of the most common types of CTCLs (Lundin et al. 2003). Patients with CD52-positive, advanced MF/SS (n = 22; median three prior therapies) received alemtuzumab 30 mg three times weekly for up to 12 weeks (after an initial dose escalation from 3 mg). The ORR was 55% (32% CR, 23% PR) (Table 2.6); in patients who had received one or two prior lines of therapy, the ORR was 80%, and in those who had received three or more prior lines of therapy it was 33%. Peripheral blood was cleared of tumor cells in 86% of patients, and tumor cells were cleared from lymph nodes and skin in 55% of patients for each disease site (Lundin et al. 2003). Erythroderma was ameliorated in 69% of patients, with 38% experiencing a CR. Patients also reported reductions in and/or disappearance of itching. Half of the patients completed all 12 weeks of treatment. The median TTF was 12 months in those who responded to therapy. Toxicities were characteristic of those seen previously with alemtuzumab in patients with CLL (i.e., infusion-related adverse events, hematologic toxicities, and infections), although the time frame for the appearance of neutropenia was somewhat delayed. The results of this trial demonstrated the activity of alemtuzumab in patients with advanced MF/SS (Lundin et al. 2003).

A subsequently reported case study of alemtuzumab treatment (10-week course) of a 32-year-old man with advanced-stage SS showed dramatic results supporting this activity. The patient had almost complete disappearance of itching after 1 week of treatment; disappearance of erythroderma and pruritis, almost complete disappearance of Sézary cells from peripheral blood and bone marrow, regression

Table 2.5 Guidelines for managing infections events. (Data from Keating et al. 2004.)

Infection	*Recommeddations*
Pneumocystis carinii pneumonia	• Prophylaxis with trimethoprim/sulfamethoxazole DS twice daily. • For patients not tolerating above regimen, other alternatives may include aerosolized pentamidine, oral dapsone, and oral atovaquone. • Prophylaxis should be continued for ≥2 months (preferably 4 months) after completion of alemtuzumab therapy, or until CD4 levels recover to $250000\,\mu L^{-1}$.
Viral infections	• Prophylaxis with agents including famciclovir, acyclovir, or valacyclovir. • Prophylaxis should be continued ≥2 months (preferably 4 months) after completion of alemtuzumab therapy, or until CD4 levels recover to $250000\,\mu L^{-1}$.
CMV reactivation	
Patients with fever of unknown origin	• Test for CMV antigen, preferably by PCR if available. • If PCR is not available, clinicians should treat all cases of fever of unknown origin with use of preemptive ganciclovir.
Symptomatic patients with positive PCR results	• Treat immediately with intravenous ganciclovir or foscarnet. • If unresponsive to ganciclovir alone, foscarnet may be added. • Hold alemtuzumab therapy until infection has cleared.
Asymptomatic patients with positive PCR results	• Test again by a second PCR test. • If patient tests positive in the second test, initiate treatment with ganciclovir and monitor with a quantitative PCR, if possible. • If unresponsive to ganciclovir alone, foscarnet may be added.
Symptomatic for pulmonary infection but negative for CMV on PCR test	• Test by bronchoscopy and lavage.

CMV = cytomegalovirus; DS = double strength; PCR = polymerase chain reaction.

of lymphadenopathy, and normal blood counts 4 months after starting treatment; there were no signs of disease at the 12-month follow-up assessment. No toxicities were noted (Gautschi et al. 2004).

In contrast, a trial of alemtuzumab in eight patients with advanced MF/SS showed less favorable results. Although the ORR was 38% (all PR), and patients reported improvements in pruritis, the response duration was less than 3 months, and a high incidence of Grade 4 hematologic toxicity and infectious complications

Table 2.6 Summary of response rates with alemtuzumab in T-cell malignancies.

No. of patients	*Disease type*	*Route of administration*	*Median no. of prior therapies (range)*	*CR [%]*	*ORR [%]*	*Median response duration, months (range)*	*Reference*
38	Primarily previously treated, refractory* T-PLL	IV	NR*	60	76	7 (4–45)	Dearden et al. (2001)
76	Primarily refractory† T-PLL	IV	2 (0–5)†	37.5	50	8.7 (0.1+ to 44.4) for patients with CR	Keating et al. (2002a)
22	Advanced mycosis fungoides/Sézary syndrome	IV	3 (1–5)	32	55	TTF 12 (5–32+)	Lundin et al. (2003)
8	Advanced, heavily pretreated mycosis fungoides/Sézary syndrome	IV	6.5 (1–17)	0	38	2.5 (2–3.5)	Kennedy et al. (2003)
14	Relapsed or refractory peripheral T-cell lymphoma	IV	2 (1–4)	21	36	6 (2–12) for patients with CR	Enblad et al. (2004)

* Two patients in this study were treatment-naïve.
† Four patients in this study were treatment-naïve.
CR = complete response; IV = intravenous; NR = not reported; ORR = overall response rate; T-PLL = T-cell prolymphocytic leukemia; TTF = time-to-treatment failure.

was observed (Kennedy et al. 2003). The authors postulated that the discrepancy between these results and those of others may be due to the more heavily pretreated patient population in this study (i.e., seven of eight patients had received at least four prior therapies).

Alemtuzumab has also been studied in patients with advanced peripheral T-cell lymphoma (PTCL), as these also have limited treatment options and poor prognoses. In a phase II study of 14 patients with relapsed/refractory PTCL who were heavily pretreated, alemtuzumab was dose escalated to 30 mg three times weekly for a maximum treatment period of 12 weeks (Enblad et al. 2004). The ORR was 36% (CR in three patients, PR in two), and CR durations were 2, 6, and 12 months in the three patients (Table 2.6). Only one patient completed all 12 weeks of treatment, and the median treatment duration was 6 weeks. Premature discontinuation occurred due to achievement of a CR (n = 3) and toxicity and/or progressive disease (n = 10). Hematologic toxicities (pancytopenia in four patients, hemophagocytosis syndrome in two) and infectious complications (CMV reactivation in six patients, pulmonary aspergillosis in two) were common, and five patients died due to serious adverse events related to alemtuzumab treatment in combination with advanced disease. Although the ORR was encouraging in this group of poor-prognosis patients, the high rates of toxicity prohibited the investigators from recommending alemtuzumab for the treatment of patients with PTCL unless they were part of clinical trials that are carefully designed and monitored (Enblad et al. 2004).

Another group of investigators used a reduced dose of alemtuzumab in patients with advanced PTCL. Ten patients with relapsed/refractory PTCL unspecified (n = 6) or MF (n = 4) were given alemtuzumab 10 mg three times weekly for 10 weeks (Zinzani et al. 2005). Patients had received a median of three prior treatments. The ORR was 60% (20% CR, 40% PR). Both CRs were in PTCL patients; three MF patients had a PR. The median duration of response was 7 months, and at this dose alemtuzumab was well tolerated (no Grade 3/4 hematologic toxicities, one CMV reactivation that was managed effectively).

A case report of alemtuzumab treatment of a heavily pretreated 74-year-old patient with CD52-positive Lennert's lymphoma, a PTCL/lymphoepithelioid cell variant, showed that after 5 weeks of treatment, there was a significant reduction in thoracic and abdominal lesions. Although the patient experienced CMV reactivation during treatment, it was managed successfully (Zeitlinger et al. 2005).

2.4.3.2 **T-Cell Prolymphocytic Leukemia (T-PLL)**

T-PLL cells also have high levels of CD52 expression. No approved treatment exists for T-PLL, and those that are used show high rates of relapse, with short median survival durations. Investigation of the activity of alemtuzumab in 39 patients with T-PLL (two naïve to therapy, 37 who had received prior treatment) showed that in 38 evaluable patients, alemtuzumab 30 mg three times weekly produced a high ORR of 76% (60% CR, 16% PR) (Dearden et al. 2001) (Table 2.6). Among patients previously resistant to chemotherapy, 34% achieved a CR. Responses

occurred at most disease sites. The median OS was 10 months for all patients, and 16 months for those who achieved a CR. Nine patients were alive 29 months after completing therapy, and one patient survived 54 months. Toxicities were characteristic of those seen with alemtuzumab treatment of patients with CLL, and alemtuzumab was well tolerated in these patients (Dearden et al. 2001). Seven patients were able to undergo high-dose chemotherapy (HDT) and autologous stem cell transplantation after alemtuzumab treatment, as flow cytometry and PCR verified that their harvested stem cells were free of T-PLL cells. Owing to these encouraging results, the investigators concluded that alemtuzumab might be an effective first-line treatment option for T-PLL (Dearden et al. 2001).

In a retrospective study that included data from 18 patients in the above trial, data from a total of 76 patients with T-PLL who had been treated with alemtuzumab were analyzed to determine its activity and safety (Keating et al. 2002a). Patients were enrolled in a compassionate-use program, and all but four (who had not received prior therapy) had failed prior therapies. Alemtuzumab 30 mg was given three times weekly for 4–12 weeks after the initial dose escalation. In the 72 pretreated patients, the ORR was 50% (37.5% CR, 12.5% PR), and three of the four previously untreated patients achieved a CR, making the total ORR 50% (Keating et al. 2002a) (Table 2.6). In the bone marrow, 39% of patients achieved a CR; in the spleen, 33% of patients with splenomegaly achieved a CR; in the lymph nodes, the CR was 32%; and 30% of patients with hepatomegaly achieved a CR. The response in the skin was 43%. Of 27 pretreated patients who achieved a CR with alemtuzumab, only one had achieved a CR and 10 a PR when on prior therapy. The median OS was 7.5 months for all patients, and 14.8 months for those who had achieved a CR. Toxicities were not different from those seen patients with CLL. The response in untreated patients suggests that alemtuzumab should be studied further in chemotherapy-naïve patients (Keating et al. 2002a). A separate study of four patients with T-PLL showed that treatment with alemtuzumab produced an ORR of 75% (Fløisand et al. 2004). Overall, the outcomes obtained in these trials have demonstrated that alemtuzumab is efficacious in patients with T-PLL.

2.4.3.3 **Adult T-Cell Leukemia**

As alemtuzumab has demonstrated tumor cell killing and prolongation of survival demonstrated in a murine model of adult T-cell leukemia (Zhang et al. 2003), and because no effective therapy exists for this malignancy, investigations of the activity of alemtuzumab in patients are warranted. A recent case report of a 55-year-old woman with refractory adult T-cell leukemia who received treatment with alemtuzumab and the nucleoside analog pentostatin as part of an ongoing clinical trial described improvements in her condition. Alemtuzumab was given at a dose of 30 mg three times weekly, and pentostatin at a dose of 4 mg m^{-2} weekly for 4 weeks, followed by dosing every other week. The regimen was well tolerated, and resolution of palpable disease, normal blood counts, and improved computed tomography (CT) scans were observed after 2 months of therapy. In addition, at

this time point and at several repeat examinations, the patient had no morphological or immunophenotypic evidence of disease in the bone marrow (Zhang et al. 2003). Hence, alemtuzumab might have promising activity in adult T-cell leukemia and should be investigated further in this patient population (Ravandi and Faderl 2005).

2.4.4 Non-Hodgkin's Lymphoma (NHL)

As described in Section 2.2, two patients with NHL were among the first to receive alemtuzumab therapy during its development process, and the compound has shown activity in blood, bone marrow, spleen, and lymph nodes in these patients (Hale et al. 1988). Relapsed or resistant low-grade NHL is difficult to treat. Rituximab is approved for this indication, and thus it is feasible that alemtuzumab might also have utility in these patients. In a phase II multicenter trial, 50 previously treated patients (25 relapsed, 25 resistant) were given alemtuzumab 30 mg three times weekly for up to 12 weeks (Lundin et al. 1998). The initial dose was either 3 or 10 mg, determined on the basis of each patient's baseline conditions. Patients completed a median of 8 weeks of treatment, with nine completing the full 12 weeks. Although 40% of patients did not respond, an ORR of 20% (4% CR, 16% PR) was obtained (Lundin et al. 1998) (Table 2.7). A CR was obtained in the blood of 94% of patients within a median of 7 days; 32% of patients had a CR in the bone marrow, splenomegaly resolved completely in 15% of patients, lymphadenopathy resolved completely in 5% of patients (and was reduced by over 50% in 11% of patients), and skin lesions completely regressed in 40% of patients. The median TTP was 4 months. Hematologic toxicities were moderate, with major Grade 4 toxicities being anemia (10%), neutropenia (28%), and thrombocytopenia (22%); however, 28% of patients had low neutrophil and/or platelet counts at baseline that improved with alemtuzumab therapy (Lundin et al. 1998). Infusion-related reactions were also common, but decreased in incidence over time. Infections included HSV reactivation, oral candidiasis, *Pneumocystis carinii* pneumonia, CMV pneumonitis, pulmonary aspergillosis, tuberculosis, and septicemia. Six patients died during treatment or follow-up for reasons including progressive disease, septicemia, pre-existing respiratory insufficiency, and pneumonia of unknown origin (Lundin et al. 1998). This study has demonstrated that, although a subset of patients with NHL responded to treatment with alemtuzumab, the response was not durable. The investigators hypothesized that the poor response in the lymph nodes and progression of lymphadenopathy in a high percentage of patients contributed to the shorter duration of response.

A study in 16 NHL patients with nonbulky disease and two with MRD showed slightly better responses (ORR = 22%, with two patients in CR alive over 4.5 years after treatment) (Table 2.7), but the trial was terminated early due to the high incidence and severity of infectious complications (Khorana et al. 2001). Thus, even in NHL patients with nonbulky disease or MRD, only a subset showed benefits with alemtuzumab treatment.

Table 2.7 Summary response rates with alemtuzumab in non-Hodgkin's lymphoma (NHL).

No. of patients	*Disease state*	*Route of administration*	*Median no. of prior therapies (range)*	*CR [%]*	*ORR [%]*	*Median response duration, months (range)*	*Reference*
50	Relapsed or refractory low-grade NHL	IV	70% received ≥2 prior therapies	4*	20	NR (time-to-progression 4 months for all responders; 10 months for patients with mycosis fungoides)	Lundin et al. (1998)
18	Previously treated nonbulky NHL	IV	2 (1–4)	17†	22	7 (5–54.+); 14+ months in 1 patient with PCR-negative CR	Khorana et al. (2001)
18	Relapsed or refractory low-grade and high-grade NHL	IV	3 (1–4)	0	44	NR	Uppenkamp et al. (2002)

* The NHL subtype for these patients was mycosis fungoides.

† Includes two patients with low-grade NHL who achieved CR, and one patient with minimal residual disease who achieved a PCR-negative CR in the bone marrow.

CR = complete response; IV = intravenous; NR = not reported; ORR = overall response rate; PCR = polymerase chain reaction.

Another study of alemtuzumab in 18 patients with relapsed/refractory NHL included two with high-grade NHL (Uppenkamp et al. 2002). Two patients were treated with a maximum of 75 mg and 240 mg alemtuzumab once weekly, and the remaining 16 received alemtuzumab 30 mg three times weekly for 6 weeks; a subset of the 16 were participants in the 50-patient, phase II, multicenter trial described above (Lundin et al. 1998). The response criteria were somewhat different, with responses classified as CR, major disease improvement (MDI), disease improvement (DI), or limited disease improvement (LDI). Responses obtained were DI (n = 2) and LDI (n = 6), for a response rate of 44%. Six of the eight responders showed a reduction of bone marrow infiltration, five a decrease in enlarged lymph nodes, and two a reduction in splenomegaly. However, the median duration of response was 3.5 months. Two patients died while on the study, and two were withdrawn due to bronchospasm. Hematologic toxicities were recorded, but few were Grade 3/4. The major treatment-limiting factor was infection, with 11 patients reporting a total of 12 different infections (Uppenkamp et al. 2002).

2.4.5 Stem Cell Transplantation (SCT)

2.4.5.1 Donor T-Cell Depletion (Prevention of GvHD) and Prevention of Graft Rejection

The Campath-1 family of antibodies was originally developed to provide a tool to deplete mature T lymphocytes from donor bone marrow allografts *in vitro* in preparation for transplantation, in the hope of reducing the risk of GvHD in transplant recipients. In an early study of 11 high-risk patients, HLA-matched sibling donor bone marrow was treated with rat monoclonal Campath-1 (autologous serum was used as the complement source) and transplanted, and no anti-GvHD prophylaxis was provided. Engraftment was rapid and successful in all patients, and over the 12-month follow-up period, none of the patients developed GvHD (Waldmann et al. 1984). These results indicated that Campath-1 and autologous human serum-provided complement could successfully remove mature T lymphocytes from allografts prior to transplantation. However, further study revealed that donor T-cell depletion increased the risk of graft rejection and disease relapse through minimization of the graft-versus-leukemia effect. Small numbers of donor T cells may be added to help restore the graft-versus-leukemia effect, although the risk of GvHD is then increased. This was illustrated in a study of 131 patients with acute leukemia (acute nonlymphocytic leukemia [ANLL] or acute lymphoblastic leukemia [ALL]) who received T-cell-depleted matched sibling allografts (121 allografts depleted using Campath-1), as 81 received donor T cells post transplant to restore graft-versus-leukemia activity (Naparstek et al. 1995). Of these patients, 39 developed acute GvHD and 21 developed chronic GvHD. The risk of developing GvHD was significantly higher in recipients of donor T cells than in nonrecipients ($P < 0.0001$). When the overall probability of relapse was examined, donor T cell administration provided no benefit over no

administration (2-year probability of relapse, 25% versus 32%, respectively, $P = 0.64$). However, patients with ALL who received donor T cells and developed GvHD had a reduced 2-year probability of relapse (14%) compared with those who received donor T cells but had no GvHD (61%), and with those who did not receive donor T cells or develop GvHD (56%) (Naparstek et al. 1995). The results suggested that donor T cells mediated the graft-versus-leukemia effect in parallel with GvHD.

A further strategy to counter the increased risk of rejection of donor T-cell-depleted allografts is to also treat the recipient with Campath-1. Results were compared in 951 patients with leukemias and other malignancies, who received HLA-matched sibling transplants in which Campath-1 (IgM and IgG2b) was used in various protocols to deplete T cells in donor bone marrow *in vitro* and/or recipient tissues *in vivo* to control GvHD and graft rejection, respectively (Hale and Waldmann 1994). The analysis showed that the lowest rate of combined complications (defined as graft failure and/or GvHD) was achieved with a protocol of Campath-1G patient treatment 5–10 days prior to chemoradiotherapy and transplant of an allograft pretreated with Campath-1M (plus complement) *in vitro* (Hale and Waldmann 1994).

This protocol was investigated in a clinical trial of 70 patients with acute myelogenous leukemia (AML) receiving HLA-matched sibling transplants. There were two control groups – one group comprising 50 patients who received Campath-1M-depleted allografts but no Campath-1G; and a second group comprising the International Bone Marrow Registry data from 459 patients who had received nondepleted grafts and conventional GvHD prophylaxis (Hale et al. 1998). The Campath-1G *in vivo*/Campath-1M *in vitro* protocol provided superior results compared with those in the first control group in terms of decreased incidence of graft rejection (6% versus 31%), acute GvHD (4% versus 20%) and transplant-related mortality (15% versus 58% at 5 years), and compared with the second control group in terms of decreased incidence of acute GvHD (4% versus 35%, respectively) and transplant-related mortality (15% versus 26% at 5 years). The incidence of graft rejection was higher in the study group compared to the second control group (6% versus 2%). Patients in the study group achieved significantly longer 5-year survival (62% versus 35%; $P = 0.001$) and leukemia-free survival (60% versus 33%; $P = 0.002$) compared to the first control group; the risk of relapse did not differ between the treatment groups. Overall survival, leukemia-free survival, and risk of relapse did not significantly differ between the study group and the second control group (Hale et al., 1998). The results of this study confirmed that T-cell depletion is highly effective in preventing GvHD, and also showed that *in-vivo* Campath-1G administration provides additional depletion of host lymphocytes to help prevent graft rejection.

2.4.5.2 **Reduced-Intensity/Non-Myeloablative Conditioning**

Nonmyeloablative conditioning regimens have been developed to reduce transplant-related mortality and to facilitate engraftment of allogeneic stem cell trans-

plants (SCT) in patients with hematologic malignancies. Existing regimens may be fludarabine-based, may also include melphalan or busulfan, or may use total body irradiation (TBI) only. Although these regimens allow for effective allogeneic engraftment with minimal nonhematologic toxicities, risks for severe acute GvHD remains high. The use of alemtuzumab as part of a novel conditioning regimen has been investigated to determine whether it could decrease the incidence of GvHD. In a study of 44 patients with hematological malignancies (14 with NHL, 10 with Hodgkin's disease, six AML, seven multiple myeloma, three hypoplastic myelodysplastic syndrome, and one each with ALL, CLL, chronic myeloid leukemia and plasma cell leukemia), alemtuzumab 20 mg was given on days −8 to −4, fludarabine 30 mg m^{-2} on days −7 to −3, and melphalan 140 mg m^{-2} on day −2 (Kottaridis et al. 2000). Patients received unmanipulated allogeneic peripheral blood stem cells from HLA-matched siblings (n = 36) or unrelated donors (n = 8), with conventional GvHD prophylaxis [cyclosporine (CsA) with or without methotrexate]. Of 43 evaluable patients, 42 achieved sustained engraftment. No instances of Grade 3/4 acute GvHD were reported, the incidence of Grade 2 acute GvHD was 5%, and only one patient developed chronic GvHD in liver and skin. The estimated OS at 12 months was 73.2% (Kottaridis et al. 2000). Thus, this novel nonmyeloablative regimen with alemtuzumab was feasible and facilitated allogeneic engraftment while minimizing the morbidity and mortality associated with GvHD.

This conditioning regimen was evaluated further in 47 patients with hematologic malignancies who received allogeneic BMT or SCT from unrelated donors, 20 of whom were mismatched for HLA class I and/or 2 alleles (Chakraverty et al. 2002). Patients were given CsA alone for GvHD prophylaxis. High rates of allogeneic engraftment were observed, with 95.7% of patients achieving sustained neutrophil recovery and engraftment (median 13 days) and 85.1% achieving platelet recovery (median 16.5 days) with independence from platelet transfusions. The results showed that a CR was achieved in 50% of the patients who had a partial response to chemotherapy at the time of transplantation. The incidence of Grade 2 acute GvHD was 14.9%, and that of Grade 3/4 GvHD 6.4%. Three of 38 evaluable patients developed limited chronic GvHD, no patient developed extensive chronic GvHD, and no patient died due to GvHD. Progression-free survival and OS at 1 year was 61.5% and 75.5%, respectively. No difference in the incidence of GvHD or overall 1-year survival was observed between patients with HLA-mismatched and HLA-matched transplants (Chakraverty et al. 2002).

Given that CMV reactivation is a frequent complication in the SCT setting, the incidence of CMV infection was investigated after the use of this alemtuzumab-containing, nonmyeloablative conditioning regimen. The pattern of CMV reactivation and outcome was investigated in 101 patients transplanted with either unmanipulated peripheral blood stem cells from a matched family donor or unmanipulated bone marrow from an unrelated donor (Chakrabarti et al. 2002). Those who were at risk for CMV infection were CMV-seropositive

pretransplant, or received grafts from CMV-seropositive donors. Patients were then screened for CMV every week from transplantation to 100 days after transplantation. If two consecutive screenings revealed CMV positivity, then ganciclovir, foscarnet, or cidofovir were administered as preemptive therapy (Chakrabarti et al. 2002). The incidence of CMV infection was 51%, with a median time to first infection of 27 days. The majority of patients (90%) who developed an infection did so within 35 days of transplantation. Three patients who developed CMV disease died. Grade 3/4 acute GvHD developed in only 3.9% of patients. The OS probability at 12 months was 71.3% and at 18 months was 65%, and the presence of CMV infection did not influence survival rates (Chakrabarti et al. 2002).

A further study compared results from 129 patients treated in two prospective trials with a nonmyeloablative conditioning regimen with fludarabine and melphalan who received GvHD prophylaxis with either CsA and alemtuzumab or CsA and methotrexate (Perez-Simon et al. 2002). The alemtuzumab-containing prophylactic regimen was associated with a higher incidence of CMV reactivation (46.6% versus 22.7%; $P = 0.018$) despite the fact that, at the initiation of the study, a larger proportion of patients in the alemtuzumab study were CMV-negative donor/recipient cases compared to the methotrexate-based study (37% versus 9.1%; $P = 0.009$). The alemtuzumab-containing regimen was more effective than the methotrexate-containing regimen in reducing the incidence of both acute GvHD (21.7% versus 45.1%; $P = 0.006$) and chronic GvHD (5% versus 66.7%, respectively; $P < 0.001$). Both regimens were capable of inducing an early and sustained engraftment. No significant differences in OS were observed between the two regimens. Thus, both nonmyeloablative treatment regimens were effective for allogeneic engraftment, but were associated with different spectra of complications (Perez-Simon et al. 2002). Although the addition of alemtuzumab to a nonmyeloablative regimen effectively reduces the incidence of GvHD, careful monitoring of patients for CMV reactivation should be performed owing to the high rates of infection observed in the above studies.

2.4.5.3 **Safety**

It is important to bear in mind that these transplantation protocols are all investigative in nature, and that detailed safety studies on specific methods have not been carried out to date. For example, in donor T-cell-depletion protocols, the posttransplant administration of donor T cells to the patient carries an increased risk of induction of GvHD, and therefore an increased risk of mortality. In the 131-patient study described above, six patients treated with donor T cells posttransplant died of GvHD. In addition, 15 patients in that same trial died from organ failure induced by the conditioning regimen (Naparstek et al. 1995). Further, as detailed above, nonmyeloablative conditioning regimens incorporating alemtuzumab show a high incidence of CMV infection posttransplant, which may also be fatal when patients develop CMV disease (Chakrabarti et al. 2002). Further studies are clearly needed to optimize transplantation treatment protocols with alemtuzumab.

2.5
The Future Outlook

2.5.1
Other Hematologic Malignancies (Multiple Myeloma, Acute Leukemias)

Because CD52 is expressed on lymphocytes, it is feasible that alemtuzumab might have applications in many other hematologic malignancies. A preliminary study in 61 patients with the plasma cell proliferative disorders multiple myeloma (n = 23), primary systemic amyloidosis (n = 29), and monoclonal gammopathy of unspecified origin (MGUS, n = 9) showed that the CD52 antigen was highly expressed on these malignant cells (Kumar et al. 2003). The percentage of the CD38+/CD45+ subset of malignant plasma cells expressing CD52 was 73%, 88%, and 80% in the MGUS, multiple myeloma, and primary systemic amyloidosis groups, respectively, suggesting that alemtuzumab may have activity in these malignancies (Kumar et al. 2003).

A case study in a 58-year-old patient with relapsed ALL suggested that administration of alemtuzumab 30 mg three times weekly for 4 weeks, with subsequent follow-up of 30 mg three times weekly every 2 months, may induce long-term remission (Laporte et al. 2004). The patient remained free of disease 16 months after treatment initiation. Although he developed cytopenia, the condition was managed using supportive care with growth factors. Another study of three patients with ALL who had relapsed after SCT showed that alemtuzumab 30 mg every other day for five doses produced clinical responses in all patients (but no CRs) (Piccaluga et al. 2005). However, the responses were not durable, and all three patients died of progressive disease within a few months of treatment. Differences in treatment protocols and patient characteristics may have accounted for these differences in response. Nonetheless, both reports indicated that alemtuzumab has activity in patients with ALL, and this should prompt further investigation in this malignancy.

2.5.2
Prevention of GvHD in Solid Organ Transplantation

The immunosuppressive properties of alemtuzumab make it an attractive candidate for use in solid organ transplantation. Although calcineurin inhibitor-based therapies with steroids are part of the standard immunosuppressive regimens used in solid organ transplantation, long-term use of these regimens may be associated with nephrotoxicity, hyperlipidemia, and secondary diabetes. Several groups have investigated the activity and safety of alemtuzumab as induction therapy in kidney transplantation protocols. In 44 patients receiving cadaveric kidney transplants, alemtuzumab 0.3 mg kg^{-1} was administered on the day of transplantation (day 0) and on postoperative day 4, with both doses preceded by IV prednisolone (Ciancio et al. 2004). The investigators assessed the feasibility of using lower (50%) doses of tacrolimus and mycophenolate mofetil (MMF) for

maintenance immunosuppression and of eliminating corticosteroid therapy after the first week post transplantation. Patients also received CMV prophylaxis (Ciancio et al. 2004). Results showed that after 1 year, this protocol achieved 100% patient and graft survival, with no requirement for corticosteroids in 38 of 44 patients. Four patients developed biopsy-proven acute graft rejection during the first 12 months post transplantation, which was successfully treated with corticosteroid-based therapies including combination therapy with bolus corticosteroid and alemtuzumab 0.3 mg kg^{-1} in two patients. No incidences of CMV or polyoma virus infections were noted. Adverse events requiring hospitalization occurred in four patients; these included atypical pneumonia, infected lymphocele, transient Epstein–Barr virus infection, and transient diarrhea (Ciancio et al. 2004).

A larger, retrospective study compared the effects of alemtuzumab induction therapy (30 mg day^{-1}) given on the day of, and the day following, kidney transplantation in 126 patients versus outcomes in 1115 historical controls who did not receive alemtuzumab. Patients in the comparison arms had received anti-CD25 monoclonal antibodies, basiliximab, or daclizumab (n = 799), polyclonal antithymocyte globulin (n = 160) or other therapies (n = 156) that included anti-CD3 monoclonal antibody, antithymocyte globulin, or non-antibody-based therapies (Knechtle et al. 2004). Alemtuzumab was given in combination with low-dose methylprednisolone (10 mg day^{-1}) and MMF (1000 mg twice daily) and either tacrolimus or CsA (initiated when serum creatinine was <3.0 mg dL^{-1}). Overall, patients who received alemtuzumab induction therapy had significantly decreased incidence of graft rejection ($P = 0.037$) and improved graft survival ($P = 0.0159$). Similarly, among the subgroup of patients who experienced delayed graft function, alemtuzumab therapy resulted in significantly decreased incidence of graft rejection ($P = 0.0096$) and improvement in graft survival ($P = 0.0119$). Importantly, the use of alemtuzumab as induction therapy may allow for reduced reliance on steroids and nephrotoxic immunosuppressive agents, without associated increases in the incidence of infections or secondary malignancies (Knechtle et al. 2004).

Recent data have demonstrated the long-term efficacy and safety of alemtuzumab induction therapy. A five-year follow-up analysis of a cohort of 33 patients who had received alemtuzumab induction therapy for renal transplantation (20 mg day^{-1} on the day of, and the day after, transplantation, followed by half-dose CsA from day 3 onwards, and no other immunosuppressants) showed that – compared with 66 controls who had not received alemtuzumab – no significant differences were seen in rates of patient survival, graft survival, graft function, acute rejection, infection, or serious adverse events (Watson et al. 2005).

A pilot study of patients receiving kidney and pancreas transplantation assessed alemtuzumab induction therapy (a single dose of 30 mg on the day of transplantation, given after a single dose of dexamethasone 100 mg) with rapid steroid elimination in comparison with rabbit antithymocyte globulin induction with a steroid-containing regimen (Sundberg et al. 2005). After transplantation, the alemtuzumab group received MMF 2 mg day^{-1} followed by tacrolimus initiated

after either a brisk diuresis or serum creatinine <4.0 mg dL^{-1}; the control group received MMF 1 mg day^{-1}, delayed tacrolimus as above, and daily steroids tapered to 20 mg day^{-1} at week 1 and to 5 mg day^{-1} at month 2. Each group contained 16 patients of the following transplantation types: deceased donor kidney transplant (n = 9), living donor kidney transplant (n = 5), simultaneous kidney/pancreas transplant (n = 1), and sequential pancreas transplant after kidney transplant (n = 1). No significant differences were seen between groups in terms of delayed kidney graft function, incidence of thrombocytopenia, incidence of infection, creatinine clearance at 6 months, incidence of acute rejection, and patient and graft survival rates after 9 and 11 months of follow-up for the alemtuzumab and control groups, respectively (Sundberg et al. 2005). Although patients in the alemtuzumab group had a significantly lower white blood cell count and absolute lymphocyte count at the follow-up evaluations (all $P < 0.05$), there was no difference in the incidence of infections.

Another group sought to determine whether the use of alemtuzumab and MMF as induction therapy and as maintenance therapy posttransplant for pancreas/kidney or solitary pancreas transplantation would allow elimination of the use of calcineurin inhibitors and steroids. Patients (n = 75) in a pilot study were given alemtuzumab 30 mg on the day of transplantation and on postoperative days 2, 14, and 42 for induction therapy. In addition, one dose of rabbit antithymocyte globulin was given on postoperative day 4, and MMF ≥2 g day^{-1} was given for at least 6 weeks (Gruessner et al. 2005). Maintenance therapy consisted of alemtuzumab 30 mg when the absolute lymphocyte count was ≥200 mm^{-3} and MMF ≥2 g day^{-1}. Results were compared with those of 266 historical controls who received antithymocyte globulin and tacrolimus-based therapy. Patient survival at 6 months for each of the three types of recipient (pancreas transplant alone, pancreas after kidney transplant, simultaneous pancreas/kidney transplant) with alemtuzumab treatment was ≥90%, and did not differ significantly from that of historical controls (Gruessner et al. 2005). Pancreas graft survival did not differ between transplant recipient types or between the alemtuzumab group and controls. No pancreas graft rejection was seen at 6 months in the simultaneous pancreas/kidney and the pancreas after kidney transplant groups; the rate of pancreas graft rejection in the pancreas transplant alone group was 15%. At 6 months, the alemtuzumab and control groups did not differ in rates of abdominal and CMV infections, and no cases of secondary malignancies post transplant were noted with alemtuzumab therapy (Gruessner et al. 2005). Although a longer follow-up time is needed, the results of this study suggests that the combination of alemtuzumab and MMF is feasible and effective in the pancreas/kidney transplantation setting, and may eliminate the adverse effects associated with use of steroids and calcineurin inhibitors.

The above-described studies illustrate that, depending upon the dosing schedule used, alemtuzumab may enable elimination of the need for long-term steroids and reduction in or elimination of calcineurin inhibitor use posttransplant. Further studies are warranted to better establish the role of alemtuzumab in the solid organ transplant setting.

2.5.3 Applications in Autoimmune Disease

Because rheumatoid arthritis (RA) is thought to be a T-lymphocyte-mediated disorder, alemtuzumab is also being evaluated for the treatment of patients with RA. Forty-one patients with active RA refractory to treatment were given alemtuzumab 100, 250, or 400 mg over 5 or 10 days, and responses to therapy were monitored for 6 months (Isaacs et al. 1996). Treatment provided improvement in symptoms, as measured in 35 evaluable patients by 50% Paulus responses (achieved by 50% of patients at 31 days, by 43% at 59 days, by 29% at 87 days, and by 20% at 178 days) and reductions in median swollen and painful joint scores (from baseline up to day 59 at the 100-mg dose, up to day 178 at the 250-mg dose, and up to day 129 [swollen] and day 178 [painful] at the 400-mg dose). The higher doses of alemtuzumab (250 and 400 mg) appeared to be associated with improved response rates and more durable responses compared to the 100-mg dose. Infusion-related adverse events were experienced by most patients, and with the exception of a rare, fatal infection, infectious events were minor (Isaacs et al. 1996). Among 31 patients with sera available for antiglobulin testing, nine showed positive antiglobulin responses. However, the development of antiglobulin was not associated with therapeutic activity or adverse events (Isaacs et al. 1996). While there is evidence of activity of alemtuzumab in RA, its role in therapy remains to be defined with further investigation. Alemtuzumab is also being investigated in other autoimmune disease, including in patients with multiple sclerosis.

References

Albitar, M., Do, K.A., Johnson, M.M., Giles, F.J., Jilani, I., O'Brien, S., Cortes, J., Thomas, D., Rassenti, L.Z., Kipps, T.J., Kantarjian, H.M., Keating, M. (2004) Free circulating soluble CD52 as a tumor marker in chronic lymphocytic leukemia and its implication in therapy with anti-CD52 antibodies. *Cancer* 101: 999–1008.

Brett, S.J., Baxter, G., Cooper, H., Rowan, W., Regan, T., Tite, J., Rapson, N. (1996) Emergence of CD52-, glycosylphosphatidylinositol-anchor-deficient lymphocytes in rheumatoid arthritis patients following Campath-1H treatment. *Int Immunol* 8: 325–334.

CAMPATH (Alemtuzumab) product information, 2004.

Chakrabarti, S., Mackinnon, S., Chopra, R., Kottaridis, P.D., Peggs, K., O'Gorman, P., Chakraverty, R., Marshall, T., Osman, H., Mahendra, P., Craddock, C., Waldmann, H., Hale, G., Fegan, C.D., Yong, K., Goldstone, A.H., Linch, D.C., Milligan, D.W. (2002) High incidence of cytomegalovirus infection after nonmyeloablative stem cell transplantation: potential role of Campath-1H in delaying immune reconstitution. *Blood* 99: 4357–4363.

Chakraverty, R., Peggs, K., Chopra, R., Milligan, D.W., Kottaridis, P.D., Verfuerth, S., Geary, J., Thuraisundaram, D., Branson, K., Chakrabarti, S., Mahendra, P., Craddock, C., Parker, A., Hunter, A., Hale, G., Waldmann, H., Williams, C.D., Yong, K., Linch, D.C., Goldstone, A.H., Mackinnon, S. (2002) Limiting transplantation-related mortality following unrelated donor stem cell transplantation

by using a nonmyeloablative conditioning regimen. *Blood*, 99: 1071–1078.

Cheson, B.D., Bennett, J.M., Grever, M., Kay, N., Keating, M.J., O'Brien, S., Rai, K.R. (1996) National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. *Blood* 87: 4990–4997.

Cheson, B.D., Bennett, J.M., Rai, K.R., Grever, M.R., Kay, N.E., Schiffer, C.A., Oken, M.M., Keating, M.J., Boldt, D.H., Kempin, S.J., et al. (1988) Guidelines for clinical protocols for chronic lymphocytic leukemia: recommendations of the National Cancer Institute-sponsored working group. *Am J Hematol* 29: 152–163.

Cheson, B.D., Horning, S.J., Coiffier, B., Shipp, M.A., Fisher, R.I., Connors, J.M., Lister, T.A., Vose, J., Grillo-Lopez, A., Hagenbeek, A., Cabanillas, F., Klippensten, D., Hiddemann, W., Castellino, R., Harris, N.L., Armitage, J.O., Carter, W., Hoppe, R., Canellos, G.P. (1999) Report of an international workshop to standardize response criteria for non-Hodgkin's lymphomas. NCI Sponsored International Working Group. *J Clin Oncol* 17: 1244.

Ciancio, G., Burke, G.W., Gaynor, J.J., Mattiazzi, A., Roohipour, R., Carreno, M. R., Roth, D., Ruiz, P., Kupin, W., Rosen, A., Esquenazi, V., Tzakis, A.G., Miller, J. (2004) The use of Campath-1H as induction therapy in renal transplantation: preliminary results. *Transplantation* 78: 426–433.

Dearden, C.E., Matutes, E., Cazin, B., Tjonnfjord, G.E., Parreira, A., Nomdedeu, B., Leoni, P., Clark, F.J., Radia, D., Rassam, S.M., Roques, T., Ketterer, N., Brito-Babapulle, V., Dyer, M.J., Catovsky, D. (2001) High remission rate in T-cell prolymphocytic leukemia with CAMPATH-1H. *Blood* 98: 1721–1726.

Dyer, M.J., Hale, G., Hayhoe, F.G., Waldmann, H. (1989) Effects of CAMPATH-1 antibodies in vivo in patients with lymphoid malignancies: influence of antibody isotype. *Blood* 73: 1431–1439.

Elter, T., Borchmann, P., Schulz, H., Reiser, M., Trelle, S., Schnell, R., Jensen, M., Staib, P., Schinkothe, T., Stutzer, H., Rech, J., Gramatzki, M., Aulitzky, W., Hasan, I., Josting, A., Hallek, M., Engert, A. (2005) Fludarabine in combination with alemtuzumab Is effective and feasible in patients with relapsed or refractory B-cell chronic lymphocytic leukemia: results of a phase II trial. *J Clin Oncol* 23: 7024–7031.

Enblad, G., Hagberg, H., Erlanson, M., Lundin, J., MacDonald, A.P., Repp, R., Schetelig, J., Seipelt, G., Osterborg, A. (2004) A pilot study of alemtuzumab (anti-CD52 monoclonal antibody) therapy for patients with relapsed or chemotherapy-refractory peripheral T-cell lymphomas. *Blood* 103: 2920–2924.

Faderl, S., Thomas, D.A., O'Brien, S., Garcia-Manero, G., Kantarjian, H.M., Giles, F.J., Koller, C., Ferrajoli, A., Verstovsek, S., Pro, B., Andreeff, M., Beran, M., Cortes, J., Wierda, W., Tran, N., Keating, M.J. (2003) Experience with alemtuzumab plus rituximab in patients with relapsed and refractory lymphoid malignancies. *Blood* 101: 3413–3415.

Ferrajoli, A., O'Brien, S.M., Cortes, J.E., Giles, F.J., Thomas, D.A., Faderl, S., Kurzrock, R., Lerner, S., Kontoyiannis, D. P., Keating, M.J. (2003) phase II study of alemtuzumab in chronic lymphoproliferative disorders. *Cancer* 98: 773–778.

Fløisand, Y., Brinch, L., Gedde-Dahl, T., Tjonnfjord, G.E. (2004) [Treatment of T-cell prolymphocytic leukemia with monoclonal anti-CD52 antibody (alemtuzumab]. *Tidsskr Nor Laegeforen* 124: 768–770.

Gautschi, O., Blumenthal, N., Streit, M., Solenthaler, M., Hunziker, T., Zenhausern, R. (2004) Successful treatment of chemotherapy-refractory Sezary syndrome with alemtuzumab (Campath-1H). *Eur J Haematol* 72: 61–63.

Gilleece, M.H. and Dexter, T.M. (1993) Effect of Campath-1H antibody on human hematopoietic progenitors in vitro. *Blood* 82: 807–812.

Ginaldi, L., De, M.M., Matutes, E., Farahat, N., Morilla, R., Dyer, M.J., Catovsky, D. (1998) Levels of expression of CD52 in normal and leukemic B and T cells: correlation with in vivo therapeutic responses to Campath-1H. *Leuk Res* 22: 185–191.

Golay, J., Manganini, M., Rambaldi, A., Introna, M. (2004) Effect of alemtuzumab on neoplastic B cells. *Haematologica* 89: 1476–1483.

Greenwood, J., Clark, M., Waldmann, H. (1993) Structural motifs involved in human IgG antibody effector functions. *Eur J Immunol* 23: 1098–1104.

Gruessner, R.W., Kandaswamy, R., Humar, A., Gruessner, A.C., Sutherland, D.E. (2005) Calcineurin inhibitor- and steroid-free immunosuppression in pancreas-kidney and solitary pancreas transplantation. *Transplantation* 79: 1184–1189.

Hale, G. (2001) The CD52 antigen and development of the CAMPATH antibodies. *Cytotherapy* 3: 137–143.

Hale, G., Waldmann, H. (1994) Control of graft-versus-host disease and graft rejection by T cell depletion of donor and recipient with Campath-1 antibodies. Results of matched sibling transplants for malignant diseases. *Bone Marrow Transplant* 13: 597–611.

Hale, G., Bright, S., Chumbley, G., Hoang, T., Metcalf, D., Munro, A.J., Waldmann, H. (1983a) Removal of T cells from bone marrow for transplantation: a monoclonal antilymphocyte antibody that fixes human complement. *Blood* 62: 873–882.

Hale, G., Swirsky, D.M., Hayhoe, F.G., Waldmann, H. (1983b) Effects of monoclonal anti-lymphocyte antibodies in vivo in monkeys and humans. *Mol Biol Med* 1: 321–334.

Hale, G., Clark, M., Waldmann, H. (1985) Therapeutic potential of rat monoclonal antibodies: isotype specificity of antibody-dependent cell-mediated cytotoxicity with human lymphocytes. *J Immunol* 134: 3056–3061.

Hale, G., Dyer, M.J., Clark, M.R., Phillips, J. M., Marcus, R., Riechmann, L., Winter, G., Waldmann, H. (1988) Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody CAMPATH-1H. *Lancet* 2: 1394–1399.

Hale, G., Zhang, M.J., Bunjes, D., Prentice, H.G., Spence, D., Horowitz, M.M., Barrett, A.J., Waldmann, H. (1998) Improving the outcome of bone marrow transplantation by using CD52 monoclonal antibodies to prevent graft-versus-host disease and graft rejection. *Blood* 92: 4581–4590.

Hale, G., Rebello, P., Brettman, L.R., Fegan, C., Kennedy, B., Kimby, E., Leach, M., Lundin, J., Mellstedt, H., Moreton, P., Rawstron, A.C., Waldmann, H., Österborg, A., Hillmen, P. (2004) Blood concentrations of alemtuzumab and antiglobulin responses in patients with chronic lymphocytic leukemia following intravenous or subcutaneous routes of administration. *Blood* 104: 948–955.

Hillmen, P., Skotnicki, A., Robak, T., Jaksic, B., Dmoszynska, A., Sirard, C., Mayer, J. (2006) Preliminary phase 3 efficacy and safety of alemtuzumab vs chlorambucil as front-lint therapy for patients with progressive B-cell chronic lymphocytic leukemia (BCLL). *J Clin Oncol* 24 (Suppl.1), 339s, Abstract 6511.

Isaacs, J.D., Manna, V.K., Rapson, N., Bulpitt, K.J., Hazleman, B.L., Matteson, E. L., St Clair, E.W., Schnitzer, T.J., Johnston, J.M. (1996) CAMPATH-1H in rheumatoid arthritis – an intravenous dose-ranging study. *Br J Rheumatol* 35: 231–240.

Keating, M.J., Cazin, B., Coutre, S., Birhiray, R., Kovacsovics, T., Langer, W., Leber, B., Maughan, T., Rai, K., Tjonnfjord, G., Bekradda, M., Itzhaki, M., Herait, P. (2002a) Campath-1H treatment of T-cell prolymphocytic leukemia in patients for whom at least one prior chemotherapy regimen has failed. *J Clin Oncol* 20: 205–213.

Keating, M.J., Flinn, I., Jain, V., Binet, J.L., Hillmen, P., Byrd, J., Albitar, M., Brettman, L., Santabarbara, P., Wacker, B., Rai, K.R. (2002b) Therapeutic role of alemtuzumab (Campath-1H) in patients who have failed fludarabine: results of a large international study. *Blood* 99: 3554–3561.

Keating, M., O'Brien, S., Kontoyiannis, D., Plunkett, W., Koller, C., Beran, M., Lerner, S., Kantarjian, H. (2002c) Results of first salvage therapy for patients refractory to a fludarabine regimen in chronic lymphocytic leukemia. *Leuk Lymphoma* 43: 1755–1762.

Keating, M., Coutre, S., Rai, K., Osterborg, A., Faderl, S., Kennedy, B., Kipps, T., Bodey, G., Byrd, J.C., Rosen, S., Dearden, C., Dyer, M.J., Hillmen, P. (2004)

Management guidelines for use of alemtuzumab in B-cell chronic lymphocytic leukemia. *Clin Lymphoma* 4: 220–227.

Kennedy, B., Rawstron, A., Carter, C., Ryan, M., Speed, K., Lucas, G., Hillmen, P. (2002) Campath-1H and fludarabine in combination are highly active in refractory chronic lymphocytic leukemia. *Blood* 99: 2245–2247.

Kennedy, G.A., Seymour, J.F., Wolf, M., Januszewicz, H., Davison, J., McCormack, C., Ryan, G., Prince, H.M. (2003) Treatment of patients with advanced mycosis fungoides and Sezary syndrome with alemtuzumab. *Eur J Haematol* 71: 250–256.

Khorana, A., Bunn, P., McLaughlin, P., Vose, J., Stewart, C., Czuczman, M.S. (2001) A phase II multicenter study of CAMPATH-1H antibody in previously treated patients with nonbulky non-Hodgkin's lymphoma. *Leuk Lymphoma* 41: 77–87.

Kirchhoff, C. (1996) CD52 is the 'major maturation-associated' sperm membrane antigen. *Mol Hum Reprod* 2: 9–17.

Knechtle, S.J., Fernandez, L.A., Pirsch, J.D., Becker, B.N., Chin, L.T., Becker, Y.T., Odorico, J.S., D'alessandro, A.M., Sollinger, H.W. (2004) Campath-1H in renal transplantation: The University of Wisconsin experience. *Surgery* 136: 754–760.

Kottaridis, P.D., Milligan, D.W., Chopra, R., Chakraverty, R.K., Chakrabarti, S., Robinson, S., Peggs, K., Verfuerth, S., Pettengell, R., Marsh, J.C., Schey, S., Mahendra, P., Morgan, G.J., Hale,G., Waldmann, H., de Elvira, M.C., Williams, C.D., Devereux, S., Linch, D.C., Goldstone, A.H., Mackinnon, S. (2000) In vivo CAMPATH-1H prevents graft-versus-host disease following nonmyeloablative stem cell transplantation. *Blood* 96: 2419–2425.

Kumar, S., Kimlinger, T.K., Lust, J.A., Donovan, K., Witzig, T.E. (2003) Expression of CD52 on plasma cells in plasma cell proliferative disorders. *Blood* 102: 1075–1077.

Laporte, J.P., Isnard, F., Garderet, L., Fouillard, L., Gorin, N.C. (2004) Remission of adult acute lymphocytic leukaemia with alemtuzumab. *Leukemia* 18: 1557–1558.

Lozanski, G., Heerema, N.A., Flinn, I.W., Smith, L., Harbison, J., Webb, J., Moran, M., Lucas, M., Lin, T., Hackbarth, M.L., Proffitt, J.H., Lucas, D., Grever, M.R., Byrd, J.C. (2004) Alemtuzumab is an effective therapy for chronic lymphocytic leukemia with p53 mutations and deletions. *Blood* 103: 3278–3281.

Lundin, J., Osterborg, A., Brittinger, G., Crowther, D., Dombret, H., Engert, A., Epenetos, A., Gisselbrecht, C., Huhn, D., Jaeger, U., Thomas, J., Marcus, R., Nissen, N., Poynton, C., Rankin, E., Stahel, R., Uppenkamp, M., Willemze, R., Mellstedt, H. (1998) CAMPATH-1H monoclonal antibody in therapy for previously treated low-grade non-Hodgkin's lymphomas: a phase II multicenter study. European Study Group of CAMPATH-1H Treatment in Low-Grade Non-Hodgkin's Lymphoma. *J Clin Oncol* 16: 3257–3263.

Lundin, J., Kimby, E., Bjorkholm, M., Broliden, P.A., Celsing, F., Hjalmar, V., Mollgard, L., Rebello, P., Hale, G., Waldmann, H., Mellstedt, H., Osterborg, A. (2002) phase II trial of subcutaneous anti-CD52 monoclonal antibody alemtuzumab (Campath-1H) as first-line treatment for patients with B-cell chronic lymphocytic leukemia (B-CLL). *Blood* 100: 768–773.

Lundin, J., Hagberg, H., Repp, R., Cavallin-Stahl, E., Freden, S., Juliusson, G., Rosenblad, E., Tjonnfjord, G., Wiklund, T., Osterborg, A. (2003) phase 2 study of alemtuzumab (anti-CD52 monoclonal antibody) in patients with advanced mycosis fungoides/Sezary syndrome. *Blood* 101: 4267–4272.

Lundin, J., Porwit-MacDonald, A., Rossmann, E.D., Karlsson, C., Edman, P., Rezvany, M.R., Kimby, E., Osterborg, A., Mellstedt, H. (2004) Cellular immune reconstitution after subcutaneous alemtuzumab (anti-CD52 monoclonal antibody, CAMPATH-1H) treatment as first-line therapy for B-cell chronic lymphocytic leukaemia. *Leukemia* 18: 484–490.

Montillo, M., Cafro, A.M., Tedeschi, A., Brando, B., Oreste, P., Veronese, S., Rossi, V., Cairoli, R., Pungolino, E., Morra, E. (2002) Safety and efficacy of subcutaneous Campath-1H for treating residual disease

in patients with chronic lymphocytic leukemia responding to fludarabine. *Haematologica* 87: 695–700.

Montillo, M., Tedeschi, A., Miqeuleiz, S., Veronese, S., Rossi, V., Cairoli, R., Intropido L., Ricci, F., Colosimo, A., Scarpati, B., Montagna, M., Nichelatti, M., Regazzi, M., Morra, E. (2006) Alemtuzumab as consolidation after a response to fludarabine is effective in purging residual disease in patients with chronic lymphocytic leukemia. *J Clin Oncol* 24: 2337–2342.

Moreton, P., Kennedy, B., Lucas, G., Leach, M., Rassam, S.M., Haynes, A., Tighe, J., Oscier, D., Fegan, C., Rawstron, A., Hillmen, P. (2005) Eradication of minimal residual disease in B-cell chronic lymphocytic leukemia after alemtuzumab therapy is associated with prolonged survival. *J Clin Oncol* 23: 2971–2979.

Naparstek, E., Or, R., Nagler, A., Cividalli, G., Engelhard, D., Aker, M., Gimon, Z., Manny, N., Sacks, T., Tochner, Z., et al. (1995) T-cell-depleted allogeneic bone marrow transplantation for acute leukaemia using Campath-1 antibodies and post-transplant administration of donor's peripheral blood lymphocytes for prevention of relapse. *Br J Haematol* 89: 506–515.

Nückel, H., Frey, U.H., Roth, A., Duhrsen, U., Siffert, W. (2005) Alemtuzumab induces enhanced apoptosis in vitro in B-cells from patients with chronic lymphocytic leukemia by antibody-dependent cellular cytotoxicity. *Eur J Pharmacol* 514: 217–224.

O'Brien, S.M., Kantarjian, H.M., Thomas, D.A., Cortes, J., Giles, F.J., Wierda, W.G., Koller, C.A., Ferrajoli, A., Browning, M., Lerner, S., Albitar, M., Keating, M.J. (2003) Alemtuzumab as treatment for residual disease after chemotherapy in patients with chronic lymphocytic leukemia. *Cancer* 98: 2657–2663.

Österborg, A., Fassas, A.S., Anagnostopoulos, A., Dyer, M.J., Catovsky, D., Mellstedt, H. (1996) Humanized CD52 monoclonal antibody Campath-1H as first-line treatment in chronic lymphocytic leukaemia. *Br J Haematol* 93: 151–153.

Österborg, A., Dyer, M.J.S., Bunjes, D., Pangalis, G.A., Bastion, Y., Catovsky, D., Mellstedt, H. (1997) phase II multicenter study of human CD52 antibody in previously treated chronic lymphocytic leukemia. *J Clin Oncol* 15: 1567–1574.

Perez-Simon, J.A., Kottaridis, P.D., Martino, R., Craddock, C., Caballero, D., Chopra, R., Garcia-Conde, J., Milligan, D.W., Schey, S., Urbano-Ispizua, A., Parker, A., Leon, A., Yong, K., Sureda, A., Hunter, A., Sierra, J., Goldstone, A.H., Linch, D.C., San Miguel, J.F., Mackinnon, S. (2002) Nonmyeloablative transplantation with or without alemtuzumab: comparison between 2 prospective studies in patients with lymphoproliferative disorders. *Blood* 100: 3121–3127.

Perkins, J.G., Flynn, J.M., Howard, R.S., Byrd, J.C. (2002) Frequency and type of serious infections in fludarabine-refractory B-cell chronic lymphocytic leukemia and small lymphocytic lymphoma: implications for clinical trials in this patient population. *Cancer* 94: 2033–2039.

Piccaluga, P.P., Martinelli, G., Malagola, M., Rondoni, M., Bonifazi, F., Bandini, G., Visani, G., Baccarani, M. (2005) Alemtuzumab in the treatment of relapsed acute lymphoid leukaemia. *Leukemia* 19: 135.

Rai, K.R., Stephenson, J. (2001) *Campath-1H: emerging frontline therapy in chronic lymphocytic leukemia*. Parthenon Publishing, Pearl River, NY, p. 62.

Rai, K.R., Freter, C.E., Mercier, R.J., Cooper, M.R., Mitchell, B.S., Stadtmauer, E.A., Santabarbara, P., Wacker, B., Brettman, L. (2002) Alemtuzumab in previously treated chronic lymphocytic leukemia patients who also had received fludarabine. *J Clin Oncol* 20: 3891–3897.

Ravandi, F. and Faderl, S. (2006) Complete response in a patient with adult T-cell leukemia (ATL) treated with combination of alemtuzumab and pentostatin. *Leuk Res* 30: 103–105.

Rawstron, A.C., Kennedy, B., Evans, P.A., Davies, F.E., Richards, S.J., Haynes, A.P., Russel, N.H., Hale, G., Morgan, G.J., Jack, A.S., Hillmen, P. (2001) Quantitation of minimal disease levels in chronic lymphocytic leukemia using a sensitive flow cytometric assay improves the prediction of outcome and can be used to optimize therapy. *Blood* 98: 29–35.

Rebello, P.R., Hale, G., Friend, P.J., Cobbold, S.P., Waldmann, H. (1999) Anti-globulin responses to rat and humanized CAMPATH-1 monoclonal antibody used to treat transplant rejection. *Transplantation* 68: 1417–1420.

Rebello, P., Cwynarski, K., Varughese, M., Eades, A., Apperley, J.F., Hale, G. (2001) Pharmacokinetics of CAMPATH-1H in BMT patients. *Cytotherapy* 3: 261–267.

Riechmann, L., Clark, M., Waldmann, H., Winter, G. (1988) Reshaping human antibodies for therapy. *Nature* 332: 323–327.

Rituxan (Rituximab) product information, 2005. Genentech, South San Francisco, CA.

Rowan, W., Tite, J., Topley, P., Brett, S.J. (1998) Cross-linking of the CAMPATH-1 antigen (CD52) mediates growth inhibition in human B- and T-lymphoma cell lines, and subsequent emergence of CD52-deficient cells. *Immunology* 95: 427–436.

Stanglmaier, M., Reis, S., Hallek, M. (2004) Rituximab and alemtuzumab induce a nonclassic, caspase-independent apoptotic pathway in B-lymphoid cell lines and in chronic lymphocytic leukemia cells. *Ann Hematol* 83: 634–645.

Sundberg, A.K., Roskopf, J.A., Hartmann, E. L., Farney, A.C., Rohr, M.S., Stratta, R.J. (2005) Pilot study of rapid steroid elimination with alemtuzumab induction therapy in kidney and pancreas transplantation. *Transplant Proc* 37: 1294–1296.

Uppenkamp, M., Engert, A., Diehl, V., Bunjes, D., Huhn, D., Brittinger, G. (2002) Monoclonal antibody therapy with CAMPATH-1H in patients with relapsed high- and low-grade non-Hodgkin's lymphomas: a multicenter phase I/II study. *Ann Hematol* 81: 26–32.

Waldmann, H., Polliak, A., Hale, G., Or, R., Cividalli, G., Weiss, L., Weshler, Z., Samuel, S., Manor, D., Brautbar, C., et al. (1984) Elimination of graft-versus-host disease by in-vitro depletion of alloreactive lymphocytes with a monoclonal rat anti-human lymphocyte antibody (CAMPATH-1). *Lancet* 2: 483–486.

Watson, C.J., Bradley, J.A., Friend, P.J., Firth, J., Taylor, C.J., Bradley, J.R., Smith, K.G., Thiru, S., Jamieson, N.V., Hale, G., Waldmann, H., Calne, R. (2005) Alemtuzumab (CAMPATH 1H) induction therapy in cadaveric kidney transplantation – efficacy and safety at five years. *Am J Transplant* 5: 1347–1353.

Wendtner, C.M., Ritgen, M., Schweighofer, C.D., Fingerle-Rowson, G., Campe, H., Jager, G., Eichhorst, B., Busch, R., Diem, H., Engert, A., Stilgenbauer, S., Dohner, H., Kneba, M., Emmerich, B., Hallek, M. (2004) Consolidation with alemtuzumab in patients with chronic lymphocytic leukemia (CLL) in first remission – experience on safety and efficacy within a randomized multicenter phase III trial of the German CLL Study Group (GCLLSG). *Leukemia* 18: 1093–1101.

Xia, M.Q., Hale, G., Lifely, M.R., Ferguson, M.A., Campbell, D., Packman, L., Waldmann, H. (1993) Structure of the CAMPATH-1 antigen, a glycosylphosphatidylinositol-anchored glycoprotein which is an exceptionally good target for complement lysis. *Biochem J* 293 (Pt 3): 633–640.

Zeitlinger, M.A., Schmidinger, M., Zielinski, C.C., Chott, A., Raderer, M. (2005) Effective treatment of a peripheral T-cell lymphoma/lymphoepitheloid cell variant (Lennert's lymphoma) refractory to chemotherapy with the CD-52 antibody alemtuzumab. *Leuk Lymphoma* 46: 771–774.

Zhang, Z., Zhang, M., Goldman, C.K., Ravetch, J.V., Waldmann, T.A. (2003) Effective therapy for a murine model of adult T-cell leukemia with the humanized anti-CD52 monoclonal antibody, Campath-1H. *Cancer Res* 63: 6453–6457.

Zinzani, P.L., Alinari, L., Tani, M., Fina, M., Pileri, S., Baccarani, M. (2005) Preliminary observations of a phase II study of reduced-dose alemtuzumab treatment in patients with pretreated T-cell lymphoma. *Haematologica* 90: 702–703.

3
Bevacizumab (Avastin)

Eduardo Díaz-Rubio, Edith A. Perez, and Guiseppe Giaccone

3.1
Introduction

3.1.1
Angiogenesis is Vital for Tumor Development

Angiogenesis, the process whereby new blood vessels are formed from pre-existing vessels, is essential for the growth and development of both normal tissues and tumors (Carmeliet 2003; Carmeliet and Jain 2000). Physiologically, angiogenesis plays a critical role in embryonic and infant growth and development, but in healthy adults this role is limited to wound healing and the menstrual cycle (Ferrara et al. 2003). The malignant progression of solid tumors is dependent on pathological angiogenesis: tumors with a volume of less than 2 mm^3 receive their nutrients by diffusion, but in order to grow any larger, tumors must establish a vascular supply. Tumor cells not only rely on angiogenesis to ensure a steady supply of nutrients and growth factors, but also use the newly formed blood vessels as a route for metastatic spread.

Angiogenesis is a complex process that is tightly controlled by factors that stimulate or inhibit the formation of new blood vessels (Carmeliet 2003; Carmeliet and Jain 2000; Jain 2003). In order to grow, tumors must trigger the development of their own blood supply, which they do by disrupting the delicate balance of pro- and anti-angiogenic factors (Ferrara et al. 2003). Tumor mutations can tip the balance in favor of pro-angiogenic factors, stimulating the development of new blood vessels in and around the lesion – this is termed the "angiogenic switch" (Bergers and Benjamin 2003). Because tumor angiogenesis results from abnormalities in the tightly controlled expression of pro- and anti-angiogenic factors, the resulting tumor vasculature has abnormal structure and function. Compared with vasculature in healthy tissues, tumor blood vessels are immature, abnormally branched, irregularly shaped, and prone to hemorrhage. Tumor vessels are also leaky, because they lack the tight endothelial layer seen in normal vasculature. This leads to a rise in interstitial fluid pressure in tumor tissue,

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which compromises the penetration of oxygen and macromolecules, such as chemotoxic agents, into the lesion.

3.1.2 Vascular Endothelial Growth Factor: A Key Angiogenic Factor

Of the different pro-angiogenic factors, vascular endothelial growth factor (VEGF) is the key mediator of both normal and tumor angiogenesis (Fig. 3.1) (Ferrara 2001; Jain 2003; Yancopoulos et al. 2000). VEGF is a homodimeric heparin-binding glycoprotein with a molecular weight of approximately 45 kDa. VEGF, also known as VEGF-A, belongs to a subfamily that includes the growth factors VEGF-B, VEGF-C, VEGF-D and platelet growth factor (Shibuya 2001). At least four main VEGF isoforms exist, the most common being $VEGF_{165}$, which consists of 165 amino acids (Ferrara 2001; Ferrara et al. 2003). VEGF binds primarily to two receptors situated on vascular endothelial cells: VEGF receptor-1, also known as Flt-1, and VEGF receptor-2, also known as Flk-1 or KDR (Ferrara et al. 2004).

Activation of the VEGF pathway through binding of VEGF to VEGF receptor-2 triggers a series of signaling events that promote vascular endothelial cell growth, migration and survival, as well as the mobilization of endothelial progenitor cells from the bone marrow into the circulation (Ferrara et al. 2003, 2004). Under normal conditions, the balanced production of VEGF and other pro-angiogenic factors ensures that blood vessels are formed when

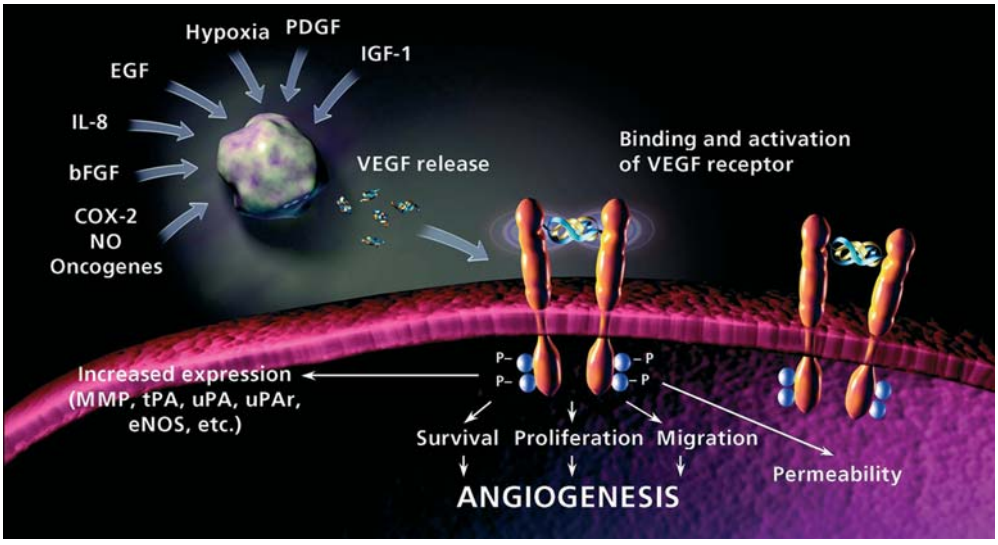

Fig. 3.1 The central role of vascular endothelial growth factor (VEGF) in angiogenesis.

and where they are physiologically required. However, in tumors the uncontrolled release of VEGF drives many of the processes central to tumor angiogenesis: it stimulates vascular endothelial cells to grow and migrate to form blood vessels; and it enables the resulting morphologically and functionally abnormal vessels to survive inside tumors. VEGF expression has also been shown to correlate with microvessel density and metastatic spread in some tumor types (Bergers and Benjamin 2003; Ferrara et al. 2004). The induction of VEGF expression appears to be characteristic of many tumor types, and studies have demonstrated that increased VEGF levels are a predictor of poor prognosis for cancer patients (Poon et al. 2001).

VEGF is also known as vascular permeability factor because initially it was shown to induce vascular permeability; in tumors, it drives the formation of holes in the walls of the vasculature and increases the permeability of blood vessels to circulating macromolecules (Ferrara et al. 2003). This allows plasma proteins to leak out of tumor blood vessels to form an extravascular matrix, which enhances the environment for subsequent endothelial cell growth. Increasing the permeability of the tumor vasculature also results in higher interstitial pressure, which reverses the normal pressure gradients within tissues and impairs the delivery of oxygen and chemotherapeutic drugs to the tumor (Jain 2002).

A variety of factors up-regulate VEGF expression (Bergers and Benjamin 2003; Ferrara 2002; Ferrara et al. 2004). Hypoxia is the primary stimulus, triggering vessel growth by signaling through hypoxia-inducible factors (HIFs), especially HIF-1α. As a tumor grows, it outstrips its blood supply and becomes hypoxic, which prompts an increase in HIF-1α levels. HIF-1α induces VEGF expression and the stimulation of further angiogenesis. In this way, VEGF production by tumors creates a positive feedback loop that supports the development of further vasculature and thus tumor growth. Other stimuli that up-regulate VEGF production include major growth factors that are frequently expressed by tumors; these include epidermal growth factor (EGF), keratinocyte growth factor 1, insulin-like growth factor 1 (IGF-1), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) (Frank et al. 1995). Hormones such as thyroid-stimulating hormone (TSH), angiotensin II and adrenocorticotropic hormone (ACTH) also induce VEGF expression (Shifren et al. 1998; Soh et al. 1996), as do inflammatory cytokines such as interleukin (IL)-1α, IL-6 and prostaglandin E_2 (Ben-Av et al. 1995; Cohen et al. 1996). In addition, mutations in tumor suppressor genes including *p53* and oncogenes such as *ras* up-regulate VEGF expression.

3.1.3
Targeting Angiogenesis and VEGF: A Rational Treatment Option

The central role of VEGF in tumor angiogenesis makes the former an attractive target for anticancer therapy. VEGF inhibition has the potential to cause regression of immature tumor blood vessels, to inhibit the development of new tumor vasculature, and to reverse the structural and functional abnormalities in existing tumor vasculature.

Importantly, inhibiting VEGF is likely to block tumor angiogenesis without compromising healthy vasculature. As angiogenesis plays a limited physiological role in healthy adults, the inhibition of VEGF should have minimal undesired effects on normal physiological processes and thus have relatively few adverse side effects.

Two additional factors make VEGF an attractive therapeutic target. First, because VEGF circulates in the blood and acts directly on vascular endothelial cells, drugs that target VEGF do not need to penetrate tumor tissue in order to inhibit tumor angiogenesis. Second, VEGF acts on endothelial cells, which are relatively stable and therefore less likely to mutate to a treatment-resistant phenotype than genetically unstable tumor cells. This makes endothelial cells a more attractive target than tumor cells for long-term therapy (Kerbel and Folkman 2002).

Two different approaches to targeting VEGF have been investigated: one approach involves targeting the VEGF molecule itself, while the other focuses on the cell-surface receptors for VEGF (Gabrilovich et al. 1999; Vitaliti et al. 2000). One disadvantage of receptor-targeted approaches is that the VEGF receptors may also bind members of the VEGF superfamily other than VEGF, and their inhibition could influence physiological systems other than angiogenesis. Targeting the VEGF molecule itself enables selective action on the best-known angiogenic factor, and is also likely to be the safer option, with minimal adverse side effects on normal physiology.

The most advanced approach to VEGF inhibition is the humanized monoclonal antibody bevacizumab (Avastin), which inhibits the VEGF ligand and is currently the only anti-angiogenic antibody approved for the treatment of cancer. Other anti-angiogenic agents in relatively advanced stages of development include sunitinib (Sutent), sorafenib (Nexavar) and PTK/ZK (vatalanib), all of which are small-molecule tyrosine kinase inhibitors targeting one or more of the VEGF receptors as well as other tyrosine kinase receptors. In order that the information regarding bevacizumab can be considered in context, the agents are briefly described at this point:

- Sunitinib is an orally active compound that inhibits VEGF receptor-2 as well as the PDGF, Flt3 and kit receptors; as such, it has the potential to inhibit both angiogenesis and tumor cell growth. Based on positive interim data from phase III trials, sunitinib has recently been submitted for regulatory approval in the USA as monotherapy for patients with refractory gastrointestinal (GI) stromal tumors and renal cell carcinoma (RCC) (Demetri et al. 2005; Motzer et al. 2005).
- Sorafenib is an oral cytostatic pan-kinase inhibitor targeting the VEGF, PDGF and Raf kinase receptors. It has recently been launched as monotherapy for the treatment of patients with advanced refractory RCC in the USA following a phase III trial demonstrating improved disease

control rates and progression-free survival over placebo (Escudier et al. 2005). Further trials of both sunitinib and sorafenib are ongoing.
- Vatalanib is an oral VEGF receptor tyrosine kinase inhibitor. Data from two phase III trials of vatalanib in combination with FOLFOX4 (5-fluorouracil [5-FU]/ leucovorin [LV] plus oxaliplatin [Eloxatin]) as either first- or second-line therapy have been reported; neither of these trials has demonstrated any benefit of the addition of vatalanib to FOLFOX4 in terms of response or progression-free survival, and it seems unlikely that there will be an overall survival benefit (Hecht et al. 2005; Schering AG 2005). Vatalanib continues to be assessed in clinical trials for colorectal cancer (CRC), RCC and non-small cell lung cancer (NSCLC).

Based on these data, it is clear that the availability of agents with anti-angiogenic activity is expanding rapidly. However, the data raise questions regarding the utility of targeting only the VEGF receptors, and whether inhibition of receptors involved in other tumor growth and survival mechanisms is required to have anticancer activity. For a number of reasons, the data mentioned do not provide answers to these issues. However, as described below, inhibition of the VEGF ligand using the humanized anti-VEGF monoclonal antibody has proved to be clinically effective in combination with chemotherapy in metastatic CRC, NSCLC and breast cancer.

3.2 The Development of Bevacizumab

3.2.1 Origin and Genetic Engineering

Bevacizumab is a humanized monoclonal antibody developed from A4.6.1, a murine antibody that targets human VEGF. A4.6.1 recognizes a particular sequence of amino acids within the human VEGF molecule (Wiesmann et al. 1997) and binds at this specific site with high affinity [dissociation constant (K_d) = 8×10^{-10} M]. Binding of A4.6.1 to VEGF prevents VEGF from binding to and activating its receptors (Ferrara et al. 2003).

As a murine protein, A4.6.1 is likely to provoke an immune response in humans and is therefore unsuitable for use in patients. To resolve this problem, the A4.6.1 antibody was humanized to form bevacizumab, which is 93% human and 7% mouse in origin. It was engineered by site-directed mutagenesis of a human DNA framework; in this process, the six regions that determine the binding specificity of A4.6.1 were transferred to a human DNA framework (Presta et al. 1997). In

order to provide equivalent binding affinity, seven amino acid residues within the framework were changed to their corresponding murine residues.

3.2.2 Mode of Action

Bevacizumab blocks the binding of all VEGF isoforms to their receptors by binding to free VEGF, thus removing VEGF from the circulation (Presta et al. 1997). By targeting circulating VEGF rather than a specific receptor, bevacizumab inhibits VEGF activity at all the receptors to which VEGF binds.

Anti-VEGF therapy inhibits the development of new tumor vasculature, which is essential for further tumor growth and metastasis. VEGF inhibition in human tumor cell lines has been shown to inhibit vascular endothelial cell proliferation and migration, and to suppress new vascular sprouting within 24 h of administration (Baluk et al. 2005). In a mouse model, anti-VEGF therapy blocked new vessel formation and caused the collapse of pre-existing vessels within 9 days (Osusky et al. 2004). Preclinical studies have demonstrated that targeting VEGF inhibits tumor angiogenesis, blocks tumor growth (Kim et al. 1993), and reduces the number and size of liver metastases in nude mice (Warren et al. 1995). The removal of anti-VEGF therapy leads to rapid regrowth of capillaries and the resumption of tumor growth (Baluk et al. 2005), suggesting that continuous VEGF inhibition is required to gain the greatest antitumor benefit.

As well as inhibiting new tumor blood vessel formation, anti-VEGF therapy causes regression of existing tumor vasculature. Throughout the early stages of tumor development, the formation of new blood vessels remains dependent upon a constant supply of VEGF (Bergers and Benjamin 2003). VEGF is a survival factor for vascular tumor cells, protecting them from apoptosis and promoting tumor growth (Harmey and Bouchier-Hayes 2002). Without a steady supply of VEGF, vascular endothelial cells undergo apoptosis, and any recently formed tumor microvasculature disintegrates. A single infusion of anti-VEGF therapy has been shown to reduce tumor microvascular density in humans (Willett et al. 2004).

VEGF inhibition may also result in the remodeling of tumor vasculature: immature vessels are pruned away, leaving only mature, normally functioning blood vessels (Jain 2001, 2005). This process leads to a more ordered tumor vasculature, a more efficient blood supply, and decreased interstitial fluid pressure inside the tumor (Jain 2001), such that chemotoxic agents and oxygen can penetrate the tumor more effectively. Thus, anti-VEGF therapy may improve the efficacy of chemotherapy and radiotherapy.

3.2.3 Preclinical Activity of Bevacizumab

Bevacizumab and A4.6.1 have demonstrated almost identical activity in preclinical tumor growth models; bevacizumab inhibited VEGF-induced proliferation of

endothelial cells *in vitro* with an ED_{50} (effective dose) of $50 \pm 5\,ng\,mL^{-1}$, while the ED_{50} for A4.6.1 is $48 \pm 8\,ng\,mL^{-1}$ (Presta et al. 1997). Both antibodies also block angiogenesis and suppress the growth of human tumor xenografts in mice to similar extents (Presta et al. 1997). Thus, in human cells and tissues, these two antibodies are pharmacologically equivalent.

Bevacizumab and A4.6.1 have also demonstrated synergistic activity in combination with chemotherapy in preclinical models. A synergistic antitumor effect was observed when bevacizumab ($2.5\,mg\,kg^{-1}$) was combined with capecitabine (Xeloda) ($360\,mg\,kg^{-1}$); the combination inhibited the growth of colorectal tumor xenografts in nude mice more effectively and for longer than either agent alone (Shen et al. 2004). Bevacizumab has also shown synergy with paclitaxel (Taxol) and trastuzumab (Herceptin), an inhibitor of the human epidermal growth factor receptor (HER)2. Several other studies have demonstrated the potential of bevacizumab in combination with standard chemotherapies. *In vitro*, bevacizumab overcame VEGF-induced protection of endothelial cells against docetaxel (Taxotere) treatment (Sweeney et al. 2001). *In vivo*, bevacizumab enhanced tumor suppression in animals when added to cisplatin (Platinol) (Kabbinavar et al. 1995), topotecan (Soffer et al. 2001), or capecitabine (Shen et al. 2004). Overall, the preclinical toxicity data indicate that both single and repeated doses of bevacizumab are likely to be well tolerated (Ryan et al. 1999). These encouraging preclinical data prompted an extensive clinical trial program, the details of which are outlined in the following section.

3.3 Clinical Trials of Bevacizumab in Key Tumor Types

The safety of bevacizumab as monotherapy (Gordon et al. 2001) and in combination with chemotherapy (Margolin et al. 2001) was assessed in two phase I trials. In the monotherapy trial, 25 patients with various types of solid tumor received 0.1, 0.3, 1, 3, or $10\,mg\,kg^{-1}$ bevacizumab on days 0, 28, 35, and 42 (Gordon et al. 2001). There were no grade 3 or 4 adverse effects, but a small number of patients reported grade 1 or 2 asthenia, headache, fever, rash and nausea. In the phase I trial of bevacizumab combined with chemotherapy, 12 patients with solid tumors received $3\,mg\,kg^{-1}$ bevacizumab weekly for 8 weeks with carboplatin (Paraplatin)/paclitaxel, doxorubicin (Adriamycin) or 5-fluorouracil (5-FU)/leucovorin (LV) (Margolin et al. 2001). Bevacizumab did not increase the frequency or severity of the anticipated adverse side effects of the concomitant chemotherapy regimens. In addition, no significant bevacizumab-related toxicity was observed. Of the 12 patients, three responded to treatment and remained on bevacizumab therapy for up to 40 weeks. No additional toxicity was observed in these patients, suggesting that long-term bevacizumab use may be well tolerated (Margolin et al. 2001).

The promising clinical activity and favorable toxicity profile demonstrated in these trials led to phase II and III trials of bevacizumab combined with standard

chemotherapy regimens in a range of indications including CRC, breast cancer, NSCLC, RCC, pancreatic cancer, and ovarian cancer. Given that anti-VEGF therapy is most likely to have an effect in earlier disease, when the tumor is most dependent on VEGF for further growth and development, bevacizumab is currently being investigated in the adjuvant setting and as first-line therapy for metastatic disease in combination with standard regimens. To date, more than 7000 patients have been treated with bevacizumab in clinical trials.

3.3.1 Colorectal Cancer

Until relatively recently, 5-FU with or without LV defined the standard of care for the first-line treatment of patients with metastatic CRC. Irinotecan (Camptosar) combined with bolus 5-FU/LV (IFL) became a new standard for chemotherapy-naïve patients with metastatic CRC based on the findings of two randomized trials. These trials demonstrated improved response rates and survival for patients treated with irinotecan combined with 5-FU/LV compared with bolus (Saltz et al. 2000) or infusional (Douillard et al. 2000) 5-FU treatment alone. Oxaliplatin was established as another agent for first-line treatment of metastatic CRC following a randomized trial (de Gramont et al. 2000) that demonstrated an improved response rate and progression-free survival in patients treated with oxaliplatin plus infusional 5-FU/LV (FOLFOX4) compared with 5-FU/LV alone. A subsequent study suggested that FOLFOX4 might improve median survival compared with IFL in patients with metastatic CRC (Goldberg et al. 2004).

While these new drugs have improved clinical outcomes for patients with CRC, the poor prognosis of this disease means that new therapies are still needed. Novel targeted therapies such as bevacizumab have been developed to improve survival without significantly increasing the toxicity of current therapies. On the basis of the evidence provided by the clinical trials of bevacizumab outlined in the following section, the US Food and Drug Administration (FDA) approved bevacizumab for the first-line treatment of metastatic CRC in combination with 5-FU-based chemotherapy in February 2004. The recommended dose is $5\,mg\,kg^{-1}$ bevacizumab given once every 14 days as an intravenous (IV) infusion until disease progression is detected. A European license was granted in January 2005 for the first-line use of bevacizumab ($5\,mg\,kg^{-1}$ once every 14 days until disease progression) in combination with 5-FU/LV, with or without irinotecan.

3.3.1.1 Efficacy of Bevacizumab in CRC

An initial phase II randomized, controlled, open-label clinical trial (AVF0780) compared bevacizumab plus 5-FU/LV with chemotherapy alone (Kabbinavar et al. 2003). In this trial, 104 patients with untreated metastatic CRC were randomized to one of three arms: control (5-FU/LV alone, n = 36); bevacizumab $5\,mg\,kg^{-1}$ every 2 weeks plus 5-FU/LV (n = 35); and bevacizumab $10\,mg\,kg^{-1}$ every 2 weeks plus 5-FU/LV (n = 33). Patients received 5-FU/LV according to the Roswell Park regimen: 5-FU $500\,mg\,m^{-2}$ and LV $500\,mg\,m^{-2}$ weekly for the first 6

weeks of each 8-week cycle. Patients in the control arm were allowed to cross over to receive bevacizumab on disease progression.

The addition of bevacizumab to 5-FU/LV improved outcomes compared with 5-FU/LV alone, with the 5 mg kg^{-1} bevacizumab dose appearing to be most effective. Bevacizumab 5 mg kg^{-1} significantly increased time to disease progression, the primary endpoint, by 73% from 5.2 months in the control arm to 9.0 months for patients treated with bevacizumab plus 5-FU/LV ($p = 0.005$). The median time to progression for patients receiving bevacizumab 10 mg kg^{-1} was 7.2 months. The risk of disease progression was 61% lower in the 5 mg kg^{-1} arm ($p = 0.002$ versus control) and 46% lower in the 10 mg kg^{-1} arm ($p = 0.052$ versus control). The tumor response rate increased from 17% in the 5-FU/LV arm to 24% in the bevacizumab 10 mg kg^{-1} arm ($p = 0.434$ versus control) and 40% in the bevacizumab 5 mg kg^{-1} arm ($p = 0.029$ versus control). Bevacizumab led to a trend towards increased median overall survival (13.8 months for the control arm and 16.1 and 21.5 months for the bevacizumab 10 mg kg^{-1} and 5 mg kg^{-1} arms, respectively), but this increase failed to reach statistical significance. This may have been due to the small sample size and the fact that patients in the 5-FU/LV arm were allowed to cross over to bevacizumab on disease progression. Based on these data, a bevacizumab dose of 5 mg kg^{-1} every 2 weeks was selected for subsequent trials.

A second phase II randomized, double-blind, controlled clinical trial (AVF2192) evaluated the efficacy of bevacizumab in combination with 5-FU/LV in patients with untreated metastatic CRC who were not optimal candidates for first-line irinotecan treatment (Kabbinavar et al. 2005a). The toxicity of irinotecan makes this agent unsuitable for some patients, in particular those with poor performance status or prior radiation therapy to the abdomen or pelvis. It should be noted that these patients are generally less healthy and tend to have a poorer prognosis than those eligible for irinotecan therapy. A total of 209 patients was randomized to receive 5-FU/LV (Roswell Park regimen) combined with either placebo ($n = 105$) or bevacizumab 5 mg kg^{-1} ($n = 104$) every 2 weeks.

Bevacizumab led to a trend towards improved median overall survival, the primary endpoint, by 29% from 12.9 months for patients receiving placebo to 16.6 months for patients in the bevacizumab arm ($p = 0.16$). Median progression-free survival increased from 5.5 months for the placebo arm to 9.2 months for the bevacizumab arm ($p = 0.0002$), while tumor response rates increased from 15.2% to 26% ($p = 0.055$). It should be noted that this trial was originally powered to identify an improvement in survival from 8.5 months in the 5-FU/LV arm to 14 months in the bevacizumab arm. Survival was considerably longer in both arms, making the trial underpowered for the overall survival endpoint. Nevertheless, the addition of bevacizumab to 5-FU/LV provides clinical benefit, as demonstrated by the observation that bevacizumab prolonged median and progression-free survival by 3.7 months when compared with 5-FU/LV alone.

A phase III randomized, double-blind, controlled clinical trial in patients with metastatic CRC (AVF2107) assessed whether combining bevacizumab with first-line irinotecan-containing chemotherapy improves overall survival compared

with chemotherapy alone (Hurwitz et al. 2004). The study was designed to compare the efficacy of bevacizumab (5 mg kg^{-1} IV every 2 weeks) combined with irinotecan (125 mg m^{-2} by IV infusion), 5-FU (500 mg m^{-2} by IV bolus) and LV (20 mg m^{-2} by IV bolus) (IFL) with that of IFL alone. A total of 923 patients was randomly assigned to one of three arms: IFL/placebo (n = 411); IFL/bevacizumab (n = 402); or 5-FU (500 mg m^{-2} by IV bolus)/LV (500 mg m^{-2} by IV infusion)/bevacizumab (n = 110). IFL was administered once weekly for the first four weeks of a 6-week cycle, while 5-FU/LV was administered once weekly for the first six weeks of an 8-week cycle. The 5-FU/LV/bevacizumab arm was a control to ensure that safety in the IFL/bevacizumab arm was acceptable. This control arm was closed after approximately 100 patients had been enrolled to each arm, and an independent review of safety data had concluded that the safety profiles of the IFL/bevacizumab and 5-FU/LV/bevacizumab arms were comparable.

Adding bevacizumab to IFL significantly increased overall survival by 30%; patients receiving IFL plus placebo had a median survival of 15.6 months, which increased to 20.3 months in patients receiving IFL plus bevacizumab ($p < 0.001$) (Fig. 3.2). Furthermore, bevacizumab increased progression-free survival by 71%, from 6.2 to 10.6 months ($p < 0.001$). The overall response rate was 44.8% for the IFL/bevacizumab arm and 34.8% for the IFL/placebo arm ($p = 0.004$) (Hurwitz et al. 2004). Furthermore, improvements in survival were seen in all prospectively defined patient subgroups based on baseline patient and tumor characteristics and irrespective of VEGF expression status (Fyfe et al. 2004; Holden et al. 2005).

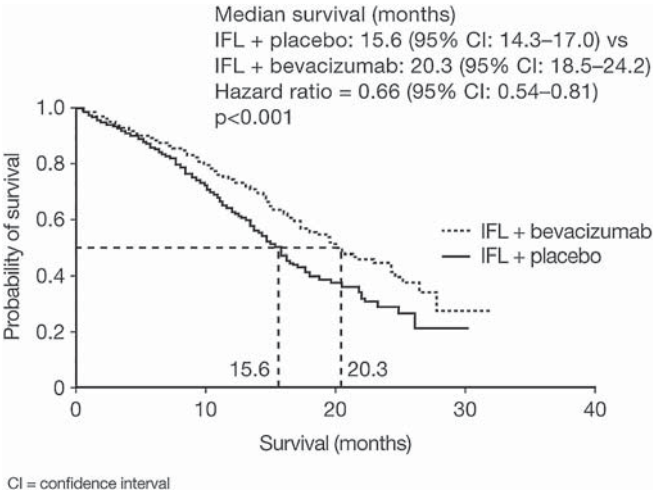

Fig. 3.2 Overall survival in patients with metastatic colorectal cancer (CRC) treated with irinotecan + 5-fluorouracil [5-FU]/leucovorin [LV] (IFL), with or without bevacizumab. (Reproduced with permission; © 2004 Massachusetts Medical Society. All rights reserved.)

Hurwitz et al. (2004) also reported that in the 25% of patients in this trial who were treated second line with oxaliplatin, overall survival was 25.1 months in the IFL plus bevacizumab arm and 22.2 months in the IFL plus placebo arm. This finding supports the conclusion of others (Grothey et al. 2004), that patients who receive several active anticancer agents survive longer than those who receive only one or two agents.

Efficacy data for the third arm of this trial, in which patients were treated with bevacizumab plus 5-FU/LV, have been reported (Hurwitz et al. 2005). Interim efficacy and safety analyses were conducted after approximately 100 patients had been randomly assigned to each treatment arm (100 to the IFL/placebo arm, 103 to the IFL/bevacizumab arm and 110 to the 5-FU/LV/bevacizumab arm). In this analysis, median overall survival was 18.3 and 15.1 months with 5-FU/LV/bevacizumab and IFL/placebo, respectively ($p = 0.25$), while progression-free survival was 8.8 and 6.8 months ($p = 0.42$) and the overall response rate was 40% and 37% ($p = 0.66$). These data confirm the activity of bevacizumab in CRC beyond combination with IFL and suggest that the 5-FU/LV/bevacizumab regimen is an active alternative treatment regimen for CRC patients.

Each of the 5-FU/LV/bevacizumab arms of these three clinical trials (Hurwitz et al. 2004; Kabbinavar et al. 2003, 2005a) recruited a relatively small number of patients. This may explain why none of these trials showed a statistically significant increase in overall survival for patients treated with bevacizumab plus 5-FU/LV, even though the increases were clinically significant. To evaluate the efficacy of the 5-FU/LV/bevacizumab regimen in a larger patient population, a combined analysis of pooled raw data from the two phase II trials and the 5-FU/LV/bevacizumab arm of the pivotal phase III trial was conducted. The combined control group comprised patients randomized to 5-FU/LV or IFL, while the bevacizumab arm included patients randomized to 5-FU/LV plus bevacizumab $5\,mg\,kg^{-1}$ every 2 weeks. The inclusion of IFL patients in the control arm biased the analysis against seeing a benefit with bevacizumab because IFL is a more effective regimen than 5-FU/LV (Kabbinavar et al. 2005a).

The combined analysis (Kabbinavar et al. 2005b) revealed that adding bevacizumab ($5\,mg\,kg^{-1}$ every 2 weeks) to 5-FU/LV significantly increased overall survival; median overall survival in the pooled 5-FU/LV/bevacizumab group was 17.9 months, compared with 14.6 months in the combined control group ($p = 0.008$). Progression-free survival also increased from 5.6 to 8.8 months ($p = 0.0001$), as did the response rate (from 24.5% to 34.1%, $p = 0.019$). The improvements in overall and progression-free survival seen with bevacizumab in this combined analysis were as good as or better than those observed when irinotecan or oxaliplatin is added to 5-FU/LV (de Gramont et al. 2000; Saltz et al. 2000).

A recent trial (Eastern Cooperative Oncology Group [ECOG] 3200) examined bevacizumab plus FOLFOX4 as second-line therapy for metastatic CRC (Giantonio et al. 2005). In this trial, 829 CRC patients previously treated with an irinotecan-based regimen received bevacizumab ($10\,mg\,kg^{-1}$ every 2 weeks), FOLFOX4 or FOLFOX4 plus bevacizumab. Adding bevacizumab to FOLFOX4 significantly improved median overall survival, which increased from 10.8 months with

FOLFOX4 alone to 12.9 months for patients who received FOLFOX4 plus bevacizumab (hazard ratio = 0.76; p = 0.0018). Progression-free survival also increased (from 4.8 months for FOLFOX4 alone to 7.2 months for FOLFOX4 plus bevacizumab, p < 0.0001), as did the response rate (from 9.2% to 21.8%, p < 0.0001). In this study, the safety profile of bevacizumab plus oxaliplatin appeared similar to that observed in other trials of bevacizumab in CRC.

3.3.1.2 Safety and Management of Bevacizumab in CRC

Bevacizumab appears to be generally well tolerated by patients with metastatic CRC (Table 3.1). The most common adverse effects observed in clinical trials of CRC that are attributable to bevacizumab therapy include hypertension, proteinuria, arterial thrombosis, wound-healing complications, and bleeding. While higher incidences of these events were reported in patients receiving

Table 3.1 Incidence of selected adverse events in clinical trials of bevazumab in combination with chemotherapy for metastatic CRC (NB: data are not adjusted for different periods of therapy).

	Kabbinavar et al. (2005a) AVF2192		*Hurwitz et al. (2004) AVF2107*		*Hurwitz et al. (2005) Third arm of AVF2107*	
Adverse events (% of patients)	***5-FU/LV + placebo (n = 104)***	***5-FU/LV + bevacizumab 5 mg kg^{-1} (n = 100)***	***IFL + placebo (n = 397)***	***IFL + bevacizumab 5 mg kg^{-1} (n = 393)***	***IFL + placebo (n = 98)***	***5-FU/LV + bevacizumab 5 mg kg^{-1} (n = 109)***
Hypertension						
Any	5	32	8.3	22.4	14.3	33.9
Grade 3	3	16	2.3	11.0	3.1	18.3
Proteinuria						
Any	19	38	21.7	26.5	25.1	34.9
Grade 3	0	1	0.8	0.8	0	1.8
Any thrombotic event	18	18	16.2	19.4	19.4	13.8
Bleeding event						
Grade 3/4	3	5	2.5	3.1	1.0	6.4
Diarrhea						
Grade 3/4	40	39	24.7	32.4	25.5	37.6
Leukopenia						
Grade 3/4	7	5	31.1	37.0	37.7	5.5
GI perforation	0	2	0.0	1.5	0	0

IFL = 5-fluorouracil [5-FU]/leucovorin [LV].

bevacizumab than in control patients, most of these adverse effects were manageable.

Hypertension is the most common adverse side effect reported in trials of bevacizumab in CRC, with 22.4 to 33.9% of all bevacizumab-treated patients reporting hypertension of any grade. However, very few grade 4 events were reported, and only 0.7% of all patients in clinical trials discontinued therapy due to hypertension. Grade 3 hypertension was reported by 11 to 18.3% of bevacizumab-treated patients. Although hypertension can occur at any time during the course of treatment, in most cases it can be effectively managed with oral anti-hypertensive agents. However, if these are not effective in lowering blood pressure, then bevacizumab treatment should be discontinued. Treatment should also be permanently discontinued in patients who develop hypertensive crises. Caution should be exercised before administering bevacizumab to patients with uncontrolled hypertension, and the patients' blood pressure should be monitored during treatment.

In CRC trials, 26.5 to 38% of bevacizumab-treated patients reported proteinuria of any grade, compared with 19 to 25.1% of control patients; proteinuria is usually asymptomatic, and grade 4 proteinuria was rarely reported. Proteinuria should be monitored with dipstick urinalysis before and during bevacizumab treatment. Proteinuria improves when bevacizumab therapy is stopped, and it is currently recommended that bevacizumab therapy be interrupted if patients develop proteinuria ≥2 g per 24 h, and discontinued if patients develop nephrotic syndrome.

In clinical trials, the overall incidence of any thromboembolism in patients with metastatic CRC was similar in bevacizumab-treated (13.8–19.4%) and control patients (16.2–19.4%). The incidence of grade 3/4 thromboembolic events was also similar in the two groups (16.0–17.6% with bevacizumab versus 14.4–18.3% with control). However, a higher incidence of arterial thromboembolic events was reported for bevacizumab-treated patients (3.3–10%) than for control patients (1.3–4.8%). In light of these findings, a retrospective analysis examined five controlled, completed clinical trials of bevacizumab to identify whether bevacizumab treatment carries an increased risk of arterial thromboembolism: three CRC trials (Hurwitz et al. 2004; Kabbinavar et al. 2003, 2005a), one NSCLC trial (Johnson et al. 2004), and one metastatic breast cancer (MBC) trial (Miller et al. 2005a) were used for the analysis.

The results revealed an increased risk of developing arterial thromboembolic events in bevacizumab-treated patients, 3.8% of whom reported an arterial thromboembolic event compared with 1.7% of patients receiving chemotherapy alone. In the phase III trial of IFL with or without bevacizumab, some patients who developed thromboembolic events were treated using anticoagulation therapy (full-dose warfarin); an analysis of data from these patients suggested that co-administration of warfarin and bevacizumab did not increase the incidence of bleeding events (Hambleton et al. 2004). The risk of developing an arterial thromboembolic event was higher for patients aged over 65 years or who had a history of such events. Patients who experience an arterial thromboembolic event should discontinue bevacizumab therapy.

One of the known roles of VEGF in adults is its involvement in wound healing. However, bevacizumab-treated patients who underwent surgery between 28 and 60 days before starting therapy did not report any wound-healing complications during bevacizumab treatment. In contrast, 10 to 20% of bevacizumab-treated patients who underwent major surgery (emergency and elective) while receiving bevacizumab treatment reported adverse events indicative of wound-healing complications. For this reason, it is recommended that bevacizumab therapy be discontinued at least 6 weeks prior to elective surgery. Therapy should also be discontinued in any patients who develop wound-healing complications that require medical intervention.

Minor mucocutaneous hemorrhage was reported relatively frequently in CRC clinical trials, with 22 to 34.2% of bevacizumab-treated patients experiencing epistaxis. This was most commonly grade 1 in severity, lasting less than 5 min, and was easily managed using standard first-aid techniques. Bevacizumab did not increase the risk of severe hemorrhagic events; the incidence of grade 3 and 4 bleeding events in bevacizumab-treated patients was similar to that in control patients. It is important to note that the risk of central nervous system (CNS) hemorrhage in patients with CNS metastases has not been fully evaluated because these patients were excluded from bevacizumab clinical trials. Bevacizumab is therefore contraindicated in patients with untreated CNS metastases. Furthermore, no information is available on the safety profile of bevacizumab in patients with congenital bleeding diathesis or acquired coagulopathy, and those receiving full-dose anticoagulation for the treatment of thromboembolism prior to starting bevacizumab treatment. These patients should be treated with bevacizumab with care, although patients who developed venous thrombosis while receiving bevacizumab therapy did not appear to be at increased risk of serious bleeding when treated with full-dose warfarin and bevacizumab concomitantly.

Finally, GI perforation is a rare but life-threatening side effect reported in patients with metastatic CRC treated with bevacizumab. Across clinical trials in CRC, GI perforation was observed in 1.5 to 2% of bevacizumab-treated patients; of these events, <0.5% were fatal. In some cases predisposing factors were present, such as intra-abdominal inflammation due to gastric ulcer disease, tumor necrosis or chemotherapy-associated colitis (Hurwitz et al. 2004; Johnson et al. 2004). Thus, bevacizumab-treated patients with metastatic CRC and an intra-abdominal inflammation may be at increased risk of developing a GI perforation. If a patient develops a GI perforation, bevacizumab should be permanently discontinued.

In summary, bevacizumab is generally well tolerated and does not increase the frequency or severity of chemotherapy-related toxicities. While some specific safety concerns have been identified, most adverse side effects are either easily manageable, or are not clinically significant.

3.3.1.3 Future Use of Bevacizumab in CRC

Clinical data indicate that bevacizumab has considerable potential in combination with existing therapeutic options; indeed, it is the only agent to date that has demonstrated a survival benefit in metastatic CRC when combined with first-line

chemotherapy. Following these encouraging data, several ongoing trials are examining bevacizumab combined with oxaliplatin-based chemotherapy as first-line therapy for metastatic CRC (e.g. studies NO16966, and DREAM). Bevacizumab may also confer clinical benefit in the adjuvant setting in view of the fact that VEGF inhibition should prevent the angiogenic switch, which is a key factor in malignant growth, but further clinical studies must be conducted are required before reaching any conclusions. Several trials are ongoing (BO17920, NSABP C-08 and QUASAR2) to examine the potential role of this agent as part of adjuvant treatment in CRC.

The phase III trial NO16966 enrolled 2,035 patients, and is designed to compare the safety and efficacy of XELOX (oxaliplatin 130 mg m^{-2} day 1, capecitabine 1000 mg m^{-2} twice daily for 14 days, every 3 weeks) with or without bevacizumab (7.5 mg kg^{-1} every 3 weeks) with that of FOLFOX4 with or without bevacizumab (5 mg kg^{-1} every 2 weeks) as first-line treatment for patients with metastatic CRC. The primary objectives of this trial are to show that the XELOX and FOLFOX4 regimens provide equivalent progression-free survival, and that adding bevacizumab to XELOX/FOLFOX4 improves progression-free survival compared with XELOX/FOLFOX4 alone. Both endpoints were met; XELOX is as effective as FOLFOX4 in terms of progression-free survival (8.0 versus 8.5 months, respectively), and the addition of bevacizumab to either chemotherapy (XELOX or FOLFOX4) significantly improved progression-free survival compared with chemotherapy alone (9.4 versus 8.0 months) (Cassidy et al., 2006).

TREE-2 is a randomized study comparing three oxaliplatin-based regimens plus bevacizumab in 223 CRC patients (Hochster et al. 2006). Patients received bolus 5 FU/LV combined with oxaliplatin (bFOL) and bevacizumab (5 mg kg^{-1} every 2 weeks), XELOX plus bevacizumab (7.5 mg kg^{-1} every 3 weeks) or a modified FOLFOX6 regimen plus bevacizumab (5 mg kg^{-1} every 2 weeks). Recently presented data indicate that combining these regimens with bevacizumab improved response rates compared with the chemotherapy regimens on their own; 53% for modified FOLFOX6 plus bevacizumab, 48% for the XELOX combination, and 41% for the bFOL combination. The addition of bevacizumab also improved time to progression (9.9, 10.3 and 8.3 months) and overall survival (26.0, 27.0 and 20.7 months) compared with modified FOLFOX6, XELOX and bFOL alone.

The toxicity of chemotherapy regimens remains an issue, and the OPTIMOX or "stop-and-go" approach has been developed to reduce the cumulative sensory neurotoxicity observed with oxaliplatin (de Gramont et al. 2004). Given that bevacizumab increases progression-free survival and treatment duration, cumulative neurotoxicity is likely to become an issue when bevacizumab is combined with oxaliplatin-based regimens. To examine the OPTIMOX approach as a way to limit toxicity and maintain efficacy, the phase III DREAM trial will recruit 640 patients with metastatic CRC. Patients will be randomized to one of three arms in which chemotherapy is paused and reintroduced: modified FOLFOX7 or XELOX4 plus bevacizumab (with bevacizumab plus erlotinib (Tarceva) during chemotherapy pause) or modified FOLFOX7 plus bevacizumab (with bevacizumab alone during chemotherapy pause).

BRiTE and First BEAT are two large studies assessing bevacizumab safety and efficacy in large patient populations; 1,987 and 1,927 patients with metastatic CRC, receiving bevacizumab with first-line chemotherapy (regimen choice at physician's discretion) were recruited, respectively. The safety profile of bevacizumab in these studies appears to be consistent with the prospective randomised clinical trials (Cunningham et al. 2006; Hedrick et al. 2006); bevacizumab-related serious adverse effects were reported in 9–12% of the study populations, GI perforations were uncommon, and no new safety signals were identified. The estimated median progression-free survival of 10.2 months observed in BRiTE compares favourably with prospective randomised clinical trials (Kozloff et al., 2006).

In the adjuvant setting, Roche is currently conducting an open-label phase III trial BO17920 of bevacizumab treatment of colon cancer (Fig. 3.3). This trial will recruit 3450 patients with high-risk stage II or III colon cancer (more than half have been enrolled to date) randomized to three treatment arms: FOLFOX4 for 24 weeks; FOLFOX4 plus bevacizumab ($5\,mg\,kg^{-1}$ every 2 weeks) for 24 weeks followed by 24 weeks of bevacizumab monotherapy ($7.5\,mg\,kg^{-1}$ every 3 weeks); and XELOX plus bevacizumab ($7.5\,mg\,kg^{-1}$ every 3 weeks) for 24 weeks followed by 24 weeks of bevacizumab monotherapy ($7.5\,mg\,kg^{-1}$ every 3 weeks). The primary endpoint of the study is disease-free survival.

In the USA, the National Surgical Adjuvant Breast and Bowel Project (NSABP) is conducting a phase III trial to assess the efficacy of bevacizumab added to FOLFOX6 (NSABP C-08). The trial aims to recruit 2700 patients who will receive modified FOLFOX6 (every 2 weeks for 12 cycles) either alone or combined with bevacizumab ($5\,mg\,kg^{-1}$ every 2 weeks for a year). The primary endpoint of the study is disease-free survival.

Initial data from clinical trials to date suggest that bevacizumab is effective when combined with any standard chemotherapy regimen for the first-line treatment of metastatic CRC, including regimens containing 5-FU, capecitabine, irinotecan, or oxaliplatin. Data from a number of ongoing trials of first-line bevacizumab with regimens such as FOLFOX, XELOX, and FOLFIRI (5-FU/LV plus irinotecan) will be reported during 2006, and will confirm the safety and efficacy of bevacizumab as part of these first-line treatment regimens. Numerous other

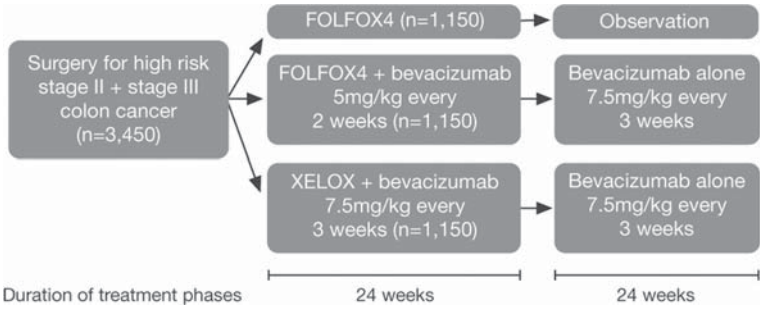

Fig. 3.3 The BO17920 trial design.

ongoing trials will determine the efficacy and safety of bevacizumab in combination with other chemotherapy regimens and biological therapies in this setting. Based on its mechanism of action, bevacizumab may be most effective as adjuvant therapy, and a number of ongoing phase III trials involving >6000 patients are designed to produce evidence to demonstrate this.

3.3.2 Non-Small Cell Lung Cancer

Patients with NSCLC have a poor prognosis and high mortality. The standard treatment for advanced NSCLC is platinum-based chemotherapy, which to date has demonstrated only modest survival benefit. The median overall survival with first-line platinum-based chemotherapy is only 8 to 10 months for advanced NSCLC patients, and novel treatment strategies are certainly urgently needed in this condition. Targeted therapies such as bevacizumab have the potential to improve patient survival without significantly adding to the toxicity of conventional chemotherapy. Indeed, bevacizumab plus carboplatin/paclitaxel is now the ECOG standard of care for patients with nonsquamous NSCLC (Sandler et al. 2005).

3.3.2.1 Efficacy of Bevacizumab in NSCLC

In the first randomized phase II trial of bevacizumab in NSCLC, patients with previously untreated advanced or recurrent NSCLC received chemotherapy (carboplatin/paclitaxel) alone or with bevacizumab at a dose of either 7.5 or 15 $mg\,kg^{-1}$ every 3 weeks (Johnson et al. 2004). The primary efficacy endpoints were time to disease progression and tumor response rate, while the secondary efficacy endpoints were overall survival and duration of response. In order to be eligible, patients had to be chemotherapy naïve, have histologically confirmed stage IIIB (with pleural effusion), stage IV or recurrent NSCLC, and have an ECOG performance status of 0, 1, or 2. In this trial, 99 patients were randomly assigned to one of three treatment arms: carboplatin/paclitaxel alone (n = 32); carboplatin/paclitaxel plus 7.5 $mg\,kg^{-1}$ bevacizumab every 3 weeks (n = 32); or carboplatin/paclitaxel plus 15 $mg\,kg^{-1}$ bevacizumab every 3 weeks (n = 35). Paclitaxel (200 $mg\,m^{-2}$) was administered over 3 h every 3 weeks, and carboplatin was given at an area under the concentration–time curve (AUC) of 6. Patients received up to six cycles of carboplatin/paclitaxel. On completion of the planned chemotherapy, patients without progressive disease were allowed to continue on bevacizumab. Patients in the chemotherapy-alone arm were permitted to receive bevacizumab (15 $mg\,kg^{-1}$ every 3 weeks) on disease progression.

In the 15 $mg\,kg^{-1}$ bevacizumab arm, time to disease progression was significantly increased to 7.4 months from 4.2 months in controls, which represents a 46% reduction in the risk of progression ($p = 0.023$). An apparent, but nonsignificant, improvement of overall survival was also observed: patients receiving chemotherapy plus bevacizumab 15 $mg\,kg^{-1}$ every 3 weeks survived for 17.7 months, compared with 14.9 months for control patients ($p = 0.63$). The lack of

significance was not surprising, given the small number of patients enrolled in this study. Furthermore, 19 of the 32 control patients crossed over to single-agent bevacizumab on disease progression, which may have positively influenced survival in the control arm. Indeed, mean survival in the control arm of this study (14.9 months) was considerably higher than that in several other comparable trials of carboplatin/paclitaxel (8–10 months) (Kelly et al. 2001; Schiller et al. 2002).

Bevacizumab at a dose of 7.5 mg kg^{-1} every 3 weeks did not improve time to progression or survival compared with the control arm (4.3 and 4.2 months, respectively). In this arm, five severe bleeding events associated with squamous cell histology occurred, which may have decreased median survival (10/32 patients in this arm had squamous cell histology, compared with 3/34 patients in the 15 mg kg^{-1} arm). A more detailed discussion of the bleeding episodes is presented below.

These data provided the basis for a phase III trial (E4599) to further investigate bevacizumab at a dose of 15 mg kg^{-1} every 3 weeks. In this trial, patients with previously untreated advanced nonsquamous NSCLC were randomized to receive carboplatin/paclitaxel alone or with bevacizumab at a dose of 15 mg kg^{-1} every 3 weeks (Sandler et al. 2005). The primary endpoint of this study was overall survival, while the secondary endpoints included response rate, time to disease progression, and tolerability. In order to be eligible, patients had to be chemotherapy-naïve, have histologically confirmed stage IIIB (with pleural effusion), stage IV or recurrent nonsquamous NSCLC, and have an ECOG performance status of 0 or 1. They also needed to have adequate hematologic, renal and hepatic function. Patients with squamous cell histology, brain metastases and hemoptysis were excluded from this trial because the phase II trial had identified squamous histology as a risk factor for severe hemoptysis.

A total of 878 patients has been recruited and randomly assigned to two treatment arms: 444 patients received carboplatin/paclitaxel alone, and 434 received carboplatin/paclitaxel plus bevacizumab 15 mg kg^{-1} every 3 weeks. Patients in the control arm are not permitted to cross over to bevacizumab on disease progression. The results presented in Table 3.2 are from the second planned interim analysis after 469 deaths; the final analysis is planned at 650 deaths.

Table 3.2 Interim results of the E4599 phase III trial of carboplatin/paclitaxel with or without bevacizumab.

	Carboplatin/ paclitaxel	***Carboplatin/ paclitaxel + bevacizumab***	***p-value***
No. of patients	350	357	
Complete response, n (%)	0 (0)	5 (1.4)	
Partial response, n (%)	35 (10.0)	92 (25.8)	
Overall response rate, n (%)	35 (10.0)	97 (27.2)	<0.0001
Median survival (months)	10.2	12.5	0.0075
Progression-free survival (months)	4.5	6.4	<0.0001

The interim analysis conducted demonstrated that adding bevacizumab to chemotherapy significantly increased overall survival compared with chemotherapy alone: patients receiving bevacizumab had a median survival of 12.5 months, compared with 10.2 months for control patients (hazard ratio = 0.77; $p = 0.0075$). In addition, bevacizumab significantly increased progression-free survival (6.4 months for patients receiving bevacizumab versus 4.5 months for control patients; $p < 0.0001$) as well as response rate (27.2% versus 10%; $p < 0.0001$).

In summary, the preliminary results of this phase III trial demonstrate that adding bevacizumab to carboplatin/paclitaxel in patients with nonsquamous NSCLC provides a statistically and clinically significant survival advantage. The final data, which confirm the preliminary efficacy results, will be published soon. Following these positive results, the regimen of bevacizumab plus carboplatin/paclitaxel was adopted as the ECOG standard of care for first-line therapy in patients with nonsquamous NSCLC.

Recent clinical trials have also assessed the efficacy and safety of bevacizumab in combination with other biological agents in the absence of chemotherapy. A phase I/II trial evaluated the dose-limiting toxicity, efficacy and tolerability of bevacizumab in combination with erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (Herbst et al. 2005; Sandler et al. 2004). The primary endpoint of phase I of this study was to establish dose-limiting toxicity, while that of phase II was to assess the efficacy and tolerability of the bevacizumab and erlotinib combination. The secondary endpoints included overall response rate and pharmacokinetics. To be eligible, patients had to have histologically confirmed stage IIIB (with pleural effusion), stage IV or recurrent nonsquamous NSCLC, and have a Karnofsky performance status of at least 70%. They also needed to have adequate hematologic, renal and hepatic function, and have relapsed after at least one platinum-based chemotherapy regimen for recurrent or metastatic disease. Patients with squamous cell histology, CNS metastases and hemoptysis were excluded from the trial, as were those who had prior anti-VEGF and/or anti-HER1/EGFR therapy, recent major surgery or radiation therapy.

A total of 40 patients was recruited; in a two-stage design, 12 patients were assigned to the dose-finding study in phase I, and a further 28 patients were recruited to assess the efficacy and tolerability of bevacizumab and erlotinib at the established dose in the second-line setting. Patients in phase I were assigned to one of three dose regimens: erlotinib (100 mg day^{-1} for 3 weeks) plus bevacizumab (7.5 mg kg^{-1} once every 3 weeks), erlotinib (100 mg day^{-1} for 3 weeks) plus bevacizumab (15 mg kg^{-1} once every 3 weeks), or erlotinib (150 mg day^{-1} for 3 weeks) plus bevacizumab (15 mg kg^{-1} once every 3 weeks). The dose chosen for further investigation in phase II was erlotinib 150 mg day^{-1} plus bevacizumab 15 mg kg^{-1} every 3 weeks.

The results of this trial indicate that combined bevacizumab and erlotinib therapy shows some early evidence of antitumor activity in patients with advanced or recurrent nonsquamous NSCLC. All 40 patients were available for efficacy assessment as determined by RECIST (Response Evaluation Criteria in Solid Tumors). No patient had a complete response, but eight patients had a partial response (20%; 95% CI: 7.6–32.4%) and 26 had stable disease (65%;

95% CI: 50.2–79.8%). The median overall survival for all patients was 12.6 months, and progression-free survival was 7.0 months.

A phase II trial evaluated bevacizumab (15 mg/kg every 3 weeks) combined with chemotherapy (docetaxel 75 mg/m^2 or pemetrexed 500 mg/m^2) or with erlotinib (150 mg daily) in patients with recurrent or refractory NSCLC (Herbst et al. 2006). Median progression-free survival was 3 months for chemotherapy alone, 4.8 months for bevacizumab plus chemotherapy (HR versus chemotherapy alone = 0.66), and 4.4 months for bevacizumab plus erlotinib (HR versus chemotherapy alone = 0.72). Median overall survival was 8.6 months for chemotherapy alone, 12.6 months for bevacizumab plus chemotherapy (HR versus chemotherapy alone = 0.74), and 13.7 months for bevacizumab plus erlotinib (HR versus chemotherapy alone = 0.76). Further investigation of the combination of bevacizumab and erlotinib as treatment for NSCLC is warranted.

3.3.2.2 Safety and Management of Bevacizumab in NSCLC

Most bevacizumab-related adverse side effects observed in NSCLC trials are similar in incidence and severity to those reported for CRC, and should be managed in the same manner. Severe and sometimes fatal hemoptysis has been observed in patients with advanced NSCLC, precluding the use of bevacizumab in patients with squamous cell NSCLC.

Patients with NSCLC treated with bevacizumab are at increased risk of severe bleeding events. In the phase II trial of carboplatin/paclitaxel with or without one of two doses of bevacizumab (Johnson et al. 2004), six of the 66 bevacizumab-treated patients (9%) experienced a major, life-threatening bleeding event (pulmonary hemorrhage and/or hemoptysis), and four of these died. A retrospective multivariate analysis revealed that patients with squamous cell histology and/or tumors located in the center of the chest close to major blood vessels were at greatest risk of developing serious bleeding events. In some cases, these major hemorrhagic events were preceded by cavitation or necrosis of the tumor.

As a result of these findings, the entry criteria for future NSCLC trials were adjusted to exclude patients with squamous cell histology (ca. 30% of NSCLC patients). By excluding patients with squamous cell histology, the incidence of severe bleeding events was reduced in the pivotal phase III trial (Sandler et al. 2005): eight of the 420 bevacizumab-treated patients (1.9%) in this trial experienced grade ≥3 hemoptysis, compared with 9% of patients in the phase II trial. Combining bevacizumab and erlotinib also appeared to reduce the incidence of these events (Herbst et al. 2005): no serious dose-limiting toxicities were reported in this trial, and no hemoptysis was observed.

In summary, a specific safety recommendation for clinical trials of NSCLC is the exclusion of patients with squamous cell histology, as this is associated with a greater risk of severe bleeding events. Patients requiring medical intervention for the management of a bleeding event should discontinue bevacizumab. Otherwise, the side-effect profile of bevacizumab is similar to that observed in trials in other indications. It is important to note that GI perforations are uncommon (<1%) in patients with NSCLC treated with bevacizumab. Furthermore, trials

of bevacizumab in NSCLC add data demonstrating that bevacizumab can be combined with many chemotherapy regimens as well as with other targeted therapies.

3.3.2.3 Future Use of Bevacizumab in NSCLC

Conventional chemotherapy regimens have reached a plateau in the treatment of NSCLC, and it is unlikely that new combinations of chemotoxic agents will offer significant improvements in patient survival. Thus, there is a clinical need for more effective therapy to treat this disease. Both VEGF and EGFR inhibition have been demonstrated to extend survival in NSCLC, leading to the expectation that dual inhibition will have greater efficacy than inhibition of single agents. Therefore, combinations of bevacizumab with other biologic agents may be effective in the treatment of NSCLC. To examine the therapeutic benefit of bevacizumab in combination with chemotherapy and/or biologic agents, three phase III trials of first-line bevacizumab in NSCLC are currently under way.

A phase III trial (BO17704) will evaluate the efficacy and safety of gemcitabine (Gemzar)/cisplatin with or without bevacizumab as first-line therapy in advanced nonsquamous NSCLC. In this trial, 1,150 patients will be randomised (1:1:1) to receive gemcitabine/cisplatin with or without bevacizumab (either 7.5 or 15 $mg\,kg^{-1}$ every 3 weeks). Recruitment is now complete and results are expected in 2007. The primary endpoint of the study is progression-free survival, and secondary endpoints include overall survival and response rate. No cross-over is allowed.

Two phase III trials will assess the combination of bevacizumab and erlotinib. The first will investigate erlotinib with or without bevacizumab in advanced nonsquamous NSCLC. In this trial, 650 patients will be enrolled to assess the efficacy of bevacizumab combined with erlotinib versus erlotinib alone after the failure of standard first-line therapy. The primary endpoint is overall survival, and secondary endpoints will include progression-free survival, response rate, safety, and pharmacokinetics. The second trial will evaluate the efficacy of first-line bevacizumab with or without erlotinib in 1,150 patients with stage IIIB/IV non-squamous NSCLC. The primary endpoint is progression-free survival, while the secondary endpoint is the safety of bevacizumab plus erlotinib.

3.3.3 Breast Cancer

VEGF expression is inversely correlated with overall survival in patients with breast cancer (Gasparini et al. 1997, 1999; Linderholm et al. 2001), providing a rationale for inhibiting VEGF in MBC. Several phase II and III trials have investigated the efficacy and safety of bevacizumab therapy in breast cancer.

3.3.3.1 Efficacy of Bevacizumab in Breast Cancer

In a phase II dose-escalation study of bevacizumab in patients with MBC (AVF0776g), 75 patients were treated with one of three doses of bevacizumab (3,

10, or 20 mg kg^{-1}) every 2 weeks (Cobleigh et al. 2003). After 22 weeks of bevacizumab therapy, 16% of patients had stable disease or an ongoing objective response. The objective response rate was 5.6%, 12.2% and 6.3% for bevacizumab doses of 3, 10, and 20 mg kg^{-1}, respectively. The highest numerical response rate was seen at a dose of bevacizumab 10 mg kg^{-1} every 2 weeks. This observation, combined with a higher incidence of severe headaches and nausea at the 20 mg kg^{-1} dose level, led the authors to suggest bevacizumab 10 mg kg^{-1} every 2 weeks as an appropriate dose for future trials. Whilst no formal phase III studies comparing the benefit of different doses of this agent have been conducted, Roche is planning a multicenter phase III trial (AVADO) that will examine the efficacy of different bevacizumab doses in patients with MBC (see Section 3.3.3.3).

In the AVF2119 phase III trial, 462 patients with MBC were randomly assigned to receive capecitabine alone (2500 mg m^{-2} day^{-1} twice daily on Days 1 through 14, every 3 weeks) or the same dose of capecitabine plus bevacizumab (15 mg kg^{-1} every 3 weeks) (Miller et al. 2005a). The patients enrolled into this trial had been pretreated with anthracyclines and taxanes in the adjuvant and/or metastatic setting. The primary endpoint was progression-free survival as determined by an independent review facility (IRF), while secondary endpoints included progression-free survival based on investigator assessment, objective response rate as determined by both IRF and investigators, and overall survival.

Combining bevacizumab with capecitabine did not increase progression-free survival, which was 4.86 months for the combination arm and 4.17 months for the capecitabine arm ($p = 0.857$). Additionally, bevacizumab did not improve overall survival when added to capecitabine in this pretreated patient population (15.1 versus 14.5 months). There are a number of potential explanations for these observations, including the concept that other angiogenic growth factors come into play in later stage disease, making VEGF inhibition a less effective strategy. However, it was interesting that the objective response rate observed in patients receiving bevacizumab with capecitabine was significantly higher than that reported for patients receiving capecitabine alone, as assessed by both the IRF (19.8% versus 9.1%, $p = 0.001$) and the investigators (30.2% versus 19.1%, $p = 0.006$).

Given the mechanism of action of bevacizumab and the decreased relative influence of VEGF on tumor growth as breast cancer progresses, the authors suggest that anti-angiogenic agents may be more effective as first-line treatment for breast cancer. To test this theory, ECOG has conducted a randomized, open-label phase III intergroup trial (E2100) in collaboration with members of the North American Breast Intergroup to study the efficacy of bevacizumab combined with weekly paclitaxel as first-line treatment for MBC (Miller et al. 2005b). The results of this trial were presented at the 13th European Cancer Conference (ECCO) 2005 and the 28th San Antonio Breast Cancer Symposium (SABCS) 2005 following a data cut-off date of 27 September 2005. A total of 715 patients was randomized to one of two arms: paclitaxel alone (90 mg m^{-2} weekly for 3 weeks followed by one week without treatment); or the same dose of paclitaxel plus bevacizumab 10 mg kg^{-1} every 2 weeks. Interim analysis demonstrated that pro-

gression-free survival (the primary endpoint) improved from 6.11 months for patients treated with paclitaxel alone to 11.4 months for those receiving paclitaxel plus bevacizumab ($p < 0.0001$). The addition of bevacizumab to paclitaxel also led to a twofold increase in response rate for all patients (13.8% to 29.9%, $p < 0.0001$) and for patients with measurable disease only (16% to 37.7%, $p < 0.0001$) (Table 3.3). Assessment of overall survival requires a longer follow-up, as the differences are not yet statistically different between the two arms.

Additional phase II data have been reported for bevacizumab in combination with vinorelbine (Navelbine) (Burstein et al. 2002), docetaxel (Ramaswamy and Shapiro 2003), erlotinib (Dickler et al. 2004; Rugo et al. 2005), and letrozole (Femara) (Traina et al. 2005) (Table 3.4). In summary, bevacizumab has been shown to have clinical activity in breast cancer, and may soon play a role as part of standard treatment regimens.

Table 3.3 Interim results of the E2100 trial (paclitaxel with and without bevacizumab in metastatic breast cancer).

	Paclitaxel	***Paclitaxel + bevacizumab***	***p-value***
No. of patients	350	365	
Overall response rate (%)			
• All patients	13.8	29.9	<0.0001
• Patients with measurable disease	16.0	37.7	<0.0001
Progression-free survival (months)	6.11	11.4	<0.0001
Overall survival (months)	25.2	28.4	0.12

Table 3.4 Interim response rates of patients treated with bevacizumab in combination with other agents in phase II clinical trials of metastatic breast cancer.

	Burstein et al. (2002)	***Ramaswamy and Shapiro (2003)***	***Rugo et al. (2005)***	***Traina et al. (2005)***
Bevacizumab plus	Vinorelbine	Docetaxel	Erlotinib	Letrozole
Total no. of patients enrolled	56	18	37	23
No. of patients with				
• Complete response	1	0	0	0
• Partial response	16	10	1	2
• Stable disease	25	6	11	13
• Progressive disease	12	0	25	3
• Too early for assessment	1	0	0	5
• Withdrew due to toxicity	1	2	0	0

3.3.3.2 Safety and Management of Bevacizumab in Breast Cancer

Bevacizumab was generally well tolerated in clinical trials of MBC. Some adverse side effects occurred more frequently in bevacizumab-treated patients than control patients, but these did not limit therapy as they consisted mainly of manageable hypertension and proteinuria. More bleeding events were reported in bevacizumab-treated patients than control patients, but these were mainly grade 1/2 epistaxis. Serious hemorrhagic events, such as those observed in the NSCLC trials, were rare and did not occur more frequently in bevacizumab-treated patients than in control patients in MBC trials.

Thromboembolic events were infrequent in breast cancer trials, and occurred at similar rates in bevacizumab-treated and control patients. In the phase II dose-escalation trial, two patients developed axillary/subclavian vein thrombosis, but no cases of thrombosis in the lower extremities or pulmonary emboli were seen. Future phase III studies will further examine whether bevacizumab increases the risk of thromboembolic events.

In the phase III trial of bevacizumab combined with capecitabine (Miller et al. 2005a), 3% of bevacizumab-treated patients reported congestive heart failure (CHF) or cardiomyopathy, compared with 1% of patients in the control group (capecitabine alone). The severity of these incidents ranged from asymptomatic declines in left ventricular ejection fraction (LVEF) to symptomatic CHF requiring hospitalization. All of the bevacizumab-treated patients had previously been treated with anthracyclines, and some had received left chest wall radiation. In addition, three of the seven affected patients had a baseline LVEF <50%. In the phase II dose-escalation trial, two of 75 patients developed CHF; both of these had also received prior anthracyclines and chest wall radiation, and one patient had metastatic involvement of the pericardium. Due to the small number of events, the association between cardiac dysfunction, prior anthracycline exposure and bevacizumab treatment remains unclear. Cardiac dysfunction will continue to be monitored in clinical trials in patients with breast cancer.

In summary, bevacizumab is generally well tolerated in breast cancer, producing similar adverse events to those reported for other indications. Ongoing trials will improve our understanding of the potential role of this agent in breast cancer.

3.3.3.3 Future Use of Bevacizumab in Breast Cancer

The results of the phase III trial of bevacizumab combined with capecitabine indicate that patients with tumors refractory to chemotherapy may not represent the optimal setting for anti-angiogenic agents. Therefore, future trials of MBC will focus on patients with less-advanced disease.

Ongoing and planned clinical trials of bevacizumab in breast cancer include the AVADO trial (BO17708). This randomized, double-blind, placebo-controlled phase III trial conducted by Roche will compare the efficacy of docetaxel in combination with bevacizumab or placebo. A total of 705 patients who have received no prior chemotherapy for MBC will be recruited and randomized to one of three arms: docetaxel ($100\,mg\,m^{-2}$ every 3 weeks) plus placebo; docetaxel

plus bevacizumab (7.5 mg kg^{-1} every 3 weeks); or docetaxel plus bevacizumab (15 mg kg^{-1} every 3 weeks). The primary endpoint of this trial is progression-free survival.

Perez and colleagues in the North Central Cancer Treatment Group (NCCTG) have recently completed accrual to a phase II study of docetaxel 75 mg m^{-2} plus capecitabine 1650 mg m^{-2} per day for 14 days plus bevacizumab 15 mg kg^{-1}, with cycles repeated every 3 weeks. Data are expected during 2006.

In the USA, a pilot safety phase II trial of bevacizumab plus chemotherapy in lymph node-positive breast cancer (E2104) will evaluate the incidence of cardiotoxicity when bevacizumab is added to an anthracycline-based regimen in the adjuvant setting. Patients must have undergone surgery to remove the tumor 4 to 12 weeks before joining the trial, and have received no prior chemotherapy or radiotherapy. The investigators will randomize 204 patients to one of two treatment arms: doxorubicin (60 mg m^{-2})/cyclophosphamide (Cytoxan) (600 mg m^{-2}) with or without bevacizumab 10 mg kg^{-1} every 2 weeks (four cycles). Patients in both arms will then receive paclitaxel (175 mg m^{-2}) plus bevacizumab 10 mg kg^{-1} every 2 weeks (four cycles) followed by 18 or 22 cycles of bevacizumab alone. The primary endpoint is to determine the incidence of cardiac dysfunction, while the secondary endpoints are to determine changes in LVEF in these patients and the noncardiac toxicity of this regimen.

The benefit of bevacizumab plus chemotherapy in the neoadjuvant setting will be assessed by a second double-blind, placebo-controlled phase II US trial (TORI B-02). Patients will receive a single dose of either bevacizumab or placebo (7.5 or 15 mg kg^{-1} every 3 weeks), followed by a six-cycle treatment phase of docetaxel, doxorubicin and cyclophosphamide plus either bevacizumab or placebo. Patients who received bevacizumab in the first phase of the study will then receive bevacizumab alone until disease progression, while patients who received placebo will receive no further therapy.

3.4 The Potential of Bevacizumab

As tumors cannot progress without a blood supply, angiogenesis plays a central role in the growth and development of cancer. In addition, in patients with solid tumors and hematological diseases, VEGF overexpression correlates with poor prognosis and survival rates. Consequently, bevacizumab offers the potential to increase survival without adding to the toxicity of conventional therapy across all tumor types.

A number of recently completed and ongoing clinical trials indicate that bevacizumab has the potential to be used across multiple tumor types. This agent significantly improves the survival of patients receiving first-line therapy for metastatic CRC, with proven efficacy when combined with 5-FU/LV with or without irinotecan. Ongoing clinical trials in metastatic CRC are assessing the efficacy of bevacizumab in the adjuvant setting and when combined first line with

other chemotherapy regimens, including oxaliplatin- and capecitabine-based regimens. Clinical benefit has also been demonstrated for bevacizumab when combined with standard first-line chemotherapy regimens in patients with metastatic NSCLC (Sandler et al. 2005) and breast cancer (Miller et al. 2005b). It is important to note that the role of bevacizumab in patients pretreated with chemotherapy has not been clearly established.

The future potential of bevacizumab therapy is illustrated by the number of tumor types for which data are already available and in which phase III trials are planned or ongoing. These include RCC, pancreatic cancer, and ovarian cancer.

Anti-VEGF therapy is a rational approach in RCC because many patients with RCC have a mutation of the von Hippel-Lindau (VHL) gene that causes VEGF overexpression, resulting in hypervascularization. Bevacizumab monotherapy has been shown to increase time to disease progression in patients with pretreated metastatic RCC (Yang et al. 2003). In addition, a phase II trial in RCC combining bevacizumab and erlotinib achieved a 25% response rate (Hainsworth et al. 2005a), which is impressive in a disease where responses are uncommon even with standard therapy (interferon-α2a). Two ongoing phase III trials are evaluating the impact of bevacizumab plus interferon or interferon alone as first-line therapy on overall survival.

Bevacizumab has also demonstrated activity in a number of trials in pancreatic cancer. A phase I trial combining bevacizumab with capecitabine and radiotherapy in pancreatic cancer patients reported partial or minor responses in 40% of patients (Crane et al. 2005). A phase II trial of bevacizumab combined with gemcitabine as first-line treatment for metastatic pancreatic cancer achieved a response rate of 19% (Kindler et al. 2004). Two phase III trials of bevacizumab in pancreatic cancer are ongoing. The first trial, BO17706, will examine overall survival in 600 previously untreated patients treated with gemcitabine plus erlotinib with or without bevacizumab 5 mg kg^{-1} every 2 weeks. The second trial, CALGB 80303, will compare overall survival in 530 previously untreated patients receiving either gemcitabine (1000 mg m^{-2} weekly for 3 weeks of a 4-week cycle) plus bevacizumab (10 mg kg^{-1} every 2 weeks) or gemcitabine alone.

In a phase II trial of ovarian cancer, bevacizumab monotherapy (15 mg kg^{-1} every 3 weeks) produced a response rate of 17.7% and a 6-month progression-free survival rate of 38.7%, which compares favorably with historical control data (Burger et al. 2005). However, another phase II trial of single-agent bevacizumab was halted after five GI perforations were reported in the first 11 patients; these may have occurred because the study population consisted of patients with more advanced disease.

Two phase III trials are planned in ovarian cancer. In the first trial, ICON 7, 500 patients with International Federation of Gynecology and Obstetrics (FIGO) stage IIb–IV epithelial ovarian carcinoma will receive carboplatin and paclitaxel with or without bevacizumab 7.5 mg kg^{-1} every 3 weeks. In the second trial, GOG 0218, patients with FIGO stage III/IV ovarian or peritoneal carcinoma will be randomized to one of three arms. All patients will receive six cycles of chemo-

therapy (paclitaxel 175 mg m^{-2}, carboplatin AUC 6 every 3 weeks). Arm 1 patients will receive placebo with the chemotherapy, followed by placebo alone. Patients in arms 2 and 3 will receive bevacizumab (15 mg kg^{-1} every 3 weeks) with the chemotherapy, followed by placebo alone for arm 2 and bevacizumab alone for arm 3 for the duration of the 15 months of treatment.

As well as these tumor types in which large phase III trials are already ongoing or planned, encouraging data are emerging from clinical studies of bevacizumab alone or in combination with other agents for the treatment of hematological malignancies, including non-Hodgkin's lymphoma and acute myelogenous leukemia (Karp et al. 2004; Stopeck et al. 2005), prostate cancer (Rini et al. 2005), melanoma (Carson et al. 2003), hepatocellular carcinoma (Schwartz et al. 2004; Zhu et al. 2005) and gastric cancer (Shah et al. 2005).

3.5 Conclusions

Anti-angiogenic agents, of which bevacizumab is the most well-developed and well-understood, have the potential to improve patient survival with fewer adverse side effects than conventional chemotherapy regimens. Bevacizumab has been demonstrated to significantly improve survival in first-line therapy for metastatic CRC and NSCLC. Progression-free survival or time to progression for patients with MBC or RCC is improved by the addition of bevacizumab to first-line therapy. Ongoing trials are examining the efficacy and tolerability of bevacizumab with the objective of optimizing its use to maximize the benefit for patients. It appears likely that bevacizumab will become a key element of first-line regimens for a variety of tumor types, whether in combination with chemotherapy, immunotherapy, radiotherapy, or other novel agents. Future and ongoing clinical trials will assess the efficacy of bevacizumab in combination with a range of chemotherapy regimens, as well as in combination with other biological therapies (Chen 2004). Several phase I/II clinical studies of combined inhibition of the EGFR and the VEGF signaling pathways have already reported encouraging findings (Hainsworth et al. 2005b; Herbst et al. 2005). These trials will elucidate the efficacy and safety of bevacizumab as both first-line therapy for metastatic disease and in the adjuvant setting.

Bevacizumab is generally well tolerated, and serious adverse events are rare. Across all clinical trials to date – irrespective of tumor type – the most frequently observed side effects related to bevacizumab are hypertension, proteinuria and minor bleeding events; these are usually mild to moderate in severity, and clinically manageable using standard therapies or first-aid techniques. The most serious bevacizumab-related adverse events reported in clinical trials are GI perforations (for GI and ovarian cancers), severe hemorrhagic events (for NSCLC), and arterial thromboembolic events. These events occur in a small minority of patients, and research to further identify risk factors for these events is ongoing.

In summary, bevacizumab is an anti-angiogenic agent which has been proven to improve clinical outcomes, including survival and progression-free survival, in patients with some malignancies. It is already approved for clinical use first line in patients with metastatic CRC, and data are currently under evaluation to determine its availability as part of first-line use in NSCLC and MBC.

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References

Baluk, P., Hashizume, H., McDonald, D.M. (2005) Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev* 15: 102–111.

Ben-Av, P., Crofford, L.J., Wilder, R.L., Hla, T. (1995) Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis. *FEBS Lett* 372: 83–87.

Bergers, G. and Benjamin, L.E. (2003) Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 3: 401–410.

Burger, R.A., Sill, M., Monk, B.J., Greer, B., Sorosky, J. (2005) phase II trial of bevacizumab in persistent or recurrent epithelial ovarian cancer (EOC) or primary peritoneal cancer (PPC): a gynecologic oncology group (GOC) study. *J Clin Oncol* 23: 457S–457S.

Burstein, H.J., Parker, L.M., Savoie, J., Younger, J., Kuter, I., Ryan, P.D., Garber, J.E., Campos, S.M., Shulman, L.N., Harris, L.N., Gelman, R., Winer, E. (2002) phase II trial of the anti-VEGF antibody bevacizumab in combination with vinorelbine for refractory advanced breast cancer. *Breast Cancer Res Treat* 79: Abstract 446.

Carmeliet, P. (2003) Angiogenesis in health and disease. *Nat Med* 9: 653–660.

Carmeliet, P. and Jain, R.K. (2000) Angiogenesis in cancer and other diseases. *Nature* 407: 249–257.

Carson, W.E., Biber, J., Shah, N., Reddy, K., Kefauver, C., Leming, P.D., Kendra, K., Walker, M. (2003) A phase 2 trial of a recombinant humanized monoclonal anti-vascular endothelial growth factor (VEGF) antibody in patients with malignant melanoma. *Proc Am Soc Clin Oncol* 22: 715 (Abstract 2873).

Cassidy, J., Clarke, S., Díaz-Rubio, E., Scheithauer, W., Figer, A., Wong, R., Koski, S., Lichinitser, M., Yang, T.-S., Saltz, L. (2006) First efficacy and safety results from XELOX-1/NO16966, a randomised 2 × 2 factorial phase III trial of XELOX vs. FOLFOX4 + bevacizumab or placebo in first-line metastatic colorectal cancer (MCRC). *Ann Oncol* 17(Suppl. 9): abstract LBA3.

Chen, H.X. (2004) Expanding the clinical development of bevacizumab. *Oncologist* 9(Suppl. 1): 27–35.

Cobleigh, M.A., Langmuir, V.K., Sledge, G.W., Miller, K.D., Haney, L., Novotny, W.F., Reimann, J.D., Vassel, A. (2003) A phase I/II dose-escalation trial of bevacizumab in previously treated metastatic breast cancer. *Semin Oncol* 30(5 Suppl. 16): 117–124.

Cohen, T., Nahari, D., Cerem, L.W., Neufeld, G., Levi, B.Z. (1996) Interleukin 6 induces the expression of vascular endothelial growth factor. *J Biol Chem* 271: 736–741.

Crane, C., Ellis, L., James, A., Henry, X., Douglas, E., Linus, H., Eric, T., Chaan, N., Jeffrey, L., Robert, W. (2005) phase I trial

of bevacizumab (BV) with concurrent radiotherapy (RT) and capecitabine (CAP) in locally advanced pancreatic adenocarcinoma (PA). *Proceedings WCGC* (Abstract O-009).

de Gramont, A., Figer, A., Seymour, M., Homerin, M., Hmissi, A., Cassidy, J., Boni, C., Cortes-Funes, H., Cervantes, A., Freyer, G., Papamichael, D., Le Bail, N., Louvet, C., Hendler, D., de Braud, F., Wilson, C., Morvan, F., Bonetti, A. (2000) Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *J Clin Oncol* 18: 2938–2947.

de Gramont, A., Cervantes, A., Andre, T., Figer, A., Lledo, G., Flesch, M., Mineur, L., Russ, G., Quinaux, E., Etienne, P.-L. (2004) OPTIMOX study: FOLFOX 7/LV5FU2 compared to FOLFOX 4 in patients with advanced colorectal cancer. *J Clin Oncol* 22(Suppl.): 251s (Abstract 3525).

Demetri, G.D., van Oosterom, A.T., Blackstein, M., Garrett, C., Shah, M., Heinrich, M., McArthur, G., Judson, I., Baum, C.M., Casali, P.G. (2005) phase III, multicenter, randomized, double-blind, placebo-controlled trial of SU11248 in patients (pts) following failure of imatinib for metastatic GIST. *J Clin Oncol* 23(Suppl.): 308s (Abstract 4000).

Dickler, M., Rugo, H., Caravelli, J., Brogi, E., Sachs, D., Panageas, K., Flores, S., Moasser, M., Norton, L., Hudis, C. (2004) phase II trial of erlotinib (OSI-774), an epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitor, and bevacizumab, a recombinant humanized monoclonal antibody to vascular endothelial growth factor (VEGF), in patients (pts) with metastatic breast cancer (MBC). *J Clin Oncol* 22(Suppl.): 127s (Abstract 2001).

Douillard, J.Y., Cunningham, D., Roth, A.D., Navarro, M., James, R.D., Karasek, P., Jandik, P., Iveson, T., Carmichael, J., Alakl, M., Gruia, G., Awad, L., Rougier, P. (2000) Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet* 355: 1041–1047.

Escudier, B., Szczylik, C., Eisen, T., Stadler, W.M., Schwartz, B., Shan, M., Bukowski, R.M. (2005) Randomized phase III trial of the Raf kinase and VEGFR inhibitor sorafenib (BAY 43-9006) in patients with advanced renal cell carcinoma (RCC). *J Clin Oncol* 23(Suppl.): 380s (Abstract LBA4510).

Ferrara, N. (2001) Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* 280: C1358–C1366.

Ferrara, N. (2002) VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* 2: 795–803.

Ferrara, N., Gerber, H.P., LeCouter, J. (2003) The biology of VEGF and its receptors. *Nat Med* 9: 669–676.

Ferrara, N., Hillan, K.J., Gerber, H.P., Novotny, W. (2004) Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov* 3: 391–400.

Frank, S., Hubner, G., Breier, G., Longaker, M.T., Greenhalgh, D.G., Werner, S. (1995) Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. *J Biol Chem* 270: 12607–12613.

Fyfe, G.A., Hurwitz, H., Fehrenbacher, L., Cartwright, T., Cartwright, J., Heim, W., Berlin, J., Kabbinavar, F., Holmgren, E., Novotny, W. (2004) Bevacizumab plus irinotecan/5-FU/leucovorin for treatment of metastatic colorectal cancer results in survival benefit in all pre-specified patient subgroups. *J Clin Oncol* 22(Suppl.): 274s (Abstract 3617).

Gabrilovich, D.I., Ishida, T., Nadaf, S., Ohm, J.E., Carbone, D.P. (1999) Antibodies to vascular endothelial growth factor enhance the efficacy to cancer immunotherapy by improving endogenous dendritic cell function. *Clin Cancer Res* 5: 2963–2970.

Gasparini, G., Toi, M., Gion, M., Verderio, P., Dittadi, R., Hanatani, M., Matsubara, I., Vinante, O., Bonoldi, E., Boracchi, P., Gatti, C., Suzuki, H., Tominaga, T. (1997) Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. *J Natl Cancer Inst* 89: 139–147.

Gasparini, G., Toi, M., Miceli, R., Vermeulen, P.B., Dittadi, R., Biganzoli, E., Morabito, A., Fanelli, M., Gatti, C., Suzuki, H., Tominaga, T., Dirix, L.Y., Gion, M. (1999) Clinical relevance of vascular endothelial growth factor and thymidine phosphorylase in patients with node-positive breast cancer treated with either adjuvant chemotherapy or hormone therapy. *Cancer J Sci Am* 5: 101–111.

Giantonio, B.J., Catalano, P.J., Meropol, N.J., O'Dwyer, P.J., Mitchell, E.P., Alberts, S.R., Schwartz, M.A., Benson, A.B. (2005) High-dose bevacizumab improves survival when combined with FOLFOX4 in previously treated advanced colorectal cancer: results from the Eastern Cooperative Oncology Group (ECOG) study E3200. *J Clin Oncol* 23(Suppl.): 1s (Abstract 2).

Goldberg, R.M., Sargent, D.J., Morton, R.F., Fuchs, C.S., Ramanathan, R.K., Williamson, S.K., Findlay, B.P., Pitot, H.C., Alberts, S.R. (2004) A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. *J Clin Oncol* 22: 23–30.

Gordon, M.S., Margolin, K., Talpaz, M., Sledge, G.W.J., Holmgren, E., Benjamin, R., Stalter, S., Shak, S., Adelman, D. (2001) phase I safety and pharmacokinetic study of recombinant human anti-vascular endothelial growth factor in patients with advanced cancer. *J Clin Oncol* 19: 843–850.

Grothey, A., Sargent, D., Goldberg, R.M., Schmoll, H.J. (2004) Survival of patients with advanced colorectal cancer improves with the availability of fluorouracil-leucovorin, irinotecan, and oxaliplatin in the course of treatment. *J Clin Oncol* 22: 1209–1214.

Hainsworth, J.D., Sosman, J.A., Spigel, D.R., Edwards, D.L., Baughman, C., Greco, A. (2005a) Treatment of metastatic renal cell carcinoma with a combination of bevacizumab and erlotinib. *J Clin Oncol* 23: 7889–7896.

Hainsworth, J.D., Sosman, J.A., Spigel, D.R., Patton, J.F., Thompson, D.S., Sutton, V., Hart, L.L., Yost, K., Greco, F.A. (2005b) Bevacizumab, erlotinib, and imatinib in the treatment of patients (pts) with advanced renal cell carcinoma (RCC): a Minnie Pearl Cancer Research Network phase I/II trial. *J Clin Oncol* 23(Suppl.): 388s (Abstract 4542).

Hambleton, J., Novotny, W.F., Hurwitz, H., Fehrenbacher, L., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Kabbinavar, F., Holmgren, E. (2004) Bevacizumab does not increase bleeding in patients with metastatic colorectal cancer receiving concurrent anticoagulation. *J Clin Oncol* 22(Suppl.): 252s (Abstract 3528).

Harmey, J.H. and Bouchier-Hayes, D. (2002) Vascular endothelial growth factor (VEGF), a survival factor for tumour cells: implications for anti-angiogenic therapy. *BioEssays* 24: 280–283.

Hecht, J.R., Trarbach, T., Jaeger, E., Hainsworth, J., Wolff, R., Lloyd, K., Bodoky, G., Borner, M., Laurent, D., Jacques, C. (2005) A randomized, double-blind, placebo-controlled, phase III study in patients (Pts) with metastatic adenocarcinoma of the colon or rectum receiving first-line chemotherapy with oxaliplatin/5-fluorouracil/leucovorin and PTK787/ZK 222584 or placebo (CONFIRM-1). *J Clin Oncol* 23(Suppl.): 2s (Abstract LBA3).

Hedrick, E., Kozloff, M., Hainsworth, J., Badarinath, S., Cohn, A., Flynn, P., Dong, W., Suzuki, S., Sugrue, M., Grothey, A. (2006) Safety of bevacizumab plus chemotherapy as first-line treatment of patients with metastatic colorectal cancer: updated results from a large observational registry in the us (BRiTE). *J Clin Oncol* 24(Suppl.): 155s (Abstract 3536).

Herbst, R.S., Fehrenbacher, L., Belani, C.P., Bonomi, P.D., Hart, L., Melnyk, O., Sandler, A., Lin, M., O'Neill, V.J. (2006) A phase II, multicenter, randomized clinical trial to evaluate the efficacy and safety of bevacizumab (Avastin) in combination with either chemotherapy (docetaxel or pemetrexed) or erlotinib hydrochloride (Tarceva) compared with chemotherapy alone for treatment of recurrent or refractory non-small-cell lung cancer. *Eur J Cancer Suppl* 4: 20 (Abstract 53).

Herbst, R.S., Johnson, D.H., Mininberg, E., Carbone, D.P., Henderson, T., Kim, E.S., Blumenschein, G., Jr., Lee, J.J., Liu, D.D.,

Truong, M.T., Hong, W.K., Tran, H., Tsao, A., Xie, D., Ramies, D.A., Mass, R., Seshagiri, S., Eberhard, D.A., Kelley, S.K., Sandler, A. (2005) Phase I/II trial evaluating the anti-vascular endothelial growth factor monoclonal antibody bevacizumab in combination with the HER-1/epidermal growth factor receptor tyrosine kinase inhibitor erlotinib for patients with recurrent non-small-cell lung cancer. *J Clin Oncol* 23: 2544–2555.

Hochster, H.S., Hart, L.L., Ramanathan, R.K., Hainsworth, J.D., Hedrick, E.E., Childs, B.H. (2006) Safety and efficacy of oxaliplatin/fluoropyrimidine regimens with or without bevacizumab as first-line treatment of metastatic colorectal cancer (mCRC): Final analysis of the TREE-Study. *J Clin Oncol* 24(Suppl.): 148s (Abstract 3510).

Holden, S., Ryan, E., Kearns, A., Holmgren, E., Hurwitz, H. (2005) Benefit from bevacizumab (BV) is independent of pretreatment plasma vascular endothelial growth factor-A (pl-VEGF) in patients (pts) with metastatic colorectal cancer (mCRC). *J Clin Oncol* 23(Suppl.): 259s (Abstract 3555).

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., Ferrara, N., Fyfe, G., Rogers, B., Ross, R., Kabbinavar, F. (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 350: 2335–2342.

Hurwitz, H.I., Fehrenbacher, L., Hainsworth, J.D., Heim, W., Berlin, J., Holmgren, E., Hambleton, J., Novotny, W. F., Kabbinavar, F. (2005) Bevacizumab in combination with fluorouracil and leucovorin: an active regimen for first-line metastatic colorectal cancer. *J Clin Oncol* 23: 3502–3508.

Jain, R.K. (2001) Normalizing tumor vasculature with anti-angiogenic therapy: a new paradigm for combination therapy. *Nat Med* 7: 987–989.

Jain, R.K. (2002) Tumor angiogenesis and accessibility: role of vascular endothelial growth factor. *Semin Oncol* 29: 3–9.

Jain, R.K. (2003) Molecular regulation of vessel maturation. *Nat Med* 9: 685–693.

Jain, R.K. (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 307: 58–62.

Johnson, D.H., Fehrenbacher, L., Novotny, W.F., Herbst, R.S., Nemunaitis, J.J., Jablons, D.M., Langer, C.J., DeVore, R.F., III, Gaudreault, J., Damico, L.A., Holmgren, E., Kabbinavar, F. (2004) Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. *J Clin Oncol* 22: 2184–2191.

Kabbinavar, F.F., Wong, J.T., Ayala, R.E., Wintroub, A.B., Kim, K.J., Ferrara, N. (1995) The effect of antibody to vascular endothelial growth factor and cisplatin on the growth of lung tumors in nude mice. *Proc Am Assoc Cancer Res* 36: 488 (Abstract 2906).

Kabbinavar, F., Hurwitz, H.I., Fehrenbacher, L., Meropol, N.J., Novotny, W.F., Lieberman, G., Griffing, S., Bergsland, E. (2003) phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. *J Clin Oncol* 21: 60–65.

Kabbinavar, F.F., Schulz, J., McCleod, M., Patel, T., Hamm, J.T., Hecht, J.R., Mass, R., Perrou, B., Nelson, B., Novotny, W.F. (2005a) Addition of bevacizumab to bolus fluorouracil and leucovorin in first-line metastatic colorectal cancer: results of a randomized phase II trial. *J Clin Oncol* 23: 3697–3705.

Kabbinavar, F.F., Hambleton, J., Mass, R.D., Hurwitz, H.I., Bergsland, E., Sarkar, S. (2005b) Combined analysis of efficacy: the addition of bevacizumab to fluorouracil/leucovorin improves survival for patients with metastatic colorectal cancer. *J Clin Oncol* 23: 3706–3712.

Karp, J.E., Gojo, I., Pili, R., Gocke, C.D., Greer, J., Guo, C., Qian, D., Morris, L., Tidwell, M., Chen, H., Zwiebel, J. (2004) Targeting vascular endothelial growth factor for relapsed and refractory adult acute myelogenous leukemias: therapy with sequential 1-beta-d-arabinofuranosylcytosine, mitoxantrone, and bevacizumab. *Clin Cancer Res* 10: 3577–3585.

Kelly, K., Crowley, J., Bunn, P.A., Jr., Presant, C.A., Grevstad, P.K., Moinpour, C.M., Ramsey, S.D., Wozniak, A.J., Weiss, G.R., Moore, D.F., Israel, V.K., Livingston, R.B., Gandara, D.R. (2001) Randomized phase III trial of paclitaxel plus carboplatin versus vinorelbine plus cisplatin in the treatment of patients with advanced non-small-cell lung cancer: a Southwest Oncology Group trial. *J Clin Oncol* 19: 3210–3218.

Kerbel, R. and Folkman, J. (2002) Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* 2: 727–739.

Kim, K.J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H.S., Ferrara, N. (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 362: 841–844.

Kindler, H.L., Friberg, G., Stadler, W.M., Singh, D.A., Locker, G., Nattam, S., Kozloff, M., Kasza, K., Vokes, E.E. (2004) Bevacizumab (B) plus gemcitabine (G) in patient (pts) with advanced pancreatic cancer (PC): updated results of a multi-center phase II trial. *J Clin Oncol* 22(Suppl.): 315s (Abstract 4009).

Kozloff, M., Hainsworth, J., Badarinath, S., Cohn, A., Flynn, P., Steis, R., Dong, W., Suzuki, S., Sugrue, M., Grothey, A. (2006) Efficacy of bevacizumab plus chemotherapy as first-line treatment of patients with metastatic colorectal cancer: updated results from a large observational registry in the us (BRiTE). *J Clin Oncol* 24(Suppl.): 155s (Abstract 3537).

Linderholm, B.K., Lindahl, T., Holmberg, L., Klaar, S., Lennerstrand, J., Henriksson, R., Bergh, J. (2001) The expression of vascular endothelial growth factor correlates with mutant p53 and poor prognosis in human breast cancer. *Cancer Res* 61: 2256–2260.

Margolin, K., Gordon, M.S., Holmgren, E., Gaudreault, J., Novotny, W., Fyfe, G., Adelman, D., Stalter, S., Breed, J. (2001) phase Ib trial of intravenous recombinant humanized monoclonal antibody to vascular endothelial growth factor in combination with chemotherapy in patients with advanced cancer: pharmacologic and long-term safety data. *J Clin Oncol* 19: 851–856.

Miller, K.D., Chap, L.I., Holmes, F.A., Cobleigh, M.A., Marcom, P.K., Fehrenbacher, L., Dickler, M., Overmoyer, B.A., Reimann, J.D., Sing, A.P., Langmuir, V., Rugo, H.S. (2005a) Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. *J Clin Oncol* 23: 792–799.

Miller, K.D., Wang, M., Gralow, J., Dickler, M., Cobleigh, M.A., Perez, E.A.S., Davidson, N.E. (2005b) Eastern Cooperative Oncology Group. A randomised phase III trial of paclitaxel versus paclitaxel plus bevacizumab as first-line therapy for locally recurrent or metastatic breast cancer. Presented at the 41st Annual Meeting of the American Society of Clinical Oncology, Orlando, Fl, USA, May 13–17.

Motzer, R.J., Rini, B.I., Michaelson, M.D., Redman, B.G., Hudes, G.R., Wilding, G., Bukowski, R.M., George, D.J., Kim, S.T., Baum, C.M. (2005) phase 2 trials of SU11248 show antitumor activity in second-line therapy for patients with metastatic renal cell carcinoma (RCC). *J Clin Oncol* 24(Suppl.): 380s (Abstract 4508).

Osusky, K.L., Hallahan, D.E., Fu, A., Ye, F., Shyr, Y., Geng, L. (2004) The receptor tyrosine kinase inhibitor SU11248 impedes endothelial cell migration, tubule formation, and blood vessel formation in vivo, but has little effect on existing tumor vessels. *Angiogenesis* 7: 225–233.

Poon, R.T.-P., Fan, S.-T., Wong, J. (2001) Clinical implications of circulating angiogenic factors in cancer patients. *J Clin Oncol* 19: 1207–1225.

Presta, L.G., Chen, H., O'Connor, S.J., Chisholm, V., Meng, Y.G., Krummen, L., Winkler, M., Ferrara, N. (1997) Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res* 57: 4593–4599.

Ramaswamy, B. and Shapiro, C.L. (2003) phase II trial of bevacizumab in combination with docetaxel in women with advanced breast cancer. *Clin Breast Cancer* 4: 292–294.

Rini, B., Weinberg, V., Fong, L., Small, E. (2005) A phase 2 study of prostatic acid phosphatase-pulsed dendritic cells

(APC8015; Provenge) in combination with bevacizumab in patients with serologic progression of prostate cancer after local therapy. Presented at the Prostate Cancer Symposium, Orlando, Fl, USA, February 17–19 (Abstract 251).

Rivera, F., Cunningham, D., Michael, M., DiBartolomeo, M., Kretzschmar, A., Berry, S., Mazier, M., Lutiger, B., Van Cutsem, E. (2006) Preliminary safety of bevacizumab with first-line Folfox, CapOx, Folfiri and Capecitabine for mCRC-First BEAT. Presented at 8th World Congress on Gastrointestinal Cancer 2006 (Abstract O-017).

Rugo, H.S., Dickler, M.N., Scott, J.H., Moore, D.H., Melisko, M., Yeh, B.M., Caravelli, J., Brogi, E., Hudis, C., Park, J.W. (2005) Change in circulating endothelial cells (CEC) and tumor cells (CTC) in patients (pts) receiving bevacizumab and erlotinib for metastatic breast cancer (MBC) predicts stable disease at first evaluation. *J Clin Oncol* 23(Suppl.): 10s (Abstract 525).

Ryan, A.M., Eppler, D.B., Hagler, K.E., Bruner, R.H., Thomford, P.J., Hall, R.L., Shopp, G.M., O'Neill, C.A. (1999) Preclinical safety evaluation of rhuMABVEGF, an antiangiogenic humanized monoclonal antibody. *Toxicol Pathol* 27: 78–86.

Saltz, L.B., Cox, J.V., Blanke, C., Rosen, L.S., Fehrenbacher, L., Moore, M.J., Maroun, J. A., Ackland, S.P., Locker, P.K., Pirotta, N., Elfring, G.L., Miller, L.L. (2000) Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. *N Engl J Med* 343: 905–914.

Sandler, A.B., Blumenschein, G.R., Henderson, T., Lee, J., Truong, M., Kim, E., Mass, B., Garcia, B., Johnson, D.H., Herbst, R.S. (2004) phase I/II trial evaluating the anti-VEGF MAb bevacizumab in combination with erlotinib, a HER1/EGFR-TK inhibitor, for patients with recurrent non-small cell lung cancer. *J Clin Oncol* 22(Suppl.): 127s (Abstract 2000).

Sandler, A.B., Gray, R., Brahmer, J., Dowlati, A., Schiller, J.H., Perry, M.C., Johnson, D.H. (2005) Randomized phase II/III trial of paclitaxel (P) plus carboplatin I with or without bevacizumab (NSC # 704865) in patients with advanced non-squamous non-small cell lung cancer (NSCLC): an Eastern Cooperative Oncology Group (ECOG) trial – E4599. *J Clin Oncol* 23(Suppl.): 2s (Abstract LBA4).

Schering AG. (2005) Schering and Novartis's vatalanib unlikely to meet phase II cancer trial endpoint. Press Release Posted on 28 July 2005.

Schiller, J.H., Harrington, D., Belani, C.P., Langer, C., Sandler, A., Krook, J., Zhu, J., Johnson, D.H. (2002) Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* 346: 92–98.

Schwartz, J.D., Schwartz, M., Goldman, J., Lehrer, D., Coll, D., Kinkabwala, M., Wadler, S. (2004) Bevacizumab in hepatocellular carcinoma in patients without metastasis and without invasion of the portal vein. *J Clin Oncol* 22(Suppl.): 355s (Abstract 4088).

Shah, M.A., Ilson, D., Ramanathan, R.K., Levner, A., D'Adamo, D., Schwartz, L., Casper, E., Schwartz, G.K., Kelsen, D.P. (2005) A multicenter phase II study of irinotecan (CPT), cisplatin (CIS), and bevacizumab (BEV) in patients with unresectable or metastatic gastric or gastroesophageal junction (GEJ) adenocarcinoma. *J Clin Oncol* 23(Suppl.): 314s (Abstract 4025).

Shen, B.Q., Stainton, S., Li, D., Pelletier, N., Zioncheck, T.F. (2004) Combination of Avastin and Xeloda synergistically inhibits colorectal tumor growth in a COLO205 tumor xenograft model. *Proc Am Assoc Cancer Res* 45: 508 (Abstract 2203).

Shibuya, M. (2001) Structure and function of VEGF/VEGF-receptor system involved in angiogenesis. *Cell Struct Funct* 26: 25–35.

Shifren, J.L., Mesiano, S., Taylor, R.N., Ferrara, N., Jaffe, R.B. (1998) Corticotropin regulates vascular endothelial growth factor expression in human fetal adrenal cortical cells. *J Clin Endocrinol Metab* 83: 1342–1347.

Soffer, S.Z., Moore, J.T., Kim, E., Huang, J., Yokoi, A., Manley, C., O'Toole, K., Stolar, C., Middlesworth, W., Yamashiro, D.J., Kandel, J.J. (2001) Combination antiangiogenic therapy: increased efficacy in a murine model of Wilms tumor. *J Pediatr Surg* 36: 1177–1181.

Soh, E.Y., Sobhi, S.A., Wong, M.G., Meng, Y.G., Siperstein, A.E., Clark, O.H., Duh, Q. Y. (1996) Thyroid-stimulating hormone promotes the secretion of vascular endothelial growth factor in thyroid cancer cell lines. *Surgery* 120: 944–947.

Stopeck, A.T., Bellamy, W., Unger, J., Rimsza, L., Iannone, M., Fisher, R.I., Miller, T.P. (2005) phase II trial of single agent bevacizumab (Avastin) in patients with relapsed, aggressive non-Hodgkin's lymphoma (NHL): Southwest Oncology Group Study S0108. *J Clin Oncol* 23: 583s.

Sweeney, C.J., Miller, K.D., Sissons, S.E., Nozaki, S., Heilman, D.K., Shen, J., Sledge, G.W.J. (2001) The antiangiogenic property of docetaxel is synergistic with a recombinant humanized monoclonal antibody against vascular endothelial growth factor or 2-methoxyestradiol but antagonized by endothelial growth factors. *Cancer Res* 61: 3369–3372.

Traina, T.A., Dickler, M.N., Caravelli, J.F., Yeh, B.M., Brogi, E., Scott, J., Moore, D., Panageas, K., Flores, S.A., Norton, L., Park, J., Hudis, C., Rugo, H. (2005) A phase II trial of letrozole in combination with bevacizumab, an anti-VEGF antibody, in patients with hormone receptor-positive metastatic breast cancer. *Breast Cancer Res Treat* 94: Abstract 2030.

Vitaliti, A., Wittmer, M., Steiner, R., Wyder, L., Neri, D., Klemenz, R. (2000) Inhibition of tumor angiogenesis by a single-chain antibody directed against vascular endothelial growth factor. *Cancer Res* 60: 4311–4314.

Warren, R.S., Yuan, H., Matli, M.R., Gillett, N.A., Ferrara, N. (1995) Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 95: 1789–1797.

Wiesmann, C., Fuh, G., Christinger, H.W., Eigenbrot, C., Wells, J.A., de Vos, A.M. (1997) Crystal structure at 1.7 A resolution of VEGF in complex with domain 2 of the Flt-1 receptor. *Cell* 91: 695–704.

Willett, C.G., Boucher, Y., di Tomaso, E., Duda, D.G., Munn, L.L., Tong, R.T., Chung, D.C., Sahani, D.V., Kalva, S.P., Kozin, S.V., Mino, M., Cohen, K.S., Scadden, D.T., Hartford, A.C., Fischman, A.J., Clark, J.W., Ryan, D.P., Zhu, A.X., Blaszkowsky, L.S., Chen, H.X., Shellito, P.C., Lauwers, G.Y., Jain, R.K. (2004) Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nat Med* 10: 145–147.

Yancopoulos, G.D., Davis, S., Gale, N.W., Rudge, J.S., Wiegand, S.J., Holash, J. (2000) Vascular-specific growth factors and blood vessel formation. *Nature* 407: 242–248.

Yang, J.C., Haworth, L., Sherry, R.M., Hwu, P., Schwartzentruber, D.J., Topalian, S.L., Steinberg, S.M., Chen, H.X., Rosenberg, S.A. (2003) A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med* 349: 427–434.

Zhu, A.X., Sahani, D., Norden-Zfoni, A., Holalkere, N.S., Blaszkowsky, L., Ryan, D. P., Clark, J., Taylor, K., Heymach, J.V., Stuart, K. (2005) A phase II study of gemcitabine, oxaliplatin in combination with bevacizumab (GEMOX-B) in patients with hepatocellular carcinoma. Presented at: 2005 Gastrointestinal Cancers Symposium; 27–29 January; Hollywood, Fl. Abstract 131. Available at: http://www.asco.org. Accessed 15 February.

4
Cetuximab (Erbitux, C-225)

Norbert Schleucher and Udo Vanhoefer

4.1
Introduction

Cetuximab is a chimeric monoclonal IgG1 antibody (Fig. 4.1) targeting the epidermal growth factor receptor (EGF-R).

The EGF-R (HER1, ErbB1) is one member of the erbB family consisting of four members, HER1 to HER4. HER2 is a truncated receptor, while HER3 has a point mutation in the ATP binding site resulting in a nonfunctional tyrosine kinase. The EGF-R is a transmembrane protein activated by various ligands such as transforming growth factor alpha (TGF-α), amphiregulin, betacellulin, or epidermal growth factor (EGF). Activation causes dimerization, homo-dimerization with another EGR-R, or hetero-dimerization with another member of the erbB family, and phosphorylization on the internal tyrosine-kinase domain. Activation of the internal tyrosine kinase domain induces a signal transduction network leading to cellular proliferation, dedifferentiation and protection from apoptosis, and further to angiogenesis, migration, invasion, and metastases. One important pathway here is the activation of phosphoinosin-3-kinase (PI3K) and the serine-threonine kinase AKT, causing a suppression of the PTEN tumor suppressor gene. One the other hand, protein kinase C (PKC) and mitogen-activated protein (MAP) kinase are activated by G-protein-dependent mechanisms. Thus, a variety of transcriptional factors are induced, leading to neoplastic proliferation as a post-translational effect.

The expression of EGF-R is usually determined by immunohistochemical staining. Thus, EGF-R is seen to be expressed in gastrointestinal malignancies, especially colorectal cancer (CRC), gynecologic tumors (breast cancer and ovarian cancer), genitourinary tumors (prostate-, bladder- and renal cell cancer; RCC), non-small cell lung cancer (NSCLC), and in squamous cell cancer of the head and neck (see Table 4.1). In general, EGF-R is expressed in approximately 60 to 80% of these malignancies.

Immunohistochemical staining of EGF-R expression on the cellular surface is limited by the receptor recycling. EGF-R is internalized and re-expressed by either

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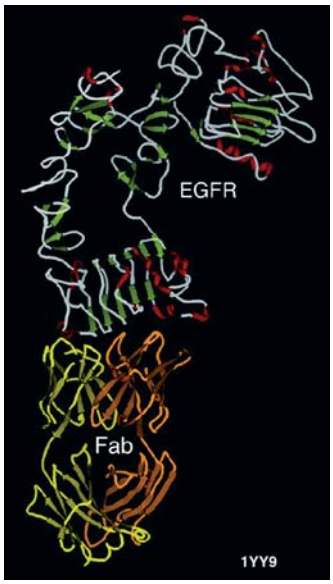

Fig. 4.1 Three-dimensional structure of the Fab fragment of cetuximab with its antigen, according to X-ray crystallographic data [49].

Table 4.1 EGFR expression in human malignancies.

Tumor	*EGF-R expression [%]*	*Reference(s)*
Colorectal cancer	25–82	Salomon (1995); Messa (1998) Goldstein (2001), Cunningham (2003)
Head and neck	80–100	Salomon (1995); Grandis (1996)
Pancreatic cancer	30–95	Salomon (1995); Uegaki (1997) Abbruzzese (2001)
NSCLC	40–81	Fujino (1996); Rusch (1997); Fontanini (1998); Gatzemeier (2003)
Renal cell cancer	50–90	Salomon (1995); Yoshida (1997)
Breast cancer	14–91	Klijn (1992); Beckman (1996); Bucci (1997); Walker (1999)
Ovarian cancer	35–70	Bartlett (1996); Fischer-Colbrie (1997)
Malignant glioma	40–63	Salomon (1995); Watanabe (1996); Rieske (1998)
Bladder cancer	31–48	Salomon (1995); Chow (1997)

NSCLC = non-small cell lung cancer.

smooth pits or coated pits. After internalization, the receptor may be degraded, with the consequent formation of multivesicular bodies, while the EGF-R is degraded in the lysosomes. In the case of recycling, a Golgi vesicle transports a recycled receptor to the cellular surface [1,2].

The expression of EGF-R has been inversely correlated with the prognosis of cancer patients; hence, patients with EGF-R-positive tumors have in general shorter survival times compared to patients with EGF-R-negative tumors [3–5]. In 1999, Inada evaluated 40 patients with esophageal cancer who underwent surgery; the 5-year survival among the EGF-R positive group was approximately 40%, compared to 70% in the EGF-R-negative group [6]. In general, patients with EGF-R-positive tumors are diagnosed at an advanced stage of the disease.

4.2 Preclinical Activity

In preclinical settings, cetuximab shows anti-tumor activity in a variety of cancer cell lines, the activity being comparable to that of conventional cytotoxic drugs. Furthermore, cetuximab can enhance the antitumor activity of irinotecan, platinum-derivatives, and fluoropyrimidines.

In human HT29 colon carcinoma nude mice xenografts, cetuximab induced a threefold reduction in tumor volume compared to untreated mice, and showed an almost equivalent antitumor activity as irinotecan. In this cell line the combination of cetuximab and irinotecan was synergistic such that no tumor growth was measured. Moreover, the combination of both drugs was even synergistic in irinotecan-refractory HT29 colon carcinoma xenografts, inducing a fourfold reduction in tumor growth compared to the irinotecan-treated controls [7].

Cisplatin resistance was reverted by the murine precursor drug M-225 in an A431 squamous cell cancer cell line in nude mice [8]. In this cisplatin-resistant cell line, a more than fourfold reduction in tumor volume resulted following addition of the noncytotoxic antibody M-225 to cisplatin treatment.

Cetuximab showed activity against RCC both *in-vitro* and *in-vivo*. In the RCC cell lines A498, Caki-1, SK-RC-4, SK-RC-29 and SW839, DNA synthesis was inhibited in a dose-dependent manner by treatment with cetuximab, while in Caki-1 ascites xenografts the survival of mice treated with cetuximab was prolonged compared to that of untreated mice [9].

In prostate cancer, cetuximab showed antitumor activity either alone or in combination with doxorubicin, with a significant reduction of tumor growth (DU145 and PC-3 xenografts) [10].

Cetuximab showed preclinical activity in p53 wild-type HepG2 hepatocellular carcinoma, and also induced cell cycle arrest in the G_0/G_1 phase, an increase of expression of the cyclin-dependent kinase inhibitors p21 (Waf1/CIP1) and p27 (Kip1), and a decrease in cyclin D1 expression. The combination with erlotinib, fluvastatin or doxorubicin resulted in synergistic antiproliferative effects [37].

Similar cell-cycle effects were seen in various cetuximab-sensitive cell lines of NSCLC, whereby synergistic effects were described for the combination of cetuximab with cisplatin, paclitaxel, or radiation [38].

Differential results were seen in pancreatic cancer cell lines. For example, after exposure to cetuximab the sensitivity of the MiaPaCa-2 pancreatic cancer cell line

to gemcitabine and radiation was increased, but in BxPC-3 the antiproliferative effects of gemcitabine and radiation were independent of cetuximab exposure. In MiaPaCa-2 the expression of the pro-apoptotic BAX was up-regulated, while resistance in BxPC-3 cells was mediated by an alternate pathway of Ras-MAPK activation [39]. Likewise, additive cytotoxic effects of cetuximab plus gemcitabine were observed in the human pancreatic carcinoma L3.6pl nude mouse xenografts [40].

The combination of cetuximab with EGF-R tyrosine-kinase inhibitors (gefitinib, erlotinib) is of clinical and preclinical interest. For example, in head and neck-, vulvar-, prostate- and NSCLC -cancer cell lines the activity of the combination was higher than the single-agent activity of these drugs [47].

The contribution by radiosentization was demonstrated by Milas in the A431 cell line in nude mice, when the addition of three doses of the C-225 antibody to 18 Gy radiation resulted in an impressive delay in tumor growth [11].

4.3 Clinical Data I: Outcome of Monotherapy

The single-agent activity of cetuximab in patients with refractory CRC was demonstrated in two phase II studies by Saltz [12] (in 57 patients) and Lenz (in 346 patients), and also in a randomized phase II study by Cunningham [14] (in 111 patients). In each of these studies, cetuximab induced 10% of the objective remissions according to RECIST criteria, and approximately 35% of stable diseases. The median overall survival ranged from 6.9 to 8.7 months. In these studies the patients were heavily pretreated and refractory against fluoropyrimidines, irinotecan, and oxaliplatin, while EGF-R positivity was mandatory. In another study, Lenz et al. [13] included nine patients without EGF-R expression, and two of these responded to cetuximab monotherapy. The resultant toxicity was usually mild and consisted of skin rash (6% grade 3/4) and asthenia (11% grade 3/4) [12–14].

Single-agent activity in patients with cisplatin-refractory head and neck cancer was evaluated by Trigo (n = 103) [15], wherein objective remissions and disease stabilizations were observed in 12.6% and 45.2% of patients, respectively. The median overall survival was 5.9 months, and toxicity due to cetuximab was generally mild, with grade 3 toxicities according to NCI-CTC criteria generally below 5% (grade 3 skin rash 1%, fever 2%, fatigue 4%). Similar data were presented by Vermorken at al. (16).

In recurrent NSCLC, single-agent therapy with cetuximab is effective in salvage treatment. For example, when 66 patients were entered into a phase II study (71% pretreated with a platinum derivative, 25% with docetaxel), partial remissions were obtained in 4.5% of patients, while 30.3% achieved disease stabilization. The median survival was 8.1 months; toxicity was generally mild, with 6.1% of patients reporting skin rash, 13.6% fatigue, and 15.2% dyspnea grade 3/4 [17].

Table 4.2 Cetuximab Grade 3 and 4 toxicities (%) in various studies.

	Cetuximab combination with					
	Mono [14]	***Irinotecan [14]***	***Irinotecan [18]***	***IFL [19]***	***FOLFIRI [21]***	***FOLFOX [22]***
Diarrhea	1.7	21.2	22	28	14	26
Asthenia	10.4	13.7	14	10	n.s.	9
Skin rash	5.2	9.4	8	19	7	21
Neutropenia	0	9.5	17	28	17	14
Nausea/vomiting	4.3	7.1	6	n.s.	11	5
Anemia	2.7	4.8	n.s.	n.s.	n.s.	n.s.
Abdominal pain	5.2	3.3	n.s.	n.s.	n.s.	n.s.
Thrombopenia	0.9	0.5	n.s.	n.s.	n.s.	n.s.
Hypersensitivity	3.5	0	3	0	4.3	n.s.

n.s. = not stated, due to abstract publication. For other abbreviations, see text.

In metastatic RCC, when 54 patients were treated with cetuximab in the first-line setting, 7% tumor responses (partial and minor responses) and 25% disease stabilizations for a minimum of 6 months were observed, in association with a favorable toxicity profile [36].

4.4 Clinical Data II: Outcome of Combination Therapy

4.4.1 Colorectal Cancer

In metastatic CRC the efficacy of the combination cetuximab + irinotecan was evaluated in 138 patients by Saltz et al. [12], and in 218 patients by Cunningham et al. [14] (the BOND [Bowel Oncology with Cetuximab Antibody] study). Both studies included patients with EGF-R positive tumors that were refractory to chemotherapy with 5-fluorouracil (5-FU), irinotecan, and oxaliplatin. Notably, in the BOND study more than 65% of patients were pretreated with all three drugs. The objective response rate was 23% in both studies, and the disease control rate 30 to 56%. In the BOND study the median time to disease progression was 4.1 months compared to 1.5 months for monotherapy ($p < 0.0001$). The median overall survival was 8.6 months compared to 6.9 months for single-agent treatment ($p = 0.48$). The grade 3 and 4 toxic effects encountered were manageable, and included diarrhea (22%), asthenia/fatigue (14%), neutropenia (10–17%), and skin rash (10–11%) [14,18]. Cetuximab was approved for the treatment of

metastatic EGF-R positive colorectal cancer in combination with irinotecan (not for monotherapy in Europe) following the failure of irinotecan and fluoropyrimide-based therapies.

Cetuximab was introduced into the first line treatment of metastatic CRC in a variety of phase II studies, and combined with all relevant combination regimes. Rosenberg et al. [19] evaluated the combination of cetuximab with weekly irinotecan, bolus 5-FU and leucovorin (LV) (the IFL regimen). A total of 29 patients was included, and the partial response rate was 48%, with an additional 41% disease stabilization. The toxicity was related to the IFL bolus regimen and consisted of grade 3 and 4 diarrhea and neutropenia in 28% of cases. A skin rash was observed in 21% of the patients [19]. Folprecht et al. [20] reported the results of a phase I/II study with a combination of cetuximab and weekly irinotecan/infusional 5-FU/LV (the AIO-irinotecan regimen) in 21 patients. The response was 67% and the stabilization rate of diseases was 29%. The median time to disease progression and median overall survival were impressive at 9.9 months and 33 months, respectively [20]. The combination of cetuximab with biweekly irinotecan and 5-FU/LV (FOLFIRI, CRYSTAL study) was described by Rougier et al. in 42 patients. Here, partial remissions were achieved in 46% of patients, and disease stabilization in 41%. The rates of high grade skin rash, diarrhea and neutropenia were 12%, 14%, and 12%, respectively [21]. Of major clinical interest was the combination of cetuximab with oxaliplatin and 5-FU/LV (FOLFOX-4 regimen). In this study (the ACROBAT study), the rate of complete response was 10%, but partial remissions were seen in 69% and 12% of patients were stable; this resulted in a clinical benefit rate of 91% (verified by an independent reviewer). Ten of 42 patients underwent metastatic surgery due to major response, and nine of these were completely (R0) resected. Grade III/IV toxicity was tolerable, with 33% of patients reporting skin-rash, 26% diarrhea, 26% neurotoxicity due to oxaliplatin, 21% neutropenia, and 16% mucositis [22].

The efficacy of cetuximab in combination with FOLFOX-4 in the second-line treatment of metastatic CRC after failure of an irinotecan-based first-line chemotherapy was analyzed in a randomized phase III trial (the EXPLORE study). The preliminary data after treatment of 102 patients showed a partial response rate of 20% in the experimental arm compared to 15.4% for FOLFOX-4 alone. Stable diseases were seen in 44% of patients versus 50% for patients treated with FOLFOX alone. Median progression-free survival and toxicity were similar in both arms [23]. The combination of cetuximab and irinotecan in second-line chemotherapy of CRC is currently under investigation (EPIC study). Following the analysis of data acquired from 400 patients, the combination appears to be feasible, though data relating to efficacy are not yet available. The study is ongoing until 1300 patients have been enrolled [24].

The efficacy of the antibody-combination of cetuximab and bevacizumab, with or without irinotecan, was analyzed by Saltz and colleagues in a randomized phase II trial (BOND-2 study) which included 81 patients with metastatic and refractory CRC [43]. The objective response rate for cetuximab and bevacizumab was 20%, with a progression-free survival of 5.6 months. The addition of

irinotecan resulted in a response rate of 37% and a progression-free survival of 7.9 months. The expression of EGF-R was not mandatory in this study [43].

Recently, an adjuvant study with FOLFOX-4 plus cetuximab has been initiated in CRC, as has an investigation into the first-line treatment of a metastatic setting in a randomized phase III study (FOLFOX-4 + cetuximab versus FOLFOX-4 alone; the OPUS study). An additional study evaluating the efficacy of cetuximab in patients with EGF-R-negative tumors (the OPERA study) has also been initiated; the rationale here is the observation of Chung et al., who described a 25% response rate in patients with EGF-R-negative tumors [44].

4.4.2 Head and Neck Cancer

The combination of cetuximab and cisplatin/carboplatin was evaluated in recurrent squamous cell cancer of head and neck refractory to platinum-based therapy. Baselga and Kies analyzed 96 and 79 patients, respectively, in two nonrandomized phase II trials [25,26]. The results were similar in both studies, with 10% partial remissions and 55% stable disease. The median survival ranged from 5.2 to 6.0 months, and grade 3/4 toxicities were less than 10% [25,26]. In another trial, 56 patients with nasopharyngeal cancer refractory to radiation and progressive within 12 months after a platinum-based chemotherapy were treated with cetuximab and carboplatin [27]. A partial response of 17% was observed, and 47% were stable. The main toxicities observed (grade 3/4) were skin rash (13%), anemia (18%), thrombocytopenia (11%), and asthenia (9%) [27].

4.4.3 Non-Small Cell Lung Cancer (NSCLC)

In first-line chemotherapy of NSCLC (adenocarcinoma and squamous cell carcinoma) the addition of cetuximab to cisplatin and vinorelbine probably enhances the antitumor activity (LUCAS study). A total of 86 patients was randomized, and the confirmed response rate was 31% for the cetuximab/cisplatin/vinorelbin combination compared to 20% in the conventional arm of the study. The stable disease rate and time to progression were similar in both arms. Leukopenia (64% versus 51%), asthenia (17% versus 5%), infections (12% versus 5%), and skin rash (5% versus 0%) were enhanced in the cetuximab arm [28]. The combination of cetuximab with carboplatin/paclitaxel or carboplatin/gemcitabine is also feasible [29,30].

In second-line therapy of NSCLC, cetuximab was combined with docetaxel in 47 patients progressive within 3 months after first-line chemotherapy and with EGF-R-positive tumors. The partial response rate was 28% and the rate of stable diseases 18%. The most common grade 3 toxicities were skin rash (19%), infection (21%), and fatigue (21%). Interestingly, four patients showed allergic reactions that led to a discontinuation of treatment [31].

4.4.4
Other Tumors

A phase II trial of cetuximab plus gemcitabine in advanced pancreatic cancer showed 12% partial responses and 63% stable disease, with a one-year overall survival rate of 32% and a median overall survival of 7.1 months. Grade III/IV toxicities were neutropenia 39%, asthenia 22%, abdominal pain 22%, and thrombocytopenia 17% [41].

In advanced ovarian cancer, primary peritoneal carcinoma and cancer of the Fallopian tube, cetuximab was combined with carboplatin and paclitaxel (six cycles) followed by a cetuximab maintenance (6 months). This schedule was safely applied to 25 patients, with clinical responses being shown in 68% (17/25) [32].

4.5
Clinical Data III: Cetuximab and Radiotherapy/Chemo-Radiotherapy

In advanced squamous cell carcinoma of the head and neck, the combination of cetuximab and high-dose radiation prolongs survival compared to radiotherapy alone. In a phase III study, 424 patients were randomized to standard treatment, which consisted of 70–76 Gy radiation, or to the experimental arm to which cetuximab was added to the radiation for 8 weeks [33]. The median overall survival was 54 months in the radiotherapy/cetuximab group compared with 28 months in patients receiving radiotherapy alone ($p = 0.02$). The 2- and 3-year survival rates were 62% and 57% compared to 55% and 44%, respectively. Cetuximab did not enhance the toxicity of radiotherapy. Based on these data, cetuximab in combination with radiotherapy was approved for head and neck cancer in 2006.

The combination of cetuximab, cisplatin and 70-Gy radiation was also evaluated [35]. Here, 21 patients received eight doses of standard-dose cetuximab, cisplatin $100\,mg\,m^{-2}$ in Weeks 1 and 4, and concomitant radiotherapy. The 2-year overall survival rate was 76%, but 46% of patients reported grade 4 toxicities (anaphylaxis, anorexia, arrhythmia, bacteremia, hypokalemia, hyponatremia, myocardial infarction and mucositis), including two toxic deaths. Thus, this treatment schedule was not recommended due to problems of toxicity.

In NSCLC the combination of cetuximab with carboplatin/paclitaxel and radiation appears to be feasible with dominant hematologic toxicity (50% grade 3/4 blood/bone marrow toxicity). The efficacy data are not yet available, however [34].

4.5.1
The PARC Study

A current randomized phase II trial in advanced pancreatic cancer is comparing simultaneous to sequential cetuximab in combination with gemcitabine-based chemoradiation therapy (the PARC study) [42].

4.5.1.1 Dosing Schedule

Cetuximab treatment is started with a loading dose of 400 mg m^{-2} as a 2-h infusion, followed by a maintenance dose of 250 mg m^{-2} as a 1-h infusion once weekly until disease progression. This dosing schedule does not require dose reduction in combination with radiotherapy [48] or chemotherapy for CRC [14,18–24], head and neck cancer [26,27], and NSCLC [28–31]. The dosing schedule for cetuximab in combination with chemoradiotherapy is currently under investigation.

Due to the possibility of allergic reactions, premedication with an antihistaminic drug (e.g., 2 mg clemastine or 50 mg diphenhydramine) is recommended. The use of steroids is possible without inhibiting the efficacy of cetuximab, but in general this is not necessary.

Cetuximab is a low-emetogenic drug; therefore, the anti-emetic treatment depends on the emetogenicity of the concomitant chemotherapy, and usually consists of 5-HT_3 antagonists.

4.5.1.2 Determinants of Cetuximab Efficacy

The efficacy of cetuximab treatment in CRC is independent of the EGF-R staining activity [14,18]. In the BOND study, the response rates for cetuximab/irinotecan were 21 to 25% for patients with weak, moderate or strong EGF-R staining. For the monotherapy with cetuximab the response rates were more heterogeneous, but not different.

One important clinical predictor of cetuximab activity is the skin rash. In the BOND study, patients without skin reactions did not respond to monotherapy, and 6% responded to the combination of cetuximab and irinotecan. Patients with skin rash responded to the combination in 26% of cases, and in 13% of cases to the monotherapy. In the combination arm the median survival was 9.1 months for patients with skin rash compared to 3.0 months for those without skin toxicity [14].

Currently, few data are available regarding the molecular determinants of cetuximab efficacy. High gene expression levels of vascular endothelial growth factor (VEGF) have been associated with cetuximab resistance. The combination of low gene expression levels of cyclooxygenase-2, EGF-R and IL-8 is a significant predictor for overall survival in refractory CRC. Patients with the low gene expression combination showed a 13.5 months survival after cetuximab treatment compared to 2.3 months for patients with high gene expression levels. These finding were independent of skin toxicity. Patients with lower levels of EGF-R messenger-RNA had a longer overall survival than patients who showed a higher EGF-R messenger-RNA levels (7.3 months versus 2.2 months). Interestingly, patients with lower expression of cyclooxygenase-2 had a significantly higher rate of skin toxicity due to cetuximab treatment [45]. Another potential prognostic molecular marker may be the polymorphism of the cyclin D1 gene A870G [46].

The impact of EGF-R mutations is currently under investigation.

4.5.1.3 Treatment of Skin Toxicity

The treatment of skin rash is not evidence-based and consists mainly of treatment elements from juvenile acne. In the acute exudative phase, desinfective or

antibiotic ointments can be used (e.g., benzoyl peroxide 5%, metronidazole gel, erythromycin 2% gel). Grade II or III skin toxicity mostly requires systemic antibiotic treatment, for example doxycycline (1–2 × 100 mg daily), tetracycline (2 × 250 mg), minocycline (2 × 50 mg), or the use of gyrase-inhibitors. Fatty ointments should not be used during the exudative phase, but in the xerotic phase skin care with fatty lotions is necessary; these include dexpanthenol-containing ointments, urea lotions, or bath oils. Antibiotic therapy is only required in case of superinfections, while antihistaminic drugs can be used for pruritus. It is also important to protect the skin from sunshine.

The treatment of nail toxicity (paronychia) consists of ichthyol ointments, local antibiotic treatment and puncture of abscesses. In the worst case the nail must be extracted.

References

1 Resat, H., Ewald, J.A., Dixan, D.A., et al. (2003) An integrated model of epidermal growth factor receptor trafficking and signal transduction. Biophys J 85: 730–745.

2 Wiley, H.S. (2003) Trafficking of the ErbB receptors and its influence on signalling. Exp Cell Res 284: 78–88.

3 Goldstein, N.S., Armin, M. (2001) Epidermal growth factor receptor immunohistochemical reactivity in patients with American Joint Committee on Cancer stage IV colon adenocarcinoma: implication for a standardized scoring system. Cancer 92: 1331–1346.

4 De Jong, K.P., Stellema, R., Karrenheld, A., et al. (1998) Clinical relevance of transforming growth factor alpha, epidermal growth factor receptor, p53 and Ki67 in colorectal liver metastases and corresponding primary tumours. Hepatology 28: 971–979.

5 Mayer, A., Takimoto, M., Fritz, E., et al. (1993) The prognostic significance of proliferating cell nuclear antigen, epidermal growth factor receptor, and mdr gene expression in colorectal cancer. Cancer 71: 2454–2460.

6 Inada, S., Koto, T., Futami, K., et al. (1999) Evaluation of malignancy and the prognosis of esophageal cancer based on an immunohistochemical study (p53, E-cadherin, epidermal growth factor receptor). Surg Today 29: 493–503.

7 Prewett, M.C., Hooper, A.T., Bassi, R., et al. (2002) Enhanced antitumour activity of anti-epidermal growth factor receptor monoclonal antibody IMC-C225 in combination with irinotecan (cpt-11) against human colorectal tumor xenografts. Clin Cancer Res 8: 994–1003.

8 Fan, Z., Baselga, J., Masui, H., et al. (1993) Antitumour effect of anti-epidermal growth factor receptor monoclonal antibodies plus cis-diamminedichloroplatinum on well established a431 cell xenografts. Cancer Res 53: 4637–4642.

9 Prewett, M.C., Rothman, M., Waksal, H., et al. (1998) Mouse-human chimeric anti-epidermal growth factor receptor antibody C225 inhibits the growth of human renal cell carcinoma xenografts in nude mice. Clin Cancer Res 4: 2957–2966.

10 Prewett, M.C., Rockwell, P., Rockwell, R.F., et al. (1996) The biological effects of C225, a chimeric monoclonal antibody to the EGF-R, on human prostate carcinoma. J Immunother Emphasis Tumor Immunol 19: 419–427.

11 Milas, L., Mason, K., Hunter, N., et al. (2000) In vivo enhancement of tumour radioresponse by C225 antiepidermal

growth factor receptor antibody. Clin Cancer Res 6: 701–708.
12 Saltz, L., Meropol, N.J., Needle, M.N., et al. (2004) phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J Clin Oncol 22: 1201–1208.
13 Lenz, H.J., Mayer, R.J., Gold, P.J., et al. (2004) Activity of cetuximab in patients with colorectal cancer refractory to both irinotecan and oxaliplatin. Proc Am Soc Clin Oncol 23: Abstract 3510.
14 Cunningham, D., Humblet, Y., Siena, S., et al. (2004) Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan refractory metastatic colorectal cancer. N Engl J Med 35: 337–345.
15 Trigo, J., Hitt, R., Koralewski, P., et al. (2004) Cetuximab monotherapy is active in patients with platinum refractory recurrent/metastatic squamous cell carcinoma of the head and neck: results of a phase II study. Proc Am Soc Clin Oncol 23: Abstract 5502.
16 Vermorken, J.B., Bourhis, J., Trigo, I., et al. (2005) Cetuximab in recurrent/ metastatic squamous cell carcinoma of head and neck refractory to first line platinum based therapies. Proc Am Soc Clin Oncol 24: Abstract 5505.
17 Lilenbaum, R., Bonomi, P., Ansari, R., et al. (2005) A phase II trial of cetuximab as therapy for recurrent non-small cell lung cancer. Proc Am Soc Clin Oncol 24: Abstract 7036.
18 Saltz, L., Rubin, M., Hochster, H., et al. (2001) Cetuximab (IMC-C225) plus irinotecan (CPT-11) is active in CPT-11 refractory colorectal cancer that expresses epidermal growth factor receptor. Proc Am Soc Clin Oncol 20: Abstract 7.
19 Rosenberg, A.H., Loehrer, P.J., Needle, M.N., et al. (2002) Erbitux (IMC-C225) plus weekly irinotecan, fluorouracil and leucovorin in colorectal cancer that expresses the epidermal growth factor receptor. Proc Am Soc Clin Oncol 21: Abstract 536.
20 Folprecht, G., Lutz, M., Schöffski, P., et al. (2005) Cetuximab and irinotecan/ 5-FU/FA (AIO) is active and safe in the first line treatment of epidermal growth factor receptor expressing metastatic colorectal cancer. World Conference on Gastrointestinal Cancer, Abstract 053.
21 Rougier, P., Raoul, J.L., Van Laethem, J.L., et al. (2004) Cetuximab + FOLFIRI as first line treatment for metastatic colorectal cancer. Proc Am Soc Clin Oncol 23: Abstract 3513.
22 Cervantes, A., Casado, E., van Cutsem, E., et al. (2005) Phase II study of cetuximab plus FOLFOX-4 in first line setting for epidermal growth factor receptor expressing metastatic colorectal cancer. Eur J Cancer Suppl. Vol. 3(2), p 181.
23 Polikoff, J., Mitchell, E.P., Badarinath, S., et al. (2005) Cetuximab plus FOLFOX for colorectal cancer (EXPLORE): preliminary efficacy analysis of a randomized phase III trial. Proc Am Soc Clin Oncol 24: Abstract 3574.
24 Sobrero, A., Scheithauer, W., Laurel, J., et al. (2005) Cetuximab plus irinotecan for metastatic colorectal cancer: safety analysis of the first 400 patients in a randomized phase III trial (EPIC). Proc Am Soc Clin Oncol 24: Abstract 3580.
25 Kies, M.S., Arquette, M.A., Nabell, L., et al. (2002) Final report of the efficacy and safety of the anti-epidermal growth factor antibody Erbitx (IMC-C225), in combination with cisplatin in patients with recurrent sqaumous cell carcinoma of the head and neck refractory to cisplatin containing chemotherapy. Proc Am Soc Clin Oncol 21: Abstract 925 update.
26 Baselga, J., Trigo, J.M., Bourhis, J., et al. (2004) phase II multicenter study of the antiepidermal growth factor receptor monoclonal antibody cetuximab in combination with platinum-based chemotherapy in patients with platinum-refractory metastatic and/ or recurrent squamous cell carcinoma of the head and neck. J Clin Oncol 23: 5568–5577.
27 Chan, A.T.C., Hsu, M.M., Goh, B.C., et al. (2003) A phase II study of cetuximab in combination with carboplatin in patients with recurrent or metastatic nasopharyngeal carcinoma

who failed to a platinum based chemotherapy. Proc Am Soc Clin Oncol 22: Abstract 2000.
28 Rosell, R., Daniel, C., Ramlau, R., et al. (2004) Randomized phase II study of cetuximab in combination with cisplatin © and vinorelbine (V) vs. CV alone in the first line treatment of patients with epidermal growth factor expressing advanced non-small cell lung cancer. Proc Am Soc Clin Oncol 23: Abstract 7012.
29 Kelly, K., Hanna, N., Rosenberg, A., et al. (2003) A multi-centered phase I/II study of cetuximab in combination with paclitaxel and carboplatin in untreated patients with stage IV non-small cell lung cancer. Proc Am Soc Clin Oncol 22: Abstract 2592.
30 Robert, F., Blumenschein, G., Dicke, K., et al. (2003) phase Ib/IIa study of anti-epidermal growth factor receptor antibody, cetuximab in combination with gemcitabine/ carboplatin in patients with advanced non-small cell lung cancer. Proc Am Soc Clin Oncol 22: Abstract 2587.
31 Kim, E.S., Mauer, A.M., Tran, H.T., et al. (2003) A phase II stud of cetuximab, an epidermal growth factor receptor blocking antibody, in combination with docetaxel in chemotherapy refractory/ resistant patients with advanced non-small cell lung cancer: final report. Proc Am Soc Clin Oncol 22: Abstract 2581.
32 Aghajanian, C., Sabbatini, P., De Rosa, F., et al. (2005) A phase II study of cetuximab/ paclitaxel/ carboplatin for the initial treatment of advanced stage ovarian, primary peritoneal and fallopian tube cancer. Proc Am Soc Clin Oncol 24: Abstract 5047.
33 Bonner, J.A., Giralt, J., Harari, P.M., et al. (2004) Cetuximab prolongs survival in patients with locoregional advanced squamous cell carcinoma of head and neck: a phase III study of high-dose radiation therapy with or without cetuximab. Proc Am Soc Clin Oncol 23: Abstract 5507.
34 Werner-Wasik, M., Swann, S., Curran, W., et al. (2005) A phase II study of cetuximab in combination with chemoradiation in patients with stage IIIA/ B non-small cell lung cancer: An interim overall toxicity report of the RTOG 0324 trial. Proc Am Soc Clin Oncol 24: Abstract 7135.
35 Pfister, D.G., Aliff, T.B., Kraus, D.H., et al. (2003) Concurrent cetuximab, cisplatin, and concomitant boost radiation therapy for locoregionally advanced, squamous cell head and neck cancer: preliminary evaluation of a new combined-modality paradigm. Proc Am Soc Clin Oncol 22: Abstract 1993.
36 Gunnett, K., Motzer, R., Amato, R., et al. (1999) phase II study of anti-epidermal growth factor receptor antibody C225 alone in patients with metastatic renal cell carcinoma. Proc Am Soc Clin Oncol 18: Abstract 1309.
37 Huether, A., Hopfner, M., Baradari, V., et al. (2005) EGF-R blockade by cetuximab alone or as combination therapy for growth control of hepatocellular cancer. Biochem Pharmacol 70: 1568–1578.
38 Raben, D., Helfrich, B., Chan, D.C., et al. (2005) The effects of cetuximab alone and in combination with radiation and/ or chemotherapy in lung cancer. Clin Cancer Res 11(2 Pt.1): 795–805.
39 Huang, Z.Q., Buchsbaum, D.J., Raisch, K.P., et al. (2003) Differential responses by pancreatic carcinoma cell lines to prolonged exposure to Erbitux (IMC-C225) anti-EGF-R antibody. J Surg Res 111: 274–283.
40 Bruns, C.J., Harbison, M.T., Davis, D.W., et al. (2000) Epidermal growth factor receptor blockade with C225 plus gemcitabine results in regression of human pancreatic carcinoma growing orthotopically in nude mice by antiangiogenic mechanisms. Clin Cancer Res 6: 1936–1948.
41 Xiong, H.S., Rosenberg, A., Lo Buglio, A., et al. (2004) Cetuximab, a monoclonal antibody targeting the epidermal growth factor receptor, in combination with gemcitabine for advanced pancreatic cancer: a multicenter phase II trial. J Clin Oncol 22: 2610–2616.
42 Krempien, R., Muenter, M.W., Huber, P.E., et al. (2005) Randomized phase II – study evaluating EGF-R targeting therapy

with cetuximab in combination with radiotherapy and chemotherapy for patients with locally advanced pancreatic cancer – PARC study protocol. BMC Cancer 5: 131.

43 Saltz, L., Lenz, H., Hochster, H., et al. (2005) Randomized phase II trial of cetuximab, bevacizumab/ irinotecan versus cetuximab/ bevacizumab in irinotecan refractory colorectal cancer. Proc Am Soc Clin Oncol 24: Abstract 3508.

44 Chung, K.Y., Shia, J., Kemeny, N.E., et al. (2005) Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J Clin Oncol 23: 1–8.

45 Vallböhmer, D., Zhang, W., Gordon, M., et al. (2005) Molecular determinants of cetuximab efficacy. J Clin Oncol 23: 3536–3544.

46 Zhang, W., Yun, J., Press, A., et al. (2004) Association of cyclin D1 gene A870G polymorphism and clinical outcome of EGF-R positive metastatic colorectal cancer patients treated with epidermal growth factor receptor inhibitor cetuximab. Proc Am Soc Clin Oncol 23: Abstract 3518.

47 Huang, S., Armstrong, E.A., Benavente, S., et al. (2004) Dual-agent molecular targeting of the epidermal growth factor receptor. Cancer Res 64: 5355–5362.

48 Robert, F., Ezekiel, M.P., Spencer, S.A., et al. (2001) phase I study of anti-epidermal growth factor receptor antibody cetuximab in combination with radiation therapy in patients with advanced head and neck cancer. J Clin Oncol 19: 3234–3243.

49 Li, S., Schmitz, K.R., Jeffrey, P.D., Wiltzius, J.J.W., Kussie, P., Ferguson, K.M. (2005) Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. Cancer Cell 7: 301–311.

5
Efalizumab (Raptiva)

Karlheinz Schmitt-Rau and Sigbert Jahn

5.1
Introduction

Efalizumab is a humanized monoclonal antibody to lymphocyte function-associated antigen (LFA-1). LFA-1 belongs to the family of the β_2 integrins and is expressed on the surface of T cells (CD4+ cells, T-helper cells). It is involved in several T-cell activities, such as T-cell activation and migration (Janeway et al. 2001; Kuypers and Roos 1989), as well as T-cell adhesion during cellular interactions that are important to the inflammatory processes (Lo et al. 1989).

LFA-1, Mac-1 and p159,95 – all of which are members of the β_2 integrin family – are heterodimeric molecules consisting of a β-subunit (CD18), common to all three molecules, which is linked noncovalently to the respective a-chain CD11a (LFA-1), CD11b (Mac-1), and CD11c (p159,95). T-cells mainly express LFA-1 (CD11a/CD18).

The ligands for LFA-1 are intercellular adhesion molecules (ICAM), and include:

- ICAM-1, which is expressed on leucocytes, vascular endothelium cells and epithelial cells, including keratinocytes (Dustin et al. 1986);
- ICAM-2, which is expressed on resting endothelium and lymphocytes (de Fougerolles et al. 1991); and
- ICAM-3, which is expressed on monocytes and resting lymphocytes (de Fougerolles et al. 1994).

ICAM expression can be triggered by proinflammatory mediators, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (Janeway et al. 2001). Monoclonal antibodies (mAbs) to LFA-1 inhibit LFA-1/ICAM interaction, leading to the inhibition of several T-cell-dependent immune functions. Results derived from animal models imply that inhibiting LFA-1/ICAM interaction by anti-CD11a antibodies might have a clinical benefit in certain T-cell-dependent diseases.

A humanized monoclonal anti-CD11a immunoglobulin (Ig)G1 antibody, efalizumab, could therefore show clinical efficacy in psoriasis.

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5.2 Development and Characterization of the Antibody

Efalizumab (rhuMAb CD11a, hu1124) is a full-length, IgG1 kappa isotype antibody composed of two identical light chains each consisting of 214 amino acid residues, and two heavy chains each consisting of 451 residues. Each light chain is covalently coupled through a disulfide link to a heavy chain. The two heavy chains are covalently coupled to each other via inter-chain disulfide bonds consistent with the structure of human IgG1. The molecular weight of intact efalizumab is 148 841 Da.

Originally developed as a murine anti-CD11a monoclonal antibody (MHM24; Hildreth et al. 1983), efalizumab has been prepared by substituting human DNA sequences using genetic engineering methods to reduce immunogenicity. This results in a "humanized" mAb (HuIgG1) in which the complementarity-determining regions (CDRs) of the murine antibody – which are important for antigen recognition – are preserved.

Previous studies on murine MHM24 have shown that, similar to other anti-CD11a antibodies, it is able to inhibit T-cell function (Hildreth and August 1985; Dougherty and Hogg 1987).

The consensus sequences for the human heavy chain subgroup III (V_H-CH1) and the light chain subgroup k 1 were used as the framework for the humanization; subsequently, several humanized variants were made and screened for binding as Fabs. To construct the first Fab variant of humanized MHM24, all six CDR residues were transferred from the murine antibody to the human framework.

Further variants were constructed by targeted exchange of either framework residues, or residues within CDRs using the first variant (Fab-1), as a template. For that purpose, both light and heavy chains were completely sequenced for each variant. Plasmids containing the sequences were then transformed to *Escherichia coli* for protein expression (Werther et al. 1996).

All variants were tested for CD11a binding in the Jurkat cell assay. V_L and V_H domains of the variant with optimal binding characteristics were then transferred to human IgG1 constant domains, producing the full-length intact humanized antibody (Werther et al. 1996).

Several *in-vitro* assays were performed to compare efalizumab with its parent murine antibody MHM24, including the keratinocyte cell-adhesion assay and the mixed lymphocyte response assay (MLR). The results showed that, in these assays, efalizumab was equally effective as MHM24. In addition, the apparent K_d values, as determined by saturation binding using peripheral blood mononuclear cells (PBMCs) of two human donors, were similar for both MHM24 and efalizumab (0.16 ± 0.01 and 0.13 ± 0.02 versus 0.11 ± 0.08 and 0.18 ± 0.03, respectively) (Werther et al. 1996).

5.3
Efalizumab in the Treatment of Psoriasis

5.3.1
Psoriasis: Prevalence, Characteristics, and Therapeutic Options

Psoriasis is one of the most common dermatological diseases. Although there is a great variation in the prevalence of psoriasis in different countries, due mainly to environmental and genetic factors, it can be said that the condition affects approximately 2–3% of the world's population (Jung and Moll 2003). Moreover, between 20 to 25% of these people suffer from moderate-to-severe forms of the disease (Weinstein and Menter 2003). The majority of patients (75%) show the first signs of disease manifestation before the age of 40 years, with a peak in the second decade of life (Gollnick and Bonnekoh 2001). These patients usually have a family history of psoriasis, their disease is more severe, and it is characterized by frequent relapses.

The most common form of psoriasis, with a prevalence of about 70%, is plaque psoriasis or "psoriasis vulgaris" (Jung and Moll 2003). Plaque psoriasis is characterized by hyperkeratosis, parakeratosis, and the presence of inflammatory lesions in the skin. There is a predilection for symmetrical involvement of the scalp, elbows, knees, and lower back, although it can occur anywhere on the body. Other, less frequent, morphologies of psoriasis include guttate, inverse, pustular, and erythrodermic forms. These may occur individually, concomitantly or sequentially.

There is agreement today that immunological mechanisms play an important role in the pathogenesis of many chronic relapsing inflammatory skin diseases such as psoriasis (Weinstein and Menter 2003; Schön and Boehncke 2005). Evidence of the pivotal role played by T cells in the pathology of psoriasis is accumulating, based on the following points:

- Activated T cells are found in psoriatic lesions (Bos et al. 1983; Ferenczi et al. 2000).
- T cells have the ability to induce the altered keratinocyte growth and differentiation pattern typical of psoriasis. This has been demonstrated in a SCID (severely compromised immunodeficient) mouse model by injecting autologous immunoctyes into the dermis of mice that have received grafts of human skin. Plaques typical of those seen in psoriasis are observed when immunocytes from a patient with psoriasis are injected into a mouse possessing a graft of symptom-free skin from the same patient (Gilhar et al. 1997; Wrone-Smith and Nickoloff 1996).
- T-cell-targeted immune suppressive drugs, such as cyclosporine (Lebwohl et al. 1998), and antibodies against the CD25 receptor (Gottlieb et al. 1995) and CD4 (Prinz et al. 1991), have been shown to improve psoriasis.

- In bone marrow transplantation, psoriasis can be transferred from a donor suffering from the disease to a healthy recipient. Also, psoriasis can be "cured" when bone marrow is transplanted from a healthy donor to a person with psoriasis (Gollnick and Bonnekoh 2001).
- When symptomless prepsoriatic human skin was engrafted onto AGR129 mice, deficient in type I and type II interferon receptors and for the recombination activating gene 2, resident human T cells in the skin grafts underwent local proliferation, demonstrating the importance of resident immune cells in the development of psoriasis (Boyman et al. 2004).

Today, a whole battery of treatment options is available for the treatment of psoriasis (Fig. 5.1). The mild forms of psoriasis are usually treated with topical preparations, including vitamin D_3 analogs, corticosteroids, and retinoids. When the disease becomes severe, phototherapeutic regimens are applied. Finally, there is the option to use an oral immune suppressive drug, such as methotrexate, cyclosporine, oral retinoids, or fumaric acid esters, either as monotherapy or in combination. As the majority of patients develop their disease before the age of 40 years, many of those with moderate-to-severe psoriasis will require decades of continuous systemic treatment or phototherapy. Unfortunately, none of the available therapies can be used chronically, because of long-term safety and toxicity problems.

Recent advances in the understanding of T-cell interactions in the pathogenesis of psoriasis have led to the development of several biological substances, such as the targeted T-cell modulator efalizumab, for continuous immune therapy of this disease, without the safety problems of the traditional systemic preparations.

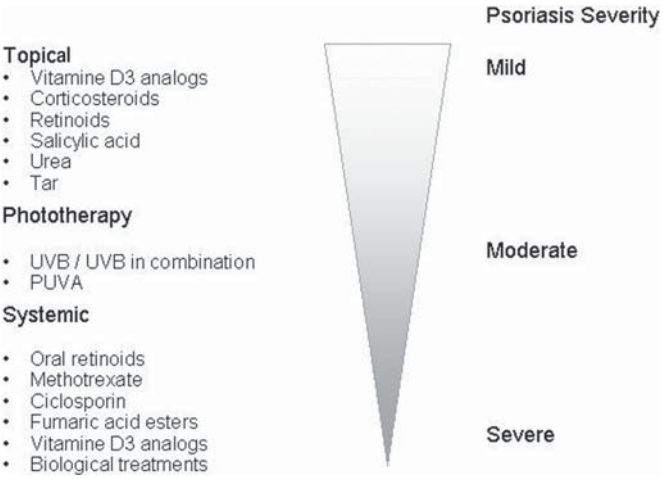

Fig. 5.1 Therapeutic options in the treatment of psoriasis.

5.3.2 Pathogenesis of Psoriasis

5.3.2.1 T-Cell Activation

The first step in the immune pathogenic cascade of psoriasis is the capture and processing of auto-antigen by Langerhans cells or dendritic cells ("antigen-presenting cells"; APCs) in the epidermis or dermis, respectively. Although the nature of the antigen is still unknown, there is some evidence that in genetically predisposed subjects keratinocyte-derived peptides may be involved (Bos and De Rie 1999; Valdimarsson et al. 1995).

The APC–antigen complex migrates to a skin-draining lymph node, where the antigen is presented via major histocompatibility complex II (MHC II) on the surface of the APC to the T-cell receptor (TCR) of the specific naïve CD4+ cell. Interaction of the APC-MHC-II/antigen–TCR complex is, however, not sufficient for T-cell activation.

The initial binding of T cells and APCs, as well as stabilization of the cell pair, is mediated by LFA-1 on the T cell and ICAM-1 on the APC. This stabilized structure has been referred to as the "immunological synapse" (Grakoui et al. 1999; Fig. 5.2), the formation of which is followed by delivery of the antigen-specific signal (signal 1) and a co-stimulatory signal (signal 2), which are also mediated by LFA-1 and ICAM-1. Delivery of signals 1 and 2 is followed by binding of cytokines that induce T cells to proliferate (i.e., to undergo clonal expansion) and to differentiate (Krueger 2002). Proliferation is thought to be largely mediated by the cytokine interleukin-2 (IL-2) (Janeway et al. 2001).

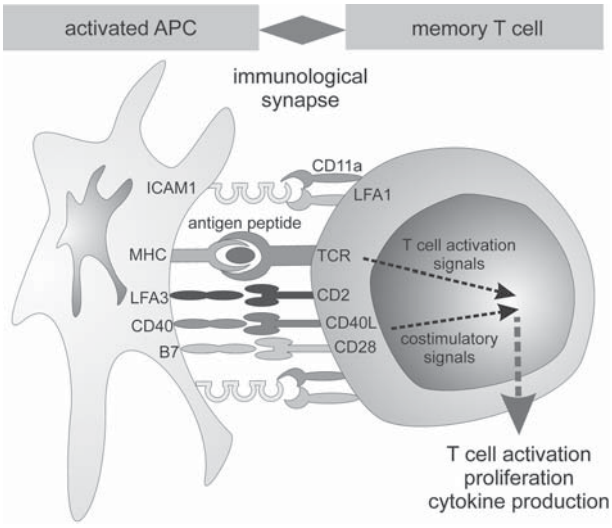

Fig. 5.2 The "immunological synapse".

5.3.2.2 T-Cell Migration and Extravasation

Following activation, T cells leave the lymph node and start trafficking via the bloodstream to the dermal vessels (Fig. 5.3). Stimulation by keratinocyte-derived cytokines (IL-8, CCL27) leads to increased expression of adhesion molecules, including E-selectin and ICAM-1, in the post-capillary venules of inflamed skin. E-selectin is the target for cutaneous lymphocyte antigen (CLA) on the T-cell surface. Binding of CLA to E-selectin, as well as interaction of the lymphocyte chemokine receptor (CCR10) with its ligand CCL27, slows circulating lymphocytes and causes them to "roll" along the endothelial wall (Fuhlbrigge et al. 2002; Homey et al. 2002).

As a result of increased exposure to chemokines, the affinity of LFA-1 for ICAM-1 is increased, probably mediated by a conformational change in the LFA-1 molecule. Bound T cells flatten and pass through the epithelium into the surrounding tissue – a process known as diapedesis (Bradley and Watson 1996). Once they have left the venule, T cells respond to chemokines, drawing them towards the site of inflammation in the dermis, and from there into the epidermis.

5.3.2.3 T-Cell Reactivation

Following transmigration from the circulation into dermal and epidermal tissue, memory T cells are brought into contact with antigen-presenting dendritic and Langerhans cells, as well as with keratinocytes, which – probably due to genetic

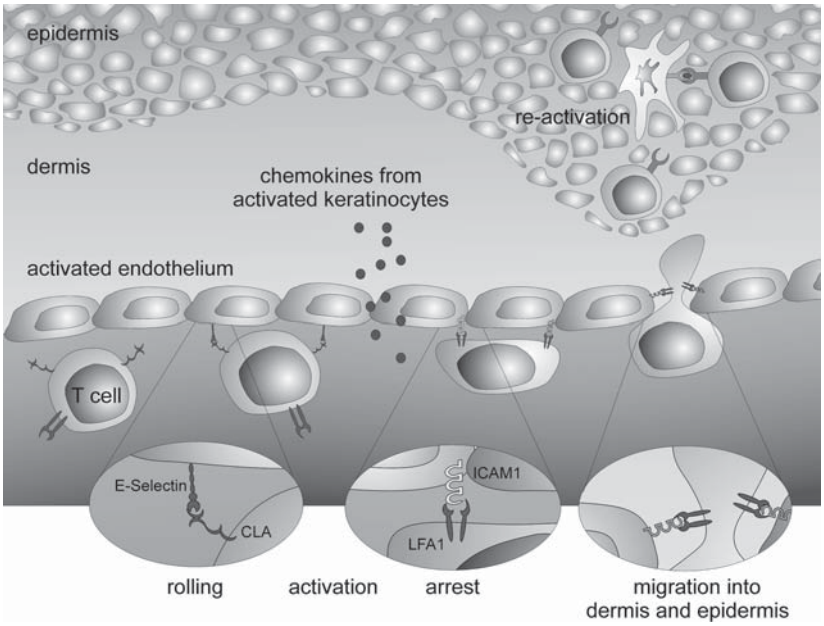

Fig. 5.3 T-cell migration, extravasation, and reactivation.

alterations – are also able to act as APCs (see Fig. 5.3). This interaction leads to the reactivation of T cells and is followed by increased production and secretion of cytokines, including IL-2, IFN-γ and TNF-α, which show the TH-1 cytokine pattern typically observed in psoriasis:

- IFN-γ leads to the induction of ICAM-1 in keratinocytes and endothelium, and consequently supports binding of lymphocytes to keratinocytes.
- TNF-α induces keratinocyte proliferation and stimulates keratinocyte-induced IL-8 production. This leads to chemotaxis of lymphocytes and neutrophils into the epidermis. TNF-α is also able to induce activation and proliferation of endothelial cells.

However, the exact mechanism by which (re-)activated T cells cause psoriatic lesions is not known. Direct lymphocyte/keratinocyte interactions might be involved (Bos and De Rie 1999), or cytokines produced during T-cell reactivation may be responsible for the most prominent alteration in psoriatic skin, namely hyperproliferation (Prinz et al. 1994).

In summary, a combination of different mechanisms may be responsible for the changes observed histologically and clinically in psoriatic skin. These include:

- increased proliferation of keratinocytes (hyperkeratosis);
- neutrophil and mast-cell migration into epidermis and dermis, respectively;
- increased production of defensins by activated keratinocytes; and
- increased angiogenesis.

5.3.3 Efalizumab: The Mechanism of Action

As described above, efalizumab is a humanized monoclonal antibody against CD11a, the a-subunit of LFA-1. Binding of efalizumab to CD11a leads to blockade of the interaction between LFA-1 and ICAM-1, the blockade of this interaction having several consequences (Krueger 2002; Jullien et al. 2004), including:

- the activation of T lymphocytes in lymph nodes;
- inhibition of extravasation of circulating lymphocytes in inflammatory skin;
- blockade of T-lymphocyte reactivation in skin by APCs; and
- reduction of the keratinocyte interaction with activated T-lymphocytes.

By inhibiting these processes, efalizumab may be able to prevent abnormal cytokine production, keratinocyte hyperproliferation and abnormal keratinocyte differentiation, which are characteristic of the psoriatic phenotype.

5.4 Pharmacology and Toxicology of Efalizumab

5.4.1 Preclinical Studies

A number of preclinical studies have been conducted with efalizumab in order to evaluate its pharmacodynamic and pharmacokinetic properties, as well as its toxicity (Menter and Griffiths 2004).

In-vitro studies performed with efalizumab have shown that it is able to bind to human and chimpanzee leucocytes, to inhibit lymphocyte binding to ICAM-1 on keratinocytes, and to inhibit T-cell proliferation. In murine and chimpanzee *in-vivo* models, efalizumab was able to down-regulate LFA-1 expression on lymphocytes, while efalizumab has been shown to have two-compartment kinetics, with nonlinear elimination in monkeys.

Anti-mouse CD11a antibodies have been used to study developmental toxicity in mice. In these studies, doses were administered at up to 30 times the equivalent recommended clinical dose of $1\,mg\,kg^{-1}$. However, no adverse effects were observed on mating, fertility, or reproductive parameters; neither was there any evidence of maternal toxicity, embryotoxicity, or teratogenicity.

Similarly to other immunoglobulins, anti-mouse CD11a antibody is secreted in the milk of lactating mice that are exposed to the antibody during gestation and lactation. Furthermore, a significant reduction was observed in the ability of their offspring to generate an antibody response at 11 weeks of age, which was – at least partially – reversible at Week 25. There were, however, no adverse effects on behavior, growth and reproductive function of the offspring.

Immunization of chimpanzees exposed to high doses of efalizumab (>10 times the clinical dose) with tetanus toxoid produced an impaired antibody response compared with control animals. However, to date no animal reproduction studies and long-term carcinogenicity studies have been conducted with efalizumab.

5.4.2 Pharmacodynamics

The pharmacodynamic properties of efalizumab were investigated in several phase I and phase II studies following intravenous and subcutaneous administration, either as a single dose or repeated weekly administration.

In the single-dose intravenous study, doses of 0.03 to $10\,mg\,kg^{-1}$ were administered (Gottlieb et al. 2000). Within 24 h, treatment with efalizumab reduced the level of CD11a expression on T cells to 25% of pretreatment levels, and this suppression persisted as long as efalizumab was present in the circulation. CD11a expression returned to baseline within 7 to 10 days following clearance of efalizumab, without showing any signs of lymphocyte depletion. The total white blood cell (WBC) count was slightly increased within about 8 h of efalizumab administration; circulating lymphocyte counts were increased (to about twofold the Upper

Limit of Normal) by day 7. Following multiple weekly dosing, lymphocyte levels remained elevated but returned to baseline after efalizumab clearance. This elevation of the lymphocyte count is probably due to demargination – the blocked entry of efalizumab-bound cells to tissues.

In order to achieve the full pharmacodynamic effect, intravenous doses exceeding $0.3\,mg\,kg^{-1}$ were necessary (Gottlieb et al. 2000). Complete saturation and maintenance of CD11a binding site down-regulation on lymphocytes required weekly intravenous doses of $0.6\,mg\,kg^{-1}$, which corresponds to an efalizumab plasma concentration of $5\,\mu g\,mL^{-1}$.

Several histologic changes were observed in psoriatic plaques following efalizumab administration. A marked reduction of keratin-16, corresponding to decreased disease activity, was noted. Keratinocyte ICAM-1 levels were also reduced, indicating reduced cytokine-mediated inflammation. Furthermore, a significant thinning of the epidermis and restoration of normal skin was observed after 28 days of treatment, in concordance with reductions of over 50% in cutaneous T-cell infiltration and reduced CD11a availability (Gottlieb et al. 2002). These data demonstrate that, by reducing CD11a on the surface of circulating and cutaneous T cells, efalizumab is able to reverse both the histological signs of inflammation and the pathological hyperplasia characteristic of plaque psoriasis.

In general, the effects of subcutaneous efalizumab on lymphocytes were comparable to those observed after intravenous dosing. Subcutaneous doses of $1\,mg\,kg^{-1}$ per week (or above) produced the required efalizumab plasma concentrations of $5\,\mu g\,mL^{-1}$ for binding site down-regulation and saturation. In addition to reduced CD11a expression on the surface of CD3+ T cells, binding of efalizumab also causes a reduced expression of other adhesion molecules, such as CD11b, L-selectin or β_7-integrin (Vugmeyster et al. 2004). The down-modulation of these adhesion molecules likely contributes to the anti-adhesive effects of efalizumab. There is also a decrease of $\alpha\beta$+ T-cell receptors and of TCR-associated co-receptors, such as CD4, CD8 or CD2. The inhibition of TCR-mediated activation therefore seems also to play a role in efalizumab's mode of action.

5.4.3 Pharmacokinetics

The pharmacokinetic properties of subcutaneous efalizumab were determined in an open, multicenter, phase I study of 70 patients suffering from moderate-to-severe plaque psoriasis. Patients received weekly doses of either $1\,mg\,kg^{-1}$ (n = 33) or $2\,mg\,kg^{-1}$ (n = 37) efalizumab for 12 weeks (Mortensen et al. 2005).

5.4.3.1 Absorption

After subcutaneous administration of efalizumab, peak plasma concentrations are reached after 2 to 3 days. The average estimated bioavailability was about 50% at the recommended dose level of subcutaneous efalizumab, $1.0\,mg\,kg^{-1}$ per week.

5.4.3.2 Distribution

Steady-state serum concentrations of efalizumab were achieved after four weekly doses of efalizumab at 1 mg kg^{-1}, and after eight weeks in patients receiving 2 mg kg^{-1}. At this dose level (with an initial dose of 0.7 mg kg^{-1} in the first week), the mean efalizumab plasma trough values were 9.1 ± 6.7 µg mL^{-1} in the 1 mg kg^{-1} group and 23.5 ± 12.2 µg mL^{-1} in the 2 mg kg^{-1} group. The volumes of distribution of the central compartment after single intravenous doses were 110 mL kg^{-1} at a dose level of 0.03 mg kg^{-1}, and 58 mL kg^{-1} at a dose of 10 mg kg^{-1} (Bauer et al. 1999).

5.4.3.3 Biotransformation

The metabolism of efalizumab is through internalization followed by intracellular degradation as a consequence of either binding to cell surface CD11a, or through endocytosis. The expected degradation products are small peptides and individual amino acids which are eliminated by glomerular filtration. Cytochrome P450 enzymes, as well as conjugation reactions, are not involved in the metabolism of efalizumab (Coffey et al. 2004).

5.4.3.4 Elimination

Efalizumab is cleared by dose-dependent nonlinear saturable elimination (Gottlieb et al. 2000). The mean steady-state clearance is 24.3 ± 18.5 and 15.7 ± 12.6 mL kg^{-1} per day for the 1 and 2 mg kg^{-1} per week groups, respectively. The elimination half-life was about 6.21 ± 3.11 days for the 1 mg kg^{-1} per week group, and 7.4 ± 2.5 days for the 2 mg kg^{-1} per week group. T_{end} at steady-state is 25.5 ± 1.6 days and 44 ± 10 days at dose levels of 1 and 2 mg kg^{-1} per week, respectively.

Efalizumab shows dose-dependent nonlinear pharmacokinetics, which can be explained by its saturable specific binding to cell-surface receptors CD11a. Clearance was more rapid at lower doses, suggesting a receptor-mediated mechanism at drug levels below 10 µg mL^{-1} (Gottlieb et al. 2000).

In a population pharmacokinetic analysis of 1088 patients, body weight was found to be the most significant covariate affecting efalizumab clearance. Other covariates such as baseline Psoriasis Area and Severity Index (PASI), baseline lymphocyte count and age had modest effects on clearance; however, gender and ethnic origin had no effect (Sun et al. 2005).

Additional pharmacokinetic data are available from an open-label extended-treatment trial in which patients who responded to an initial treatment of efalizumab (2 mg kg^{-1} for 12 weeks) received the drug in a maintenance phase for up to 33 months at a dose of 1 mg kg^{-1} (Gottlieb et al. 2003). Pharmacokinetic analysis of each 12-week treatment period for up to 15 months showed that steady-state trough levels remained constant during continuous efalizumab dosing. There was no evidence of efalizumab accumulation or alteration of the pharmacokinetic profile of efalizumab during long-term continuous dosing (Gottlieb et al. 2003).

5.5
Clinical Development of Efalizumab

The therapeutic efficacy, safety and tolerability, and the changes in health-related quality of life (HRQoL) of subcutaneous efalizumab have been evaluated in more than 3000 adult patients with moderate-to-severe plaque psoriasis. Five large, randomized, placebo-controlled, double-blind phase III studies have been conducted in which all patients included had a minimum affected body surface area (BSA) of 10%, and a PASI of ≥12. The presence of guttate, pustular or erythrodermic psoriasis as the sole or predominant form of psoriasis was an exclusion criterion. Patients were randomized to receive placebo or efalizumab, administered subcutaneously at doses of 1 or 2 mg kg^{-1} once weekly for 12 weeks, with an initial conditioning dose of 0.7 mg kg^{-1}. Patients could receive low-dose topical corticosteroids concomitantly with efalizumab; however, no other concomitant systemic therapies were allowed.

The primary endpoint was the proportion of patients with a ≥75% improvement in the PASI score (PASI 75) relative to baseline when assessed after a 12-week treatment course. Secondary endpoints included the Physician's Global Assessment of change (PGAc), the static PGA (sPGA), the proportion of patients with a ≥50% improvement in PASI score (PASI 50) relative to baseline after 12 weeks of treatment, the proportion of subjects who achieved a rating of "minimal" or "clear" on a static global assessment by the physician, and the overall lesion severity (OLS). Additional secondary endpoints were the improvement in the Dermatology Life Quality Index (DLQI) and the Psoriasis Symptom Assessment (PSA).

The physician-assessed PASI is a complex scoring system to quantify the efficacy of psoriasis treatments. The system takes into account the affected BSA, as well as the severity of erythema, scaling and infiltration in each of the four body areas; head, trunk, upper and lower extremities. The combined score has a numerical range of 0 to 72, with higher values indicating more serious disease. Although PASI is used by regulatory authorities (e.g., the US FDA and the European EMEA) as a precondition for the approval of new anti-psoriasis drugs, its relevance in clinical practice has been questioned by many experts (Carlin et al. 2004; Langley et al. 2004). Due to its complexity, the PASI measurement is therefore not frequently used in clinical practice.

In addition to the first 12 weeks of treatment, extended treatment and retreatment periods have been studied in addition to an open-label long-term study in which patients received continuous efalizumab for up to 3 years.

5.5.1
Clinical Efficacy

5.5.1.1 Randomized, Placebo-Controlled, Double-Blind Studies

In one clinical study, 498 patients received subcutaneous efalizumab (1 or 2 mg kg^{-1} per week) or placebo for 12 weeks (Leonardi et al. 2005a). After 12 weeks of

treatment, significantly more patients who received efalizumab showed at least 75% PASI improvement in both dose groups compared with patients receiving placebo (39% and 27% in the 1 and 2 mg kg^{-1} groups respectively, versus 2% after placebo). Some 61% and 51% of efalizumab-treated patients achieved at least a 50% PASI improvement in the 1 and 2 mg kg^{-1} groups compared with 15% in the placebo group.

In another study, 556 patients were randomized to receive placebo (n = 187) or efalizumab 1 mg kg^{-1} per week (n = 369) (Gordon et al. 2003). The mean PASI improvement relative to baseline at Week 12 in this study was 52% in the efalizumab group versus 19% in the placebo group (Gordon et al. 2003). A significant improvement in PASI was seen at Week 4, and was further improved through to Week 12. Comparable improvements were observed in sPGA ("minimal" or "clear"; 26% versus 3%, respectively) and PGA ("excellent" or "cleared"; 33% versus 5%, respectively).

The efficacy of efalizumab after 12 weeks was confirmed in the other phase III trials conducted in North America (Lebwohl et al. 2003; Papp et al. 2006) and in the European CLEAR (Clinical Experience acquired with Raptiva) study (Sterry et al. 2004). The most important results of these studies are summarized in Table 5.1. As can been seen, increasing the dose to 2 mg kg^{-1} per week (Lebwohl et al. 2003; Leonardi et al. 2005a) did not result in any additional benefit for the patients.

The CLEAR study, conducted in 103 centers in 19 countries, evaluated the efficacy and safety of efalizumab in patients with chronic plaque psoriasis, and was similar to the other phase III studies. However, this trial included a prospectively defined patient cohort for which at least two existing systemic therapies were unsuitable or inadequate due to contraindications, lack of efficacy, or intolerance. The results of this study demonstrate that even more severely affected patients, for whom treatment options are limited, showed treatment benefits with efalizumab, comparable to those seen in the broader population (Sterry et al. 2004).

5.5.1.2 **Extended Treatment and Re-Treatment**

In order to investigate the effect of extended treatment, efalizumab patients who did not achieve PASI 75 at week 12 were re-randomized to receive efalizumab 1 mg kg^{-1} per week or placebo for another 12 weeks (Leonardi et al. 2005b). At Week 24, patients who received extended treatment showed improved responses; 20.2% achieved PASI 75 compared with 6.7% who received placebo during Weeks 13 to 24.

In another randomized, double-blind, placebo-controlled 12-week study (Menter et al. 2005), patients had the option to enter a second 12-week, open-label, extended efalizumab treatment period. At Week 24, 66.6% (n = 368) and 43.8% (n = 368) of the efalizumab patients achieved PASI 50 and PASI 75 responses, respectively.

In the CLEAR study (Ring et al. 2005), extended treatment of patients without PASI 75 response at first treatment (FT) in Week 12 maintained and improved

Table 5.1 The 12-weeK efficacy of efalizumab in randomized, placebo-controlled, double-blind studies.

Study protocol/ treatment	*Gordon et al. (2003) (n = 187/369) [%]*	*Lebwohl et al. (2003) (n = 122/232/243) [%]*	*Leonardi et al. (2005a) (n = 170/162/166) [%]*	*Sterry et al. (2004) (n = 264/529) [%]*	*Papp et al. (2006) (n = 236/450) [%]*
PASI 75 Placebo	4	5	2	4	3
PASI 75 Efalizumab 1 mg kg^{-1} per week	27	22	39	31	24
PASI 75 Efalizumab 2 mg kg^{-1} per week		28	27		
PASI 50 Placebo	14	16	15	14	14
PASI 50 Efalizumab 1 mg kg^{-1} per week	59	52	61	54	52
PASI 50 Efalizumab 2 mg kg^{-1} per week		57	51		
sPGA minimal or clear Placebo	3	3	3	3	4
sPGA minimal or clear 1 mg kg^{-1} per week	26	19	32	26	20
sPGA minimal or clear 2 mg kg^{-1} per week		23	22		

PASI = Psoriasis Area and Severity Index; sPGA = static Physician's Global Assessment of change.
n = number of patients in each treatment arm.

response. Among FT efalizumab patients with >50% but <75% improvement at FT Week 12, almost half (47.5%) showed PASI 75 response after a second 12-week period. Among FT efalizumab patients with PGA ratings of "good" at FT Week 12, 43.9% had ratings of "excellent" or "cleared" by FT Week 12.

When patients who received placebo during the first 12 weeks of treatment were switched to efalizumab in Weeks 13 to 24, a rapid improvement in their PASI was observed; 60.3% achieved PASI 50 and 24.1% PASI 75 at the end of the 12-week open-label efalizumab treatment phase (Menter et al. 2005).

The clinical benefit of efalizumab treatment is gradually lost after discontinuation of treatment. The median time to relapse (loss of ≥50% of PASI improvement) has been shown to be between 58 days (Ring et al. 2005) and 84 days (Lebwohl et al. 2003). When patients are re-treated at a dose of $1\,mg\,kg^{-1}$ after relapse, efalizumab re-establishes disease control: at the end of the re-treatment period after 12 weeks, PASI scores in these patients showed mean improvements of 62% from baseline (Ring et al. 2005).

5.5.1.3 **Long-Term Efficacy**

An open-label trial was conducted to evaluate the safety, tolerability and efficacy of continuous subcutaneous efalizumab therapy for 3 years in patients who achieved a PASI 50 response or an sPGA grade of "mild", "minimal" or "clear" after an initial 12-week treatment period (Gottlieb et al. 2005). During the first 12-week treatment period, patients received $2.0\,mg\,kg^{-1}$ efalizumab each week. During the third month, patients were also randomized to receive either fluocinolone acetonide (Synalar/Medicis) or white petrolatum in addition to efalizumab.

The patients (n = 339) who entered this study had experienced psoriasis for a mean of 17.9 years, and over half (57.5%) of them had a history of prior systemic therapy. Patients in this study population also had significant disease: the mean baseline PASI score was 19.8 and the mean BSA affected by psoriasis was 31.5%.

At FT Week 12, patients who had at least a 50% improved PASI score or an sPGA grading of "mild", "minimal" or "clear" could enter the maintenance treatment period, which was analyzed in 12-week treatment segments. During the maintenance period, patients received $1.0\,mg\,kg^{-1}$ efalizumab weekly. In the case of relapse – which was defined as the loss of 50% of the PASI improvement achieved at Week 12 – the patient ended participation in the current 12-week segment and started the next segment at $2.0\,mg\,kg^{-1}$ per week. During months 4 to 15, the dose for relapsed patients could be increased to a maximum of $4\,mg\,kg^{-1}$ per week, if clinically indicated. After month 15, further dose escalation was not allowed.

In this study the impact of efalizumab on PASI was analyzed in two ways. The primary analysis was based on the intent-to-treat (ITT), and comprised all 339 patients who entered the study. Patients who discontinued treatment during the first 3 months were classified as PASI nonresponders at 3 months and for subsequent periods. For patients who discontinued treatment during the maintenance

treatment, the last available PASI assessment was carried forward to the end of the current 12-week segment, after which the patient was considered to be a nonresponder. As a secondary analysis, an analysis of the "as-treated" population (i.e., the number of patients decreased over time due to study discontinuations) was also performed. Use of these two populations provides a reliable estimate of the range of efficacy.

At Week 12, 86% (290/339) of the patients showed a 50% improved PASI score or an sPGA grading of "mild", "minimal", or "clear" and were eligible to enter the maintenance phase; 41% and 13% of patients achieved PASI 75 and PASI 90, respectively. At month 36, the PASI 75 responses were 45% in the ITT population and 73% in the as-treated population (n = 113). PASI 90 responses at month 36 were 25% for ITT, and 40% for as-treated patients (n = 115). The PASI 75 and PASI 90 results of the ITT population are illustrated graphically in Fig. 5.4. More than 50% of patients maintained or improved their response during efalizumab therapy. Moreover, more than half of the patients who achieved a PASI 75 response at 33 and 36 months had a PASI 90 response and were therefore virtually clear of their disease.

5.5.2 Safety and Tolerability

The most frequent symptomatic adverse drug reactions (ADRs) observed during efalizumab therapy were mild-to-moderate acute flu-like symptoms, including headache, fever, chills, nausea, and myalgia. In the efalizumab group, the incidence of acute adverse events was highest following the first dose (27.4% versus 21.2% of patients), and decreased with each subsequent dose (Papp et al. 2006). These reactions were generally less frequent from the third and subsequent

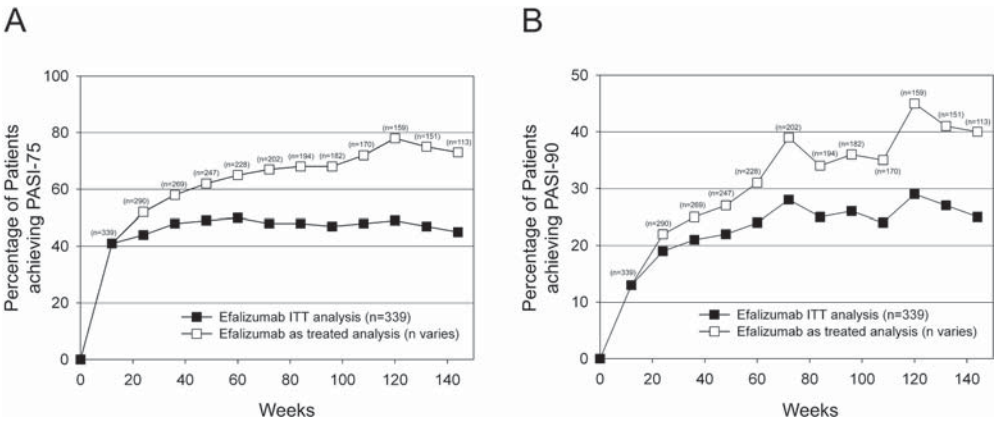

Fig. 5.4 Percentages of patients with PASI 75 and PASI 90 during efalizumab long-term treatment. Data are based on "Intent-to-treat" (ITT) and "As-treated" analyses.

weekly injections, and occurred at similar rates to those seen in the placebo group (3.7% and 3.9%, respectively). The incidence of acute adverse events in other placebo-controlled trials was similar.

Antibodies to efalizumab were detected in 2–6% of patients (Gordon et al. 2003; Lebwohl et al. 2003; Leonardi et al. 2005b). There were no differences in pharmacokinetics, clinically noteworthy adverse events or clinical efficacy between patients with or without antibody responses.

About 40% of patients developed sustained asymptomatic lymphocytosis (this was related to the drug's mechanism of action) during efalizumab therapy (Papp et al. 2005). Typical values were less than threefold the ULN, and lymphocyte counts returned to baseline after therapy discontinuation.

Eight patients (0.3%) developed thrombocytopenia, with a cell count of $<52 \times 10^3 \mu L^{-1}$ reported (Leonardi 2004). One patient was lost to follow-up, but in the remaining seven patients the symptoms were resolved with conservative management of patients. Causality between the administration of efalizumab and thrombocytopenia is likely. Platelet monitoring is recommended. The rate of psoriasis-related ADRs was 2.2% in the efalizumab-treated patients and 0.8% in the placebo group (Papp et al. 2006). Serious psoriasis-related adverse events included erythrodermic, pustular and guttate subtypes (Papp et al. 2006). Exacerbation or flare-up of psoriatic arthritis was observed in 1.6% of efalizumab-treated patients compared to 1.3% in the placebo group (Papp et al. 2006). A recent analysis of pooled data from phase II, III, and IV clinical trials showed that both efalizumab-treated patients and placebo subjects showed a similar incidence of arthropathy during the first 12-week treatment. However, there was no increase in the incidence of arthropathy among efalizumab-treated patients during an extended long-term treatment of up to 3 years (Pincelli and Casset-Semanez 2005).

As with other protein products, efalizumab is potentially immunogenic. During the placebo-controlled clinical studies, the percentage of patients experiencing an adverse event suggestive of hypersensitivity – including urticaria, rash and allergic reactions – was comparable between the efalizumab and placebo groups (Gordon et al. 2003).

Approximately 4.5% of patients developed sustained elevation of alkaline phosphatase throughout efalizumab therapy, compared with 1% of those receiving placebo. All values were between 1.5- and 3-times the ULN, and returned to baseline levels after therapy discontinuation (Serono Europe 2004). About 5.7% of patients developed elevated levels of alanine aminotransferase during efalizumab therapy compared to 3.5% after placebo. However, all such occurrences were asymptomatic, and values above 2.5 ULN were no more frequent in the efalizumab group than in the placebo group. All values returned to baseline levels upon therapy discontinuation (Serono Europe 2004).

Therapies that alter T-lymphocyte function have been associated with an increased risk of developing serious infections. In clinical trials, infection rates were similar between efalizumab- and placebo-treated patients. High rates of malignancy have been associated with therapies that affect the immune system, but in these clinical trials the overall incidences of malignancy were similar

among efalizumab- and placebo-treated patients. In addition, the incidences of specific tumors in patients treated with efalizumab were in line with those observed in control psoriasis populations. Among psoriasis patients who received efalizumab at any dose level, the overall incidence of malignancies of any type was 1.8 per 100 patient-years after efalizumab treatment, compared to 1.6 per 100 patient-years for placebo-treated patients. There appears to be no evidence of a risk of developing malignancy exceeding that expected among the psoriasis population (Leonardi 2004).

Continuous treatment with efalizumab for 36 months was generally well tolerated, and no new adverse events were reported (Gottlieb et al. 2005). The adverse event profiles associated with long-term efalizumab therapy showed no evidence for end-organ damage or cumulative toxicity. The incidence of infection-related adverse events observed during this trial (Fig. 5.5) appeared to be similar to that observed during placebo-controlled trials (28.9% and 26.3% of efalizumab-treated and placebo-treated patients, respectively; see Fig. 5.5). There were no opportunistic infections, no tuberculosis, no demyelination, no congestive heart failure, and no deaths during the 3-year study. There was also no evidence of increased risk of opportunistic infections (Langley et al. 2005).

During continuous therapy, the incidence of psoriasis adverse events and of malignancies did not increase over time (Gottlieb et al. 2005) (Fig. 5.6). Whilst the majority of malignancies were nonmelanoma skin carcinomas, other malignancy events observed included one lymphoma, two carcinomas of the colon, two lung carcinomas, and one melanoma.

Based on clinical development data, there seems to be no evidence of risk of developing malignancy. Further data from post-marketing exposure, however, are still needed to confirm this observation.

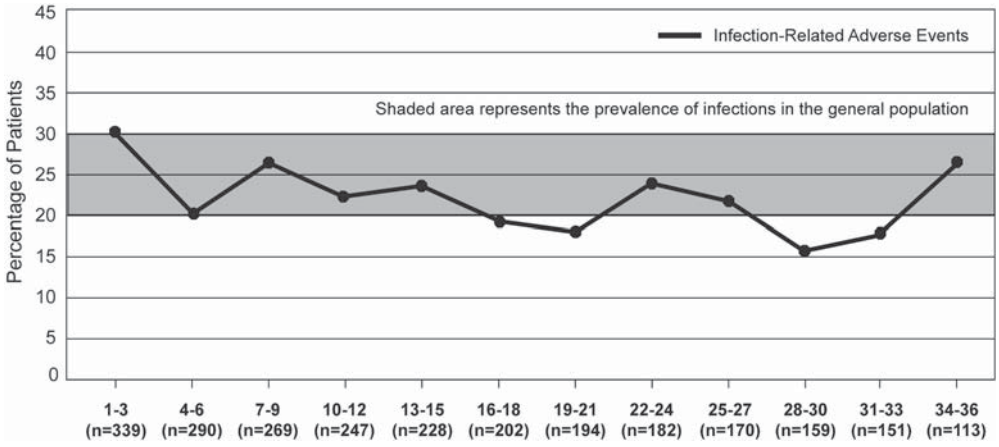

Fig. 5.5 Infection-related adverse events during 3 years' continuous therapy with efalizumab.

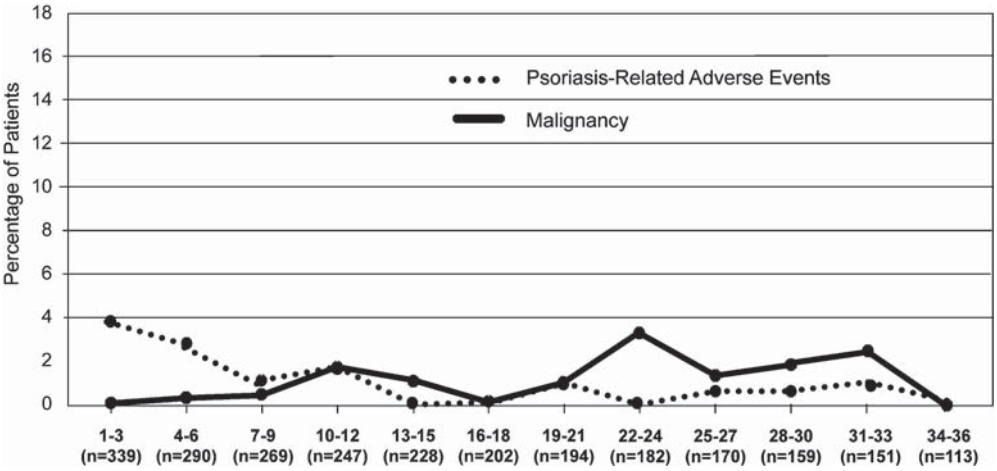

Fig. 5.6 Psoriasis-related adverse events and malignancies during 3 years' continuous therapy with efalizumab.

5.5.3 Health-Related Quality of Life (HRQoL)

It is well known that psoriasis may lead to a reduced quality of life, psychosocial distress, and even disability. The impact of psoriasis on the patients' physical and psychological function has been shown to exceed that of other diseases, such as heart disease, cancer, diabetes, or arthritis (Rapp et al. 1999).

In order to evaluate the effect of efalizumab treatment on HRQoL and disease burden, the results of patient-reported outcomes of three phase III, randomized double-blind, placebo-controlled, multicenter clinical trials were pooled and analyzed (Menter et al. 2004a). A total of 1242 patients with moderate-to-severe plaque psoriasis, receiving either efalizumab ($1\,mg\,kg^{-1}$) or placebo for 12 weeks was included in the analyses. The methods used were dermatology-related quality of life (DLQI), PSA, and an itch scale. Scores were assessed prior to treatment and at the end of the 12-week treatment period, comparing results of the active treatment group with those of the placebo group. Further score assessments were made at the end of an extended treatment period at Week 24. The extension period was performed open label, with the former placebo patients receiving active treatment of $1\,mg\,kg^{-1}$ efalizumab for Weeks 13 to 24.

Baseline data on the disease burden of moderate-to-severe psoriasis indicate a substantial burden across all items of the DLQI. More than 60% of the patients in both the active treatment and placebo groups reported that they were bothered "a lot" or "very much" by itchy, sore painful skin, or by being embarrassed or self-conscious. Over one-third of patients (36%) reported problems with their social life, and about 20% had problems with their partner or had sexual difficulties. In the PSA, more than 80% of patients reported suffering from itching or scaling skin.

Twelve-week treatment with efalizumab led to significant improvements in patient-reported outcomes compared with placebo. Across all measures – DLQI, PSA severity, PSA frequency and itch – the proportion of patients with improvement was at least twofold greater in the active treatment group than in placebo-treated patients (Menter et al. 2004a). Moreover, improvements were maintained during the additional 12-week extended treatment period. Patients randomized to placebo in the FT course and switched to efalizumab for Weeks 13 to 24 showed similar improvements in DLQI and symptom assessments as the efalizumab group during the first treatment phase (Menter et al. 2004a).

5.6
Practical Considerations for Therapy with Efalizumab

Efalizumab gained regulatory approval in the US in 2003 for the continuous treatment of adult patients with moderate-to-severe plaque psoriasis who are eligible for systemic therapy. In 2004, the European Commission approved the drug for adult patients with moderate-to-severe chronic plaque psoriasis who have failed to respond to, or have contraindications to, or are intolerant of other systemic therapies, including cyclosporine, methothrexate, and PUVA (psoralen + UV-A).

Efalizumab is provided as a lyophilized powder in vials containing 150 mg of the drug substance; before injection it is reconstituted in water for injections. After an initial single 0.7 mg kg^{-1} dose, weekly injections of 1.0 mg kg^{-1} should be given (maximum single dose not to exceed a total of 200 mg). Continuous therapy can be given to those patients in Europe who have responded to treatment (PGA of "good" or better) after 12 weeks of initial treatment.

5.6.1
Managing Patients during Long-Term Efalizumab Treatment

Although efalizumab is well tolerated by the majority of patients, some aspects require special attention (Hamilton 2005; Leonardi et al. 2005b).

Patients may develop transient localized papular eruptions, typically within 4 to 8 weeks after the start of therapy. These 2- to 4-mm inflammatory papules are observed at sites not previously affected by the disease – usually the neck, torso, or flexural areas. Discontinuation of efalizumab is not required in those cases. "Treating through" with efalizumab – alternately combining efalizumab with a topical corticosteroid – will resolve the issue in most cases.

Patients may develop generalized inflammatory exacerbations. This is an infrequent complication, generally observed in nonresponders at 6 to 10 weeks after the initiation of therapy. Patients experiencing such an exacerbation should be transferred to alternative systemic therapies to control the flare.

Patients may develop rebound after discontinuation of efalizumab therapy (defined as PASI >125% of baseline or new widespread pustular, erythrodermic or more inflammatory psoriasis occurring within 8 weeks of discontinuing treatment in responding patients). As efalizumab is approved for continuous therapy, this should normally not occur in patients who were responders during the first 12 weeks of treatment. However, should discontinuation be necessary (for any reason), patients are advised to consult their physician at the first signs of a relapse. Efalizumab re-treatment should then be initiated. Patients who did not respond within the first 12 weeks of treatment should be given alternative systemic therapies to prevent rebound. Therapies with the highest success rates at preventing rebound are cyclosporine, methotrexate and systemic corticosteroids (Menter et al. 2004b; Papp et al. 2005).

5.6.2 Other Concerns

Efalizumab is contraindicated in patients with a history of malignancies. As for other anti-psoriatic therapies for systemic use that alter T-lymphocyte functions, efalizumab may affect host defenses against infections. As a consequence, efalizumab should not be used in patients with active tuberculosis or other severe infections, as well as in immune-deficient patients. Patients developing an infection during treatment with efalizumab should be monitored, and efalizumab – according to the severity of the infection – should be discontinued.

Pregnant and breastfeeding women should not use efalizumab; women of childbearing potential should be advised to use appropriate contraception. Immunoglobulins are known to cross the placental barrier. Although preclinical studies have shown no evidence of efalizumab-associated adverse events during the course of pregnancies, or of any harm to fetuses or infants, animal studies indicate a reversible impairment of immune function of offspring exposed to CD11a. Additionally, immunoglobulins are known to be secreted in human milk.

In a trial characterizing immune responses to the neoantigen phiX174, single doses of efalizumab significantly attenuated the secondary immune response at doses recommended for treating psoriasis (Gottlieb et al. 2002). Therefore, in cases of necessary vaccination, efalizumab treatment should be stopped 8 weeks before and be re-initiated 2 weeks after vaccination.

As several cases of thrombocytopenia have occurred during efalizumab treatment, periodic assessment of platelet counts is recommended upon initiating and during efalizumab treatment. It is recommended that assessments be performed monthly during the first 12 weeks of treatment, and every 3 months during continued treatment. In cases of clinical symptoms such as echymoses, spontaneous bruising or bleeding from mucocutaneous tissues, efalizumab should be stopped immediately, a platelet count should be performed, and appropriate symptomatic treatment should be instituted immediately.

5.7 Summary

Psoriasis is a chronic, debilitating disease that may affect patients for several decades. Until recently, no satisfactory systemic treatment was available that showed both efficacy and long-term safety, especially for those patients suffering from moderate-to-severe forms of the condition.

Developments in understanding the pathophysiology of inflammatory diseases such as psoriasis, together with the progress made in biotechnology in designing compounds that specifically target pathogenic molecules within the immune system, has opened a new horizon for the treatment of psoriasis. One of these compounds, efalizumab – a humanized, monoclonal antibody against the CD11a subunit of the lymphocyte function associated antigen-1 (LFA-1) – has been shown in clinical trials to be effective and well tolerated in the treatment of patients with moderate-to-severe psoriasis. Although more data are required to determine the safety profile of continuous therapy, such a strategy appears to be successful for maintaining tight control of psoriasis symptoms, as demonstrated in a recent phase III study in which patients received efalizumab continuously over 3 years (Gottlieb et al. 2005).

References

Bauer, R.J., Dedrick, R.L., White, M.L., Murray, M.J., Garovoy, M.R. (1999) Population pharmacokinetics and pharmacodynamics of the anti-CD11a antibody hu1124 in human subjects with psoriasis. *J Pharmacokinet Biopharm* 27: 397–400.

Bos, J.D., De Rie, M.A. (1999) The pathogenesis of psoriasis: Immunological facts and speculations. *Immunol Today* 20: 40–46.

Bos, J.D., Hulsebosch, H.J., Krieg, S.R., Bakker, P.M., Cormane, R.H. (1983) Immunocompetent cells in psoriasis: In situ immunophenotyping by monoclonal antibodies. *Arch Dermatol Res* 275: 181–189.

Boyman, O., Hefti H., Conrad, C., Nickoloff, B.J., Suter M., Nestle, F.O. (2004) Spontaneous development of psoriasis in a new animal model shows an essential role for resident T cells and tumor necrosis factor-α. *J Exp Med* 199: 731–736.

Bradley, L.M., Watson, S.R. (1996) Lymphocyte migration into tissue: The paradigm derived from CD4 subsets. *Curr Opin Immunol* 8: 312–320.

Carlin, C.S., Feldman, S.R., Krueger, J.G., Menter, A., Krueger, G. (2004) A 50% reduction in the Psoriasis Area and Severity Index (PASI 50) is a clinically significant endpoint in the assessment of psoriasis. *J Am Acad Dermatol* 50: 859–866.

Coffey, G.P., Stefanich, E., Palmieri, S., Eckert, R., Padilla-Eager, J., Fielder, P., Pippig, S. (2004) In vitro internalization, intracellular transport and clearance of efalizumab (Raptiva) by humanT-cells. *J Pharmacol Exp Ther* 310: 896–904.

de Fougerolles, A.R., Stacker, S.A., Schwalting, R., Springer, T.A. (1991) Characterization of ICAM-2 and evidence for a third counter-receptor for LFA- I. *J Exp Med* 174: 253–267.

de Fougerolles, A.R., Qin, X., Springer, T.A. (1994) Characterization of the function of

intercellular adhesion molecule (ICAM)-3 and comparison with ICAM-I and ICAM-2 in immune responses. *J Exp Med* 179: 619–629.

Dougherty, G.J., Hogg, N. (1987) The role of monocyte lymphocyte function-associated antigen I (LFA-1) in accessory cell function. *Eur J Immunol* 17: 943–947.

Dustin, M.L., Rothlein, R., Bhan, A.K., Dinarello, C.A., Springer, T.A. (1986) Induction of IL-1 and IFN-γ: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-I). *J Immunol* 137: 245–254.

Ferenczi, K., Burack. L., Pope, M., Krueger, J.G., Austin, L.M. (2000) CD69, HLA-DR and the IL-2R identify persistently activated T cells in psoriasis vulgaris lesional skin: Blood and skin comparisons by flow cytometry. *J Autoimmun* 14: 63–78.

Fuhlbrigge, R.C., King, S.L., Dimitroff, C.J., Kupper, T.S., Sackstein, R. (2002) Direct real-time observation of E- and P-selectin-mediated rolling on cutaneous lymphocyte-associated antigen immobilized on Western blots. *J Immunol* 168: 5645–5651.

Gilhar, A., David, M., Ullmann, Y., Berkutski, T., Kalish, R.S. (1997) T-lymphocyte dependence of psoriatic pathology in human psoriatic skin grafted to SCID mice. *J Invest Dermatol* 109: 283–288.

Gollnick, H., Bonnekoh, B. (2001) *Psoriasis – Pathogenese, Klinik und Therapie*. Uni-Med Verlag AG, Bremen.

Gordon, K.B., Papp, K.A., Hamilton, T.K., Walicke, P.A., Dummer, W., Li, N., Bresnahan, B.W., Menter, A. (2003) Efalizumab for patients with moderate to severe plaque psoriasis. A randomised controlled trial. *JAMA* 290: 3073–3080.

Gottlieb, S.L., Gilleaudeau, P., Johnson, R., Estes, L., Woodworth, T.G., Gottlieb, A.B., Krueger, J.G. (1995) Response of psoriasis to a lymphocyte-selective toxin (DAB389IL-2) suggests a primary immune, but not keratinocyte, pathogenic basis. *Nat Med* 1: 442–447.

Gottlieb, A., Krueger, J.G., Bright, R., Ling, M., Lebwohl, M., Kang, S., Feldman, S., Spellman, M., Wittkowski, K., Ochs, H.D. (2000) Effects of administration of a single dose of a humanized monoclonal antibody to CD11a on the immunobiology and clinical activity of psoriasis. *J Am Acad Dermatol* 42: 428–435.

Gottlieb, A.B., Krueger, J.B., Wittkowski, K. (2002) Psoriasis as a model for T-cell mediated disease: immunobiologic and clinical effects of treatment with multiple doses of efalizumab, an anti CD 11a antibody. *Arch Dermatol* 138: 591–600.

Gottlieb, A.B., Hamilton, T.K., Walicke, P.A., Li, N., Joshi, A., Garovoy, M., Gordon, K.B. (2003) Efficacy, safety, and pharmacokinetic outcomes observed in patients with plaque psoriasis during long-term efalizumab therapy: results from an open-label trial. International Investigative Dermatology Meeting April 30–May 4, 2003 Miami Beach Florida, Poster 379.

Gottlieb, A.B., Gordon, K.B., Lebwohl, M.G., Caro, I., Walicke, P.A., Li, N., Leonardi, G. (2004) Extended efalizumab therapy sustains efficacy without increasing toxicity in patients with moderate to severe chronic plaque psoriasis. *J Drugs Dermatol* 3: 614–624.

Gottlieb, A.B., Gordon, K.B., Hamilton, T.K., Leonardi, C. (2005) Maintenance of efficacy and safety with continuous efalizumab therapy in patients with moderate to severe plaque psoriasis: final phase IIIb study results. *J Am Acad Dermatol* 52: P4.

Grakoui, A., Bromley, S.K., Sumen, C., Davis, M.M., Shaw, A.S., Allen, P.M., Dustin, M.L. (1999) The immunological synapse: a molecular machine controlling T cell activation. *Science* 285: 221–227.

Hamilton, T.K. (2005) Clinical considerations of efalizumab therapy in patients with psoriasis. *Semin Cutan Med Surg* 24: 19–27.

Hildreth, J.E.K., August, J.T. (1985) The human lymphocyte function-associated (HLFA) antigen and a related macrophage differentiation antigen (HMac-1): functional effects of subunit-specific monoclonal antibodies. *J Immunol* 134: 3272–3280.

Hildreth, J.E.K., Gotch, F.M., Hildreth, P.D.K., McMichael, A.J. (1983) A human lymphocyte-associated antigen involved in cell-mediated lympholysis. *Eur J Immunol* 13: 202–208.

Homey, B., Alenius, H., Muller, A., Soto, H., Bowman, E.P., Yuan, W., McEnvoy, L., Lauerman, A.I., Assmann, T., Bunemann, E., Lehto, N., Wolff, H., Yen, D., Marxhausen, H., To, W., Sedgwick, J., Ruzicka, T., Lehmann, P., Zlotnik, A. (2002) CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nat Med* 8: 157–165.

Janeway, C.A., Travers, P., Walport, M., Shlomchick, M. (2001) *Immunobiology. The Immune System in Health and Disease*. 5th edn. Garland Publishing.

Jullien, D., Prinz J.C., Langley, R.G.B., Caro, I., Dummer, W., Joshi, A., Dedrick, R., Natta, P. (2004) T-cell modulation for the treatment of chronic plaque psoriasis with efalizumab (Raptiva): Mechanisms of action. *Dermatology* 208: 297–306.

Jung, E.G., Moll, I. (2003) *Dermatologie*. Georg Thieme Verlag, Stuttgart.

Krueger, J.G. (2002) The immunologic basis for the treatment of psoriasis with new biologic agents. *J Am Acad Dermatol* 46: 1–23.

Kuypers, T., Roos, D. (1989) Leukocyte membrane adhesion proteins LFA-I, CR3 and p150.95: a review of functional and regulatory aspects. *Res Immunol* 140: 461–486.

Langley, R.G., Ellis, C.N. (2004) Evaluating psoriasis with Psoriasis Area and Severity Index, Psoriasis Global Assessment, and Lattice System Physician's Global Assessment. *J Am Acad Dermatol* 51: 563–569.

Langley, R.G., Carey, W.P., Rafal, E.S., Tyring, S.K., Caro, I., Wang, X., Wetherill, G., Gordon, K.B. (2005) Incidence of infection during efalizumab therapy for psoriasis: analysis of the clinical trial experience. *Clin Ther* 27: 1317–1328.

Lebwohl, M., Tyring, S.K., Hamilton, T.K., Toth, D., Glazer, S., Tawfik, N.H., Walicke, P., Dummer, W., Wang, X., Garovoy, M.R., Pariser, D. (2003) A novel targeted T-cell modulator, efalizumab, for plaque psoriasis. *N Engl J Med* 349: 2004–2013.

Leonardi, C.L. (2004) Efalizumab in the treatment of psoriasis. *Derm Ther* 17: 393–400.

Leonardi, C.L., Papp, K.A., Gordon, K.B., Menter, A., Feldman, S.R., Caro, I., Walicke, P.A., Compton, P.G., Gottlieb, A.B. (2005a) Extended efalizumab therapy improves chronic plaque psoriasis: Results from a randomized phase III trial. *J Am Acad Dermatol* 52: 425–433.

Leonardi, C.L., Menter, A., Sterry, W., Bos, J.D., Papp, K.A. (2005b) Long-term management of chronic plaque psoriasis: guidelines for continuous therapy with efalizumab. 14th EADV Fall Symposium, London, UK, 12–16 October, 2005.

Lo, S., van Seventer, G, Levin, S., Wright S. (1989) Two leukocyte receptors (CD11a/CD18 and CD11b/CD18) mediate transient adhesion to endothelium by binding to different ligands. *J Immunol* 143: 3325–3329.

Menter, A., Griffiths, C. (2004) *Efalizumab and psoriasis*. Science Press Ltd., London.

Menter, A., Kosinski, M., Bresnahan, B.W., Papp, K.A., Ware, J.E. (2004a) Impact of efalizumab on psoriasis-specific patient reported outcomes. Results from three randomized, placebo-controlled clinical trials of moderate to severe plaque psoriasis. *J Drugs Dermatol* 3: 27–38.

Menter, A., Kardatzke, D., Rundle, A.C., Garovoy, M.R., Leonardi, C.L. (2004b) Incidence and prevention of rebound upon efalizumab discontinuation. Poster Presentation, International Psoriasis Symposium (IPS), Toronto, 2004.

Menter, A., Gordon, K., Carey, W., Hamilton, T., Glazer, S., Caro, I., Li, N., Gulliver, W. (2005) Efficacy and safety observed during 24 weeks of efalizumab therapy in patients with moderate to severe plaque psoriasis. *Arch Dermatol* 141: 31–38.

Mortensen, D.L., Walicke, P.A., Wang, X., Kwon, P., Kuebler, P., Gottlieb, A.B., Krueger, J.G., Leonardi, C.L., Miller, B., Joshi, A. (2005) Pharmacokinetics and pharmacodynamics of multiple weekly subcutaneous efalizumab doses in patients with plaque psoriasis. *J Clin Pharmacol* 45: 286–298.

Papp, K.A., Toth, D., Rosoph, L. (2005) Investigational study comparing different transitioning therapies for patients discontinuing treatment with efalizumab. 14th EADV Fall Symposium, London, UK, 12–16 October, 2005.

Papp, K.A., Bressinck, R., Fretzin, S., Goffe, B., Kempers, S., Gordon, K.B., Caro, I.,

Walicke, P.A., Wang, X., Menter, A. (2006) The safety of efalizumab in adults with chronic moderate to severe plaque psoriasis: a phase IIIb, randomized, controlled trial. *Int J Dermatol* 45: 605–614.

Pincelli, P., Casset-Semanez, F. (2005) Efalizumab therapy and incidence of arthropathy adverse events: analysis of pooled data from phase II, III, IV clinical trials. Psoriasis from Gene to Clinic, 4th International Congress, London, UK, 1–3 December, 2005.

Prinz, J.C., Braun-Falco, O., Meurer, M., Daddona, P., Reiter, C., Rieber, P., Riethmuller, G. (1991) Chimaeric CD4 monoclonal antibody in treatment of generalised pustular psoriasis. *Lancet* 338: 320–321.

Prinz, J.C., Gross, B., Vollmer, S., Trommler, P., Strobel, I., Meurer, M., Plewig, G. (1994) T cell clones from psoriasis skin lesions can promote keratinocyte proliferation in vitro via secreted products. *Eur J Immunol* 24: 593–598.

Rapp, S.R., Feldman, S.R., Exum, M.L. (1999) Psoriasis causes as much disability as other major medical diseases. *J Am Acad Dermatol* 41: 410–417.

Ring, J., Dubertret, L., May, T., Chimenti, S., Sterry, W. (2005) The safety and efficacy of efalizumab patients with chronic plaque psoriasis. Results from retreatment and extended treatment phases following a placebo-controlled trial, 14th EADV Fall Symposium, London, UK 12–16 October, 2005, Poster P06.87.

Schön, M.P., Boehncke, W.-H. (2005) Medical progress: Psoriasis. *N Engl J Med* 352: 1899–1912.

Serono Europe Ltd. (2004) Raptiva (Efalizumab). Summary of Product Characteristics.

Sterry, W., Dubertret, L., Papp, K., Chimenti, S., Larsen, C.G. (2004) Efalizumab for patients with moderate to severe chronic plaque psoriasis: results of the international, randomized, controlled phase III Clinical Experience Acquired with Raptiva (CLEAR) trial. *J Invest Dermatol* 123: A64.

Sun, Y., Yu, J., Joshi, A., Compton, P., Kwon, P., Bruno, R.A. (2005) Population pharmacokinetics of Efalizumab (humanized monoclonal CD 11 antibody) following long term subcutaneous weekly dosing in psoriasis subjects. *J Clin Pharmacol* 45: 468–476.

Valdimarsson, H., Baker, B.S., Jonsdottir, I., Powles, A., Fry, L. (1995) Psoriasis: A T-cell-mediated autoimmune disease induced by streptococcal superantigens? *Immunol Today* 16: 145–149.

Vugmeyster, Y., Kikuch, T., Lowes, M.A., Chamian, F., Kagen, M., Gilleaudeau, P., Lee, E., Howell, K., Bodary, S., Dummer, W., Krueger, J.G. (2004) Efalizumab (anti-CD11a)-induced increase in peripheral blood leukocytes in psoriasis patients is preferentially mediated by altered trafficking of memory CD8+ T cells into lesional skin. *Clin Immunol* 113: 38–46.

Weinstein, G.D., Menter A. (2003) An overview of psoriasis. In: Weinstein, G.D., Gottlieb, A.B. (Eds.), *Therapy of Moderate to Severe Psoriasis*. Marcel Dekker Inc., New York, pp. 1–29.

Werther, W.A., Gonzalez, T.N., O'Connor, S.J., McCabe, S., Chan, B., Hotaling, T., Champe, M., Fox, J.A., Jardieu, P.M., Berman, P.W., Prestal, L.C. (1996) Humanization of an anti-lymphocyte function-associated antigen (LFA)-1 monoclonal antibody and reengineering of the humanized antibody for binding to rhesus LFA-1. *J Immunol* 157: 4986–4995.

Wrone-Smith, T., Nickoloff, B.J. (1996) Dermal injection of immunocytes induces psoriasis. *J Clin Invest* 98: 1878–1887.

6
^{99m}Tc-Fanolesomab (NeutroSpec)

Christopher J. Palestro, Josephine N. Rini, and Charito Love

6.1
Introduction

Nuclear medicine plays an important role in the evaluation of patients suspected of harboring infection. Among the available radionuclide techniques, *in-vitro*-labeled autologous leukocyte imaging currently is the "gold standard" for imaging most infections in the immunocompetent population. There are, unfortunately, several disadvantages to the technique. The *in-vitro* labeling process is labor-intensive, not always available, and involves the direct handling of blood products. For musculoskeletal infection, the need to perform complementary marrow and/or bone imaging adds complexity and expense to the procedure, and is also an inconvenience to patients [1]. A satisfactory *in-vivo* method of labeling leukocytes would overcome many of these problems, and consequently several such methods have been investigated, including peptides and antigranulocyte antibodies/antibody fragments [2–9]. One of these agents is ^{99m}Tc-fanolesomab (NeutroSpec; Palatin Technologies, Cranberry, New Jersey, USA) – an antigranulocyte antibody that binds to CD-15 receptors present on human leukocytes. This radiolabeled antibody is injected directly into patients, labeling leukocytes *in vivo* and eliminating the disadvantages of *in-vitro* labeling [10].

6.2
The Agent

Fanolesomab is a murine monoclonal M class antibody with a molecular weight of approximately 900 kDa. It was originally raised against stage-specific embryonic antigen (SSEA)-1 in mice immunized with murine embryonal carcinoma F9 cells. The antibody exhibits a high affinity (association constant $K_d = 10^{-11}$ M) for the carbohydrate moiety 3-fucosyl-*N*-acetyl lactosamine contained in the CD15 antigen, which is expressed on human neutrophils, eosinophils, and lymphocytes [11,12].

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6.3 Pharmacokinetics/Dosimetry

Fanolesomab binds to CD 15 receptors expressed on neutrophils in greater proportion than to any other cell type. Less than 1% of activity is bound to lymphocytes and platelets, and only about 4% is associated with erythrocytes. The affinity of fanolesomab for the CD15 receptor on neutrophils is 1.6×10^{-11} M. The concentration bound at 50% of maximum binding is 0.44 to 1.05×10^{-8} M, and the leukocyte labeling efficiency is 80 ± 4%. The activity bound to the recovered granulocyte fraction is 40.7 ± 8.2%. Binding is up-regulated with neutrophil activation, and increases proportionately with increasing numbers of circulating neutrophils [10–13].

Following intravenous injection, ^{99m}Tc-fanolesomab is rapidly distributed with a mean distribution half-life of 0.3 h [13]. The mean linear elimination half-life for blood clearance is 8 h (monoexponential half-life = 5.7 h) [14].

Excretion is primarily through the kidneys, although the radioactivity excreted in the urine is not antibody-bound [13]. The renal excretion fraction varies from 31% to 49% over 26 to 31 h (mean = 39.5 ± 6.5%) [15].

Bone marrow activity peaks shortly after injection, and has a longer elimination time compared to background tissues. Most of the radioactivity accumulates in the liver, followed by the spleen and bladder. Peak liver activity consists of 45 to 50% of the injected dose, decreasing to 25 to 40% of the injected dose by 24 h. Spleen activity peaks at 5 to 12% of the injected dose, and drops by half within 24 h. A low level of activity is seen in the testes. Several tissues known to contain surface receptors identical to SSEA-1/CD15, such as the salivary glands, brain, breasts, eyes and ovaries, are not visualized, and activity does not cross the blood–brain barrier. In contrast to leukocytes that are labeled *in vitro*, transient retention of activity in the lungs following ^{99m}Tc-fanolesomab injection has not been observed [14].

The dose-limiting organ is the spleen, which receives an estimated 0.064 mGy/MBq (0.24 rads/mCi), an amount that is considerably lower than the estimated 90 mGy (9 rads) for the typical dose of 18.5 MBq (0.5 mCi) for ^{111}In-labeled leukocytes [15]. Differences in the absorbed radiation doses between males and females are minimal.

There is a rapid accumulation of radioactivity in target sites as early as 10 min after injection [13]. Uptake of ^{99m}Tc-fanolesomab in infection is governed by two factors. First, the antibody presumably binds to circulating neutrophils, which eventually migrate to the focus of infection. Second, the agent also binds to neutrophils and neutrophil debris which express CD-15 receptors, and already are sequestered at a site of infection [11,16].

6.4 Biodistribution

The biodistribution of ^{99m}Tc-fanolesomab includes the blood pool, which decreases over time, the reticuloendothelial system (liver, spleen, bone marrow), and the

kidneys and urinary tract. Gastrointestinal (GI) tract activity is variable. In one investigation of 46 patients, no GI tract activity was identified up to 2 h after injection, while in another investigation of 17 patients, none was observed up to 24 h after injection [12,16]. In a study of 10 normal volunteers, colonic activity was seen only at 24 h after injection [15]. In our experience, the distribution of ^{99m}Tc-fanolesomab in the gastrointestinal tract is similar to that of ^{99m}Tc-HMPAO-labeled leukocytes (unpublished data). Small bowel activity has been observed within 3 to 4 h after injection, and colonic activity – the intensity of which is variable – is usually present by 24 h after injection. Gallbladder activity has also been observed on occasion, though whether this activity reflects the hepatobiliary excretion of unbound radiolabeled antibody, or perhaps hydrophilic technetium complexes, is uncertain (Figs. 6.1–6.3).

The axial and appendicular bone marrow usually are well seen. In adults, hematopoietically active marrow normally is confined to the axial and proximal appendicular skeletons; however, generalized marrow expansion, which is a response to systemic processes such as anemia, tumor and myelophthisic states, and localized marrow expansion, which is a response to local stimuli such as fracture, surgery, orthopedic hardware, and the neuropathic joint, can alter the usual distribution. As with *in-vitro*-labeled leukocytes, the distribution of marrow activity on ^{99m}Tc-fanolesomab images varies in response to these conditions (Fig. 6.4).

Granulating wounds, such as tracheostomy, colostomy/ileostomy, and percutaneous feeding gastrostomy sites, as well as surgical incisions healing by secondary intention, incite an intense neutrophil response. Uptake of ^{99m}Tc-fanolesomab, as with *in-vitro*-labeled leukocytes, is a normal finding in these conditions and should not be mistaken for infection (Figs. 6.5 and 6.6).

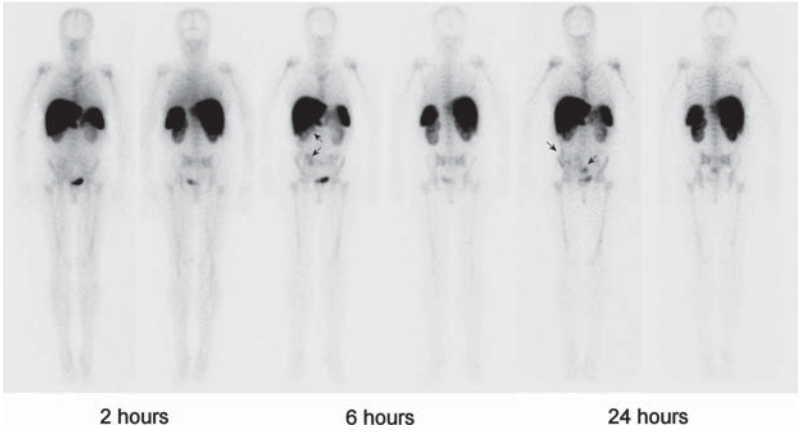

Fig. 6.1 Normal anterior and posterior whole-body images performed at multiple time points after injection of ^{99m}Tc-fanolesomab. At 2 h activity is present in the blood pool, genitourinary tract, and reticuloendothelial system. Over time, the blood pool activity decreases, and bone marrow activity becomes more prominent. Colonic activity (arrows) is seen at 6 and 24 h, but not at 2 h.

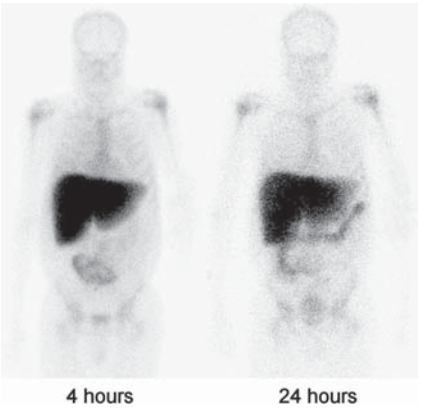

Fig. 6.2 Small bowel activity is present in the right lower quadrant at 4 h after injection. By 24 h most of this activity has passed into the colon.

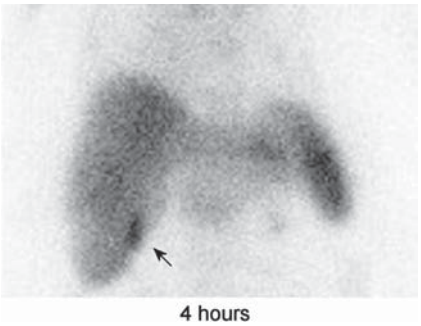

Fig. 6.3 Gallbladder activity (arrow) occasionally can be observed on ^{99m}Tc-fanolesomab studies; its presence cannot automatically be attributed to infection.

6.5 Technique

Fanolesomab is supplied as a lyophilized sterile unit dose kit containing 250 µg of antibody that can be reconstituted and labeled instantly. For the labeling procedure, 40 mCi ^{99m}Tc-pertechnetate ($^{99m}TcO_4^-$) in 0.20–0.35 mL solution is added to the vial and incubated for 30 min at 37°C. It should be noted that a high specific activity is required for satisfactory labeling. If ^{99m}Tc-fanolesomab is prepared using $^{99m}TcO_4^-$ that is more than a few hours old, then free $^{99m}TcO_4^-$ may be observed (Fig. 6.7). In order to stabilize the radiolabeled antibody and to dilute the solution, a sufficient volume of 500 mg mL^{-1} ascorbic acid is added to the vial to bring the final volume to 1.0 mL. Prior to patient administration, the preparation should be tested for radiochemical purity using instant thin-layer chromatography, and should be used only if the percentage of unbound ^{99m}Tc is not more than 10%. The preparation must be kept at room temperature and used within 6 h after reconstitution. The recommended dose to be administered to an adult is 75 to 125 µg of fanolesomab bound to 370–740 MBq (10–20 mCi) $^{99m}TcO_4^-$. For

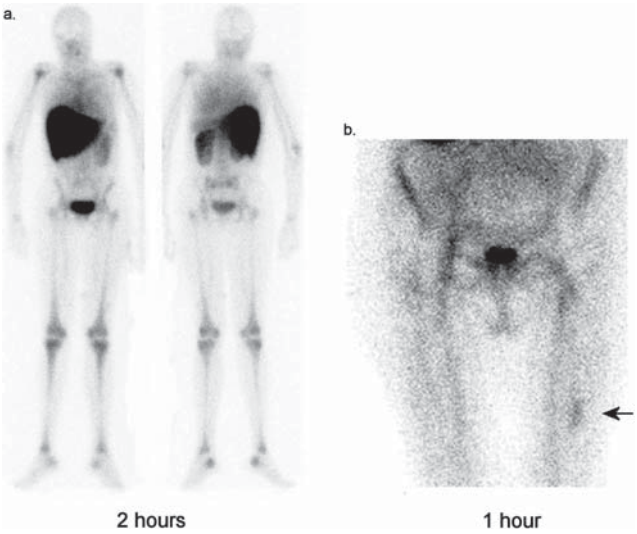

Fig. 6.4 (a) There is diffusely increased activity in the appendicular marrow, which is compatible with generalized marrow expansion, in a patient with sickle-cell disease. Note also the absence of splenic activity, which is secondary to the functional asplenia typical of this entity. (b) There is focally increased activity (arrow) at the distal tip of the femoral component of a left hip prosthesis. For reasons that are not clear, implantation of a prosthetic joint frequently produces localized marrow expansion.

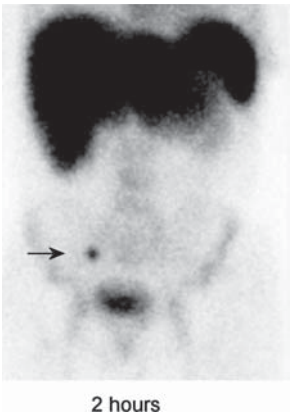

Fig. 6.5 Physiologic accumulation of ^{99m}Tc-fanolesomab at an ileostomy site (arrow) in the right lower quadrant of the abdomen.

children, 3.7 MBq (0.1 mCi) kg^{-1}, to a maximum of 740 MBq (20 mCi), of ^{99m}Tc-fanolesomab is administered [10].

The imaging procedure is well established for patients with suspected appendicitis. Dynamic imaging over the lower abdomen beginning at the time of injection, followed by static images, up to 90 min post-injection, is recommended [17]. Preliminary investigations suggest that for diabetic pedal

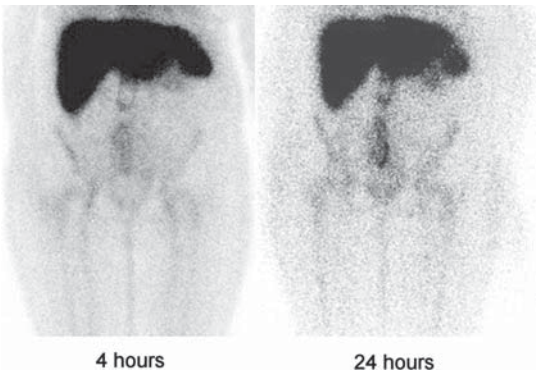

Fig. 6.6 Physiologic accumulation of ^{99m}Tc-fanolesomab in a midline abdominal wall incision healing by secondary intention. Uptake is better delineated at 24 h than at 4h.

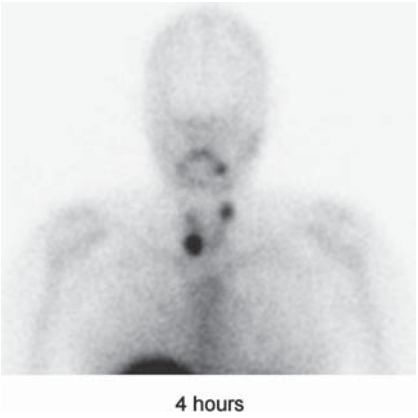

Fig. 6.7 Note the presence of activity in the oral cavity and in the multinodular thyroid gland. High specific activity is required for satisfactory labeling. If fanolesomab is labeled with $^{99m}TcO_4^-$ that is more than a few hours old, free pertechnetate may be seen, as this image illustrates.

osteomyelitis, 1-h fanolesomab images are comparable to 24-h ^{111}In-labeled leukocyte images [18].

Optimal imaging protocols for other sites of infection have not been established, though in some cases dramatic changes have been observed between images obtained at 3 to 4 h and those obtained 24 h after injection of ^{99m}Tc-fanolesomab (Figs. 6.8 and 6.9).

6.6 Indications

6.6.1 Appendicitis

Accurate and timely diagnosis of acute appendicitis can be clinically challenging. The typical presentation, including vague epigastric or periumbilical pain that

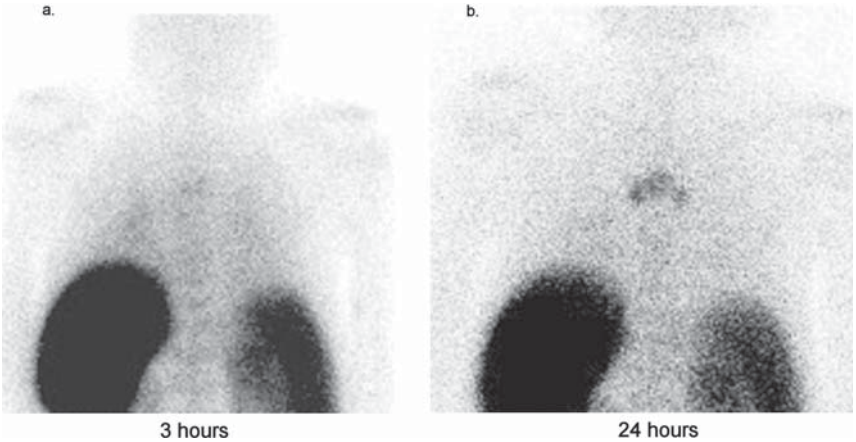

Fig. 6.8 (a) Posterior image of the thorax demonstrates questionable bilateral pulmonary activity, but is otherwise unremarkable. (b) When the image was repeated the next day, however, abnormal activity is clearly seen within the mid-thoracic spine. Osteomyelitis was subsequently confirmed on magnetic resonance imaging.

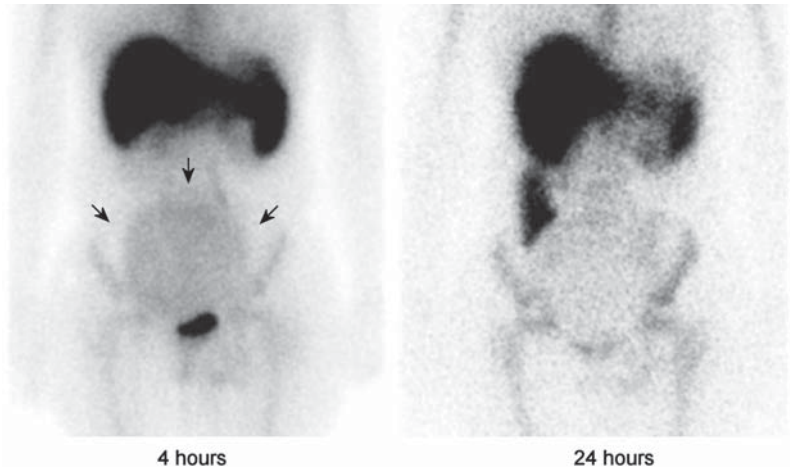

Fig. 6.9 Blood pool activity, present in a large uterine fibroid (arrows) on the initial image, has cleared by 24 h. It would be difficult to exclude infection of this lesion on the basis of the 4-h image alone.

subsequently localizes to the right lower quadrant and is associated with localized right lower quadrant tenderness, anorexia, nausea, fever and leukocytosis, is found in only about 50 to 60% of patients, and is even less common in very young and very old patients, and women of childbearing age. Consequently, appendicitis is one of the most commonly misdiagnosed entities in Emergency Departments.

Almost 30% of pediatric patients ultimately diagnosed with acute appendicitis were originally misdiagnosed, with the result that complications occurred in up to 40% of these individuals [19,20].

Individuals with an atypical or equivocal presentation for acute appendicitis usually are observed in-hospital with frequent examination and imaging studies, or are discharged and advised to return if symptoms worsen. Computed tomography (CT), with an accuracy of approximately 94% when using oral and intravenous contrast is the imaging study of choice for diagnosing appendicitis. The disadvantages of CT include the time delay required for luminal contrast opacification to reach the area of the appendix, lower test sensitivity in patients with low body fat content, and the potential for contrast reactions [10].

Although generally not appreciated by the imaging community, appendicitis can be diagnosed accurately with *in-vitro*-labeled leukocyte imaging. In a study of 100 children with an equivocal presentation, Rypins et al. [21] found that the test was 97% sensitive and 94% specific for diagnosing appendicitis. The negative predictive value of the test was 98%. The negative laparotomy rate in this series was only 4%, compared to the current standard of about 12%. Disadvantages, however, included limited availability, lengthy preparation time (2 h or more), hazards of *ex-vivo* leukocyte labeling and, in the case of small children, the amount of blood required for the labeling process. Consequently, despite the favorable results obtained with labeled leukocyte imaging, this test has not gained widespread use for diagnosing appendicitis.

In contrast to the *in-vitro*-labeled leukocyte procedure, ^{99m}Tc-fanolesomab does not require specially trained personnel, is supplied in kit form, and can be formulated in less than 1 h. Recent studies have found that this antigranulocyte antibody is a valuable diagnostic adjunct in atypical appendicitis and may, in fact, serve as a screening test for acute appendicitis [17,22]. Its efficacy for diagnosing appendicitis was assessed in a phase II study in 49 patients with equivocal signs and symptoms [22]. The sensitivity, specificity and accuracy of the test were 100%, 83%, and 92%, respectively. The positive predictive value was 87% and the negative predictive value 100%.

A large multicenter phase III trial was conducted to assess the efficacy of ^{99m}Tc-fanolesomab for diagnosing acute appendicitis in patients with an equivocal presentation, to evaluate its safety, and to assess its potential impact on the clinical management of these patients [17]. Fifty-nine of 200 patients enrolled in the trial had histopathologically confirmed acute appendicitis. The sensitivity, specificity, and accuracy of ^{99m}Tc-fanolesomab were found to be 91%, 86%, and 87%, respectively, while the positive and negative predictive values of the test were 73% and 96%, respectively. The diagnosis of appendicitis was made within 90 min after injection in all cases; in fact, studies became positive within 8 min in 50% of the patients with acute appendicitis. Similar results were obtained in the 48 pediatric patients studied, with sensitivity, specificity, and accuracy of ^{99m}Tc-fanolesomab of 91%, 86%, and 88%, respectively. Positive and negative predictive values were 67% and 97%, respectively. The high negative predictive value is particularly valuable, because a negative result means that acute appendicitis is very unlikely,

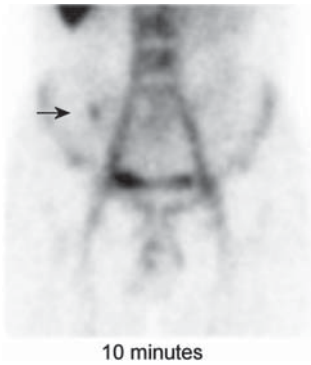

Fig. 6.10 Focal ^{99m}Tc-fanolesomab accumulation in the right lower quadrant of the abdomen (arrow) of a patient with acute appendicitis.

thereby reducing unnecessary time in the hospital for observation, as well as unnecessary surgery. The antibody was well tolerated with no serious adverse events reported. Finally, there was a significant improvement in making the appropriate management decision, both in patients with and in those without appendicitis, after the scan.

The imaging protocol for suspected appendicitis is simple. Planar imaging is carried out over a 90-min period. Single photon emission computed tomography (SPECT) imaging usually is not needed. Abnormal right lower quadrant activity in the "appendicitis zone" that persists over time is the hallmark appearance of appendicitis (Fig. 6.10) [22].

6.6.2 Osteomyelitis

Initial results indicate that ^{99m}Tc-fanolesomab accurately diagnoses osteomyelitis in the appendicular skeleton. A phase II trial was undertaken to assess the accuracy of the antibody for diagnosing osteomyelitis and to compare it with ^{111}In-labeled leukocyte imaging and three-phase bone scintigraphy [23]. A total of 24 patients (10 men, 14 women, aged 48 to 91 years) was enrolled in whom indications included: infected joint replacement (n = 12), diabetic pedal osteomyelitis (n = 8) and long bone osteomyelitis (n = 4). Patients were imaged at multiple time points up to 2 h after tracer injection. There were 11 cases of osteomyelitis. Bone scintigraphy, not surprisingly, was sensitive (100%) but not specific (38%). The 2-h ^{99m}Tc-fanolesomab images were sensitive (91%) and moderately specific (69%), and were comparable in accuracy to 24-h ^{111}In-labeled leukocyte images (91% sensitivity, 62% specificity) (Fig. 6.11). When interpreted together with bone images, the sensitivity and specificity of both the antibody and ^{111}In-labeled leukocytes improved to 100% and 85%, and 100% and 77%, respectively. The performance of the antigranulocyte antibody in this investigation was comparable to that of ^{111}In-labeled leukocytes and, when combined with bone imaging, was more accurate for diagnosing osteomyelitis than any of the other tests.

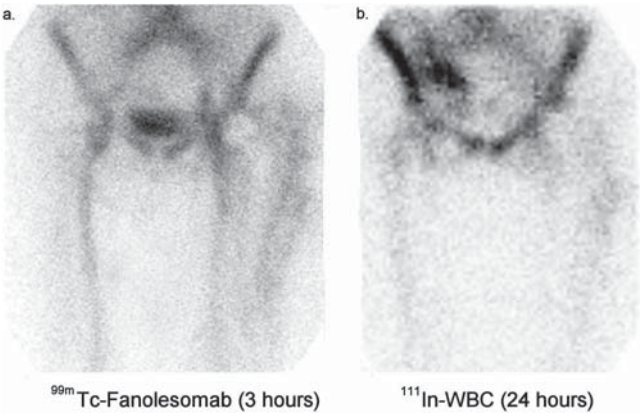

Fig. 6.11 (a) Diffusely increased activity along the lateral aspect of the infected left hip prosthesis at 3 h after injection. (b) A similar, though less obvious, abnormality is present on the ^{111}In-labeled leukocyte image obtained at about 24 h after injection.

The role of ^{99m}Tc-fanolesomab in the diagnosis of osteomyelitis in diabetic patients with pedal ulcers also has been studied [18]. In a phase II investigation, 25 diabetic patients with pedal ulcers (22 in the forefoot, three in the midfoot) underwent ^{99m}Tc-fanolesomab, ^{111}In-labeled leukocyte, and three-phase bone imaging. The 1-h antibody, 24-h labeled leukocyte and three-phase bone images were interpreted separately and classified as either positive or negative for osteomyelitis. Antibody and labeled leukocyte images also were interpreted together with the bone images. The sensitivity, specificity, and accuracy of ^{99m}Tc-fanolesomab alone were 90%, 67%, and 76%, respectively, similar to those obtained with labeled leukocyte imaging alone (80%, 67%, and 72%) (Fig. 6.12). The antibody was as sensitive as – and significantly more specific ($p = 0.004$) than – three-phase bone imaging (90% sensitivity, 38% specificity). Interpreting the antibody together with the bone scan did not improve the results.

Although these initial reports about the value of ^{99m}Tc-fanolesomab for diagnosing osteomyelitis are encouraging, there are nevertheless many issues that are not resolved. In the vast majority of diabetic patients studied, the area of concern involved the forefoot. Patients with open, granulating, surgical incisions were excluded from this investigation, with only those patients receiving antibiotic therapy for less than 7 days being eligible for entry into the study. Consequently, the utility of this agent in the neuropathic joint, in patients with healing surgical incisions, and in patients receiving antibiotics for more than one week is not known.

When performing *in-vitro*-labeled leukocyte studies for osteomyelitis, it is often necessary to perform complementary bone marrow imaging to facilitate the differentiation of labeled leukocyte uptake in bone marrow from uptake in infection [24]. Although the need for marrow imaging has not been confirmed, as the

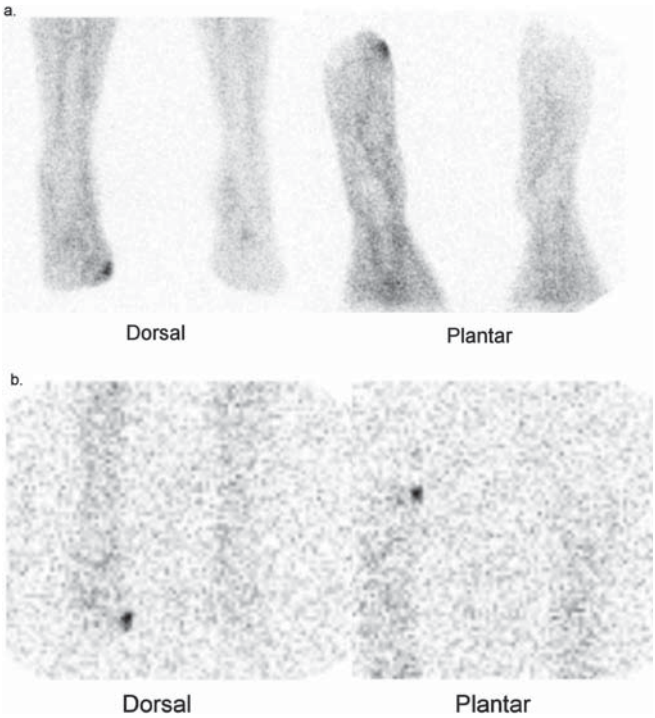

Fig. 6.12 (a) At 1 h after injection, there is focal accumulation of ^{99m}Tc-fanolesomab in the right great toe of a diabetic patient with osteomyelitis of this digit. (b) The uptake pattern is nearly identical on the ^{111}In-labeled leukocyte study of the same patient obtained at about 24 h after injection.

normal distribution of ^{99m}Tc-fanolesomab includes the bone marrow, it is reasonable to conclude that marrow imaging will be necessary (Fig. 6.13).

Finally, because it is essentially another method for performing labeled leukocyte imaging, it is likely that, although data are not available, ^{99m}Tc-fanolesomab will probably not be useful for diagnosing spinal osteomyelitis [25].

6.6.3 Other Infections

Few data are available demonstrating the utility of ^{99m}Tc-fanolesomab for diagnosing infections other than appendicitis or osteomyelitis [12,13,16,26–28]. Thakur et al. [12] studied 12 patients with clinical evidence of active inflammatory processes at the time of imaging, and all 12 had unequivocally positive images within 3 h after injection. In another investigation, 46 patients suspected of having infection underwent fanolesomab imaging at multiple time points up to 2 h after injection [13]. In some patients, additional imaging was performed at various

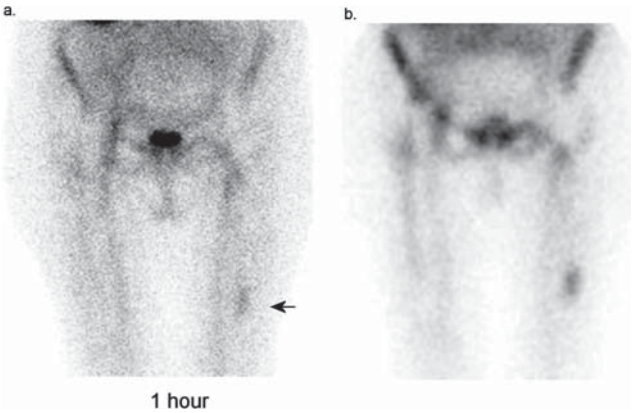

Fig. 6.13 (a) Focally increased activity at the distal tip of the femoral component of a left hip replacement (arrow). It is not possible, based on the ^{99m}Tc-fanolesomab images alone, to determine whether this uptake represents infection or localized marrow expansion. (Same patient as illustrated in Fig. 6.4b). (b) An identical focus is present on the ^{99m}Tc-sulfur colloid marrow image, confirming that the activity on the antibody image represents marrow, not infection.

time points up to 24 h. Indications for imaging included appendicitis (n = 19), osteomyelitis (n = 5), abdominal infection/inflammation (n = 9), pulmonary (n = 5), and miscellaneous (n = 8). The sensitivity, specificity, and accuracy of the test in this population were 95%, 100%, and 96%, respectively, and the positive and negative predictive values were 100% and 75%, respectively. Thirty-three scans were found to be diagnostic within 10 min, and the remainder within 2 h, after injection. Gratz et al. [16] compared ^{99m}Tc-fanolesomab to ^{99m}Tc-HMPAO-labeled leukocytes in 17 patients, with 23 foci of infection, all within soft tissue or bone. The sensitivity and specificity of the antibody were 95% and 96%, respectively, compared to 91% and 82%, respectively, for ^{99m}Tc-HMPAO-labeled leukocyte imaging.

The present authors' clinical experience with ^{99m}Tc-fanolesomab, in a variety of conditions, has been favorable. This agent has been used, in lieu of ^{111}In-labeled leukocytes, to diagnose abscesses, prosthetic vascular graft infections, and to identify the source of an "occult" infection. Generally, the results have been satisfactory (Figs. 6.14 and 6.15) [26–28], although under certain circumstances ^{111}In-labeled leukocytes may have been preferable to ^{99m}Tc-fanolesomab. For example, unlike ^{111}In-labeled leukocytes, fanolesomab normally accumulates in the genitourinary and GI tracts, making detection of infection in these systems more difficult to diagnose with this agent. Therefore, in the setting of occult infection, or when genitourinary or GI tract infections are suspected, ^{111}In-labeled leukocytes, rather than ^{99m}Tc-fanolesomab, should most likely be used (Fig. 6.16).

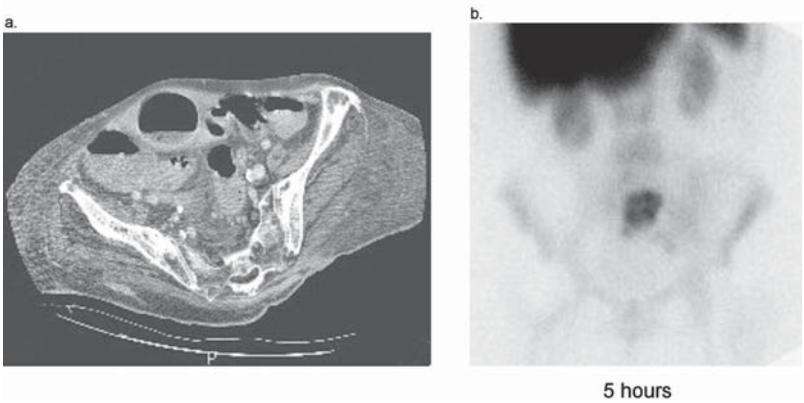

Fig. 6.14 (a) A large cystic mass with an air/fluid level in the lower abdomen was identified on a CT scan performed on a patient with fever and abdominal pain. (b) The intense accumulation of ^{99m}Tc-fanolesomab within this lesion confirmed the presence of infection.

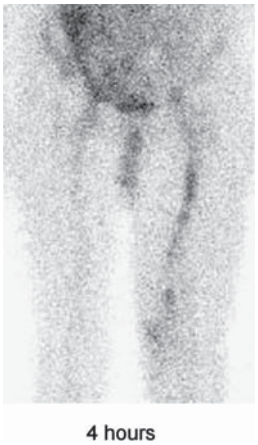

Fig. 6.15 A linear area of activity extending along the medial aspect of the left thigh can be appreciated in a patient with an infected left lower-extremity prosthetic vascular graft.

6.7 Adverse Side Effects and Safety

The chemotactic function of neutrophils is not affected by the degree of receptor occupancy. At 10% receptor occupancy by the antibody, the phagocytic and adherence functions are substantially diminished, while at 4–5% occupancy no effect on neutrophil function is observed. At least 1mg of fanolesomab is needed to saturate 4% of the estimated 5.1×10^5 CD15 surface receptors present on a neutrophil [11,12]. The usual dose injected contains 75 to 125 µg of antibody, which is considerably less than the amount needed to produce pharmacological effects [12,29].

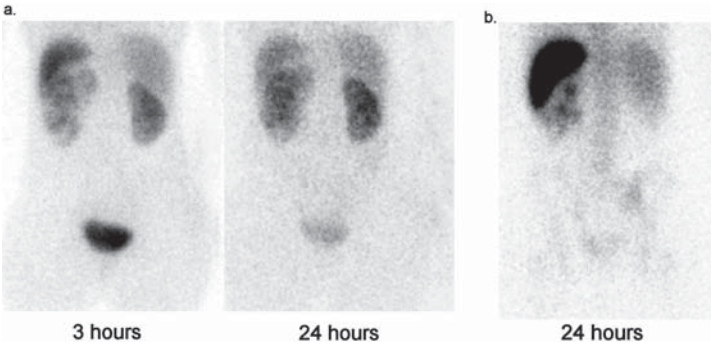

Fig. 6.16 (a) A febrile 56-year-old woman underwent ^{99m}Tc-fanolesomab imaging for occult infection. Posterior images showed only a large left kidney. (b) A ^{111}In-labeled autologous leukocyte study performed 2 days (24 h post injection) later clearly demonstrated abnormal labeled leukocyte accumulation in an infected left kidney. Urine cultures grew *Candida albicans*.

There is a transient decrease in the number of circulating white blood cells within 20 min after injection of fanolesomab; typically, the relative number of granulocytes decreases from 57.2 ± 10.5 to 35.3 ± 17.7% of total circulating leukocytes. The effects on the other blood cells are qualitatively similar, but less pronounced. Recovery is rapid, averaging about 20 min, and there have been no clinical complaints associated with this phenomenon [14,16].

Murine monoclonal antibodies may result in the development of human anti-mouse antibody (HAMA) in individuals to whom these agents are administered. One study to assess formation of HAMA and safety of fanolesomab administration was undertaken in 30 healthy subjects [13]. Each subject received 125 µg antibody labeled with decayed $^{99m}TcO_4^-$, with blood samples being obtained before and at 3–4 weeks and 3–4 months after antibody injection. There was no statistically significant elevation of HAMA titers following injection; neither were any adverse reactions or changes in vital signs observed.

An investigation of 30 healthy volunteers was undertaken to evaluate the safety and the extent of induction of HAMA response following a second injection of fanolesomab [30]. Subjects were injected on two separate occasions, 3 weeks apart, with 125 µg of fanolesomab labeled with decayed $^{99m}TcO_4^-$. HAMA assays were performed on blood samples drawn prior to each injection, and at 1 and 4 weeks after the second injection. Five subjects (17%) exhibited induction of HAMA; two of these were considered marginal and three moderate responses. Seven subjects (23%) experienced adverse events, most of which were coincidental to fanolesomab administration. None of the adverse events was serious or severe, and the investigators concluded that repeated fanolesomab injections, at clinically useful doses, did not appear to induce a strong HAMA response or to present a risk for serious adverse events.

In pre-market studies submitted to the United States Food and Drug Administration (FDA), there were relatively few safety concerns. Among 523 patients and normal volunteers enrolled in various clinical trials, only 49 adverse events were reported by 37 patients (7%). Flushing (2%) and dyspnea (1%) were the most frequently reported adverse events; less-common adverse events included syncope, dizziness, hypotension, chest pressure, paresthesia, nausea, and burning at the injection site. Four of the 49 adverse events were severe: hypotension, worsening of sepsis, chest pressure and decreased O_2 saturation. Two patients enrolled in clinical trials of post-surgical infection or abscess died following injection, although no relationship could be determined between ^{99m}Tc-fanolesomab and these fatalities [31].

At the end of 2005, approximately 18 months after its introduction, ^{99m}Tc-fanoleosmab was withdrawn from the United States market as a result of post-marketing reports of serious and life-threatening cardiopulmonary events following its administration. The onset of these events generally occurred within minutes after injection, and included two deaths attributed to cardiopulmonary failure within 30 min after injection. Additional cases of serious cardiopulmonary events, including cardiac arrest, hypoxia, dyspnea and hypotension, required resuscitation with fluids, vasopressors, and oxygen. However, there is no evidence that patients who already safely received the drug face any long-term risks.

6.8 Summary

The antigranulocyte antibody ^{99m}Tc-fanolesomab labels human leukocytes *in vivo*, rapidly, and accurately diagnoses appendicitis in patients with an equivocal presentation. Preliminary investigations suggest that ^{99m}Tc-fanolesomab is comparable to *in-vitro*-^{111}In-labeled leukocytes for diagnosing diabetic pedal osteomyelitis. Although at present the data are limited, it is likely that ^{99m}Tc-fanolesomab may also be used to accurately diagnose other infections. Based on results of clinical trials conducted to date, the drug appeared to be safe, but as a result of post-marketing reports of untoward (and sometimes severe) adverse events following its administration, ^{99m}Tc-fanolesomab was withdrawn from the US market, and its future remains uncertain.

References

1 Love, C., Palestro, C.J. (2004) Radionuclide imaging of infection. J Nucl Med Tech 32: 47–57.

2 Palestro, C.J., Weiland, F.L., Seabold, J.E., et al. (2001) Localizing infection with a technetium labeled peptide: initial results. Nucl Med Commun 22: 695–701.

3 Becker, W., Goldenberg, D.M., Wolf, F. (1994) The use of monoclonal antibodies and antibody fragments in the imaging of infectious lesions. Semin Nucl Med 24: 142–153.

4 Becker, W., Blair, J., Behr, T., et al. (1994) Detection of soft tissue infections and

osteomyelitis using a technetium-99m-labeled anti-granulocyte monoclonal antibody fragment. J Nucl Med 35: 1436–1443.
5 Becker, W., Saptogino, A., Wolf, F.G. (1992) The single late Tc-99m-granulocyte antibody scan in inflammatory diseases. Nucl Med Commun 13: 186–1920.
6 Gratz, S., Schipper, M.L., Dorner, J., et al. (2003) Leukoscan for imaging infection in different clinical settings: A retrospective evaluation and extended review of the literature. Clin Nucl Med 28: 267–276.
7 Becker, W., Palestro, C.J., Winship, J., et al. (1996) Rapid diagnosis of infections with a monoclonal antibody fragment (Leukoscan). Clin Orthop 329: 263–272.
8 Hakki, S., Harwood, S.J., Morrissey, M.A., Camblin, J.G., Laven, D.L., Webster, Jr. W.B. (1997) Comparative study of monoclonal antibody scan in diagnosing orthopaedic infection. Clin Orthop 335: 275–285.
9 Deveillers, A., Garin, E., Polard, J.L., et al. (2000) Comparison of Tc-99m-labelled antileukocyte fragment Fab' and Tc-99m-HMPAO leukocyte scintigraphy in the diagnosis of bone and joint infections: a prospective study. Nucl Med Commun 21: 747–753.
10 Love, C., Palestro, C.J. (2003) 99mTc-fanolesomab. IDrugs 6: 1079–1085.
11 Thakur, M.L., Richard, M.D., White, F.W., III. (1988) Monoclonal antibodies as agents for selective radiolabeling of human neutrophils. J Nucl Med 29: 1817–1825.
12 Thakur, M.L., Marcus, C.S., Henneman, P., et al. (1996) Imaging inflammatory diseases with neutrophil-specific technetium-99m-labeled monoclonal antibody anti-SSEA-1B. J Nucl Med 37: 1789–1795.
13 Thakur, M.L., Marcus, C.S., Kipper, S.L., et al. (2001) Imaging infection with LeuTech. Nucl Med Commun 22: 513–519.
14 Mozley, P.D., Thakur, M.L., Alavi, A., et al. (1999) Effects of a 99mTc-labeled murine immunoglobulin M antibody to CD15 antigens on human granulocyte membranes in healthy volunteers. J Nucl Med 40: 2107–2114.
15 Mozley, P.D., Stubbs, J.B., Dresel, S.H., et al. (1999) Radiation dosimetry of a 99mTc-labeled IgM murine antibody to CD15 antigens on human granulocytes. J Nucl Med 40: 625–630.
16 Gratz, S., Behr, T., Herrmann, A., Dresing, K. (1998) Intraindividual comparison of 99mTc-labelled anti-SSEA-1 antigranulocyte antibody and 99mTc-HMPAO labeled white blood cells for the imaging of infection. Eur J Nucl Med 25: 386–393.
17 Rypins, E.B., Kipper, S.L., Weiland, F., et al. (2002) 99mTc anti-CD15 monoclonal antibody (LeuTech) imaging improves diagnostic accuracy and clinical management in patients with equivocal presentation of appendicitis. Ann Surg 235: 232–239.
18 Palestro, C.J., Caprioli, R., Love, C., et al. (2003) Rapid diagnosis of pedal osteomyelitis in diabetics with a technetium-99m labeled monoclonal antigranulocyte antibody. J Foot Ankle Surg 42: 2–8.
19 Sarosi, Jr., G.A., Turnage, R.H. (2002) Appendicitis. In: Feldman, M., Tschumy, Jr., W.O., Friedman, L.S., Sleisenger, M.H. (Eds.), Sleisenger & Fordtran's Gastrointestinal and Liver Disease. 7th edition. St Louis: W.B. Saunders, pp. 2089–2099.
20 Kipper, S.L. (1999) The role of radiolabeled leukocyte imaging in the management of patients with acute appendicitis. Q J Nucl Med 43: 83–92.
21 Rypins, E.B., Kipper, S.L. (1997) 99mTc-hexamethylpropyleneamine oxime (Tc-WBC) scan for diagnosing acute appendicitis in children. Am Surg 63: 878–881.
22 Kipper, S.L., Rypins, E.B., Evans, D.G., et al. (2000) Neutrophil-specific 99mTc-labeled anti-CD15 monoclonal antibody imaging for diagnosis of equivocal appendicitis. J Nucl Med 41: 449–455.
23 Palestro, C.J., Kipper, S.L., Weiland, F.L., et al. (2002) Osteomyelitis: Diagnosis with 99mTc-labeled antigranulocyte antibodies compared with diagnosis with indium-111-labeled leukocytes-

initial experience. Radiology 223: 758–764.
24 Palestro, C.J., Love, C., Tronco, G.G., et al. (2006) Combined labeled leukocyte and technetium-99m sulfur colloid marrow imaging for diagnosing musculoskeletal infection: principles, technique, interpretation, indications and limitations. RadioGraphics 26: 859–870.
25 Palestro, C.J., Kim, C.K., Swyer, A.J., Vallabhajosula, S., Goldsmith, S.J. (1991) Radionuclide diagnosis of vertebral osteomyelitis: Indium-111-leukocyte and technetium-99m-methylene diphosphonate bone scintigraphy. J Nucl Med 32: 1861–1865.
26 Rini, J.N., Love, C., Zia, N., et al. (2005) Initial clinical experience with Tc-99m-fanolesomab in patients with suspected infection. Radiological Society of North America 91st Scientific Assembly and Annual Meeting Program; (P)312 [abstract].
27 Rini, J.N., Love, C., Zia, N., et al. (2005) Tc99m-fanolesomab: a new diagnostic agent for the detection of infection. Radiological Society of North America 91st Scientific Assembly and Annual Meeting Program; (P)806 [abstract].
28 Tronco, G.G., Love, C., Rini, J.N., et al. Diagnosing prosthetic vascular graft infection with the antigranulocyte antibody ^{99m}Tc-fanolesomab. Nucl Med Commun (in press).
29 Thakur, M.L., Lee, J., DeFulvio, M.D., et al. (1990) Human neutrophils: Evaluation of adherence, chemotaxis, and phagocytosis, following interaction with radiolabeled antibodies. Nucl Med Commun 11: 37–43.
30 Line, B.R., Breyer, R.J., McElvany, K.D., et al. (2004) Evaluation of human anti-mouse antibody response in normal volunteers following repeated injections of fanolesomab (NeutroSpec), a murine anti-CD15 IgM monoclonal antibody for imaging infection. Nucl Med Commun 25: 807–811.
31 Package insert: NeutroSpec [Kit for the preparation of technetium (99m Tc) fanolesomab].

7
Gemtuzumab Ozogamicin (Mylotarg)

Matthias Peipp and Martin Gramatzki

7.1
Introduction

Monoclonal antibodies (mAbs) have proven their usefulness as biotherapeutics across a spectrum of diseases, including cancer, infection and immune disorders, as shown by the growing list of mAbs that have been approved by the US Food and Drug Administration (FDA) and by the European Medicines Agency (EMEA) [1,2]. Oncology has been a major area of interest for mAb-based therapeutics, because various antigens have been identified that are overexpressed on certain types of cancer cells compared with normal tissues. Even so, translation into clinically effective therapeutics required that many challenges be overcome. The initial application of murine antibodies was limited by immunogenicity, short serum half-lives, and a lack of efficient interaction with human immune effector cells [3]. Today, however, the introduction of chimeric, humanized and fully human antibodies has overcome these limitations, and such agents can now be produced on a routine basis through protein engineering (humanization), display technologies (phage-, ribosomal-display), or by immunization of transgenic animals carrying human immunoglobulin genes [4–6]. Several agents such as Rituximab, Trastuzumab and Cetuximab have been approved for clinical applications, and demonstrate varying activity in different tumor types [2]. Although these unmodified mAbs show some therapeutic potency, the effects are often not curative. Present knowledge about the clinically relevant mechanisms of action for mAbs is rather limited, but several lines of evidence point to an important role of Fc receptors [7,8]. Therefore, different approaches to improve the therapeutic efficacy of antibody therapeutics by enhancing Fc/FcR interaction have been followed (see Volume I: Fc engineering).

A conceptionally different approach to enhance the efficacy of antibody therapeutics represents arming mAbs with drugs, toxins, or radionuclides [1]. Although such agents are simple in principle, deriving activities from them has been a major challenge because the mAb-conjugated drugs can decompose or decay before being delivered to the target site, the pharmacokinetics of the mAb carriers

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may be suboptimal, the conjugation process can disturb the binding characteristics, the chemical linkers used may have inappropriate stability, and the drugs may therefore not be released in active states or in quantities needed to achieve therapeutic efficacy. Despite these complex requirements, progress has now been made, and the FDA has recently approved three mAb conjugates for cancer therapy [1]. Whilst two of these mAb conjugates are murine radiolabeled antibodies, the third mAb conjugate is a humanized, CD33-specific IgG4 mAb conjugated to a calicheamicin derivative – gemtuzumab ozogamicin – which is used for the treatment of leukemia [9], and is reviewed in this chapter.

7.2 CD33 as a Target Antigen in Acute Leukemia Therapy

One important factor that contributes to the success of antibodies and antibody conjugates as drugs represents the choice of an appropriate target antigen for intervention in the pathophysiology of disease [2,3,10].

Approximately 85 to 90% of adult and pediatric acute myeloid leukemia (AML) cases and 15 to 25% of acute lymphoblastic leukemia (ALL) cases are considered CD33-positive, as defined by the presence of antigen on at least 20 to 25% of leukemic blasts. Importantly, AML blasts express large amounts of CD33 compared to normal tissue [11,12]. The presence of CD33 on AML blasts motivated the development of mAbs against this myeloid cell-surface antigen for the treatment of AML [13,14]. CD33 is a 67 kDa type 1 transmembrane sialoglycoprotein, and a founding member of a rapidly evolving immunoglobulin superfamily subset of sialic acid-binding immunoglobulin-related lectins (siglecs; siglec-3) [15,16]. It was identified by the murine monoclonal antibody anti-MY9 [17], and the human CD33 gene has been mapped to chromosome 19q13.3 and most closely resembles the genes for two adhesion receptors, the myelin-associated glycoprotein and the B-cell antigen CD22 [18,19].

Physiologically, CD33 expression is restricted to early multilineage hematopoietic progenitors, myelomonocytic precursors, and to more mature myeloid cells, macrophages, monocytes and dendritic cells. CD33 is highly expressed on granulocyte precursors, but its expression decreases with maturation and differentiation. It is also expressed on liver cells, but importantly it is absent from normal pluripotent hematopoietic stem cells [20–26].

The natural ligand of CD33 and its biologic functions are unknown. The cytoplasmic tail of CD33 has two tyrosine residues in sequences that closely resemble immunoreceptor tyrosine-based inhibitory motifs (ITIMs). When phosphorylated, these tyrosine motifs provide docking sites for Src homology-2 (SH2) domain-containing tyrosine phosphatases (SHP-1 and SHP-2) [27–29]. CD33 activation and association with SHP-1 may result in inhibitory signals, and may affect the function of neighboring membrane receptors. Recent *in-vitro* studies using genetically modified myeloid cell lines demonstrated that mutations in the ITIM-like motifs impair internalization of antibody-bound CD33, suggesting that structural and functional variations altering this pathway might affect the

susceptibility to CD33-targeted therapies [30]. The expression pattern, its overexpression on tumor cells, and the efficient internalization make CD33 an ideal target for immunoconjugates.

7.3 Gemtuzumab Ozogamicin

Gemtuzumab ozogamicin (GO; Mylotarg, CMA-676; Wyeth Laboratories, Philadelphia, PA, USA) consists of a recombinant humanized immunoglobulin G4 (IgG4) CD33 monoclonal antibody (hP67.6) conjugated to the antitumor antibiotic calicheamicin-γ1, *N*-acetyl-γ1-calicheamicin dimethyl hydrazide (NAc-gamma calicheamicin DMH; Fig. 7.1). GO was very potent and selectively cytotoxic to HL-60 leukemia cells in tissue culture, and low doses given to mice bearing HL-60 xenografts resulted in long-term, tumor-free survivors. In addition, GO selectively inhibited leukemia colony formation by marrow cells from a significant proportion of AML patients [31,32].

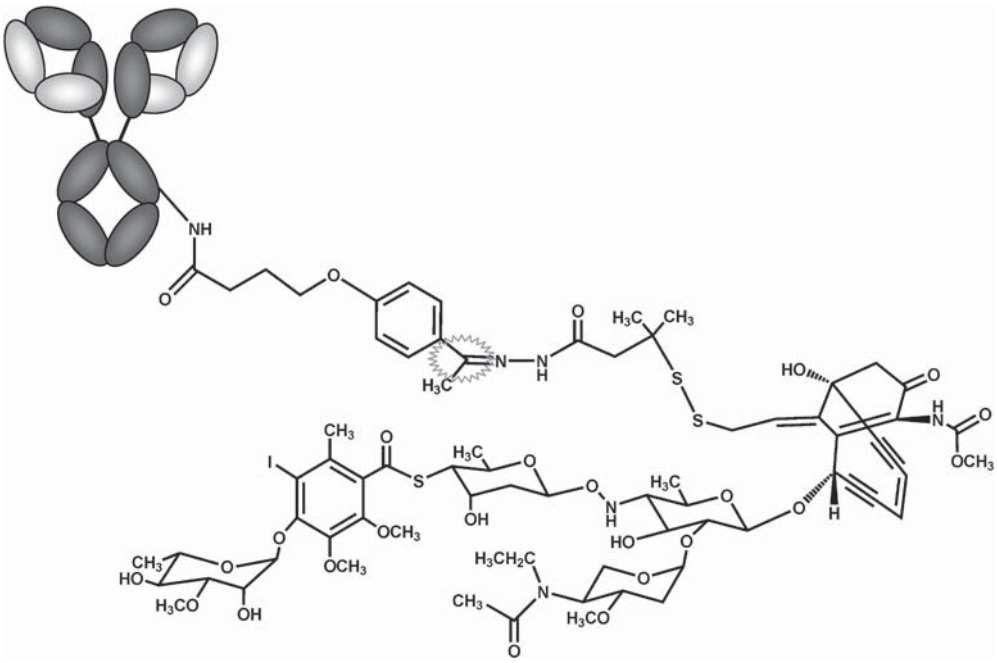

Fig. 7.1 The chemical structure of gemtuzumab ozogamicin GO. The designed hybrid conjugate contains both a lysine attachment to the antibody through amide formation, and a hydrazone linkage (indicated by the gray star-shaped motif). Hydrazones release the drug under acidic conditions within the lysosomes of target cells. The drug loading is 2–3 mol mol^{-1}.

7.3.1 The IgG4 Moiety

The human IgG4 component of GO is not cytotoxic *in vitro*, nor does it induce complement-mediated (CDC) or antibody-dependent cellular cytotoxicity (ADCC; See Chapter 8, Vol I, FC engineering). Therefore, the antibody moiety is utilized primarily as vehicle to transport the cytotoxic drug to CD33-positive leukemia cells.

7.3.2 Calicheamicin

The calicheamicins are highly potent anti-tumor antibiotics of the enediyne family. Originally isolated from a broth extract of the soil microorganism *Micromonospora echinospora* ssp. *calichensis*, the calicheamicins were detected in a screen for potent DNA-damaging agents [33]. The parent of this family of xenobiotics, chalicheamicin γI^1, has been shown to bind to double-stranded DNA in a sequence-specific manner [33–36]. Upon binding, it cleaves the sugar backbone of the DNA, even at sub-picomolar concentrations. The major DNA contact surface of the antibiotic is an aryltetrasaccharide moiety that interacts with the minor groove of the DNA duplex (Fig. 7.2) [34,37–39]. The enediyne portion of the antibiotic appears to contribute to the drug–DNA interaction by increasing binding

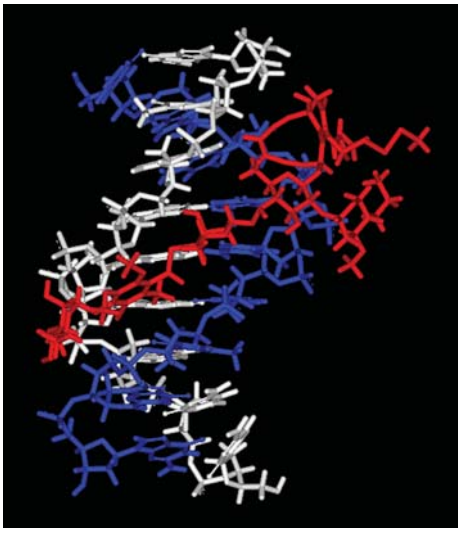

Fig. 7.2 Calicheamicin binding to the minor groove of DNA. Solution structure of the calicheamicin $\gamma 1^I$-DNA hairpin duplex complex containing a central (T-C-C-T)·(A-G-G-A) segment based on a combined analysis of NMR and molecular dynamics calculations. The DNA backbone and base pairs are shown in white and blue. Calicheamicin is shown in red [92,93]. [RSCB Protein Data Bank Accession Number 2PIK].

energy and widening the spectrum of target sequences [40]. One of the preferred target sequences is the tetranucleotide TCCT [36]; others include TCTC and TTTT [34,41,42]. Structural and chemical analyses have shown that the carbohydrate tail of chalicheamycin is orientated towards the 3′ end of the TCCT surface, and that the iodine of the aryltetrasaccharide plays a critical role in the sequence-selective recognition of DNA [34,41]. In addition, the chalicheamycin oligosaccharide was demonstrated to inhibit transcription factor binding to DNA [43,44]. Due to their unique mode of action and potency, several analogs of the calicheamicins have been tested in preclinical models as potential anti-tumor agents. Since the therapeutic range is limited by the high toxicity, their potency makes them ideal for antibody-targeted chemotherapy. Calicheamicin-conjugates may offer significant advantages over previous antibody-targeted agents, because they are approximately 1000-fold more cytotoxic than other clinically used anti-cancer agents [31], although they may have much lower immunogenicity than for example protein toxins such as ricin or pseudomonas exotoxin A.

7.3.3
The Design of Antibody-Chalicheamicin Conjugates: Humanization and Choice of Linker

Earlier studies with chalicheamicin conjugates of the murine, CD33 antibody P67.6 demonstrated that a site of hydrolytic release, as supplied by a hydrazone, was necessary for potent and selective cytotoxicity in tissue culture, and for a maximum efficacy in xenograft models of AML in athymic mice [45]. A carbohydrate-based conjugate was chosen that was made by oxidizing the naturally occurring carbohydrate residues in murine P67.6 with periodate, and reacting the resultant aldehydes with a hydrazide derivative of calicheamicin, NAc-gamma calicheamicin DMH [46]. In order to minimize the potential for immune responses in human trials, the P67.6 antibody has been humanized by CDR-grafting [31]. However, the periodate oxidation required when preparing a humanized hP67.6 calicheamicin carbohydrate conjugate resulted in a virtually total loss of immunoreactivity. Subsequent experiments indicated that an oxidatively sensitive methionine residue near the CDR that originated with the human sequences might be the reason for this sensitivity to oxidation. This necessitated the design of a new class of conjugates in which the designed hybrid conjugates contain both lysine attachment to the antibody through amide formation, which obviates the need for oxidation of the carbohydrates, and a hydrazone linkage, which allows for the necessary hydrolytic release. The most optimal conjugates were made with unsubstituted acetophenones, and the 4-(4-acetylphenoxy)butanoic acid) linker, which appeared to afford the most favorable balance between hydrolytic stability in physiological buffers (pH 7.4) and efficient drug release at the pH of lysosomes (~4). The final product, gemtuzumab ozogamicin, consists of a 1:1 mixture of unconjugated hP67.6 and hP67.6 conjugated to four to six moles of NAc-gamma calicheamicin, thus providing an average drug loading ratio of two to three drug molecules to one antibody molecule [47].

7.4 Mechanisms of Action

After antibody binding, CD33-antibody complexes are rapidly internalized and translocated into lysosomes [12,48,49]. In the acidic environment of the lysosome, the acid-hydrolyzable linker is efficiently cleaved [31], and the chalicheamicin moiety is released and subsequently reduced to a highly reactive 1,4-dehydrobenzene-diradical species, through the action of glutathione [36,50]. This diradical species binds within the minor groove of DNA and abstracts hydrogen atoms from the deoxyribose backbone. The resulting radicals scavenge oxygen and initiate a sequence of events that lead to site-specific single- and double-stranded DNA scission [12,51,52]. The mitochondrial pathway of apoptosis appears to be predominantly utilized for GO-induced cell death. GO treatment of primary AML samples and AML cell lines causes the loss of mitochondrial membrane potential and activation of caspase 3, while overexpression of Bcl-2 or Bcl-X_L inhibits GO-induced cytotoxicity [53,54]. *In-vitro* exposure to GO also causes G_2/M cell cycle arrest in susceptible myeloid leukemia cell lines, with activation of cyclin B1 and phosphorylation of checkpoint kinases 1 and 2 (Chk1; Chk2) [53,55]. In summary, calicheamicin-induced DNA damage stimulates a strong DNA damage response in the cell [50,56,57], resulting in permanent growth arrest, cell death, or temporary arrest followed by DNA repair. Thus, multiple pathways appear to interact during GO-induced DNA damage, resulting in either cytotoxicity or cell survival [12].

7.5 Potential Mechanisms of Resistance

Several potential mechanisms of resistance have been postulated from clinical and *in-vitro* observations. Different members of the ATP-binding cassette (ABC) superfamily of transporter proteins such as Pgp (MDR1; ABCB1) and multidrug resistance protein 1 (MRP1; ABCC1) have been demonstrated to mediate *in-vitro* resistance to GO [12,55,58–60], whereas the ABC transporter breast cancer resistance protein (BCRP; ABCG2), which is variably expressed in AML, does not confer resistance to GO [12,61,62]. In clinical phase II trials, Pgp expression on AML blasts correlated with treatment failure [9,58]. Although multidrug resistance inhibitors, such as cyclosporine A (CsA) and PK11195 have been shown to increase *in-vitro* GO sensitivity in AML cells [54], incorporating the Pgp inhibitor CsA in GO-containing regimes as induction, salvage or post-remission therapy in AML did not appear to increase rates of response and survival [63,64].

Alternative resistance mechanisms include reduced GO-binding capacity to leukemic blasts [65], altered pharmacokinetics, high levels of tumor load in the peripheral blood [65,66], overexpression of anti-apoptotic proteins, such as bcl-2 or bcl-X_L [54], or resting state of cell cycle [67,68]. Therefore, it is postulated that combinations of GO with myeloid cytokines that stimulate AML blast

proliferation or agents that down-modulate anti-apoptotic proteins (e.g., Bcl-2 antisense) might enhance drug sensitivity and clinical efficacy [12,69–71].

7.6 Clinical Trials: The Data of GO

CD 33 has a wide expression on myeloid cells [72], and thus may serve as a target in many patients with AML. In contrast, hematopoietic stem cells are CD 33-negative. The group of Scheinberg [13] used lintuzumab (HuM195), a humanized (CDR-grafted) antibody, to treat various AML forms and achieved limited success. Lintuzumab could be added safely to intensive induction chemotherapy, and has also been applied as an immunoconjugate with alpha-emitting bismuth [73]. However, the first – and until now only FDA-approved targeted therapy for CD 33-positive leukemic cells is GO (Mylotarg). Following numerous unsuccessful attempts to develop immunoconjugates with mitoxantrone, American Cynamide/Wyeth succeeded in coupling the carbohydrate toxin calicheamicine to CD33. Maytansinoids such as calicheamicine have been evaluated since the 1970s for their cancer therapy properties, but due to the narrow therapeutic range [74] they did not find any role in cancer treatment. Thus, the possibility of delivering calicheamicine directly to the myeloid blast by coupling to a CD33 antibody proved to be an exciting event.

GO – which originally was named CMA-676 – had first shown efficacy in xenotransplanted nude mice [31]. In a preliminary phase I study, 40 patients with relapsed or refractory CD33-positive AML were treated with GO [32], and a promising high rate of clinical response was observed. At the highest dose level of $9\,mg\,m^{-2}$, long-term neutropenia was observed. Whilst GO was well tolerated, a syndrome of fever and chills after infusion was usually observed, though it is believed that this limited toxicity might have been due to rapid internalization and degradation of the chemical linker being confined to the lysosome.

In phase II evaluations, following a first dose of $9\,mg\,m^{-2}$, a second 2-h infusion was given 14 days later after appropriate premedication which included antihistamines [9]. A total of 142 patients with AML in first relapse and a median age of 61 years was treated in this international study, and 30% achieved remission. Due to a favorable toxicity profile, 27 patients went on to hematopoietic stem cell transplantation. Not surprisingly, GO led to grade IV myelosuppression (neutropenia and thrombocytopenia), though 28% of patients developed grade III and IV infectious complications. Approximately 20% of these patients showed liver abnormalities that might have contributed to death in two cases. Specificity of the drug was documented by minimal mucositis and complete lack of alopecia (Fig. 7.3). No antibody responses to the immunoconjugate were found. From a practical viewpoint, this therapy could in part even be given in an outpatient setting.

These data have recently been updated by Larson and co-workers for 277 patients participating in phase III trials on both sides of the Atlantic [75]. These clinical

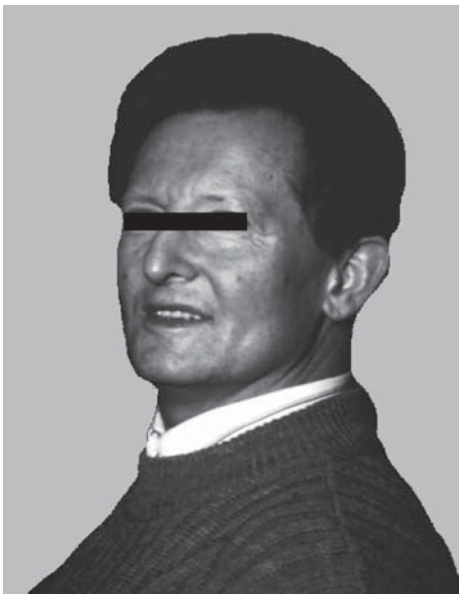

Fig. 7.3 A patient after gemtuzumab ozogamicin (GO) treatment. The specificity of GO is documented by a complete lack of alopecia. This patient received GO infusions three times for relapsed acute myeloid leukemia. No hair loss inevitable to conventional chemotherapy was seen, the patient achieved complete remission, and was latter successfully transplanted with peripheral blood stem cells from his sister.

studies clearly showed that, if remissions were achieved, they were rather short-lived and were of approximately 12 months' duration. Notably, similar clinical responses were obtained in younger and older individuals. In 2000, GO was approved by the FDA as the third antibody-based drug for cancer therapy, and the first immunoconjugate ever to be used in this manner.

The Italian GIMEMA group assessed GO in elderly patients who in general poorly tolerate the toxicity associated with chemotherapy [76]. These frail patients, who had untreated AML and were aged between 61 and 89 years, were to receive two courses of GO as induction therapy, followed by two additional infusions if complete remission (CR) was achieved. For CD33-positive patients aged less than 75 years, a median survival of 11.4 months was quite remarkable, but those patients aged over 75 years or who were CD33-negative had a dismal outcome. Whilst almost no mucositis was observed, the problems associated with neutropenia were severe. In addition, hepatotoxicity was seen. These findings confirmed the observations made by Larson and co-workers [77] in relapsed elderly patients who had little mucositis, cardiotoxicity, cerebellar toxicity or alopecia. However, when administered to elderly patients with newly diagnosed AML or myelodysplasia, GO did not seem superior to standard idarubicin combined with cytosine-arabinoside [78].

Recently, several studies conducted in the US and in Europe also documented activity of GO in children [79–81]. Among children with relapsed or refractory AML, GO treatment led to only limited toxicity of organs other than bone marrow, but significant hepatotoxicity occurred. Subsequently, among 13 patients reported by Arceci et al. who proceeded to stem cell transplantation after GO therapy, 40% developed hepatic veno-occlusive disease/sinusoidal obstruction syndrome (VOD/SOS) [79].

Not surprisingly, when trying to integrate an immunoconjugate into chemotherapy, both the scheduling and dose were difficult to determine. In monotherapy, lower doses of GO such as 6 mg m^{-2} had shown activity. Moreover, colleagues from the MD Anderson Cancer Center combined GO at 4.5 mg m^{-2} with fludarabine, cytarabine, and CsA and showed feasibility [82]. Currently, several multicenter trials are under way to identify the correct chemotherapy partner and schedule for GO in AML therapy (for reviews, see [68,83,84]).

Clearly, GO alone will not achieve longlasting remissions in the majority of patients, and therefore allogeneic bone marrow transplantation is a logical choice for consolidation therapy. Many patients have received such treatment in early pivotal studies [9,75], with the median overall survival time exceeding 18.3 months, compared to 16.5 months for autologous stem cell transplantation, 12.2 months for chemotherapy, and 11.2 months for supportive care. As CD33-positive cells are found in the sinusoids of the liver [85] and VOD/SOS is seen in patients receiving GO outside the transplant setting [86], liver toxicity was of particular concern. Wadleigh and co-workers from the Dana Faber Cancer Institute, when retrospectively comparing patients with and without GO prior to human stem cell transplantation, found VOD rates of 64% and 8%, respectively [87]. In particular, a short interval between GO treatment and stem cell transplantation appeared to be a major risk factor. Versluys et al. [88] reported on a group of children who received prophylactic defibrotide (40 mg m^{-2} per day) which may have helped to prevent graft-versus-host disease (GvHD), an experience which we have shared in adults at our center. Taking this into consideration, it appears justified to conduct additional studies to include GO into conditioning regimens such as that proposed by Bornhäuser and colleagues [89], with split doses of GO administered 21 and 14 days before allogeneic transplantation. In fact, defibrotide treatment may have positive effects even on GO-induced VOD seen outside the transplant situation [90].

7.7 Summary and Conclusions

Gemtuzumab ozogamicin was the first immunoconjugate to be approved for cancer therapy. The carbohydrate toxin calicheamicin is efficiently delivered by specific monoclonal antibodies to CD33-positive leukemic cells and, after internalization, deliberates from the acid-sensitive linker in the lysosome. Clinical studies in patients with AML have demonstrated remarkable single-agent efficacy

for GO, with few adverse side effects that were mainly caused by hepatic toxicity and the inevitable targeting of normal CD33-positive hematopoiesis. Due to the overall favorable side-effect profile, GO may be particularly helpful in elderly AML patients, or may be administered in conjunction with bone marrow transplantation. In the latter situation, however, it is vital that the occurrence of VOD/SOS is avoided.

To date, the clinical experiences with GO have stimulated the development of similarly designed immunoconjugates. For example, an additional calicheamicin immunoconjugate targeting CD22 has demonstrated potent and selective inhibition of CD22-positive B-cell lymphoma cell lines both *in vitro* and in xenografted tumor models [91]. This immunoconjugate is currently undergoing clinical trials for the treatment of lymphoma patients. Nonetheless, it remains to be seen how many immunoconjugates against other internalizing antigens can be successfully developed, and what the ultimate role of these therapeutics will be in the clinic.

References

1 Wu, A.M., Senter, P.D. (2005) Arming antibodies: prospects and challenges for immunoconjugates. Nat Biotechnol 23: 1137–1146.

2 Carter, P.J. (2006) Potent antibody therapeutics by design. Nat Rev Immunol 6: 343–357.

3 Glennie, M.J., van de Winkel, J.G. (2003) Renaissance of cancer therapeutic antibodies. Drug Discov Today 8: 503–510.

4 Rondot, S., Koch, J., Breitling, F., Dubel, S. (2001) A helper phage to improve single-chain antibody presentation in phage display. Nat Biotechnol 19: 75–78.

5 Lonberg, N. (2005) Human antibodies from transgenic animals. Nat Biotechnol 23: 1117–1125.

6 Hanes, J., Schaffitzel, C., Knappik, A., Pluckthun, A. (2000) Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display. Nat Biotechnol 18: 1287–1292.

7 Nimmerjahn, F., Ravetch, J.V. (2005) Divergent immunoglobulin g subclass activity through selective Fc receptor binding. Science 310: 1510–1512.

8 Weng, W.K., Levy, R. (2003) Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. J Clin Oncol 21: 3940–3947.

9 Sievers, E.L., Larson, R.A., Stadtmauer, E.A., et al. (2001) Efficacy and safety of gemtuzumab ozogamicin in patients with CD33-positive acute myeloid leukemia in first relapse. J Clin Oncol 19: 3244–3254.

10 Waldmann, T.A. (2003) Immunotherapy: past, present and future. Nat Med 9: 269–277.

11 Jilani, I., Estey, E., Huh, Y., et al. (2002) Differences in CD33 intensity between various myeloid neoplasms. Am J Clin Pathol 118: 560–566.

12 Linenberger, M.L. (2005) CD33-directed therapy with gemtuzumab ozogamicin in acute myeloid leukemia: progress in understanding cytotoxicity and potential mechanisms of drug resistance. Leukemia 19: 176–182.

13 Feldman, E.J., Brandwein, J., Stone, R., et al. (2005) Phase III randomized multicenter study of a humanized anti-CD33 monoclonal antibody, lintuzumab, in combination with chemotherapy, versus chemotherapy alone in patients with refractory or first-relapsed acute myeloid leukemia. J Clin Oncol 23: 4110–4116.

14 Caron, P.C., Dumont, L., Scheinberg, D.A. (1998) Supersaturating infusional humanized anti-CD33 monoclonal antibody HuM195 in myelogenous leukemia. Clin Cancer Res 4: 1421–1428.

15 Crocker, P.R. (2002) Siglecs: sialic-acid-binding immunoglobulin-like lectins in cell-cell interactions and signalling. Curr Opin Struct Biol 12: 609–615.

16 Freeman, S.D., Kelm, S., Barber, E.K., Crocker, P.R. (1995) Characterization of CD33 as a new member of the sialoadhesin family of cellular interaction molecules. Blood 85: 2005–2012.

17 Griffin, J.D., Linch, D., Sabbath, K., Larcom, P., Schlossman, S.F. (1984) A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leuk Res 8: 521–534.

18 Peiper, S.C., Ashmun, R.A., Look, A.T. (1988) Molecular cloning, expression, and chromosomal localization of a human gene encoding the CD33 myeloid differentiation antigen. Blood 72: 314–321.

19 Simmons, D., Seed, B. (1988) Isolation of a cDNA encoding CD33, a differentiation antigen of myeloid progenitor cells. J Immunol 141: 2797–2800.

20 Andrews, R.G., Singer, J.W., Bernstein, I.D. (1989) Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scatter properties. J Exp Med 169: 1721–1731.

21 Brashem-Stein, C., Flowers, D.A., Smith, F.O., Staats, S.J., Andrews, R.G., Bernstein, I.D. (1993) Ontogeny of hematopoietic stem cell development: reciprocal expression of CD33 and a novel molecule by maturing myeloid and erythroid progenitors. Blood 82: 792–799.

22 Andrews, R.G., Takahashi, M., Segal, G.M., Powell, J.S., Bernstein, I.D., Singer, J.W. (1986) The L4F3 antigen is expressed by unipotent and multipotent colony-forming cells but not by their precursors. Blood 68: 1030–1035.

23 Andrews, R.G., Torok-Storb, B., Bernstein, I.D. (1983) Myeloid-associated differentiation antigens on stem cells and their progeny identified by monoclonal antibodies. Blood 62: 124–132.

24 Gao, Z., McAlister, V.C., Williams, G.M. (2001) Repopulation of liver endothelium by bone-marrow-derived cells. Lancet 357: 932–933.

25 Tchilian, E.Z., Beverley, P.C., Young, B.D., Watt, S.M. (1994) Molecular cloning of two isoforms of the murine homolog of the myeloid CD33 antigen. Blood 83: 3188–3198.

26 Robertson, M.J., Soiffer, R.J., Freedman, A.S., et al. (1992) Human bone marrow depleted of CD33-positive cells mediates delayed but durable reconstitution of hematopoiesis: clinical trial of MY9 monoclonal antibody-purged autografts for the treatment of acute myeloid leukemia. Blood 79: 2229–2236.

27 Paul, S.P., Taylor, L.S., Stansbury, E.K., McVicar, D.W. (2000) Myeloid specific human CD33 is an inhibitory receptor with differential ITIM function in recruiting the phosphatases SHP-1 and SHP-2. Blood 96: 483–490.

28 Taylor, V.C., Buckley, C.D., Douglas, M., Cody, A.J., Simmons, D.L., Freeman, S.D. (1999) The myeloid-specific sialic acid-binding receptor, CD33, associates with the protein-tyrosine phosphatases, SHP-1 and SHP-2. J Biol Chem 274: 11505–11512.

29 Ulyanova, T., Blasioli, J., Woodford-Thomas, T.A., Thomas, M.L. (1999) The sialoadhesin CD33 is a myeloid-specific inhibitory receptor. Eur J Immunol 29: 3440–3449.

30 Walter, R.B., Raden, B.W., Kamikura, D.M., Cooper, J.A., Bernstein, I.D. (2005) Influence of CD33 expression levels and ITIM-dependent internalization on gemtuzumab ozogamicin-induced cytotoxicity. Blood 105: 1295–1302.

31 Hamann, P.R., Hinman, L.M., Hollander, I., et al. (2002) Gemtuzumab ozogamicin, a potent and selective anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Bioconj Chem 13: 47–58.

32 Sievers, E.L., Appelbaum, F.R., Spielberger, R.T., et al. (1999) Selective ablation of acute myeloid leukemia using antibody-targeted chemotherapy: a phase I study of an anti-CD33 calicheamicin immunoconjugate. Blood 93: 3678–3684.

33 Lee, M.D., Dunne, T.S., Siegel, M.M., Chang, C.C., Morton, G.O., Borders, D.B. (1987) Calichemicins, a novel family of antitumor antibiotics. 1. Chemistry and partial structure of calichemicin. gamma.1I. J Am Chem Soc 109: 3464–3466.

34 Nicolaou, K.C., Tsay, S.C., Suzuki, T., Joyce, G.F. (1992) DNA-carbohydrate interactions. Specific binding of the calicheamicin.gamma.1I oligosaccharide with duplex DNA. J Am Chem Soc 114: 7555–7557.

35 Zein, N., Poncin, M., Nilakantan, R., Ellestad, G.A. (1989) Calicheamicin gamma 1I and DNA: molecular recognition process responsible for site-specificity. Science 244: 697–699.

36 Zein, N., Sinha, A.M., McGahren, W.J., Ellestad, G.A. (1988) Calicheamicin gamma 1I: an antitumor antibiotic that cleaves double-stranded DNA site specifically. Science 240: 1198–1201.

37 Aiyar, J., Danishefsky, S.J., Crothers, D.M. (1992) Interaction of the aryl tetrasaccharide domain of calicheamicin. gamma.1I with DNA: influence on aglycon and methidiumpropyl-EDTA. cntdot.iron(II)-mediated DNA cleavage. J Am Chem Soc 114: 7552–7554.

38 Walker, S., Gupta, V., Kahne, D., Gange, D. (1994) Analysis of hydroxylamine glycosidic linkages: Structural consequences of the NO bond in calicheamicin. J Am Chem Soc 116: 3197–3206.

39 Walker, S., Valentine, K.G., Kahne, D. (1990) Sugars as DNA binders: a comment on the calicheamicin oligosaccharide. J Am Chem Soc 112: 6428–6429.

40 Uesugi, M., Sugiura, Y. (1993) New insights into sequence recognition process of esperamicin A1 and calicheamicin gamma 1I.: origin of their selectivities and 'induced fit' mechanism. Biochemistry 32: 4622–4627.

41 Gomez-Paloma, L., Smith, J.A., Chazin, W.J., Nicolaou, K.C. (1994) Interaction of calicheamicin with duplex DNA: Role of the oligosaccharide domain and identification of multiple binding modes. J Am Chem Soc 116: 3697–3708.

42 Li, T., Zeng, Z., Estevez, V.A., Baldenius, K.U., Nicolaou, K.C., Joyce, G.F. (1994) Carbohydrate-minor groove interactions in the binding of calicheamicin. gamma.1I to duplex DNA. J Am Chem Soc 116: 3709–3715.

43 Ho, S.N., Boyer, S.H., Schreiber, S.L., Danishefsky, S.J., Crabtree, G.R. (1994) Specific inhibition of formation of transcription complexes by a calicheamicin oligosaccharide: a paradigm for the development of transcriptional antagonists. Proc Natl Acad Sci USA 91: 9203–9207.

44 Liu, C., Smith, B.M., Ajito, K., et al. (1996) Sequence-selective carbohydrate-DNA interaction: dimeric and monomeric forms of the calicheamicin oligosaccharide interfere with transcription factor function. Proc Natl Acad Sci USA 93: 940–944.

45 Hamann, P.R., Hinman, L.M., Beyer, C.F., et al. (2002) An anti-CD33 antibody-calicheamicin conjugate for treatment of acute myeloid leukemia. Choice of linker. Bioconj Chem 13: 40–46.

46 Rodwell, J.D., Alvarez, V.L., Lee, C., et al. (1986) Site-specific covalent modification of monoclonal antibodies: in vitro and in vivo evaluations. Proc Natl Acad Sci USA 83: 2632–2636.

47 Bross, P.F., Beitz, J., Chen, G., et al. (2001) Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. Clin Cancer Res 7: 1490–1496.

48 McGrath, M.S., Rosenblum, M.G., Philips, M.R., Scheinberg, D.A. (2003) Immunotoxin resistance in multidrug resistant cells. Cancer Res 63: 72–79.

49 Press, O.W., Shan, D., Howell-Clark, J., et al. (1996) Comparative metabolism and retention of iodine-125, yttrium-90, and indium-111 radioimmunoconjugates by cancer cells. Cancer Res 56: 2123–2129.

50 Elmroth, K., Nygren, J., Martensson, S., Ismail, I.H., Hammarsten, O. (2003) Cleavage of cellular DNA by calicheamicin gamma1. DNA Repair (Amst.) 2: 363–374.

51 Krishnamurthy, G., Brenowitz, M.D., Ellestad, G.A. (1995) Salt dependence of calicheamicin-DNA site-specific interactions. Biochemistry 34: 1001–1010.

52 Walker, S., Landovitz, R., Ding, W.D., Ellestad, G.A., Kahne, D. (1992) Cleavage behavior of calicheamicin gamma 1 and calicheamicin T. Proc Natl Acad Sci USA 89: 4608–4612.

53 Amico, D., Barbui, A.M., Erba, E., Rambaldi, A., Introna, M., Golay, J. (2003) Differential response of human acute myeloid leukemia cells to gemtuzumab ozogamicin in vitro: role of Chk1 and Chk2 phosphorylation and caspase 3. Blood 101: 4589–4597.

54 Walter, R.B., Raden, B.W., Cronk, M.R., Bernstein, I.D., Appelbaum, F.R., Banker, D.E. (2004) The peripheral benzodiazepine receptor ligand PK11195 overcomes different resistance mechanisms to sensitize AML cells to gemtuzumab ozogamicin. Blood 103: 4276–4284.

55 Naito, K., Takeshita, A., Shigeno, K., et al. (2000) Calicheamicin-conjugated humanized anti-CD33 monoclonal antibody (gemtuzumab zogamicin, CMA-676) shows cytocidal effect on CD33-positive leukemia cell lines, but is inactive on P-glycoprotein-expressing sublines. Leukemia 14: 1436–1443.

56 Martensson, S., Nygren, J., Osheroff, N., Hammarsten, O. (2003) Activation of the DNA-dependent protein kinase by drug-induced and radiation-induced DNA strand breaks. Radiat Res 160: 291–301.

57 Zhao, B., Konno, S., Wu, J.M., Oronsky, A.L. (1990) Modulation of nicotinamide adenine dinucleotide and poly(adenosine diphosphoribose) metabolism by calicheamicin gamma 1 in human HL-60 cells. Cancer Lett 50: 141–147.

58 Linenberger, M.L., Hong, T., Flowers, D., et al. (2001) Multidrug-resistance phenotype and clinical responses to gemtuzumab ozogamicin. Blood 98: 988–994.

59 Matsui, H., Takeshita, A., Naito, K., et al. (2002) Reduced effect of gemtuzumab ozogamicin (CMA-676) on P-glycoprotein and/or CD34-positive leukemia cells and its restoration by multidrug resistance modifiers. Leukemia 16: 813–819.

60 Walter, R.B., Raden, B.W., Hong, T.C., Flowers, D.A., Bernstein, I.D., Linenberger, M.L. (2003) Multidrug resistance protein attenuates gemtuzumab ozogamicin-induced cytotoxicity in acute myeloid leukemia cells. Blood 102: 1466–1473.

61 Abbott, B.L. (2003) ABCG2 (BCRP) expression in normal and malignant hematopoietic cells. Hematol Oncol 21: 115–130.

62 Walter, R.B., Raden, B.W., Thompson, J., et al. (2004) Breast cancer resistance protein (BCRP/ABCG2) does not confer resistance to gemtuzumab ozogamicin and calicheamicin-gamma1 in acute myeloid leukemia cells. Leukemia 18: 1914–1917.

63 Tsimberidou, A., Cortes, J., Thomas, D., et al. (2003) Gemtuzumab ozogamicin, fludarabine, cytarabine and cyclosporine combination regimen in patients with CD33+ primary resistant or relapsed acute myeloid leukemia. Leuk Res 27: 893–897.

64 Tsimberidou, A., Estey, E., Cortes, J., et al. (2003) Gemtuzumab, fludarabine, cytarabine, and cyclosporine in patients with newly diagnosed acute myelogenous leukemia or high-risk myelodysplastic syndromes. Cancer 97: 1481–1487.

65 van Der Velden, V.H., te Marvelde, J.G., Hoogeveen, P.G., et al. (2001) Targeting of the CD33-calicheamicin immunoconjugate Mylotarg (CMA-676) in acute myeloid leukemia: in vivo and in vitro saturation and internalization by leukemic and normal myeloid cells. Blood 97: 3197–3204.

66 van der Velden, V.H., Boeckx, N., Jedema, I., et al. (2004) High CD33-antigen loads in peripheral blood limit the efficacy of gemtuzumab ozogamicin (Mylotarg) treatment in acute myeloid leukemia patients. Leukemia 18: 983–988.

67 Jedema, I., Barge, R.M., van der Velden, V.H., et al. (2004) Internalization and cell cycle-dependent killing of leukemic cells by Gemtuzumab Ozogamicin: rationale for efficacy in CD33-negative malignancies with endocytic capacity. Leukemia 18: 316–325.

68 Tsimberidou, A.M., Giles, F.J., Estey, E., O'Brien, S., Keating, M.J., Kantarjian, H. M. (2006) The role of gemtuzumab

ozogamicin in acute leukaemia therapy. Br J Haematol 132: 398–409.
69 Leone, G., Rutella, S., Voso, M.T., Fianchi, L., Scardocci, A., Pagano, L. (2004) In vivo priming with granulocyte colony-stimulating factor possibly enhances the effect of gemtuzumab-ozogamicin in acute myeloid leukemia: results of a pilot study. Haematologica 89: 634–636.
70 Moore, J., Seiter, K., Kolitz, J., et al. (2006) A phase II study of Bcl-2 antisense (oblimersen sodium) combined with gemtuzumab ozogamicin in older patients with acute myeloid leukemia in first relapse. Leuk Res 30: 777–783.
71 Rutella, S., Bonanno, G., Procoli, A., et al. (2006) Granulocyte colony-stimulating factor enhances the in vitro cytotoxicity of gemtuzumab ozogamicin against acute myeloid leukemia cell lines and primary blast cells. Exp Hematol 34: 54–65.
72 Repp, R., Schaekel, U., Helm, G., et al. (2003) Immunophenotyping is an independent factor for risk stratification in AML. Cytometry B Clin Cytom 53: 11–19.
73 Jurcic, J.G., Larson, S.M., Sgouros, G., et al. (2002) Targeted alpha particle immunotherapy for myeloid leukemia. Blood 100: 1233–1239.
74 Chabner, B.A., Levine, A.S., Johnson, B.L., Young, R.C. (1978) Initial clinical trials of maytansine, an antitumor plant alkaloid. Cancer Treat Rep 62: 429–433.
75 Larson, R.A., Sievers, E.L., Stadtmauer, E.A., et al. (2005) Final report of the efficacy and safety of gemtuzumab ozogamicin (Mylotarg) in patients with CD33-positive acute myeloid leukemia in first recurrence. Cancer 104: 1442–1452.
76 Amadori, S., Suciu, S., Stasi, R., et al. (2005) Gemtuzumab ozogamicin (Mylotarg) as single-agent treatment for frail patients 61 years of age and older with acute myeloid leukemia: final results of AML-15B, a phase 2 study of the European Organisation for Research and Treatment of Cancer and Gruppo Italiano Malattie Ematologiche dell'Adulto Leukemia Groups. Leukemia 19: 1768–1773.
77 Larson, R.A., Boogaerts, M., Estey, E., et al. (2002) Antibody-targeted chemotherapy of older patients with acute myeloid leukemia in first relapse using Mylotarg (gemtuzumab ozogamicin). Leukemia 16: 1627–1636.
78 Estey, E.H., Thall, P.F., Giles, F.J., et al. (2002) Gemtuzumab ozogamicin with or without interleukin 11 in patients 65 years of age or older with untreated acute myeloid leukemia and high-risk myelodysplastic syndrome: comparison with idarubicin plus continuous-infusion, high-dose cytosine arabinoside. Blood 99: 4343–4349.
79 Arceci, R.J., Sande, J., Lange, B., et al. (2005) Safety and efficacy of gemtuzumab ozogamicin in pediatric patients with advanced CD33+ acute myeloid leukemia. Blood 106: 1183–1188.
80 Brethon, B., Auvrignon, A., Galambrun, C., et al. (2006) Efficacy and tolerability of gemtuzumab ozogamicin (anti-CD33 monoclonal antibody, CMA-676, Mylotarg) in children with relapsed/refractory myeloid leukemia. BMC Cancer 6: 172.
81 Reinhardt, D., Diekamp, S., Fleischhack, G., et al. (2004) Gemtuzumab ozogamicin (Mylotarg) in children with refractory or relapsed acute myeloid leukemia. Onkologie 27: 269–272.
82 Tsimberidou, A.M., Estey, E., Cortes, J.E., et al. (2003) Mylotarg, fludarabine, cytarabine (ara-C), and cyclosporine (MFAC) regimen as post-remission therapy in acute myelogenous leukemia. Cancer Chemother Pharmacol 52: 449–452.
83 Burnett, A.K., Mohite, U. (2006) Treatment of older patients with acute myeloid leukemia – new agents. Semin Hematol 43: 96–106.
84 Fenton, C., Perry, C.M. (2005) Gemtuzumab ozogamicin: a review of its use in acute myeloid leukaemia. Drugs 65: 2405–2427.
85 Rajvanshi, P., Shulman, H.M., Sievers, E.L., McDonald, G.B. (2002) Hepatic sinusoidal obstruction after gemtuzumab ozogamicin (Mylotarg) therapy. Blood 99: 2310–2314.
86 Giles, F.J., Kantarjian, H.M., Kornblau, S.M., et al. (2001) Mylotarg (gemtuzumab

ozogamicin) therapy is associated with hepatic venoocclusive disease in patients who have not
received stem cell transplantation. Cancer 92: 406–413.
87 Wadleigh, M., Richardson, P.G., Zahrieh, D., et al. (2003) Prior gemtuzumab ozogamicin exposure significantly increases the risk of veno-occlusive disease in patients who undergo myeloablative allogeneic stem cell transplantation. Blood 102: 1578–1582.
88 Versluys, B., Bhattacharaya, R., Steward, C., Cornish, J., Oakhill, A., Goulden, N. (2004) Prophylaxis with defibrotide prevents veno-occlusive disease in stem cell transplantation after gemtuzumab ozogamicin exposure. Blood 103: 1968.
89 Hanel, M., Thiede, C., Helwig, A., et al. (2003) Successful combination of anti-CD33 antibody (gemtuzumab ozogamicin) and minimal conditioning before second allografting in recurrent acute myeloid leukaemia. Br J Haematol 120: 1093–1094.
90 Lannoy, D., Decaudin, B., Grozieux de Laguerenne, A., et al. (2006) Gemtuzumab ozogamicin-induced sinusoidal obstructive syndrome treated with defibrotide: a case report. J Clin Pharmacol Ther 31: 389–392.
91 Di Joseph, J.F., Armellino, D.C., Boghaert, E.R., et al. (2004) Antibody-targeted chemotherapy with CMC-544: a CD22-targeted immunoconjugate of calicheamicin for the treatment of B-lymphoid malignancies. Blood 103: 1807–1814.
92 Ikemoto, N., Kumar, R.A., Ling, T.T., Ellestad, G.A., Danishefsky, S.J., Patel, D. J. (1995) Calicheamicin-DNA complexes: warhead alignment and saccharide recognition of the minor groove. Proc Natl Acad Sci USA 92: 10506–10510.
93 Kumar, R.A., Ikemoto, N., Patel, D.J. (1997) Solution structure of the calicheamicin gamma 1I-DNA complex. J Mol Biol 265: 187–201.

8
Infliximab (Remicade)

Maria Wiekowski and Christian Antoni

8.1
Antibody Characteristics

Infliximab was developed as a potential therapeutic agent for various inflammatory chronic diseases that are believed to be driven by the proinflammatory cytokine tumor necrosis factor alpha (TNF-α). Infliximab (cA2) is a human–murine chimeric monoclonal antibody that potently binds and neutralizes the soluble TNF-α homotrimer and its membrane-bound precursor. It does not neutralize TNF-β (lymphotoxin α), a related cytokine that utilizes the same receptors as TNF-α.

Infliximab was genetically engineered by fusing the variable region of the murine antibody A2 that binds with high selectivity and specificity human TNF-α to the constant region of human IgG1 κ immunoglobulin using recombinant DNA technology. It had been expected that the chimeric antibody had a better immunogenic and pharmacokinetic profile than the murine antibody [1].

The chimeric product cA2 binds soluble TNF-α in its monomeric [2] and trimeric form with an affinity of K_d 100 pM for the latter [3]. The binding of TNF monomers might slow down or even prevent the formation of bioactive trimeric TNF. The binding affinity for the membrane-bound form is about twofold higher (K_d 46 pM), as determined from a cell line expressing membrane-bound recombinant human TNF-α [3]. Comparison of binding affinities of cA2 FAB fragments demonstrated 50-fold higher binding affinity of the dimeric $F(ab')_2$ fragment over the monomeric FAB fragment, indicating bivalent interaction with the ligand [3]. The stability of the infliximab–TNF-α complex is further demonstrated by its slow dissociation rate – in fact, in an *in-vitro* assay no dissociation was observed within 4 h from soluble or within 2 h from membrane-bound TNF-α [2].

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8.2 Preclinical Characterization

In vitro, cA2 inhibited TNF-induced mitogenesis and interleukin (IL)-6 secretion by human fibroblasts, and blocked expression of adhesion molecules on endothelial cells [4].

The *in-vivo* activity of infliximab was demonstrated in a transgenic mouse that overexpresses human TNF-α and dies prematurely of wasting. In this model, twice-weekly injections of infliximab ($2\,mg\,kg^{-1}$) allowed 93% of the animals to survive by reversing the TNF-induced lethal wasting syndrome [4].

The effect of infliximab on apoptosis was also tested in an animal model. Severely compromised immunodeficient (SCID) mice, which lack T and B lymphocytes as well as NK cells, were reconstituted with a human monocytic cell line (THP-1) or a human T-cell line (Jurkat). Following intraperitoneal injection of infliximab, apoptotic cell death was shown to occur within 1 h of injection [3,5]. Similarly, the induction of apoptosis has been reported for synovial macrophages from rheumatoid patients treated with infliximab [6]. Currently it is unknown which pathway of apoptosis infliximab engages.

8.3 Pharmacokinetics

Infliximab is administered to patients by intravenous (IV) infusion as an induction therapy (infusions at Weeks 0, 2, and 6), and then every 8 weeks thereafter. The pharmacokinetics of infliximab were determined in clinical trials for various indications; those from a phase II clinical trial in psoriasis are shown graphically in Fig. 8.1 [7]. The highest serum levels of infliximab were observed in patients

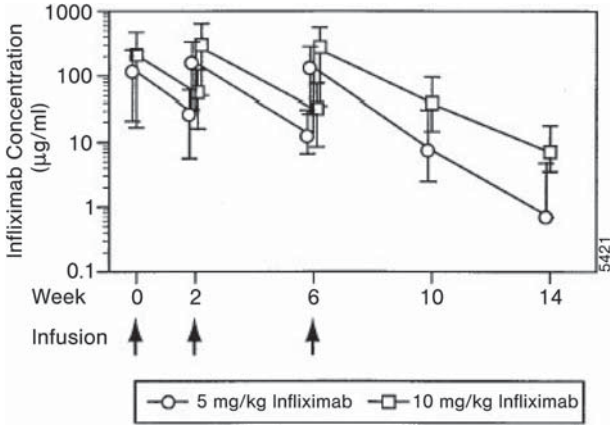

Fig. 8.1 Infliximab pharmacokinetics in a phase II clinical trial of psoriasis.

at Week 2, immediately after the second dose, with median concentrations of 158.14 and 298.89 $mg\,mL^{-1}$ for the 5 and 10 $mg\,kg^{-1}$ dose groups, respectively. The serum infliximab concentrations were directly proportional to the administered dose. The lowest serum concentrations (0.67 $mg\,mL^{-1}$) were observed at Week 14 for the 5 $mg\,kg^{-1}$ group (range from <0.1 to 6.09 $mg\,mL^{-1}$), while the mean serum level for the 10 $mg\,kg^{-1}$ group was 7.11 $mg\,mL^{-1}$ at Week 14.

Infliximab is distributed primarily in the intravascular space; the median elimination half-life of infliximab was 7.62 days (interquartile range 6.62 to 10.15 days) for the 5 $mg\,kg^{-1}$ dose group and 9.97 (interquartile range 6.17 to 10.14 days) for the 10 $mg\,kg^{-1}$ dose group [7]. In patients with Crohn's disease, infliximab was found to have a median half-life of ~9.5 days [8].

The relationship between serum infliximab concentrations and clinical improvement was monitored in the ATTRACT trial for rheumatoid arthritis. Doses of 3 or 10 $mg\,kg^{-1}$ infliximab resulted in maximal serum concentrations that were directly proportional to the intravenous dose, with serum concentrations of 68.8 and 219.1 $\mu g\,mL^{-1}$ identified at 1 h post infusion [9]. The trough concentrations were also found to depend on the amount and frequency of dosing; in the ATTRACT trial, median trough levels were comparable in patients receiving 10 $mg\,kg^{-1}$ every 8 weeks and those receiving 3 $mg\,kg^{-1}$ every 4 weeks [9].

When the trough serum levels of infliximab at Week 54 were correlated with clinical response it became apparent that a higher ACR-N response was significantly associated with a higher trough serum concentration of infliximab [9]. In general, in rheumatoid arthritis (RA), a trough concentration of >1.0 $\mu g\,mL^{-1}$ is associated with a good therapeutic response [9], and the clinical response declined rapidly when serum infliximab levels fell below this threshold [10].

8.4 Clinical Response

8.4.1 Therapeutic Indications

In Europe, infliximab is currently (2006) approved for the treatment of six chronic inflammatory conditions:

- For the reduction of signs and symptoms, as well as improvement in physical function, in patients with RA.
- Severe and active Crohn's disease in patients who do not respond to corticosteroid and/or immunosuppressant therapy, or those with fistulating, active Crohn's disease.
- In ulcerative colitis, in patients who do not respond to conventional therapy.
- In patients with ankylosing spondylitis who have severe axial symptoms, elevated serological markers of

inflammatory activity, and who have responded inadequately to conventional therapy.
- In patients with active and progressive psoriatic arthritis, alone or in combination with methotrexate (MTX); in patients who have responded inadequately to disease-modifying anti-rheumatic drugs.
- In patients with moderate to severe plaque psoriasis who are intolerant of other systemic therapies.

8.4.1.1 Crohn's Disease

Crohn's disease is a chronic inflammatory disorder of the gastrointestinal (GI) tract. Originally, the condition was treated with corticosteroids, until infliximab as a TNF-α blocker presented a significant advancement in the treatment of this disease.

In the ACCENT I trial, Crohn's disease patients experienced a clinical response following one infusion of infliximab, and were then randomized at Week 2 to receive either placebo or infliximab at Weeks 2 and 6, and at every 8 weeks thereafter. Evaluation at Week 30 demonstrated that 39% and 45% of patients achieved remission after infliximab doses of 5 and 10 mg kg^{-1}, respectively, while only 21% of those in the placebo group were in remission. Evaluation at Week 54 confirmed the sustained efficacy of infliximab. In addition, the time to loss of response was significantly longer among infliximab-treated patients than in the placebo group. In addition, the number of patients receiving infliximab who had been able to discontinue corticosteroid treatment at Week 54 was threefold higher than in the placebo group [11]. The benefit of disease control through infliximab therapy was associated with reduced hospitalizations and surgeries, increased employment, and a normalized quality of life [12].

The efficacy of infliximab in inducing response and remission in Crohn's disease triggered the question of whether the disease would be better controlled by continuous infliximab exposure (maintenance treatment; infusions every 8 weeks) or by treatment only upon relapse of the disease (episodic treatment). Consequently, a clinical study showed that maintenance treatment resulted in better clinical responses, higher rates of mucosal healing and lower hospitalization rates, and also fewer incidences of development of antibodies to infliximab [13].

An important advancement in the management of Crohn's disease was achieved with a clinical study that compared the efficacy of the widely used immunosuppressors azathioprine (AZA) or 6-mercaptorpurine (6-MP) to the efficacy of the immunosuppressors in combination with infliximab. In steroid-dependent Crohn's patients, almost twice as many receiving the combination therapy were in clinical remission without steroids at Weeks 12, 24, and 54 as compared to the AZA/6-MP monotherapy group [14].

In addition, infliximab has been shown to be effective in inducing and maintaining complete and durable closures of draining fistulas (ACCENT II) [15] which occur in 17 to 43% of patients with Crohn's disease. This effect also

resulted in reduced hospitalizations and surgeries when compared with placebo [16].

Thus, while infliximab offered a new and highly efficacious treatment entity for Crohn's disease, there was concern originally that such treatment might result in a higher incidence of intestinal strictures or obstructions. In order to address these and other safety concerns, registries were implemented that followed Crohn's disease patients receiving infliximab or other therapies besides TNF-α blockers for several years.

Indeed, the analysis of data obtained from the observational TREAT (the Crohn's Therapy, Resource, Evaluation and Assessment Tool) registry and also ACCENT I, have indicated that symptoms such as intestinal strictures are associated with other factors of Crohn's disease, including disease duration, ileal disease, and new corticosteroid use [17].

8.4.1.2 **Rheumatoid Arthritis**

The effect of repeated doses of infliximab in rheumatoid arthritis was first assessed in a double-blind, placebo-controlled study conducted during the late 1990s and involving 101 patients with active RA, despite treatment with low doses of MTX. Infliximab was administered in the described induction scheme (infusions at Weeks 0, 2, 6 followed by 8-week infusions) up to Week 14 at doses of 1, 3, and 10 $mg\,kg^{-1}$ body weight, with or without combined MTX treatment. The clinical response was evaluated at Week 26. Independent of concomitant treatment with MTX, 60% of patients receiving either 3 or 10 $mg\,kg^{-1}$ responded to the treatment with a median duration of over 18 weeks, while the lowest dose of 1 $mg\,kg^{-1}$ induced a response only when given in combination with MTX.

Pharmocokinetic analysis of antibody concentrations in serum showed that the response to infliximab treatment correlated with sustained antibody levels in the circulation [18]. The rapid induction of response to infliximab in combination with MTX compared to MTX alone was demonstrated in a phase III trial with 428 patients who had active RA despite MTX treatment. A response to 3 or 10 $mg\,kg^{-1}$ infliximab treatment was detected as early as 2 weeks after the first infusion. Maintenance treatment with 8-week infusions maintained the response for over 30 weeks [19]. During this trial, two fatal infections of tuberculosis and coccidiomyosis occurred, which then led to an awareness of the risk that patients might encounter when treated with TNF-α blockers (see below). However, the benefits still greatly outweighed the risk for patients, as infliximab treatment not only greatly improved the quality of life but also preserved the structure of the joint. While at Week 54 joint damage had increased in the MTX group (as determined by radiography), no radiographic progression was observed in the infliximab group [20]. Infliximab was found to have a significant benefit when erosions and joint-space narrowing were examined, and when the hands and feet were examined separately [20]. The deceleration of joint damage was even reduced in patients who did not show a clinical response.

The benefit of infliximab treatment was sustained, when treatment was extended to 102 weeks. Patients experienced significant, clinically relevant

improvements in physical function and quality of life, inhibition of progressive joint damage and sustained improvement in the signs and symptoms of RA [21,22]. A subanalysis of the patient cohort in this study highlighted the importance for early intervention to slow the progression of physical damage, as greater joint damage at baseline was associated with poorer physical function at baseline and also less improvement in physical function after treatment [21]. In addition, patients treated with infliximab in combination with MTX showed minimal disease progression on radiologic assessments, and improvements in physical function as measured by the health assessment questionnaire (HAQ) score. In contrast, those patients treated only with MTX experienced worsening of these measures [9].

Interestingly, in some patients, an inhibition of joint destruction was even observed on treatment with infliximab when no improvement of clinical variable was seen (ACR20 nonresponders). These results suggest a significant effect of infliximab on joint damage, implying that TNF-α inhibition in RA has a much higher impact on joint destruction and cartilage degradation than on inflammation [23]. In fact, TNF-α synergizes with the receptor activator of NF-κB ligand (RANKL) in osteoclastogenesis, which results in an increase in the numbers of joint and circulating osteoclast precursors in patients with RA. A similar situation occurs in psoriatic arthritis, which might explain the highly beneficial effect of TNF-α blockade on the bone erosion seen in these patients [24].

The infliximab-induced attenuation of joint destruction suggested that the initiation of infliximab treatment early in the disease might result in a greater benefit to the patient. Thus, in the ASPIRE trial patients were enrolled who had been diagnosed with RA less than 3 years prior to the study. In combination with MTX, infliximab (3 and 6 $mg\,kg^{-1}$ bodyweight) caused greater improvement in signs and symptoms at Week 54 than MTX treatment alone [25]. While high-dose MTX monotherapy showed efficacy in RA, infliximab in combination with MTX proved to be more efficacious, especially in early RA patients with the highest swollen joint counts and an acute-phase response [26]. Indeed, the radiologic progression of structural damage over 54 weeks was significantly attenuated in patients treated with infliximab in combination with MTX. This indicated that intervention with infliximab in RA early in the disease preserves the joint before destruction and the progression of physical disability [27].

While infliximab is recommended in combination with MTX for the treatment of RA, the long-term follow-up of RA patients captured by registries has demonstrated that, in the real-life setting, both monotherapy and combination therapy are used [28].

8.4.1.3 **Ankylosing Spondylitis**

Ankylosing spondylitis (AS) is a chronic, inflammatory rheumatic disease that most frequently involves the sacroiliac joints, enthuses, and spine. A hallmark of this disease is chronic inflammatory back pain. Progressive ankylosis of the spine results in restricted mobility, disability, and a decreased quality of life which is often accompanied by unemployment.

A randomized, placebo-controlled 3-month trial in patients with AS showed that infliximab is superior to placebo in improving disease activity, function, spine mobility, peripheral arthritis, enthesitis, and quality of life [29]. This response was maintained at Weeks 54 and 102 in about 50% of patients receiving infliximab treatment [30,31]. When patients who had received infliximab for 2 years were compared to randomly selected patients on standard therapy that did not include TNF-α blockade, it became apparent that infliximab treatment reduced the number of the inflammatory spinal lesions in AS patients by 50 to 70% [32], and also decelerated radiographic progression [33]. Patients with AS receiving infliximab for 3 years showed a durable clinical response without loss of efficacy [34]. The discontinuation of treatment resulted in relapse of disease in almost all patients (41/42), but retreatment was safe and restored the response [35]. These results were confirmed in the ASSERT trial, where at Week 24 some 61% of AS patients showed an ASAS (Assessment in Ankylosing Spondylitis) 20 response compared to only 19% of placebo-treated patients [36].

8.4.1.4 **Psoriatic Arthritis**

Psoriatic arthritis (PsA) is a chronic and inflammatory arthritis that occurs in association with skin psoriasis. T cells and proinflammatory cytokines have been identified as important components of the pathogenesis of both psoriasis and PsA.

In psoriatic skin lesions and in synovial membranes of patients with PsA, the concentrations of proinflammatory cytokines such as TNF-α or IL-1 are increased [37,38]. Likewise, expression of the nuclear factor κB (NF-κB), which is regulated by proinflammatory cytokines such as TNF-α, is up-regulated in psoriatic skin [39] and synovial membranes of patients with PsA [40], which suggests that, in PsA, TNF-α triggers the inflammation in skin as well as in the synovial membrane.

Thus, it was postulated that anti-TNF-α treatment would show efficacy in PsA, as it does in RA [40]. The success of anti-TNF-α treatment in RA and the scientific rationale of the role of proinflammatory cytokines in PsA led to open and double-blind trials to investigate the efficacy of infliximab treatment in PsA.

In a small study involving six PsA patients, infusions with infliximab at Weeks 0, 2, and 6 resulted in a drastic improvement in the psoriasis area and severity index (PASI) score at Week 10; moreover, arthritis symptoms as well as skin lesions were improved [41].

In an open-label follow-up study, ten PsA patients were treated with infliximab at 0, 2, and 6 weeks and subsequently at 8-week intervals up to Week 54. Infliximab treatment resulted in an improvement of all global and peripheral assessments of arthritis [42]. Furthermore, in patients who achieved an ACR50 response at Week 6 the response was sustained until Week 54, despite lowering the dose or discontinuing infliximab treatment due to remission in some patients. The sizes of the psoriatic plaques were also visibly reduced. Magnetic resonance

imaging of the joints at Weeks 0 and 10 demonstrated a more than 80% mean reduction in inflammation compared to baseline [42].

These results were confirmed in a placebo-controlled, randomized, double-blind study for infliximab (IMPACT) in 102 PsA patients. These patients had failed at least one prior therapy with a disease-modifying anti-rheumatic drug (DMARD). Again, the ACR20 at Week 16 was 65% for infliximab-treated patients, and 10% for the placebo arm. While no placebo-treated patients achieved ACR50 or ACR70, 46% and 29% of infliximab-treated patients achieved ACR50 and ACR70, respectively. Improvements in the arthritis score occurred concomitantly with the improvements in skin lesions, and >68% of infliximab-treated patients achieved at least 75% improvement in the PASI score at Week 16 [43]. Continued therapy with infliximab resulted in a sustained improvement in articular and dermatologic manifestations of PsA through Week 50 [44].

In the IMPACT 2 study, the efficacy of infliximab in combination with MTX was confirmed in 200 PsA patients. Infliximab treatment caused a significant improvement in the ACR 20, 50, and 70 responses over placebo, and concomitant treatment with MTX did not appear to further improve the efficacy. In addition, two distinctive and common clinical manifestations of PsA – dactylitis and enthesopathy, which are not commonly included as outcomes in PsA clinical studies – were present in a substantial proportion of patients at baseline and improved significantly at Weeks 14 and 24 in those receiving infliximab [44].

An assessment of quality of life in these patients demonstrated a significant improvement of the HAQ score as early as Week 2, and this was maintained through Week 24. In addition, an improvement in the physical component summary score and the mental component summary score, as well as the raw score of the quality of life short form-36 (SF-36) assessment, and a greater improvement in both scores, correlated with a greater improvement in the ACR and PASI scores [45].

Analyses of serum samples obtained from PsA patients before and after infliximab treatment indicated that there were no changes in serum levels of TNF, but significant reductions occurred in IL-6, E-selectin, vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP)-2 expression [46]. Infliximab treatment also significantly reduced the expression of angiogenic growth factors in synovial tissue in patients with PsA, in parallel with dramatic clinical skin and joint responses; this suggested that vascular regression is one potential mechanism of infliximab therapy [47].

8.4.1.5 **Psoriasis**

The improvement of skin lesions in PsA patients treated with infliximab triggered clinical studies to test the efficacy of infliximab in psoriasis. Psoriasis is a chronic, inflammatory skin disease of which plaque psoriasis is the most common form. Psoriatic skin lesions can cause physical discomfort and emotional debilitation, as this disease can significantly affect a patient's perception of general health and social functioning.

Proof-of-concept for the efficacy of infliximab in psoriasis was obtained in a phase II trial where patients received 5 or 10 mg kg^{-1} infliximab or placebo at Weeks 0, 2, and 6. A significant improvement in the PASI score was observed as early as Week 2, and by Week 10 this was manifested in 93% and 95% improvements for the 5 and 10 mg kg^{-1} groups, respectively, compared to 11% in the placebo group [7].

Histological analysis of skin biopsies at Week 10 showed a significant decrease in epidermal T cells and a significant decrease in epidermal thickness. Similarly, keratin K16 and ICAM expression by keratinocytes was decreased to normal levels at Week 10. This study demonstrated, for the first time, the important role of TNF-α in the pathogenesis of psoriasis [7].

These results were confirmed in a phase III trial involving 249 patients; subsequently, 72% and 88% of patients treated with 3 or 5 mg kg^{-1} infliximab monotherapy, respectively, achieved 75% improvement of their baseline PASI score (PASI 75) at Week 10, compared with 6% of placebo patients. The response began to decline at 4 to 8 weeks after the last infusion, but could be regained in 38% and 64% of the patients after retreatment with 3 or 5 mg kg^{-1} infliximab, respectively, compared to 18% of placebo-treated patients [48]. Patients responding to infliximab treatment also experienced a significant improvement in their quality of life [49].

An additional study showed that continued infliximab treatment resulted in long-term maintenance of the therapeutic response for skin as well as nail lesions. When treatment with infliximab was continued every 8 weeks following the induction therapy, 82% of infliximab-treated patients achieved PASI 75 at Week 24 (compared to 4% for placebo) and 58% a PASI 90 at Week 24 (compared to 1% placebo). At Week 50, the PASI 75 response was maintained for 61% of patients, and PASI 90 for 45% [50]. Similarly, the nail psoriasis severity index (NAPSI) score decreased by 56% in infliximab-treated patients at Week 54 and was maintained through Week 50. This demonstrated that infliximab had high efficacy in improving skin as well as nail lesions, and furthermore caused a rapid onset of response in psoriasis.

8.4.1.6 **Ulcerative Colitis**

Ulcerative colitis is an inflammatory disease characterized by mucosal ulceration of the colon, rectal bleeding, diarrhea, and abdominal pain. TNF-α has been found in increased concentrations in the blood, colonic tissue and stools of patients with ulcerative colitis. In the ACT1 and ACT2 trials, the efficacy of infliximab was evaluated in patients who had not responded to corticosteroid or AZA/6-MP. About twofold more patients in the infliximab group showed a clinical response, as defined by a decrease in the Mayo score of at least 3 points, and no rectal bleeding at Weeks 8, 30, and 54 than in the placebo group. Mucosal healing also occurred in about twofold more patients, while remission rates were almost threefold higher in the infliximab group than in the placebo group [51].

Infliximab is also a safe and effective rescue therapy in patients experiencing an acute severe or moderately severe attack of ulcerative colitis and do not respond

to conventional corticosteroid treatment. Only 29% of these patients had colectomies following one infusion of infliximab compared to 67% who had received a sham infusion [52].

8.5 Safety

TNF-α is a central mediator in inflammation and immunity, and plays a crucial role in host defense. Thus, certain serious adverse events such as bacterial infections, tuberculosis and certain opportunistic infections, as well as demyelinating syndromes, have been observed with all TNF-α antagonists.

8.5.1 Serious Infections

In clinical studies, 36% of infliximab-treated patients experienced infections compared with 28% of placebo-treated patients; respiratory and urinary tract infections were the most commonly reported problems. No increased risk of serious infections was observed when infliximab was compared to placebo in the Crohn's disease studies. However, in the RA trials the incidence of serious infections, including pneumonia, was higher in patients treated with infliximab in combination with MTX than in those treated with MTX alone, especially at infliximab doses of $6\,mg\,kg^{-1}$ or greater. In the psoriasis studies, 1.5% of patients (average 41.9 weeks follow-up) receiving infliximab and 0.6% of patients (average 18.1 weeks follow-up) receiving placebo developed serious infections.

In order to determine the risk of developing serious infections, approximately 1000 patients with active RA were followed for 22 months. The risk of developing serious infections was similar in patients who received the approved infliximab dose of $3\,mg\,kg^{-1}$ in combination with MTX to those receiving MTX alone, although the risk was increased in patients receiving high doses of infliximab ($10\,mg\,kg^{-1}$) combined with MTX [53].

Opportunistic infections, such as tuberculosis (TB), atypical mycobacteria, pneumocystosis, histoplasmosis, coccidioidomycosis, cryptococcosis, aspergillosis, listeriosis and candidiasis, have also been reported to occur in patients treated with other TNF-α antagonists.

8.5.1.1 Tuberculosis

The first case of TB in an infliximab-treated patient occurred during the ATTRACT trial in RA, and since then the risk of developing TB infection or reactivation of latent TB has been recognized as a class-effect risk for TNF-α-blocking agents. Even in a country such as Sweden, where the incidence of TB is very low (5 cases in 100 000 population), the risk of TB in RA patients is about twofold higher than in the normal population, and RA patients treated with TNF antagonists have an

approximately fourfold higher risk than RA patients not treated with TNF antagonists for developing TB [54].

Thus, before, during, and after infliximab treatment it is recommended that patients are closely monitored for signs and symptoms of active TB and other serious infections. Before starting infliximab treatment the patient should be evaluated for a personal history of TB and appropriately screened using a skin test and chest X-radiography (SPC). In the case of apparent or suspected TB, patients should be treated with isoniazid (INH) before infliximab treatment can be initiated. An analysis of the Spanish registry BIOBADASER (Society of Rheumatology Database on Biologic Products) on the incidence ratio of TB indicated that the rate of TB cases in patients taking TNF-α antagonists fell by 78% after TB screening had been recommended [55]. In addition, all severe adverse events are collected in a central database by the market authorization holder, and safety updates are reported periodically to the health authorities. In addition, the TB education of rheumatologists, dermatologists and gastroenterologists is targeted to raise awareness of the risk of TB infections in patients receiving TNF-α blockers.

While serious infection is considered a risk for patients using TNF-α blockers, the long-term follow-up of Crohn's disease patients (over 5000 patient-years) has shown no correlation between the incidences of serious infections and infliximab treatment. Rather, prednisone use, narcotic analgesic use and disease activity correlated with the occurrence of infections [17].

8.5.2
Antibody Formation against Infliximab

The detection or interpretation of the analyses of antibodies to infliximab can be hindered by the presence of infliximab in the serum. Taking into account that infliximab remains in the circulation for at least 4 to 12 weeks after infusion, serum samples are usually collected for analysis at 12 weeks or later following the last infusion. When the formation of antibodies against infliximab (human antichimeric antibodies; HACA) was determined at 12 weeks after the last infusion in RA patients, HACA were detected in about 17% of all treated patients. The rate of HACA responses was inversely proportional to the dosage; thus, HACA formation occurred in 53%, 21% and 7% of patients treated with 1, 3 and 10 $mg\,kg^{-1}$ infliximab, respectively. Concomitant treatment with MTX reduced the rates to 15%, 7% and 3% for the three respective dosages. These data suggest that anti-TNF-α treatment induces a phenomenon resembling tolerance [18].

A significantly higher incidence of antibodies to infliximab was detected in Crohn's disease patients who had received episodic treatment – that is, one dose of infliximab at the start of the trial, but then only placebo until Week 14 or later until the disease worsened. Whilst 30% of patients with this treatment schedule developed HACA, only 8% who received infliximab infusions every 8 weeks did so. The incidence of antibodies to infliximab was higher in patients who did not

receive immunomodulators compared to those who did (18% versus 10%, p = 0.02) [56]. The rate of infusion reactions correlated positively with the development of HACA, but the antibody results proved to be poorly predictive of these events [56,57]. The development of antibodies to infliximab is also associated with a reduced duration of response to treatment [57]. Taken together, the results of these studies suggest that scheduled treatment in combination with an immunosuppressive drug carries the least risk of antibody formation against infliximab, and offers the best probability to maintain the response.

8.5.3 Infusion Reactions/Delayed Hypersensitivity Reactions

The highest frequency of adverse events in response to infliximab treatment are infusion reactions occurring within 2h of the infusion. Typically, these consist of fever, chills, nausea, dyspnea, and headaches; the symptoms can be controlled with drug treatment (e.g., antihistamines). Infusion reactions led to the discontinuation of treatment in approximately 3% of patients, and were considered serious in <1% [15,20,25,48]. Delayed reactions such as myalgias, arthralgias, fever, rash, pruritis, facial, hand or lip edema, dysphagia, urticaria, sore throat and headache may occur within 3 to 12 days following infliximab infusion [20].

8.5.4 Auto-Antibody Formation

The formation of auto-antibody has been noted in patients treated with infliximab for refractory spondyloarthropathy [58], RA [59], and Crohn's disease [60]. In these studies, 50 to 60% of patients developed antinuclear antibodies (ANA), and 17 to 30% developed antibodies to double-stranded (ds)-DNA. These auto-antibodies are generally not associated with clinical autoimmunity. Infliximab does not affect the formation of other non-organ-specific antibodies [60], although antibodies to antiphospholipid/anticardiolipin have been detected in RA patients upon infliximab treatment [61]. Co-medication with MTX or systemic maintenance therapy with infliximab appears to reduce the risk of auto-antibody induction or systemic maintenance therapy [58,60]. The immunopathological mechanism of auto-antibody induction after infliximab treatment is currently unknown.

8.5.5 Neurological Disorders/Demyelinating Disease

Rare cases of optic neuritis, seizures and new-onset or exacerbation of demyelinating disorders, including multiple sclerosis, have been reported after treatment with TNF-α antagonists. The discontinuation of anti-TNF-therapy resulted in

complete or partial improvement of symptoms [62]. While the association of TNF-α blockade with demyelination remains unclear, the treating physician should carefully evaluate risks and benefits before recommending infliximab treatment in patients with demyelination disorders.

8.5.6 Malignancies/Lymphoma

No apparent increase in the risk of developing malignancies has been shown for RA patients receiving anti-TNF-α therapy [63]. Current data are insufficient to establish a causal relationship between RA treatment and the development of lymphoma; indeed, an analysis of the National Data Bank for Rheumatic Diseases identified only 29 cases of lymphoma in 18 572 patients. The standardized incidence ratio (SIR) for RA patients for lymphoma was 1.9 (955 confidence interval [95% CI] 1.3–2.7), while the SIR for infliximab (with or without etanercept in patients who had received multiple TNF-α blockers) was 2.6 (95% CI 1.4–4.5). However, it remains to be seen whether the increased SIR with anti-TNF treatment is reflective of channeling bias, as patients with the highest risk of lymphoma preferentially receive anti-TNF therapy [64]. Similarly, five cases of lymphoma were identified in 1603 person-years at risk in anti-TNF-treated RA patients compared to two lymphoma cases in 3948 person-years treated with conventional anti-rheumatic therapies. This translated to a lymphoma risk of 11.5(95% CI 3.7–26.9) and 1.2 (95% CI 0.2–4.5). Thus, the apparent increase of lymphoma risk in anti-TNF-treated patients is based on only a few cases [63].

Long-term data from the TREAT registry that follows Crohn's disease patients for up to 5 years while they are being treated with standard therapy or infliximab, do not show any increased risk of malignancies. However, Crohn's disease patients treated with the immunosuppressive drugs AZA or 6-MP have a fourfold higher risk of developing lymphomas compared to the general population [65].

8.5.7 Congestive Heart Failure

In a randomized, double-blind trial in patients with moderate-to-severe heart failure (New York Heart Association class III to IV) the risk of death from any cause or hospitalization for heart failure was significantly higher in infliximab-treated patients [66]. However, post-marketing analysis of approximately 1000 Swedish RA patients of whom about 50% were treated with TNF-α blockers, suggests that treatment with TNF-α antagonists actually lowers the risk of developing cardiovascular disease [67]. Patients starting on TNF-α therapy have most likely more severe RA and might be predisposed to developing ischemic heart disease. However, the risk of developing cardiovascular disease in these patients appears to be reduced by aggressive antirheumatic therapy that includes TNF-α blockers.

8.5.8 Other Adverse Events

8.5.8.1 Hepatic Events

Hepatic events, most often seen as mild to moderate increases in alanine aminotransferase (ALT) and aspartate aminotransferase (AST), have occurred in patients treated with infliximab, but a relationship with infliximab has not been established.

8.5.8.2 Pregnancy Outcome

An analysis of the worldwide infliximab safety database (Centocor) on data collected between 1998 and 2001 has identified 96 women who were directly exposed to infliximab during pregnancy, and for whom outcome data could be collected. Live births occurred in 67% of cases, miscarriages in 15% and therapeutic termination in 19%. This outcome does not differ from that reported for the general US population [68]. While no increased risk is apparent from these data, the follow-up of larger numbers of pregnancies occurring during infliximab treatment will need to be analyzed.

8.6 Summary

Infliximab therapy has demonstrated efficacy in a wide variety of chronic inflammatory diseases, thus highlighting the central role of TNF-α in inflammation. The preservation of joint structure in RA and PsA, as well as the rapid clearance of skin lesions in psoriasis and PsA, has led to an important and impressive improvement in the quality of life in these patients. Similarly, in Crohn's disease and ulcerative colitis infliximab treatment has resulted in a reduced number of surgeries and hospitalization of patients, thus contributing to the improved quality of life in these cases. While infliximab treatment thus provides definite benefits to patients, its mechanism of action as a TNF-α blocker carries some degree of risk, which is especially apparent in the increased susceptibility of patients receiving TNF-α antagonists to serious infections. However, an improved awareness of this risk and the employment of preventive measures can in fact reduce the number of patients encountering infections. Other major concerns associated with TNF-α blockers, such as increased malignancies and lymphoma rates, are currently being addressed by the long-term follow-up of patients, although at present TNF-α blockade is considered to be a treatment paradigm for chronic inflammatory disease with a very positive benefit/risk profile.

References

1 Knight, D.M., Trinh, H., Le, J., et al. (1993) Construction and initial characterization of a mouse-human chimeric anti-TNF antibody. Mol Immunol 30: 1443–1453.

2 Scallon, B., Cai, A., Solowski, N., Rosenberg, A., Song, X.Y., Shealy, D., Wagner, C. (2002) Binding and functional comparisons of two types of tumor necrosis factor antagonists. J Pharmacol Exp Ther 301: 418–426.

3 Scallon, B.J., Moore, M.A., Trinh, H., Knight, D.M., Ghrayeb, J. (1995) Chimeric anti-TNF-alpha monoclonal antibody cA2 binds recombinant transmembrane TNF-alpha and activates immune effector functions. Cytokine 7: 251–259.

4 Siegel, S.A., Shealy, D.J., Nakada, M.T., Le, J., Woulfe, D.S., Probert, L., Kollias, G., Ghrayeb, J., Vilcek, J., Daddona, P.E. (1995) The mouse/human chimeric monoclonal antibody cA2 neutralizes TNF in vitro and protects transgenic mice from cachexia and TNF lethality in vivo. Cytokine 7: 15–25.

5 Shen, C., Maerten, P., Geboes, K., Van Assche, G., Rutgeerts, P., Ceuppens, J.L. (2005) Infliximab induces apoptosis of monocytes and T lymphocytes in a human-mouse chimeric model. Clin Immunol 115: 250–259.

6 Catrina, A.I., Trollmo, C., af Klint, E., Engstrom, M., Lampa, J., Hermansson, Y., Klareskog, L., Ulfgren, A.K. (2005) Evidence that anti-tumor necrosis factor therapy with both etanercept and infliximab induces apoptosis in macrophages, but not lymphocytes, in rheumatoid arthritis joints: extended report. Arthritis Rheum 52: 61–72.

7 Gottlieb, A.B., Masud, S., Ramamurthi, R., Abdulghani, A., Romano, P., Chaudhari, U., Dooley, L.T., Fasanmade, A.A., Wagner, C.L. (2003) Pharmacodynamic and pharmacokinetic response to anti-tumor necrosis factor-alpha monoclonal antibody (infliximab) treatment of moderate to severe psoriasis vulgaris. J Am Acad Dermatol 48: 68–75.

8 Cornillie, F., Shealy, D., D'Haens, G., Geboes, K., Van Assche, G., Ceuppens, J., Wagner, C., Schaible, T., Plevy, S.E., Targan, S.R., Rutgeerts, P. (2001) Infliximab induces potent anti-inflammatory and local immunomodulatory activity but no systemic immune suppression in patients with Crohn's disease. Aliment Pharmacol Ther 15: 463–473.

9 St Clair, E.W., Wagner, C.L., Fasanmade, A.A., Wang, B., Schaible, T., Kavanaugh, A., Keystone, E.C. (2002) The relationship of serum infliximab concentrations to clinical improvement in rheumatoid arthritis: results from ATTRACT, a multicenter, randomized, double-blind, placebo-controlled trial. Arthritis Rheum 46: 1451–1459.

10 Markham, A., Lamb, H.M. (2000) Infliximab: a review of its use in the management of rheumatoid arthritis. Drugs 59: 1341–1359.

11 Hanauer, S.B., Feagan, B.G., Lichtenstein, G.R., Mayer, L.F., Schreiber, S., Colombel, J.F., Rachmilewitz, D., Wolf, D.C., Olson, A., Bao, W., Rutgeerts, P. (2002) Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. Lancet 359: 1541–1549.

12 Lichtenstein, G.R., Yan, S., Bala, M., Hanauer, S. (2004) Remission in patients with Crohn's disease is associated with improvement in employment and quality of life and a decrease in hospitalizations and surgeries. Am J Gastroenterol 99: 91–96.

13 Rutgeerts, P., Feagan, B.G., Lichtenstein, G.R., Mayer, L.F., Schreiber, S., Colombel, J.F., Rachmilewitz, D., Wolf, D.C., Olson, A., Bao, W., Hanauer, S.B. (2004) Comparison of scheduled and episodic treatment strategies of infliximab in Crohn's disease. Gastroenterology 126: 402–413.

14 Lemann, M., Mary, J.Y., Duclos, B., Veyrac, M., Dupas, J.L., Delchier, J.C., Laharie, D., Moreau, J., Cadiot, G., Picon, L., Bourreille, A., Sobahni, I., Colombel, J.F. (2006) Infliximab plus azathioprine

for steroid-dependent Crohn's disease patients: a randomized placebo-controlled trial. Gastroenterology 130: 1054–1061.
15 Sands, B.E., Anderson, F.H., Bernstein, C.N., et al. (2004) Infliximab maintenance therapy for fistulizing Crohn's disease. N Engl J Med 350: 876–885.
16 Lichtenstein, G.R., Yan, S., Bala, M., Blank, M., Sands, B.E. (2005) Infliximab maintenance treatment reduces hospitalizations, surgeries, and procedures in fistulizing Crohn's disease. Gastroenterology 128: 862–869.
17 Lichtenstein, G.R., Feagan, B.G., Cohen, R.D., Salzberg, B.A., Diamond, R.H., Chen, D.M., Pritchard, M.L., Sandborn, W.J. (2006) Serious infections and mortality in association with therapies for Crohn's disease: TREAT registry. Clin Gastroenterol Hepatol 4: 621–630.
18 Maini, R.N., Breedveld, F.C., Kalden, J.R., Smolen, J.S., Davis, D., Macfarlane, J.D., Antoni, C., Leeb, B., Elliott, M.J., Woody, J.N., Schaible, T.F., Feldmann, M. (1998) Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor alpha monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. Arthritis Rheum 41: 1552–1563.
19 Maini, R., St Clair, E.W., Breedveld, F., Furst, D., Kalden, J., Weisman, M., Smolen, J., Emery, P., Harriman, G., Feldmann, M., Lipsky, P. (1999) Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. ATTRACT Study Group. Lancet 354: 1932–1939.
20 Lipsky, P.E., van der Heijde, D.M., St Clair, E.W., Furst, D.E., Breedveld, F.C., Kalden, J.R., Smolen, J.S., Weisman, M., Emery, P., Feldmann, M., Harriman, G. R., Maini, R.N. (2000) Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. N Engl J Med 343: 1594–1602.
21 Breedveld, F.C., Emery, P., Keystone, E., Patel, K., Furst, D.E., Kalden, J.R., St Clair, E.W., Weisman, M., Smolen, J., Lipsky, P.E., Maini, R.N. (2004) Infliximab in active early rheumatoid arthritis. Ann Rheum Dis 63: 149–155.
22 Maini, R.N., Breedveld, F.C., Kalden, J.R., Smolen, J.S., Furst, D., Weisman, M.H., St Clair, E.W., Keenan, G.F., van der Heijde, D., Marsters, P.A., Lipsky, P.E. (2004) Sustained improvement over two years in physical function, structural damage, and signs and symptoms among patients with rheumatoid arthritis treated with infliximab and methotrexate. Arthritis Rheum 50: 1051–1065.
23 Smolen, J.S., Han, C., Bala, M., Maini, R.N., Kalden, J.R., van der Heijde, D., Breedveld, F.C., Furst, D.E., Lipsky, P.E. (2005) Evidence of radiographic benefit of treatment with infliximab plus methotrexate in rheumatoid arthritis patients who had no clinical improvement: a detailed subanalysis of data from the anti-tumor necrosis factor trial in rheumatoid arthritis with concomitant therapy study. Arthritis Rheum 52: 1020–1030.
24 Ritchlin, C.T., Schwarz, E.M., O'Keefe, R.J., Looney, R.J. (2004) RANK, RANKL and OPG in inflammatory arthritis and periprosthetic osteolysis. J Musculoskelet Neuronal Interact 4: 276–284.
25 St Clair, E.W., van der Heijde, D.M., Smolen, J.S., Maini, R.N., Bathon, J.M., Emery, P., Keystone, E., Schiff, M., Kalden, J.R., Wang, B., Dewoody, K., Weiss, R., Baker, D. (2004) Combination of infliximab and methotrexate therapy for early rheumatoid arthritis: a randomized, controlled trial. Arthritis Rheum 50: 3432–3443.
26 Smolen, J.S., Van Der Heijde, D.M., St Clair, E.W., Emery, P., Bathon, J.M., Keystone, E., Maini, R.N., Kalden, J.R., Schiff, M., Baker, D., Han, C., Han, J., Bala, M. (2006) Predictors of joint damage in patients with early rheumatoid arthritis treated with high-dose methotrexate with or without concomitant infliximab: results from the ASPIRE trial. Arthritis Rheum 54: 702–710.

27 Taylor, P.C., Steuer, A., Gruber, J., Cosgrove, D.O., Blomley, M.J., Marsters, P.A., Wagner, C.L., McClinton, C., Maini, R.N. (2004) Comparison of ultrasonographic assessment of synovitis and joint vascularity with radiographic evaluation in a randomized, placebo-controlled study of infliximab therapy in early rheumatoid arthritis. Arthritis Rheum 50: 1107–1116.

28 Hyrich, K.L., Symmons, D.P., Watson, K.D., Silman, A.J. (2006) Comparison of the response to infliximab or etanercept monotherapy with the response to cotherapy with methotrexate or another disease-modifying antirheumatic drug in patients with rheumatoid arthritis: Results from the British Society for Rheumatology Biologics Register. Arthritis Rheum 54: 1786–1794.

29 Braun, J., Brandt, J., Listing, J., Zink, A., Alten, R., Golder, W., Gromnica-Ihle, E., Kellner, H., Krause, A., Schneider, M., Sorensen, H., Zeidler, H., Thriene, W., Sieper, J. (2002) Treatment of active ankylosing spondylitis with infliximab: a randomised controlled multicentre trial. Lancet 359: 1187–1193.

30 Braun, J., Brandt, J., Listing, J., Zink, A., Alten, R., Burmester, G., Golder, W., Gromnica-Ihle, E., Kellner, H., Schneider, M., Sorensen, H., Zeidler, H., Reddig, J., Sieper, J. (2003) Long-term efficacy and safety of infliximab in the treatment of ankylosing spondylitis: an open, observational, extension study of a three-month, randomized, placebo-controlled trial. Arthritis Rheum 48: 2224–2233.

31 Braun, J., Brandt, J., Listing, J., Zink, A., Alten, R., Burmester, G., Gromnica-Ihle, E., Kellner, H., Schneider, M., Sorensen, H., Zeidler, H., Sieper, J. (2005) Two year maintenance of efficacy and safety of infliximab in the treatment of ankylosing spondylitis. Ann Rheum Dis 64: 229–234.

32 Sieper, J., Baraliakos, X., Listing, J., Brandt, J., Haibel, H., Rudwaleit, M., Braun, J. (2005) Persistent reduction of spinal inflammation as assessed by magnetic resonance imaging in patients with ankylosing spondylitis after 2 yrs of treatment with the anti-tumour necrosis factor agent infliximab. Rheumatology (Oxford) 44: 1525–1530.

33 Baraliakos, X., Listing, J., Rudwaleit, M., Brandt, J., Sieper, J., Braun, J. (2005) Radiographic progression in patients with ankylosing spondylitis after 2 years of treatment with the tumour necrosis factor alpha antibody infliximab. Ann Rheum Dis 64: 1462–1466.

34 Braun, J., Baraliakos, X., Brandt, J., Listing, J., Zink, A., Alten, R., Burmester, G., Gromnica-Ihle, E., Kellner, H., Schneider, M., Sorensen, H., Zeidler, H., Sieper, J. (2005) Persistent clinical response to the anti-TNF-alpha antibody infliximab in patients with ankylosing spondylitis over 3 years. Rheumatology (Oxford) 44: 670–676.

35 Baraliakos, X., Listing, J., Brandt, J., Zink, A., Alten, R., Burmester, G., Gromnica-Ihle, E., Kellner, H., Schneider, M., Sorensen, H., Zeidler, H., Rudwaleit, M., Sieper, J., Braun, J. (2005) Clinical response to discontinuation of anti-TNF therapy in patients with ankylosing spondylitis after 3 years of continuous treatment with infliximab. Arthritis Res Ther 7: R439–R444.

36 van der Heijde, D., Dijkmans, B., Geusens, P., Sieper, J., DeWoody, K., Williamson, P., Braun, J. (2005) Efficacy and safety of infliximab in patients with ankylosing spondylitis: results of a randomized, placebo-controlled trial (ASSERT). Arthritis Rheum 52: 582–591.

37 van Kuijk, A.W., Reinders-Blankert, P., Smeets, T.J., Dijkmans, B.A., Tak, P.P. (2006) Detailed analysis of the cell infiltrate and the expression of mediators of synovial inflammation and joint destruction in the synovium of patients with psoriatic arthritis: implications for therapy. Ann Rheum Dis. (in press).

38 Olaniran, A.K., et al. (1996) Cytokine expression in psoriatic skin lesions during PUVA therapy. Arch Dermatol Res 288(8): 421–425.

39 Lizzul, P.F., Aphale, A., Malaviya, R., Sun, Y., Masud, S., Dombrovskiy, V., Gottlieb, A.B. (2005) Differential expression of phosphorylated NF-kappaB/RelA in normal and psoriatic epidermis and downregulation of NF-kappaB in

response to treatment with etanercept. J Invest Dermatol 124: 1275–1283.
40 Danning, C.L., Illei, G.G., Hitchon, C., Greer, M.R., Boumpas, D.T., McInnes, I.B. (2000) Macrophage-derived cytokine and nuclear factor kappaB p65 expression in synovial membrane and skin of patients with psoriatic arthritis. Arthritis Rheum 43(6): 1244–1256.
41 Ogilvie, A.L., Antoni, C., Dechant, C., Manger, B., Kalden, J.R., Schuler, G., Luftl, M. (2001) Treatment of psoriatic arthritis with antitumour necrosis factor-alpha antibody clears skin lesions of psoriasis resistant to treatment with methotrexate. Br J Dermatol 144: 587–589.
42 Antoni, C., Dechant, C., Hanns-Martin Lorenz, P.D., Wendler, J., Ogilvie, A., Lueftl, M., Kalden-Nemeth, D., Kalden, J.R., Manger, B. (2002) Open-label study of infliximab treatment for psoriatic arthritis: clinical and magnetic resonance imaging measurements of reduction of inflammation. Arthritis Rheum 47: 506–512.
43 Antoni, C.E., Kavanaugh, A., Kirkham, B., et al. (2005) Sustained benefits of infliximab therapy for dermatologic and articular manifestations of psoriatic arthritis: results from the infliximab multinational psoriatic arthritis controlled trial (IMPACT). Arthritis Rheum 52: 1227–1236.
44 Antoni, C., Krueger, G.G., de Vlam, K., Birbara, C., Beutler, A., Guzzo, C., Zhou, B., Dooley, L.T., Kavanaugh, A. (2005) Infliximab improves signs and symptoms of psoriatic arthritis: results of the IMPACT 2 trial. Ann Rheum Dis 64: 1150–1157.
45 Kavanaugh, A., Antoni, C.E., Gladman, D., Wassenberg, S., Zhou, B., Beutler, A., Keenan, G., Burmester, G., Furst, D.E., Weisman, M.H., Kalden, J.R., Smolen, J., van der Heijde D. (2006) The Infliximab Multinational Psoriatic Arthritis Controlled Trial (IMPACT): results of radiographic analyses after 1 year. Ann Rheum Dis 65: 1038–1043.
46 Mastroianni, A., et al. (2005) Cytokine profiles during infliximab monotherapy in psoriatic arthritis. Br J Dermatol 153(3): 531–536.
47 Canete, J.D., Pablos, J.L., Sanmarti, R., Mallofre, C., Marsal, S., Maymo, J., Gratacos, J., Mezquita, J., Mezquita, C., Cid, M.C. (2004) Antiangiogenic effects of anti-tumor necrosis factor alpha therapy with infliximab in psoriatic arthritis. Arthritis Rheum 50: 1636–1641.
48 Gottlieb, A.B., Evans, R., Li, S., Dooley, L.T., Guzzo, C.A., Baker, D., Bala, M., Marano, C.W., Menter, A. (2004) Infliximab induction therapy for patients with severe plaque-type psoriasis: a randomized, double-blind, placebo-controlled trial. J Am Acad Dermatol 51: 534–542.
49 Feldman, S.R., Gordon, K.B., Bala, M., Evans, R., Li, S., Dooley, L.T., Guzzo, C., Patel, K., Menter, A., Gottlieb, A.B. (2005) Infliximab treatment results in significant improvement in the quality of life of patients with severe psoriasis: a double-blind placebo-controlled trial. Br J Dermatol 152: 954–960.
50 Reich, K., Nestle, F.O., Papp, K., Ortonne, J.P., Evans, R., Guzzo, C., Li, S., Dooley, L.T., Griffiths, C.E. (2005) Infliximab induction and maintenance therapy for moderate-to-severe psoriasis: a phase III, multicentre, double-blind trial. Lancet 366: 1367–1374.
51 Rutgeerts, P., Sandborn, W.J., Feagan, B.G., Reinisch, W., Olson, A., Johanns, J., Travers, S., Rachmilewitz, D., Hanauer, S.B., Lichtenstein, G.R., de Villiers, W.J., Present, D., Sands, B.E., Colombel, J.F. (2005) Infliximab for induction and maintenance therapy for ulcerative colitis. N Engl J Med 353: 2462–2476.
52 Jarnerot, G., Hertervig, E., Friis-Liby, I., Blomquist, L., Karlen, P., Granno, C., Vilien, M., Strom, M., Danielsson, A., Verbaan, H., Hellstrom, P.M., Magnuson, A., Curman, B. (2005) Infliximab as rescue therapy in severe to moderately severe ulcerative colitis: a randomized, placebo-controlled study. Gastroenterology 128: 1805–1811.
53 Westhovens, R., Yocum, D., Han, J., Berman, A., Strusberg, I., Geusens, P., Rahman, M.U. (2006) The safety of infliximab, combined with background treatments, among patients with

rheumatoid arthritis and various comorbidities: a large, randomized, placebo-controlled trial. Arthritis Rheum 54: 1075–1086.

54 Askling, J., Fored, C.M., Brandt, L., Baecklund, E., Bertilsson, L., Coster, L., Geborek, P., Jacobsson, L.T., Lindblad, S., Lysholm, J., Rantapaa-Dahlqvist, S., Saxne, T., Romanus, V., Klareskog, L., Feltelius, N. (2005) Risk and case characteristics of tuberculosis in rheumatoid arthritis associated with tumor necrosis factor antagonists in Sweden. Arthritis Rheum 52: 1986–1992.

55 Carmona, L., Gomez-Reino, J.J., Rodriguez-Valverde, V., Montero, D., Pascual-Gomez, E., Mola, E.M., Carreno, L., Figueroa, M. (2005) Effectiveness of recommendations to prevent reactivation of latent tuberculosis infection in patients treated with tumor necrosis factor antagonists. Arthritis Rheum 52: 1766–1772.

56 Hanauer, S.B., Wagner, C.L., Bala, M., Mayer, L., Travers, S., Diamond, R.H., Olson, A., Bao, W., Rutgeerts, P. (2004) Incidence and importance of antibody responses to infliximab after maintenance or episodic treatment in Crohn's disease. Clin Gastroenterol Hepatol 2: 542–553.

57 Baert, F., Noman, M., Vermeire, S., Van Assche, G., Carbonez, A., Rutgeerts, P. (2003) Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. N Engl J Med 348: 601–608.

58 Sellam, J., Allanore, Y., Batteux, F., Deslandre, C.J., Weill, B., Kahan, A. (2005) Autoantibody induction in patients with refractory spondyloarthropathy treated with infliximab and methotrexate. Joint Bone Spine 72: 48–52.

59 Eriksson, C., Engstrand, S., Sundqvist, K.G., Rantapaa-Dahlqvist, S. (2005) Autoantibody formation in patients with rheumatoid arthritis treated with anti-TNF alpha. Ann Rheum Dis 64: 403–407.

60 Nancey, S., Blanvillain, E., Parmentier, B., Flourie, B., Bayet, C., Bienvenu, J., Fabien, N. (2005) Infliximab treatment does not induce organ-specific or nonorgan-specific autoantibodies other than antinuclear and anti-double-stranded DNA autoantibodies in Crohn's disease. Inflamm Bowel Dis 11: 986–991.

61 Jonsdottir, T., Forslid, J., van Vollenhoven, A., Harju, A., Brannemark, S., Klareskog, L., van Vollenhoven, R.F. (2004) Treatment with tumour necrosis factor alpha antagonists in patients with rheumatoid arthritis induces anticardiolipin antibodies. Ann Rheum Dis 63: 1075–1078.

62 Mohan, N., Edwards, E.T., Cupps, T.R., Oliverio, P.J., Sandberg, G., Crayton, H., Richert, J.R., Siegel, J.N. (2001) Demyelination occurring during anti-tumor necrosis factor alpha therapy for inflammatory arthritides. Arthritis Rheum 44: 2862–2869.

63 Geborek, P., Bladstrom, A., Turesson, C., Gulfe, A., Petersson, I.F., Saxne, T., Olsson, H., Jacobsson, L.T. (2005) Tumour necrosis factor blockers do not increase overall tumour risk in patients with rheumatoid arthritis, but may be associated with an increased risk of lymphomas. Ann Rheum Dis 64: 699–703.

64 Wolfe, F., Michaud, K. (2004) Lymphoma in rheumatoid arthritis: the effect of methotrexate and anti-tumor necrosis factor therapy in 18,572 patients. Arthritis Rheum 50: 1740–1751.

65 Kandiel, A., Fraser, A.G., Korelitz, B.I., Brensinger, C., Lewis, J.D. (2005) Increased risk of lymphoma among inflammatory bowel disease patients treated with azathioprine and 6-mercaptopurine. Gut 54: 1121–1125.

66 Chung, E.S., Packer, M., Lo, K.H., Fasanmade, A.A., Willerson, J.T. (2003) Randomized, double-blind, placebo-controlled, pilot trial of infliximab, a chimeric monoclonal antibody to tumor necrosis factor-alpha, in patients with moderate-to-severe heart failure: results of the anti-TNF Therapy Against Congestive Heart Failure (ATTACH) trial. Circulation 107: 3133–3140.

67 Jacobsson, L.T., Turesson, C., Gulfe, A., Kapetanovic, M.C., Petersson, I.F., Saxne, T., Geborek, P. (2005) Treatment with tumor necrosis factor blockers is

associated with a lower incidence of first cardiovascular events in patients with rheumatoid arthritis. J Rheumatol 32: 1213–1218.

68 Katz, J.A., Antoni, C., Keenan, G.F., Smith, D.E., Jacobs, S.J., Lichtenstein, G.R. (2004) Outcome of pregnancy in women receiving infliximab for the treatment of Crohn's disease and rheumatoid arthritis. Am J Gastroenterol 99: 2385–2392.

9
Muromonab-CD3 (Orthoclone OKT3)

Harald Becker

9.1
Introduction

Prior to the availability of cyclosporine (CsA), acute allograft rejection was a major cause of graft loss following transplantation. Often, acute renal allograft rejections in patients receiving CsA maintenance immunosuppression were not reversible with high-dose corticosteroids (Thistlethwaite et al. 1987), and consequently a more effective agent was needed. The introduction of muromonab CD3 (Orthoclone OKT3) into clinical trials – and its subsequent approval by the US Food and Drug Administration as well as by the European authorities in 1985 and 1986 for use as an anti-rejection agent for renal transplantation – was a landmark in the field of clinical transplantation of solid organs. Hence, muromonab-CD3 was the first monoclonal antibody (mAb) to be approved for clinical use in humans.

Because it is a mAb preparation, muromonab-CD3 is a homogeneous, reproducible antibody product with consistent, measurable reactivity to human T cells. Each 5-mL ampoule of Orthoclone OKT3 sterile solution contains 5 mg (1 mg mL^{-1}) of muromonab-CD3 in a clear, colorless buffered solution (pH 7.0 ± 0.5) of monobasic sodium phosphate (2.25 mg), dibasic sodium phosphate (9.0 mg), sodium chloride (43 mg), and polysorbate 80 (1.0 mg) in water for injection.

The recognition of foreign antigens forms the physiological basis of acute allograft rejection, and proteins located on the cell surfaces play a major role in this immune response. The genes that encode these proteins are termed "histocompatibility" genes, and in every species this set of genes – known as the major histocompatibility complex (MHC) class II antigens – plays a critical role in the T-cell-dependent immune responses that initiate acute allograft rejection. Host recognition of donor antigen depends on the expression of recipient T-cell antigen receptors (TcRs). The TcR is closely associated with polypeptide chains known as clusters of differentiation (CD3), and together this unit is referred to as the TcR–CD3 complex, located on the surfaces of the T cells. The TcR–CD3 (Fig. 9.1)

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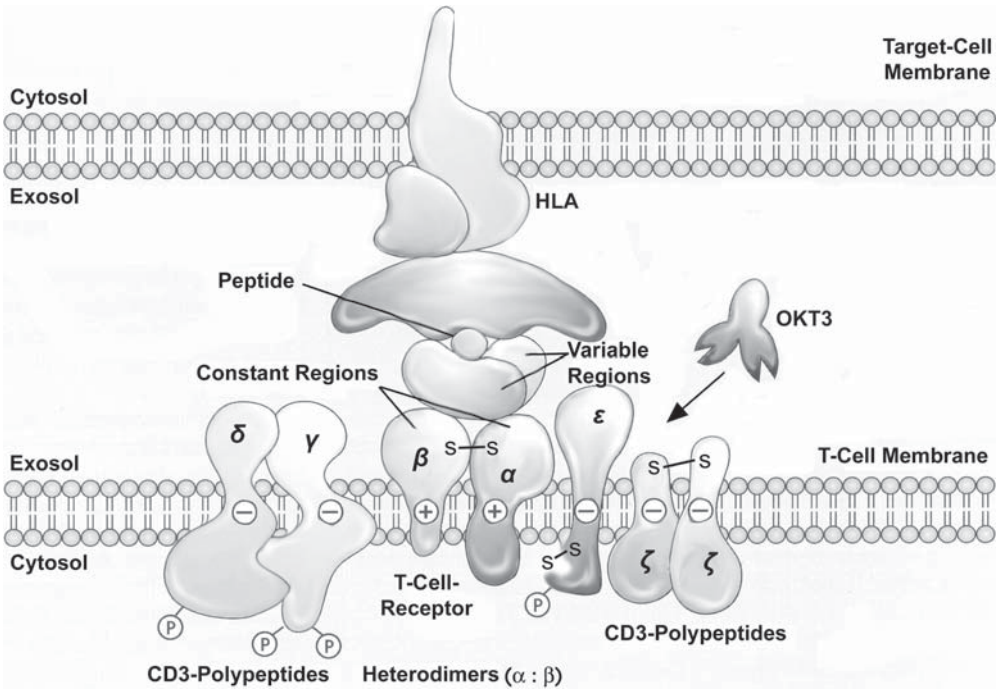

Fig. 9.1 Diagrammatic representation of the CD3 complex, T-cell antigen receptor (TCR) and site of muromonab-CD3 binding (epsilon chain of the CD3 complex). (Modified from Krensky et al. 1990.)

complex triggers the recognition of foreign antigens bound to MHC class II antigens on antigen-presenting cells (APCs), and transduces signals that subsequently induce lymphocyte activation against the allograft (Smith 1996).

9.2 Production of the Monoclonal Antibody

A mAb is an antibody derived from a single clone that is active against a single target antigen. The development of mAbs was derived from the knowledge that a single B cell (and its expanded clone) would produce a single specific antibody. The means of translating this knowledge into a reagent with practical applications in clinical transplantation occurred when Kohler and Milstein first successfully fused individual antibody-producing cells with myeloma cells, thus establishing permanent cell lines capable of secreting a single defined antibody (Kohler and Milstein 1976). In 1984, Kohler and Milstein were awarded the Nobel Prize in Medicine for their findings in this area. In theory, a mAb can be produced for any antigen against which an antibody can be produced. In the case of transplan-

tation, however, the key was to produce an antibody against the cell surface molecules that mediate acute allograft rejection.

Muromonab-CD3 was first identified by Kung and colleagues in 1979 as part of their efforts to describe T-cell subsets in humans (Kung et al. 1979). Muromonab-CD3 is a murine monoclonal antibody to the TcR–CD3 complex (CD3) on the surface of circulating human T cells which functions as an immunosuppressant. It is administered only via the intravenous route. The antibody is biochemically purified IgG_{2a} immunoglobulin with two heavy chains each of approximately 50 000 Da, and two light chains each of approximately 25 000 Da. It is directed to a glycoprotein (the 20 000-Da epsilon chain) in the human T-cell surface, which is essential for T-cell functions.

In order to produce immortal hybridomas, mice are immunized with an appropriate antigen complex such as a human T cell or a specific antigen complex such as CD 3; this increases the number of cells responding to the desired antigen. Approximately 1 month later, splenocytes are harvested and fused to murine myeloma cells, after which the hybridomas are grown in a medium which selects for their survival. The secreted antibody is tested for both, survival and specificity, and the clones producing the desired antibody are maintained and expanded (Fig. 9.2). Expansion is generally accomplished by passage through the peritoneal cavities of mice, or by sequential bulk tissue culture techniques (Chatenoud 1995).

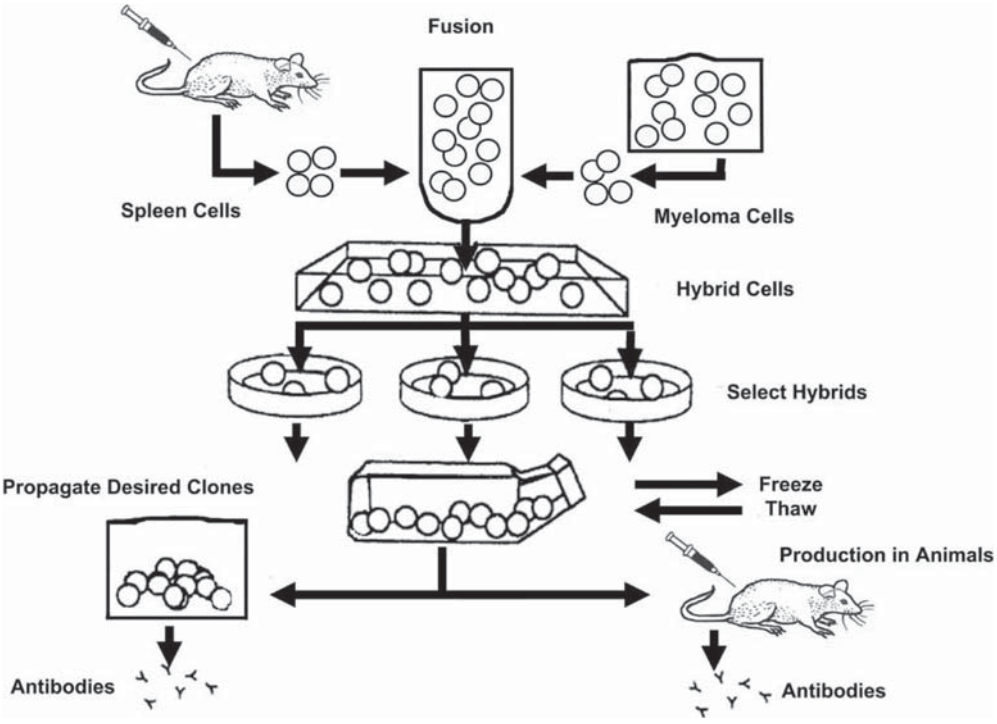

Fig. 9.2 Production of a murine monoclonal antibody (modified from Smith 1996).

The specific mAb is purified from the ascitic fluid or culture supernatant and tested to ensure the absence of microbial pathogens. The nature of the immunization process and the specificity screening employed prior to clonal expansion preclude the development of antibodies specific to platelet or red blood cell (RBC) determinants. New anti-lymphoid monoclonal antibodies considered for clinical use must be carefully tested to rule out cross-reactivity with nonlymphoid cells, as some functional surface proteins are shared by several cell types (Latham et al. 1970). Between 90 and 95% of the protein in commercially produced mAb preparations is specific antibody (Chatenoud 1995). The generation of hybridoma cell lines and the production of mAbs is described in detail elsewhere in this book (see Chapter 2, Vol I). Muromonab-CD3 solution is a homogenous, reproducible antibody product with consistent measurable reactivity to human T cells.

9.3 The Pharmacology of Muromonab-CD3

The murine mAb IgG2a specifically reacts with the TcR–CD3 complex on the surface of circulating human T cells. Muromonab-CD3 binds to a glycoprotein (the 20-kDa epsilon chain) on the CD3 complex to activate circulating T cells, and this interaction results in a transient activation of T cells with release of cytokines, and the blocking of T-cell proliferation and differentiation. As a result, almost all functional T cells are eliminated transiently from the peripheral circulation. Although T cells reappear in the circulation during the course of treatment, these cells are CD3-negative and incapable of T-cell activation. T-cell function usually returns to normal within approximately 48 h of the discontinuation of therapy (Smith 1996).

Although the mechanism by which muromonab-CD3 blocks the function of circulating T cells is incompletely understood, the results of *in-vitro* and *in-vivo* studies indicate that the primary modes of action are steric inhibition, stimulation of T-cell activation (induction of cytokine release), peripheral T-cell opsonization and depletion, and modulation of the CD3 complex (Roitt 1993). The initial effect is blockade of the TcR engagement with MHC class II antigens by steric inhibition of APCs. In addition, T-cell activation, or the expression of surface-activation markers such as interleukin (IL)-2 receptors and cellular adhesion counter-receptors, followed by profound T-cell dysfunction, occurs. The T cells are opsonized by macrophages in the reticuloendothelial system (RES), resulting in the margination of T cells into the intravascular spaces and redistribution to the lymph nodes. A rapid and concomitant decrease in the number of circulating CD3+ cells, including those that are CD2+, CD4+ or CD8+, has been observed in patients studied within minutes after the intravenous administration of muromonab-CD3 (Table 9.1). These cells are therefore unavailable to recognize transplant antigens (Kreis et al. 1991). Finally, any remaining T cells undergo TcR–CD3 modulation or internalization, during which TcR with CD3 antigens are removed

Table 9.1 Definition of surface antigens (from Bernard and Boumsell 1984).

OKT monoclonal antibody	*Recognized antigen*			*Cell population*
	CD-class	*T-class*	*Alternative*	
OKT1	CD5	T1		All T cells
OKT3	CD3	T3	LEU4	All matured peripheral T cells
OKT4	CD4	T4	LEU3a	Helper/inducer T cells
OKT6	CD1	T6		Thymocytes
OKT8	CD8	T8	LEU2a	Suppressor cytotoxic T cells
OKT11	CD2	T11	LEU5	T cells with receptor of sheep-erythrocytes

CD = cluster of differentiation; LEU = leucocytes.

from the T-cell surfaces, rendering the T cells CD3-negative and immunologically inactive (Caillat-Zucman et al. 1981).

9.3.1 Pharmacokinetic Properties of Muromonab-CD3

Plasma muromonab-CD3 concentrations vary according to the muromonab-CD3 antibody status, transplanted organ, and patient age. In renal transplant recipients receiving muromonab-CD3 5 mg once daily for 10 to 14 days, mean plasma concentrations were 996 $\mu g\,L^{-1}$ after 1 h and 104 $\mu g\,L^{-1}$ at 24 h. The mean trough steady-state serum concentrations range from 500 to 1000 $\mu g\,L^{-1}$ after 2 to 4 days. A serum level of approximately 1000 $\mu g\,L^{-1}$ is required to block cytotoxic T-cell function *in vitro*. Moreover, steady-state serum muromonab-CD3 concentrations are achieved earlier with prophylactic administration than with administration to treat transplant rejection.

There is evidence of drug accumulation after repeated doses, with muromonab-CD3 plasma elimination half-lives of approximately 18 h having been reported after administration to treat rejection, and of 36 h after prophylactic administration (Wilde et al. 1996).

Low plasma levels of muromonab-CD3 have been associated with a failure of muromonab-CD3 prophylaxis (Abramowicz et al. 1994; McDiarmid et al. 1990; Schroeder et al. 1991). The CD3+ cell level alone is not a reliable indicator for muromonab-CD3 concentrations or early sensitization (Broughan et al. 1994; Gebel et al. 1989a; McCarthy et al. 1993; Shaefer et al. 1990), and therefore the achievement of optimal dosage adjustments and efficacy requires continued surveillance for clinical signs of rejection during muromonab-CD3 prophylaxis in addition to monitoring the drug's serum levels, CD3+ cell levels, and anti-muromonab-CD3 antibody titers, as appropriate (Abramowicz et al. 1994; Gebel

et al. 1989a,b; Hammond et al. 1993; Henell et al. 1993; Moore et al. 1991; Ohman et al. 1993; Ryckman et al. 1991; Schroeder et al. 1991; Toyoda et al. 1995).

9.3.2 Pharmacodynamics of Muromonab-CD3

Muromonab-CD3 reverses graft rejection, most probably by blocking all T-cell functions and is, therefore, classified as a pan-T-cell suppressive mAb. This blockade plays a major role in acute allograft rejection, as muromonab-CD3 reacts with and blocks the function of a 20000-Da molecule (CD3) in the membrane of human T-cells. *In vivo*, muromonab-CD3 reacts with most peripheral blood T cells and T cells in body tissues, but has not been found to react with other hematopoietic elements or other tissues of the body. In all patients studied, a rapid and concomitant decrease in the number of circulating CD3+, CD4+ and CD8+ T cells was observed within minutes after the administration of muromonab-CD3 (Table 9.2). Between Days 2 and 7 after administration, increasing numbers of circulating CD4+ and CD8+ cells have been observed in patients, although CD3+ cells are not detectable. Due to their rapid clearance from the peripheral circulation, the T cells are unavailable to recognize transplant antigens. Finally, any remaining T cells undergo TcR–CD3 modulation or internalization, during which TcR with CD3 antigens are removed from the T-cell surfaces, thus rendering the T cells CD3-negative and immunologically inactive (Caillat-Zucman et al. 1981).

T-cell receptor stimulation by muromonab-CD3 *in vitro* led to a strong increase in tyrosine phosphorylation in all specimens of kidney transplant recipients and healthy controls (Muller et al. 1999). A significant release of IL-10, which plays a role in the down-modulation of the muromonab-CD3-triggered T-cell activation cascade, was observed after muromonab-CD3 administration to human renal allograft recipients (Herbelin et al. 1999). The pretreatment of renal allograft

Table 9.2 Possible mechanisms of action of muromonab-CD3. Modified from Wilde MI et al. (1996).

Accepted mechanisms	*Supposed mechanisms*
• Antigenic modulation of the CD3/T-cell receptor complex on peripheral T cells resulting in failure of antigen recognition. • Opsonization of circulating T cells and subsequent removal by the reticuloendothelial system.	• Immunomodulation of graft-infiltrating lymphocytes. • Elimination of activated CD3+ cells by induction of apoptosis. • Modulation of CD3 complex by shedding CD3 antigens of the CD3 complex. • Increasing lymphocyte adhesion molecule expression on peripheral blood lymphocytes. • Induction of cell-mediated cytolysis.

patients with doses of up to 1 μg kg^{-1} bodyweight recombinant human (rh) IL-10 was found safe and reduced the release of tumor necrosis factor alpha (TNF-α) induced by muromonab-CD3. Higher doses however, seemed to promote early sensitization to muromonab-CD3 (Wissing et al. 1997).

CD3+ cells reappear rapidly and reach pretreatment levels within a week after termination of muromonab-CD3 therapy. Increasing numbers of CD3+ cells have been observed in some patients during the second week of muromonab-CD3 therapy, possibly as a result of the development of neutralizing antibodies to the compound. Antibodies to muromonab-CD3 have been observed, occurring with an incidence of 21% (n = 43) for IgM, 86% (n = 43) for IgG, and 29% (n = 35) for IgE. The mean (± SD) time of appearance of IgG antibodies was 20 ± 2 days, while early IgG antibodies appeared by the end of the second week of treatment in 3% (n = 86) of the patients. Serum levels of muromonab-CD3 are measurable using an enzyme-linked immunosorbent assay (ELISA). During treatment with 5 mg per day for 14 days, mean serum-trough levels of muromonab-CD3 were increased during the first 3 days of administration, but then remained at steady state (mean 0.9 μg mL^{-1}) on Days 3 to 14. The levels obtained during therapy have been shown to block T-cell effector functions *in vitro*. Following *in-vivo* administration, leukocytes have been observed in cerebrospinal and peritoneal fluids, though the mechanism of this effect is not understood.

9.3.3 Activation of Human T Cells

The cytokines TNF-α, IL-2, -3, -6 and -10 and interferon gamma (IFN-γ) are all released following the administration of muromonab-CD3, associated with an acute-phase reaction involving C-reactive protein (CRP), neopterin, endothelin-1, complement, transferrin, alpha-1-proteinase inhibitor and neutrophilic granulocytes (Abramowicz et al. 1989; Chatenoud 1994; Ellenhorn et al. 1990; Gaston et al. 1991; Goumy et al. 1990; Kreis 1993; Raasveld et al. 1993). This response usually occurs after the first and possibly second and third dose(s). A similar response, particularly the release of IL-6 (Bloemena et al. 1990), may also occur later during the course of treatment if CD3+ cell levels are increased substantially. This may account for some of the late adverse events observed with muromonab-CD3. The T-cell-activating properties of muromonab-CD3 manifest clinically as first-dose events (Cytokine Release Syndrome; CRS), while T-cell receptor stimulation by muromonab-CD3 *in vitro* led to a strong increase in tyrosine phosphorylation in all specimens of kidney transplant recipients and healthy controls (Muller et al. 1999). Monocyte-dependent Fc receptor-mediated cell activation appears to be the main mechanism underlying these events (Ellenhorn et al. 1990; Gaston et al. 1991; Hoffman et al. 1992; Vossen et al. 1995; Woodle et al. 1992).

The administration of muromonab-CD3 to human renal allograft recipients led to a significant release of IL-10 that may play a major role in the down-modulation of the muromonab-CD3-triggered T-cell activation cascade (Herbelin et al. 1999).

The release of IL-6 and IL-10 may also be involved in the pathogenesis of Epstein–Barr virus (EBV)-associated lymphoproliferative disorders in transplant recipients receiving muromonab-CD3 (Goldman et al. 1992; Swinnen et al. 1993). Anti-CD3 monoclonal antibodies have also been shown to trigger T-cell mitogenesis *in vitro* (Chatenoud 1994).

Several agents, including corticosteroids and pentoxifylline, reduce muromonab-CD3-induced cytokine release (Chatenoud et al. 1990, 1991; Ferran et al. 1990; Leimenstoll et al. 1993). However, although corticosteroids can reduce first-dose cytokine-related adverse events, pretreatment with pentoxifylline does not appear to reduce the CRS associated with prophylactic muromonab-CD3 (Alegre et al. 1991; De Vault et al. 1994).

9.3.4 Immunogenicity

The time to first appearance of anti-muromonab-CD3 antibodies in heart transplant patients who received muromonab-CD3-based quadruple sequential immunosuppression varied from Day 0 to Day 35. Anti-muromonab-CD3 antibodies decreased to negative levels in 11 of 12 patients who were initially positive following treatment by 1 year post transplant. Anti-idiotype and anti-isotype antibodies occurred at similar times and titers in patients who were anti-muromonab-CD3-antibody-positive (Bhat et al. 1997).

Stimulation of the T cells of healthy persons by muromonab-CD3 has led to a biphasic increase in phosphotyrosine levels, with the first peak occurring after 15 s and the absolute maximum occurring after 3 to 5 min. Levels remained high up to 30 min and subsequently returned to baseline. Using muromonab-CD3 to stimulate the T cells of bone marrow transplant (BMT) patients led to strong increases in phosphotyrosine levels comparable to those of controls. In contrast, the response of T cells of HIV-infected patients with AIDS-syndrome was severely impaired ($p = 0.01$) (Muller et al. 1998).

The selective depletion of activated type 1-like T cells by muromonab-CD3 resulted in longlasting immune deviation that might explain the long-term effects of muromonab-CD3 treatment (Reinke et al. 1997). Complement activation – an early event after muromonab-CD3 administration – was associated with the increased expression of adhesion molecules on neutrophils, and with pulmonary hemodynamic changes (Vallhonrat et al. 1999). T-cell depletion using muromonab-CD3 resulted in a higher T-cell content and higher rates of acute graft-versus-host disease (GvHD) and posttransplant lymphoproliferative disease (PTLD) compared with T10B9 (anti-TCR). A decreased risk of relapse for patients with high-risk disease was seen with muromonab-CD3-treated grafts (Keever-Taylor et al. 2001).

Following the infusion of muromonab-CD3 (three daily doses), a statistically significant increase in the binding of the mAb anti-factor V/Va to platelets (2.2% versus 12.8%, $p = 0.04$) was seen at 15 min after the second dose (Lozano et al. 2001). In uremic patients receiving prophylactic muromonab-CD3, an increase

in the binding of anti-factor V/Va was noted, denoting an increased exposure of anionic phospholipids in platelets.

High antibody titers (≥1 : 1000) were detected in 5.8% of 12 133 serum samples from patients who received muromonab-CD3 for the treatment or prevention of transplant rejection (Carey et al. 1995). These antibodies may result in decreased muromonab-CD3 plasma concentrations and increased circulating CD3+ levels, and may preclude re-use of the agent in some patients (Chatenoud 1993a).

The two types of anti-muromonab-CD3 antibodies that may be induced are anti-idiotypic and anti-isotypic (Chatenoud 1993a, 1994; Todd and Brogden 1989). Anti-idiotypic antibodies compete with muromonab-CD3 for binding to the CD3 complex, and can neutralize the activity of muromonab-CD3. IgG – but not IgM – anti-muromonab-CD3 antibodies are able to reduce the activity of the agent. Although anti-isotypic antibodies bind to the constant portion of the muromonab-CD3 antibody molecule, they do not block the effects of the drug (Wilde et al. 1996). Anti-idiotypic antibodies to the muromonab-CD3 do not cross-react with murine antibodies of similar or different isotypes, and cross-react with only 10% of other anti-CD3 mAbs (Norman 1992).

Although variation in antibody test results between centers is significant, the incidence of anti-muromonab-CD3 antibodies and the percentage of high antibody titers (≥1 : 1000) appears to be greatest in liver or kidney transplant recipients, and least in cardiac transplant recipients (Carey et al. 1995; Kimball et al. 1993; O'Connell et al. 1991; Schroeder et al. 1994). The risk of high anti-muromonab-CD3 antibody titers also appears to be greatest in patients aged <30 years, in those who have undergone previous transplantation or muromonab-CD3 courses, and in those receiving muromonab-CD3 for rescue treatment (versus prophylaxis or first-line treatment of rejection) (Carey et al. 1995). The administration of concomitant immunosuppressants reduces the likelihood of anti-muromonab-CD3 antibody formation (Chatenoud 1993a, 1994; Kreis 1993; Norman et al. 1993; Schroeder et al. 1990; Taylor et al. 1994).

9.3.5 Interactions

Concomitant medications (azathioprine, corticosteroids, CsA) may have contributed to the neuropsychiatric, infectious, nephrotoxic, thrombotic, and/or neoplastic events reported in patients treated with muromonab-CD3. A study by Vasquez and Pollack (1997) presented clinical evidence for an interaction between muromonab-CD3 and CsA, a CYP3A4 substrate and p-glycoprotein inhibitor. The authors postulated that cytokine release by muromonab-CD3 and subsequent cytokine-induced inhibition of CYP3A4 enzyme activity might be responsible for elevations in CsA blood levels observed during muromonab-CD3 therapy. The study was designed as a single-center retrospective analysis in 33 subjects (17 subjects received combined muromonab CD3/CsA versus 16 subjects receiving CsA/anti-lymphocyte globulin [ALG]). Generally, CsA is known to demonstrate high inter-patient variability (Mendez et al. 1999), and changes in plasma protein

binding may impact its clearance and thus its plasma or blood concentrations (Strong et al. 1997). CsA is also known to have saturable blood and tissue distribution (Tanaka et al. 1999), which may cause higher variability in the estimations of blood concentrations. As these authors did not standardize their dose adjustment algorithm, the adjustments made might be arbitrary and further strengthen the fact that that there was a large variability in the CsA blood concentrations. In view of the above-mentioned limitations, it is difficult to ascertain whether the described effect is indeed true.

A summary report by Mignat (1997) provided clinically significant drug interactions with immunosuppressive agents, although there was no mention of interactions of muromonab-CD3 with either tacrolimus or CsA. The use of indomethacin by some patients who were simultaneously receiving therapy with muromonab-CD3 may have contributed to some encephalopathic and other CNS adverse events, but the mechanisms of these effects are unknown.

9.4 Therapeutic Use

Muromonab-CD3 is indicated for the treatment of acute allograft rejection in renal transplant patients, and for the treatment of steroid-resistant acute allograft rejection in cardiac and hepatic transplant patients. The effectiveness of muromonab-CD3 for prophylaxis of renal allograft rejection has not been established. The dosage of other immunosuppressive agents used in conjunction with muromonab-CD3 should be reduced to the lowest level compatible with an effective therapeutic response. The recommended dose of muromonab-CD3 to treat acute renal, steroid-resistant cardiac or steroid-resistant hepatic allograft rejection in adults is 5 mg per day in a single (bolus) IV injection given in less than 1 min, over a period of 10 to 14 days. Lower initial doses of muromonab-CD3 (<5 mg day^{-1}) may be effective and better tolerated than higher doses (Norman et al. 1991, 1994). Rejection incidences with low-dose muromonab-CD3-based induction therapy in renal transplant recipients were similar to or lower than those with higher dosages (Norman et al. 1991, 1994).

High muromonab-CD3 doses (10 mg day^{-1}) have been associated with intragraft thromboses, whilst single doses (30, 40 or 50 mg) have provided no advantages in terms of efficacy over a 5 mg day^{-1} dose, and were in fact associated with a high incidence of adverse events (Welter et al. 1990).

In pediatric patients, the initial recommended dose is 2.5 mg day^{-1} for those weighing ≤30 kg, and 5 mg day^{-1} for those weighing >30 kg; all daily doses are given as a single (bolus) IV injection in less than 1 min, and for 10 to 14 days. Daily increases in muromonab-CD3 doses (i.e., 2.5-mg increments) may be required to achieve depletion of CD3+ cells (<25 cells mm^{-3}) and ensure therapeutic muromonab-CD3 serum concentrations (>800 ng mL^{-1}). Pediatric patients may require augmentation of the muromonab-CD3 dose. In all patients with acute renal rejection, treatment should begin upon diagnosis, but for

steroid-resistant cardiac or hepatic allograft rejection the treatment should begin when a rejection has not been reversed by an adequate course of corticosteroid therapy.

Laboratory screening should be carried out prior to the administration of muromonab-CD3. Likewise, the patient's volume (fluid) status and a chest X-radiograph should be assessed to rule out volume overload, uncontrolled hypertension, or uncompensated heart failure. Patients should not weigh >3% above their minimum weight during the week prior to injection. The following investigations should be conducted prior to and during muromonab-CD3 therapy:

- Renal: Blood urea nitrogen (BUN), serum creatinine, etc.;
- Hepatic: transaminases, alkaline phosphatase, bilirubin;
- Hematopoietic: WBCs and differential, platelet count, etc.;
- Chest X-radiograph within 24 h before initiating muromonab-CD3 treatment to rule out heart failure or fluid overload.
- Blood tests: Periodic assessment of organ system functions (renal, hepatic, and hematopoietic).

In addition, during therapy with muromonab-CD3 the following parameters should be followed:

- Plasma muromonab-CD3 levels to be $\geq 800\,ng\,mL^{-1}$; and
- T-cell clearance (CD3+ T cells <25 cells mm^{-3}).

In adults, these should be monitored *periodically*; in pediatric patients, they should be monitored *daily*.

The end-points of immunosuppressive trials should include rejection incidence and severity, time to first rejection episode, and effects on organ function as well as graft and patient survival. Two types of induction therapy to prevent rejection are used in transplantation:

- Administration of an anti-lymphocyte preparation, azathioprine and corticosteroids from the time of transplantation with CsA withheld until renal function is established (sequential therapy).
- Administration of an anti-lymphocyte preparation, azathioprine, corticosteroids and low-dose CsA followed by maintenance therapy.

Muromonab-CD3 was usually administered during the immediate postoperative period, although some patients received the drug intraoperatively (Wilde et al. 1996).

Most clinical trials were randomized, but the majority were not double-blind because the first-dose reaction to muromonab-CD3 and different modes of administration of anti-lymphocyte antibodies precluded double-blinding. Therefore, small numbers of patients were involved in the studies. Dosage regimens of concomitant immunosuppressants were varied and complex, and the administration

of muromonab-CD3 was preceded by a variety of prophylactic agents, making between-study comparisons difficult. Most recipients were undergoing cadaveric transplantation. Rejection episodes were documented histologically or with appropriate laboratory tests of organ function (Wilde et al. 1996).

Factors influencing the outcome of rejection prophylaxis include:

- Primary disease and severity including baseline organ function.
- The age and gender of the recipient and donor (Wechsler et al. 1995).
- Pre-existing anti-muromonab-CD3 antibody titers.
- Delayed graft function (Howard et al. 1993; Troppmann et al. 1995).
- ABO blood group compatibility.
- Previous transplantation or blood transfusions.
- Donor/recipient cytomegalovirus (CMV) status.
- Histocompatibility antigen matching and preformed reactive anti-HLA antibodies, although the potential beneficial effects of HLA-matching, especially in those receiving anti-lymphocyte agents, are controversial (Costanco-Nordin et al. 1993; Kermann et al. 1994).
- Duration of organ cold ischemia time.
- Surgical technique.
- Postoperative patient management, including treatment of rejection.

9.4.1 Renal and or Renal-Pancreas Transplant Recipients

Some studies with muromonab-CD3-based immunosuppression and triple therapy or regimens containing other lymphocyte preparations are summarized in Table 9.3.

Muromonab-CD3 was successfully used by Chkotua et al. (2003) as effective treatment for steroid-resistant rejections in renal transplant patients with severe rejection who did not respond to the first few doses of antithymocyte globulin (ATG). In a prospective, randomized trial with thymoglobulin/daclizumab induction, tacrolimus and steroid maintenance, comparing rapamycin with mycophenolate mofetil (MMF) as immunosuppression in simultaneous pancreas renal-pancreas transplantation, five acute rejection episodes in the MMF group were steroid-resistant, but responsive to muromonab-CD3 or thymoglobulin (Ciancio et al. 2004). Another study compared the outcomes of renal transplantation with two distinct induction protocols, basiliximab versus muromonab-CD3, in the setting of CsA-based immunosuppression. Post-induction protocols included either total prednisone avoidance or prednisone sparing versus standard prednisone dosing. A total of 245 adult patients receiving kidney transplantation between 1995 and 2000 was included in the study. Treatment in group 1 was

Table 9.3 Efficacy of muromonab-CD3 (M-CD3) immunosuppression (delayed cyclosporine A) as prophylaxis of renal allograft rejection versus standard triple therapy (immediate cyclosporine A) or other anti-lymphocyte-based regimens. A selection of clinical studies. Modified from Wilde et al. (1996).

Reference	*Study design*	*No. of evaluable patients (characteristics)*	*Treatment*	*Time to initial rejection [days]*	*Actuarial graft survival [%]*	*Rejection incidence [%]*	*Actuarial patient survival [%]*	*Overall efficacy*	*Comments*
Abramowicz et al. (1994)	Randomized	56	M-CD3[a]	23 (first 3 months)	83 (overall; 3 years) 92 graft survival	32 (3 years)	94.5 (3 years)	M-CD3 ≥ triple therapy	Fewer rejections with M-CD3 corticosteroid-resistant (15 vs. 30%)
		52	Triple therapy[c]	11 (first 3 months)	75 (overall; 3 years) 79 graft survival	22 (3 years)	93 (3 years)		
Benvenisty et al. (1990)	Non-blinded	34 (delayed graft function)	M-CD3[a]		80 (1 year) 74 (2 years)	44	89	M-CD3 ≥ triple therapy	M-CD3 decreased the duration of graft non-function (9.4 vs. 14.9 days)
		40 (delayed graft function)	Triple therapy[c]		55 (1 year) 47 (2 years)	82	89		
Bock et al. (1995)	Randomized, non-blinded	51	M-CD3[a]		78 (1 year)	45	92 (1 year)	ATG > M-CD3	
		53	ATG[d]		91 (1 year)	26	96 (1 year)		
Broyer et al. (1993)	Randomized	77	M-CD3[a]		79 (1 year) 71 (2 years) 68 (3 years)	11	96	M-CD3 = ALG	
		71	ALG[b]		80 (1 year) 77 (2 years) 73 (3 years)	11	99		

Table 9.3 *Continued*

Reference	*Study design*	*No. of evaluable patients (characteristics)*	*Treatment*	*Time to initial rejection [days]*	*Actuarial graft survival [%]*	*Rejection incidence [%]*	*Actuarial patient survival [%]*	*Overall efficacy*	*Comments*
Cole et al. (1994)	Randomized	83	M-CD3[a]		81 (1 year)	31 (patients with no rejection)	95 (1 year) 89.5 (1 year)	ATG ≥ M-CD3	More steroid-resistant rejections with M-CD3 (25 vs. 12)
		83	ATG[d]		78 (1 year)	57 (patients with no rejection)			
Frey et al. (1992)	Randomized	67	M-CD3[a]	M-CD3 = ALG	87 (1 year) 83 (2 years)	36	96 (1 year) 96 (2 years)	M-CD3 = ALG	
		71	ALG[b]		84 (1 year) 80 (2 years)	45	93 (1 year) 91 (2 years)		
Hanto et al. (1994)	Randomized	59	M-CD3[a]	35	84 (1 year) 79 (2 years) 79 (2 years)	33 (37)	98 (1, 2, and 3 years)	M-CD3 = ALG	
		58	ALG[b]	29	81 (1 year) 78 (2 years) 78 (3 years)	27 (31)	96 (1, 2, and 3 years)		
Steinmuller et al. (1991)	Randomized	25	M-CD3[a]	67.5	84 (<6 months)	64	99 (<6 months)		
		26	ALG[b]	95	85 (<6 months)	35	99 (<6 months)		

a MCD3-based regimen: MCD3 + Az + MPr + Pr + CsA.
b ALG + MPr + Az + CsA.
c CsA + Az + MPr + Pr.
d ATG + MPr + Az + CsA.
Efficacy status: = indicates equivalence; > indicates significantly ($p < 0.05$) more effective than comparator; > indicates significantly ($p < 0.05$) greater efficacy than comparator in terms of at least one efficacy parameter; *$p < 0.05$ compared with comparator.
ALG = anti-lymphocyte globulin (Minnesota; horse antibody); ATG = anti-thymocyte globulin; Az = azathioprine; CsA = cyclosporine A; MPr = methylprednisolone; Pr = prednisone.

muromonab CD3 + CsA + adjunct + standard prednisone; group 2 received basiliximab + CsA + adjunct + steroid sparing; group 3 received basiliximab + CsA + adjunct + no prednisone. The demographics between all groups were similar. The incidences of acute rejection within 1 year in the respective groups were 28% versus 15% versus 16%. Thus, the authors concluded that the use of basiliximab in transplant recipients resulted in long-term patient and graft survival similar to those achieved with muromonab-CD3 (Kung et al. 2004).

9.4.2 Liver Transplant Recipients

In addition to the toxic effects of early CsA use, hepatic transplant recipients are at an increased risk of renal impairment during the early postoperative period because of intraoperative hemodynamic instability, hepatorenal syndrome and pre-existing renal or hepatic dysfunction. Studies comparing muromonab-CD3-based immunosuppression with triple therapy or regimens containing other anti-lymphocyte preparations or anti-IL-2 antibody are summarized in Table 9.4.

In a retrospective study of 156 patients with recurrent primary biliary cirrhosis after liver transplantation, 10.9% of patients experienced recurrence (Sanchez et al. 2003). Muromonab-CD3 was used for steroid-resistant rejection in 41% of recurrent primary biliary cirrhosis patients, and in 23.7% of nonrecurrent primary biliary cirrhosis patients. There was no significant difference in the development of recurrent primary biliary cirrhosis between the groups ($p = 0.36$). Arenas et al. (2003) evaluated the influence of rejection treatment (methylprednisolone) on hepatitis C virus (HCV) quasispecies after liver transplantation, with muromonab-CD3 being used in two patients for steroid-resistant rejection. Those liver transplant recipients treated for rejection had a decrease in HCV quasispecies diversity after transplantation.

9.4.3 Cardiac Transplant Recipients

The prevention of rejection is particularly important for cardiac transplant recipients because, unlike renal transplant failure, cardiac graft failure is usually fatal. Although direct comparisons are few in number, on the available evidence muromonab-CD3-based therapy appears to be similar to CsA plus prednisone or triple therapy as assessed by rejection incidence, time to first rejection episode, and graft and patient survival (see Table 9.5). The time to rejection was significantly longer with muromonab-CD3-based induction therapy than triple therapy, but there was no significant between-treatment difference in rejection incidence (Barr et al. 1990). The results of selected studies are summarized in Table 9.5. Data relating to muromonab-CD3-based therapy compared with other anti-lymphocyte-based regimens are conflicting. For example, some investigators have reported a longer time to first rejection and/or a lower incidence of rejection with muromonab-CD3 (Costanco-Nordin et al. 1990; Renlund et al. 1989), whereas

Table 9.4 Efficacy of muromonab-CD3 (M-CD3) and delayed cyclosporine A (CsA) as prophylaxis of hepatic allograft rejection versus standard triple therapy (Tr) (immediate CsA), other anti-lymphocyte-based regimens or anti-interleukin-2 monoclonal antibody-based immunosuppression. Modified from Wilde et al. (1996).

Reference	*Study design*	*No. of evaluable patients (characteristics)*	*Treatment*	*Time to initial rejection [days]*	*Actuarial graft survival [%]*	*Rejection incidence [%]*	*Actuarial patient survival [%]*	*Overall efficacy*	*Comments*
Farges et al. (1994)	Randomized, multicenter	44	M-CD3[a]	38	61 (4 years)	34 (2 weeks) 67 (1 year)	82 (1 year) 69 (4 years)	M-CD3 ≥ triple therapy	
		50	Triple therapy[b]	9	54 (4 years)	61 (2 weeks) 75 (1 year)	78 (1 year) 62 (4 years)		
McDiarmid et al. (1991a,b)	Randomized	46	M-CD3[a]	12	63 (>3 months)	28 (postop. day 0–14) 46 (<1 months) 91 (>3 months)	67 (>3 months)	M-CD3 ≥ triple therapy	Mean duration of follow-up 648 days for M-CD3 and 682 days for triple therapy
		39	Triple therapy[b]	9.5	73 (>3 months)	67 (postop. day 0–14) 31 (rejection-free < 1 month) 99 (rejection-free >3 months)	84 (>3 months)		
Mühlbacher et al. (1989)		30 58	M-CD3[a] Triple therapy[b]			56 79.5	57 (1 year) 45 (1 year)	M-CD3 > triple therapy	

Pons et al. (1993)		25 19	M-CD3[a] Triple therapy[b]		25 69	3.5 (mortal. 2 months) 26 (mortal. 2 months)		
Reding et al. (1993)	Randomized	37 (adults + children)	M-CD3[a]	86 (1 year)	81 (first 3 months)	86 (1 year)		
		35	Anti-IL-2 receptor[d]	97 (1 year)	91 (first 3 months)	100 (1 year)		
		28	Triple therapy[b]	75 (1 year)	96 (first 3 months)	79 (1 Year)		
Steininger et al. (1991)	Retrospective	63	CsA+Pred.		67 (first 3) weeks		ATG > CsA + pred./ M-CD3	Not standard regimens. Rejection advantage of ATG abolished when severe rejection observed
		32	M-CD3[a]		44			
		49	ATG[c]		24			
Cosimi AB, et al. 1990	Randomized	38	M-CD3[a]		13/39 (1/2 weeks) 68 (1 year)	84	M-CD3 ≥ triple therapy	Duration of the initial rejection-free period longer with M-CD3
		41	Triple therapy[b]		46/71 (1/2 weeks) 78 (1 year)	73		

For details of superscripts, abbreviations, etc., see Tab. 9.3.

Table 9.5 Selection of clinical studies concerning efficacy of muromonab-CD3 (M-CD3) and delayed cyclosporine A (CsA) as prophylaxis of cardiac allograft rejection versus standard triple therapy and immediate CsA or anti-lymphocyte regimens. Modified from Wilde et al. (1996).

Reference	*Study design*	*No. of evaluable patients (characteristics)*	*Treatment*	*Time to initial rejection (days)*	*Actuarial graft survival (%)*	*Rejection Incidence (%)*	*Actuarial patient survival (%)*	*Overall efficacy*
Balk et al. (1991)	Randomized	33	M-CD3[a]		91	1.33 (1 year) 80/31/28 (1, 3, 6 months)	91 (2 years)	M-CD3 = CysA + prednisone
		33	CsA + Pr		94	1.36 (1 year) 66, 33, 27 (1, 3, 6 months)	94 (2 years)	
Barr et al. (1990)	retro-spective	26	M-CD3[a]	42		0.003	88/81 (6/18 months)	M-CD3 = triple therapy
		26	Triple therapy[b]	21		0.003	92/87 (6/18 months)	
Costanzo-Nordin et al. (1990)	Randomized	12	M-CD3[a]	32		3.4		M-CD3 ≥ ATG
		11	ATG[c]	15		5		
Griffith et al. (1990)	Randomized	43	M-CD3[a]	33		0.58/pat.; 0.43/pat.; 0.2 (1; 2; >2 months)	98 (1 year) 95 (1 year)	M-CD3 ≤ ATG
		39	ATG[c]	67		0.08/pat.; 0.24/pat.; 0.2 (1; 2; >2 months)		

Ippoliti et al. (1991)	Randomized	15 15	M-CD3[a] ATG[c]			80 (6 months) 93 (6 months)	ATG ≥ M-CD3
Ladowski et al. (1993)	Randomized	30 34 15	M-CD3[a] ATG[c] ALG[d]		0.8/pat. (3 months) 1.03/pat. (1 year) 0.24/pat. (3 months) 0.26/pat. (1 year) 1.14/pat. (3 months) 1.27/pat. (1 year)	83 (1 year) 82 (1 year) 80 (1 year)	ATG ≥ ALG and M-CD3
Macdonald et al. (1993)	Randomized	20 21	M-CD3[a] ATG[c]	33 27	90 100	83 (1 year) 81 (1 year)	M-CD3 = ATG
Menkis et al. (1992)	Randomized	20 19	M-CD3[a] ALG	5.6 weeks 5.3 weeks	2.1/pat. (6 months) 1.4/pat. (6 months)	92 (2 years) 84 (2 years)	M-CD3 = ALG
Renlund et al. (1989)	Randomized	26 25	M-CD3[a] ATG[c]	76 36	1.5/pat. (6 months) 0.2/pat. (1 year) 2.2/pat. (6 months) 0.8/pat. (1 year)		

For details of superscripts, abbreviations, etc., see Tab. 9.3.

others have not observed any differences (Kirklin et al. 1990; Macdonald et al. 1993; Menkis et al. 1992; Wollenek et al. 1989) or have reported a lower incidence of rejection (Griffith et al. 1990; Ladowski et al. 1993) and/or a longer time to first rejection episode (Griffith et al. 1990; Ippoliti et al. 1991) with ATG-based therapy. No between-treatment differences in graft or patient survival have been reported (Wilde et al. 1996).

9.5 Cytokine Release Syndrome

Cytokines are small peptides that regulate cell growth and tissue homeostasis. Proinflammatory cytokines, such as IL-1, -2, and -6, INF-γ, and TNFs play an important role in allograft rejection. Understanding the cytokine response is crucial to the effective management of the transplant patient. Cytokines are multifunctional, are involved in virtually all organ systems, and have effects that are sometimes stimulatory and sometimes inhibitory. The cell-mediated event of acute rejection is stimulated by cytokines following the introduction of allogeneic organs to the transplant recipient. An enhanced production of IL-1, IL-6 and TNF-α results when APCs detect the presence of a foreign body. T-cell activation also increases the production of IL-2, which in turn produces an antigen-specific T-cell response. Other stimulated cytokines that extend the immune response include T lymphocyte-derived IL-2, IL-3, INF-γ, TNF-α, and TNF-β. The presence of adhesion molecules also promotes the proinflammatory response. The result of this cascade of events is the destruction and failure of the allograft tissue. Monoclonal antibodies such as muromonab-CD3 disrupt the cytokine process, thereby allowing for greater allograft transplantation success (Gaston 1994).

Muromonab-CD3 is directed to a glycoprotein in the human T-cell surface that is essential for T-cell function. In *in-vitro* cytolytic assays, muromonab-CD3 blocks both the generation and function of effector cells. Binding of muromonab-CD3 to T lymphocytes results in the early activation of T cells, which leads to cytokine release, followed by blocking of T-cell functions. T-cell activation results in the release of numerous cytokines/lymphokines, which are felt to be responsible for many of the acute clinical manifestations seen following muromonab-CD3 administration.

9.5.1 The Pathophysiology of the Cytokine Release Syndrome

Early investigators first attributed the fever, chills and other adverse reactions typically experienced with a dose of muromonab-CD3 to an idiosyncratic or allergic-type reaction, or to massive lymphocytolysis (Gaston 1994; Rossi et al. 1993). However, the observation that symptoms resolved after repeated doses of muromonab-CD3 challenged these theories. The current accepted etiology of the CRS involves a massive and transient release of proinflammatory cytokines into the

circulation, attributed to the mitogenic properties of muromonab-CD3 (Chatenoud et al. 1991; First et al. 1993; Rossi et al. 1993). The first dose of muromonab-CD3 induces a surge of cytokine secretion that is probably a response to the transient T-cell activation by the mAb (Gaston 1994). Several cytokines are seen in the circulation, including IL-1, IL-2, IL-3, IL-6, INF-γ, TNF, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (First et al. 1993; Jeyarajah et al. 1993). As soon as 1 h following administration of the first muromonab-CD3 dose, the release of TNF is observed in the circulation (Chatenoud 1993b; Gaston 1994). TNF-α is believed to be the key mediator of the CRS, causing the most severe symptoms of the first-dose response as well as meningeal inflammation, encephalopathy, nephropathy, and cardiopulmonary deterioration (Eason et al. 1996; Gaston, 1994; Jeyarajah et al. 1993; Rossi et al. 1993; Vincenti et al. 1993). IL-6 production also increases in the circulation approximately 6 to 48 h after muromonab-CD3 dosing (Chatenoud 1993b; Gaston 1994), while INF-γ and IL-2 peak at approximately 2 to 4 h after administration. Elevations decrease significantly with subsequent therapy. All cytokines return to values seen at baseline approximately 12 to 24 h following the first muromonab-CD3 treatment (Chatenoud 1993b).

Abramowicz et al. (1989) evaluated the presence of IL-2, INF-γ and TNF-α in the serum of renal transplant patients who had been treated prophylactically with muromonab-CD3. Beginning on the day of transplantation, nine patients received IV muromonab-CD3 (5 mg day^{-1}), azathioprine (2 mg kg^{-1} per day), and prednisolone (0.3 mg kg^{-1} per day) for 14 days, and six patients received orally on the day of transplantation CsA (6 mg kg^{-1}) and azathioprine (1 mg kg^{-1}), as well as methylprednisolone (2 mg kg^{-1}, intravenously). Muromonab-CD3 patients also received a 1 mg kg^{-1} bolus of methylprednisolone immediately preceding therapy, and 100 mg hydrocortisone 30 min later. No increase in TNF was found in patients treated with CsA, and these patients had no detectable levels of IL-2 or INF-γ. However, all nine patients treated with muromonab-CD3 experienced a significant rise in the serum levels of TNF ($p < 0.01$), IL-2 ($p < 0.01$) and INF-γ ($p < 0.01$). The TNF levels peaked at 1 h following muromonab-CD3 administration, and remained elevated compared to those in CsA-treated patients ($p < 0.05$) for 4 h. IL-2 and INF-γ first became evident in the serum at 1 h after dosing, and peaked after 2 h in eight of the nine patients. IL-2 and INF-γ were not detectable in the serum after 24 h. All patients developed a fever ($p < 0.01$ compared with CsA-treated patients). The second and third injections of muromonab-CD3 induced no significant release of cytokines, with the exception of one patient who exhibited a low level of INF at 2 h after the second injection.

Several authors have suggested that the CRS appears only after the first few injections of muromonab-CD3 (Abramowicz et al. 1991; Chatenoud 1993b; Chatenoud et al. 1991; First et al. 1993; Rossi et al. 1993; Todd et al. 1989). However, Vasquez et al. (1995) conducted a retrospective review which suggested that many patients continue to experience this syndrome subsequent to the first two doses. In a review of 83 evaluable renal transplant patients, the investigators discovered that 50% of patients experienced fever and chills at the first dose.

However, over one-third of patients developed reactions that were apparently mediated by muromonab-CD3 after doses beyond the second. Those patients who experienced the later effects were usually being treated for rejection rather than to induce immunosuppression. The authors concluded that the extension of adverse reactions to the later doses suggests that other mechanisms and/or mediators to this phenomenon might exist that have yet to be identified.

9.5.2 Symptoms of the Cytokine Release Syndrome

Most patients develop this acute clinical syndrome associated with the first few doses of muromonab-CD3 (particularly the first two to three doses). Symptoms generally resolve after 4 to 6 h, and cessation of therapy is usually not necessary (Rossi et al. 1993). The CRS has ranged from a more frequently reported mild, self-limited, "flu-like" illness to a less frequently reported severe, life-threatening shock-like reaction, which may include serious cardiovascular and CNS manifestations. The syndrome typically begins approximately 30 to 60 min after administration of a dose of muromonab-CD3 (though it may also occur later) and may persist for several hours. The frequency and severity of this symptom complex are usually greatest with the first dose. With each successive dose of muromonab-CD3, both the frequency and severity of the CRS tend to diminish. Increasing the amount of muromonab-CD3 or resuming treatment after a hiatus may result in a reappearance of the CRS.

The CRS displays a distinctive group of clinical signs and symptoms (Jeyarajah et al. 1993). Common clinical manifestations may include high fever (often spiking >40 °C), chills/rigors, headache, tremor, nausea/vomiting, diarrhea, abdominal pain, malaise, muscle/joint aches and pains, and generalized weakness. Less frequently reported adverse experiences include minor dermatologic reactions (e.g., rash, pruritus, etc.) and a spectrum of often serious, occasionally fatal, cardiorespiratory and CNS adverse experiences. A higher frequency of cardiac, gastrointestinal, and neurologic adverse events is usually observed in patients treated prophylactically than is observed in those treated for actual acute rejection (Jeyarajah et al. 1993).

Cardiorespiratory findings may include dyspnea, shortness of breath, bronchospasm/wheezing, tachypnea, respiratory arrest/distress, cardiovascular collapse, cardiac arrest, angina/myocardial infarction, chest pain/tightness, tachycardia (including ventricular), hypertension, hemodynamic instability, hypotension including profound shock, heart failure, pulmonary edema (cardiogenic and noncardiogenic), adult respiratory distress syndrome, hypoxemia, apnea, and arrhythmias.

In the initial studies of renal allograft rejection, potentially fatal severe pulmonary edema occurred in 5% of the initial 107 patients. Fluid overload was present before treatment in all of these cases. The reaction did not occur in any of the subsequent 311 patients treated with first-dose volume/weight restrictions. In subsequent trials, severe pulmonary edema has also occurred in patients who

appeared to be euvolemic. The pathogenesis of pulmonary edema may involve all or some of the following: volume overload, increased pulmonary vascular permeability, and/or reduced left ventricular compliance/contractility. During the first 1 to 3 days of muromonab-CD3 therapy, some patients have experienced an acute and transient decline in the glomerular filtration rate (GFR), there being a diminished urine output with a resultant increase in the level of serum creatinine. A massive release of cytokines appears to lead to reversible renal functional impairment and/or delayed renal allograft function. Similarly, transient elevations in hepatic transaminases have been reported following administration of the first few doses of muromonab-CD3.

In controlled clinical trials for the treatment of acute renal allograft rejection, patients treated with muromonab-CD3 plus concomitant low-dose immunosuppressive therapy (primarily azathioprine and corticosteroids) were observed to have an increased incidence of adverse experiences during the first 2 days of treatment, as compared with the group of patients receiving azathioprine and high-dose steroid therapy. During this period the majority of patients experienced pyrexia (90%; 40.0 °C or above in 19% of cases) and chills (59%). In addition, other adverse experiences occurring in 8% or more of the patients during the first 2 days of muromonab-CD3 therapy included dyspnea (21%), nausea (19%), vomiting (19%), chest pain (14%), diarrhea (14%), tremor (13%), wheezing (13%), headache (11%), tachycardia (10%), rigor (8%), and hypertension (8%). A similar spectrum of clinical manifestations has been observed in open clinical studies and in post-marketing experience involving patients treated with muromonab-CD3 for rejection following renal, cardiac, and hepatic transplantation.

9.5.3
Muromonab-CD3 and the Cytokine Release Syndrome

Thistlethwaite et al. (1988) reported the complications associated with muromonab-CD3 therapy in 122 renal transplant recipients. Muromonab-CD3 was administered to 83 patients for rejection treatment, and to 39 patients for prophylactic immunosuppression induction. Therapy for all patients was supplemented with CsA, and most received triple therapy that included CsA, prednisone, and azathioprine. Muromonab-CD3 (5 mg day^{-1}, 2.5 mg day^{-1} for children under 30 kg) was administered to 39 patients treated for induction, along with azathioprine 3 mg kg^{-1} per day and prednisone 0.25 mg kg^{-1} per day. Treatment of muromonab-CD3 continued for 7 or 14 days. Two days prior to the last muromonab-CD3 dose, CsA (10 mg kg^{-1} per day) was given, and the dose of azathioprine was reduced to 2 mg kg^{-1} per day. In the 83 patients treated for rejection therapy, methylprednisolone (5–10 mg kg^{-1}) was given up to three times a day in addition to baseline immunosuppression. A percutaneous needle biopsy of the renal allograft was performed to confirm a rejection diagnosis if adequate response was not achieved. If rejection was confirmed, muromonab-CD3 (5 mg day^{-1}, 2.5 mg day^{-1} for children under 30 kg) was administered. Adult patients received acetaminophen (paracetamol) 600 mg and diphenhydramine 50 mg prior to the first muromonab-

CD3 dose, and hydrocortisone 100 mg 30 min later. CsA was discontinued while azathioprine was reduced to 25 mg day^{-1} on the day that muromonab-CD3 was commenced. Peripheral blood CD3+ cell counts were monitored prior to muromonab-CD3 therapy, and then three times weekly.

The adverse event experienced with highest incidence in both the rejection treatment and immunosuppression groups during the 122 courses of muromonab-CD3 treatment was hyperpyrexia (89% and 92%, respectively). Most patients experienced this event during the first treatment only; however, some continued to develop fevers at later doses, and a few experienced post-dose fever spikes throughout the course of therapy. Fluid-overload contributed to hypertension and/or mild dyspnea, and tachycardia and/or hypotension were credited to relative volume depletion due to planned dialysis or forced diuresis prior to therapy. There were no reports of serious pulmonary complications. Headache (35% and 54%, respectively) and rigors (29% and 31%, respectively) were also frequently reported events. Self-limiting events included gastrointestinal symptoms (nausea/vomiting/diarrhea) and CNS effects (headache, and rare cases of aseptic meningitis or seizures). Most of these events occurred between Days 2 and 5 of treatment, and they generally resolved after 2 to 4 days. Aseptic meningitis developed in four patients (3%), three of whom also had leukocytosis revealed by lumbar puncture. Fifty-two patients (44%) experienced headaches without any other CNS sequelae. Generalized seizures were reported in eight patients (6%); these patients presented with multiple risk factors, including fever and hypercalcemia. The use of muromonab-CD3 in uremic patients during the early postoperative period tended to predispose patients to seizure activity. There were only rare reports of late adverse events to muromonab-CD3 therapy. Infectious complications were an identified late event of muromonab-CD3 therapy, and two-thirds of patients who received two or three doses experienced infections, compared to one-sixth of patients who received only one prophylactic dose.

A randomized, prospective, double-blind study was conducted by Norman et al. (1993) to compare the severity of CRS between two dosage regimens of muromonab-CD3. A total of 26 renal transplant patients was enrolled. Patients in the high-dose arm received 5 mg muromonab-CD3 for 12 days (60 mg total), while those in the low-dose arm received 1 mg muromonab-CD3 for 2 days, followed by 2 mg daily for 10 days (22 mg total). All patients also received a standard immunosuppression regimen of CsA, azathioprine, methylprednisolone, and prednisone. There were no differences in demographics or medical history between the groups, except that more men were randomly assigned to the low-dose group ($p < 0.05$). CRS side effects, including anorexia, nausea, headache, vomiting, diarrhea, weakness, dyspnea, pulmonary edema, and hallucinations, were observed with equivalent frequency between the two groups. The high-dose group also had no significantly greater degree and occurrence of pyrexia than the low-dose group. There were significantly fewer reports of hypotension in the low-dose group ($p < 0.02$). In this study, both protocols were effective, but the lesser degree of hypotension seen with the low-dose protocol improved its safety profile over the high-dose regimen.

9.5.4 Management of the Cytokine Release Syndrome

Clinical management of the CRS involves precautionary measures to prevent the serious sequelae of first-dose reactions. No treatment modality is known completely to prevent this syndrome, but the severity can be greatly reduced with careful patient management (Jeyarajah et al. 1993). Several pharmaceutical agents have been used to decrease the severity of CRS related to muromonab-CD3 therapy. Acetaminophen has been administered 30 min prior to therapy for fever reduction, and diphenhydramine is often administered in an attempt to ward off any allergic-type reaction. Methylprednisolone is used for both antipyretic and anti-inflammatory properties. Patients with fluid overload benefit from forced diuresis, hemodialysis, or hemofiltration to prevent pulmonary edema. Muromonab-CD3 should be avoided in patients who are more than 3% over their baseline bodyweight, or who demonstrate hemodynamic instability. Together with chest radiography to rule out fluid overload, these measures can dramatically reduce the likelihood of life-threatening complications. Finally, bronchospasm may be controlled by nasal oxygen or inhaled bronchodilators (Gaston 1994; Rossi et al. 1993).

9.5.4.1 Methylprednisolone

Corticosteroids have nonspecific immunosuppressive and anti-inflammatory activity, which makes this class of drug a logical choice for use in transplant patients. Research has shown that increased doses of these agents are also useful in inhibiting cytokine synthesis (Rossi et al. 1993). Goldman et al. (1989) conducted a study to evaluate the effect of high-dose methylprednisolone prior to the first muromonab-CD3 injection on levels of cytokine release. Peak levels of TNF, INF-γ, and IL-2 were compared in patients receiving an IV bolus of either 1 or 8 $mg\,kg^{-1}$ methylprednisolone 4 h prior to the administration of muromonab-CD3. The extent of TNF and INF-γ release was significantly reduced in patients receiving high-dose methylprednisolone ($p < 0.05$) compared to those receiving low-dose therapy. Additionally, only five of 15 patients pretreated at high doses required postoperative dialysis, in contrast to 14 of the 21 patients treated with low-dose methylprednisolone ($p = 0.04$). These authors recommended that high-dose methylprednisolone be administered by IV bolus preceding muromonab-CD3 therapy in kidney transplant patients.

Chatenoud et al. (1991) conducted a prospective, randomized trial to determine the optimal timeframe for administration of high-dose corticosteroids with muromonab-CD3 therapy. Three groups of patients participated in this study. Group 1 consisted of 27 renal transplant recipients who received 5 $mg\,day^{-1}$ muromonab-CD3 for 14 days for rejection prophylaxis. In addition, patients received corticosteroids (0.25 $mg\,kg^{-1}$ per day) and azathioprine (3 $mg\,kg^{-1}$ per day). All patients in Group 1 were given high-dose (1 g) methylprednisolone without any precise schedule, either at the same time as the first muromonab-CD3 injection or between 30 min and 4 h later. The six patients assigned to Group 2 received

intrafamilial renal transplant, and consequently did not receive daily corticosteroids. These patients received 5 mg day^{-1} muromonab-CD3 for 20 days and azathioprine (3 mg kg^{-1} per day). Three patients did not receive the 1-g dose of methylprednisolone, while three other patients received the same pretreatment as patients in Group 1 due to the severity of their illness. Again, no dosing schedule was followed for corticosteroid administration. Data from 12 renal transplant patients in Group 3 were included in an open randomized analysis, where the regimen was identical to that of Group 1. However, six of the patients were assigned to receive high-dose methylprednisolone concomitantly while six were assigned to receive it 1 h preceding muromonab-CD3 administration.

In Group 1, a marked increase in TNF plasma levels was observed. Levels started to decrease at 4 h after the injection of muromonab-CD3, but remained higher than pretreatment values. Subsequent injections did not produce any further increases in TNF levels. At 1 h after injection, INF-γ levels increased, and then peaked by 4 h. Although the nonprospective collection approach made the clinical data difficult to assess, a trend was recognizable: corticosteroids given 30 to 60 min prior to muromonab-CD3 decreased the CRS symptoms and decreased cytokine levels. Group 2 exhibited the highest TNF and INF-γ levels, associated with serious acute clinical syndrome. The results from patients in Group 3 confirmed that the methylprednisolone administration schedule markedly affects the activity of TNF and INF-γ. With high-dose methylprednisolone given 1 h before muromonab-CD3 therapy, TNF levels were significantly lower ($p < 0.007$) than with concomitant administration. The same effect holds true for levels of INF-γ measured at 4 h after muromonab-CD3 administration ($p < 0.003$). Although these data are encouraging, the authors state that anesthesia was given to all patients and may have contributed to the improved symptomatology. The authors recommend administering high-dose methylprednisolone 1 h prior to muromonab-CD3 therapy to decrease cytokine production and resultant CRS.

Bemelman et al. (1994) attempted to reduce the severity of CRS symptoms by manipulating the time interval between steroid and muromonab-CD3 administration in renal allograft recipients treated for acute cellular rejection. Three groups of patients were treated with 5 mg kg^{-1} muromonab-CD3 for 10 days. Group 1 ($n = 10$) received 500 mg methylprednisolone at 6 h before the first muromonab CD3 dose; Group 2 ($n = 10$) received the same dose of methylprednisolone at 1 h prior; and Group 3 ($n = 6$) received two equal doses of methylprednisolone 250 mg, one given at 6 h and one at 1 h prior to muromonab-CD3 bolus injection. All methylprednisolone doses were administered as a 20-min infusion. Patients also received basic immunosuppressive therapy consisting of CsA and prednisolone. Clinical side effects, lymphocyte counts, and plasma TNF and IL-6 levels were monitored at frequent intervals. The patients' body temperature was measured prior to the muromonab-CD3 bolus and again at 2, 4, 8, 12, 24, 36, and 48 h. Other adverse side effects were monitored by observation and questioning; patient blood pressure was assessed twice daily. In Group 3, which received steroids administered in a divided dose, the peak temperature levels were

significantly lower than in Groups 1 and 2 ($p = 0.0459$ and 0.0157, respectively), and patients experienced significantly fewer side effects ($p = 0.0392$ and 0.016, respectively). There were no differences in cumulative side effect scores or body temperature between Groups 1 and 2 during the 24-h period following muromonab-CD3 administration. The blood pressures of the three groups remained unchanged. In Group 3, the median plasma level of IL-6 was $14\,pg\,mL^{-1}$, compared to $180\,pg\,mL^{-1}$ in Group 1 and $43\,pg\,mL^{-1}$ in Group 2; in addition, peak levels were significantly decreased compared to Group 1 ($p = 0.0152$). Peak IL-6 levels occurred at 3 h in Groups 2 and 3, and were apparently postponed by 3 h in Group 1. Group 2 patients displayed significantly higher peak levels of TNF than did the other groups ($p = 0.0321$). The authors concluded that an additive effect was observed when methylprednisolone was administered in two divided doses, at 6 h and at 1 h prior to the first dose of muromonab-CD3. Although the prevention was not complete, administration of methylprednisolone in this manner aided in preventing the first-dose side effects of muromonab-CD3.

9.5.4.2 **Pentoxifylline**

A prospective study was conducted by Vincenti et al. (1993) to assess the effects of pretreatment with pentoxifylline on the cytokine-induced first-dose side effects of muromonab-CD3 in 20 renal transplant patients. Ten patients were historical controls and were also treated with muromonab-CD3. Patients were treated with $5\,mg\,day^{-1}$ muromonab-CD3 for 10 days. All patients received $7\,mg\,kg^{-1}$ prednisolone (IV) at 1 h before the first muromonab-CD3 dose; pretreatment consisted of $2\,mg\,kg^{-1}$ before the second dose and $0.5\,mg\,kg^{-1}$ before subsequent doses. Additionally, patients were administered acetaminophen 650 mg and diphenhydramine 50 mg every 6 h for 2 days. Patients were given pentoxifylline 600 mg every 8 h for the 24-h period prior to muromonab-CD3 therapy and throughout the course of treatment. Muromonab-CD3-related adverse events (fever, chills, headache, dyspnea) were rated for severity on a scale of 0 to 3 (0 = absent, 3 = severe); gastrointestinal events were graded as either +1 (present) or 0 (absent). Compared to historic controls, patients treated with pentoxifylline had significantly lower reaction scores ($p < 0.0001$). It has been hypothesized that pentoxifylline might be successful in reducing the severity of the CRS because of its ability to inhibit transcription of TNF mRNA and thus prevent the accumulation of TNF mRNA in activated monocytes (Rossi et al. 1993; Vincenti et al. 1993). In this study the first-dose reactions stimulated by muromonab-CD3 were significantly decreased by pentoxifylline. Rossi et al. (1993) have cautioned that in the study conducted by Vincenti and colleagues, pentoxifylline was used in conjunction with high-dose corticosteroids, thus making it difficult to ascertain the effects of pentoxifylline alone. Additionally, pentoxifylline has shown no direct effect on end-organ cytokine response, and does not affect TNF already in circulation. Consequently, Rossi et al. (1993) concluded that the clinical evidence for a beneficial effect remains controversial, and recommended starting pentoxifylline prior to treatment with muromonab-CD3.

9.5.4.3 Indomethacin

Various studies have shown that inhibition of the cyclooxygenase pathway decreases cytokine release, thus initiating attention to the use of indomethacin for its potential role in reducing CRS symptoms (Rossi et al. 1993). Shield et al. (1992) conducted a randomized, placebo-controlled, multicenter study to evaluate the possible effects of indomethacin on the incidence and severity of CRS symptoms during the first 48 h after muromonab-CD3 injection. Sixty patients being treated with muromonab-CD3 as either first-line or rescue therapy for renal rejection were randomly assigned to receive either indomethacin 100 mg ($n = 29$) or placebo ($n = 31$) at 1 to 2 h before muromonab-CD3, then indomethacin 50 mg or placebo respectively at 8-h intervals for a total of 48 h. Patients also received methylprednisolone and diphenhydramine at 1 to 2 h before the initial muromonab-CD3 dose. The indomethacin-treated group reported significantly fewer episodes of body pain than the placebo group (0% versus 16%, $p = 0.053$). Additionally, the indomethacin group experienced a milder febrile response ($p = 0.02$). The incidence of chills in the indomethacin group was also significantly lower (45% versus 71%, $p = 0.066$). Significantly fewer patients in the indomethacin group developed pyrexia after the second muromonab-CD3 dose than did placebo-treated patients (28% and 87%, $p = 0.001$). The severity of pyrexia ($p = 0.001$) and tachycardia ($p = 0.024$) and overall reported adverse experiences ($p = 0.05$) were all significantly lower in the indomethacin group. Although these findings are encouraging, the use of indomethacin still induced significant increases in circulating IL-1β ($p = 0.01$), TNF ($p = 0.001$) and IL-6 ($p = 0.025$) after the first dose of muromonab-CD3. The placebo group had significant increases only in TNF and GM-CSF levels ($p = 0.001$ for both). The authors concluded that, while indomethacin did not inhibit the production and circulation of cytokines, the prevalence and severity of CRS symptoms associated with muromonab-CD3 were greatly reduced during the first 48 h post dose.

9.5.4.4 Recombinant Human Soluble Tumor Necrosis Factor Receptor (TNFR:Fc)

TNFR:Fc, a dimer of the p80 TNF receptor, binds to both TNF-α and lymphotoxin (LT). This product may be more appealing as preventive therapy for muromonab-CD3-related CRS than anti-TNF mAb as it has a higher affinity for TNF, has minimal immunogenicity, and is physiologically closer to a human receptor. Eason et al. (1996) performed a prospective, randomized study in 12 renal graft recipients who were diagnosed with steroid-resistant rejection to evaluate the ability of TNFR:Fc to decrease the symptoms of CRS and promote renal function. All patients received a bolus dose of methylprednisolone 500 mg, oral acetaminophen 350 mg, and oral diphenhydramine 25 mg before the first muromonab-CD3 dose. The first two doses of muromonab-CD3 were received either alone or with TNFR:Fc (0.05 to 0.15 $mg\,kg^{-1}$), administered 30 min prior. There was a significant decrease in the frequency of chills ($p < 0.05$) in the group treated with TNFR:Fc compared to the control group. The TNFR:Fc group also had fewer symptoms ($p = 0.032$) by Day 2. TNFR:Fc returned patients to normal renal function beginning at 24 h compared to 48 h in the control group, and in addition was associated

with lower serum creatinine levels during the first 6 days of muromonab-CD3 therapy ($p = 0.032$).

9.5.4.5 Anti-TNF Monoclonal Antibodies

Studies are under way to develop new treatments to control CRS, such as nonactivating anti-CD3 antibodies. The use of the $F(ab')_2$ fragment of muromonab-CD3 antibody may show promise in reducing cytokine activation (Jeyarajah et al. 1993). Current research has demonstrated the potential of anti-TNF IgG, which may bind to TNF already released into the circulation of allograft recipients. The results from murine models of this process have demonstrated significant TNF inactivation and consequent prevention of the synthesis of other cytokines (Rossi et al. 1993).

9.6 The Consequences of Immunosuppression

9.6.1 Infections

Muromonab-CD3 is usually added to immunosuppressive therapeutic regimens, thereby augmenting the degree of immunosuppression. This increase in the total amount of immunosuppression may alter the spectrum of infections observed and increase the risk, the severity, and the morbidity of infectious complications. During the first month post transplant, patients are at greatest risk for the following infections:

- those present prior to transplant, perhaps exacerbated by posttransplant immunosuppression;
- infection conveyed by the donor organ; and
- the usual postoperative urinary tract, intravenous line-related, wound, or pulmonary infections due to bacterial pathogens.

At approximately 1 to 6 months post transplant, patients are at risk for viral infections [e.g., CMV, EBV, herpes simplex virus (HSV), etc.] which produce serious systemic disease and which also increase the overall state of immunosuppression.

Reactivation (at 1 to 4 months post transplant) of EBV and CMV has been reported. When administration of an anti-lymphocyte antibody, including Orthoclone OKT3, is followed by an immunosuppressive regimen including CsA, there is an increased risk of reactivating CMV and an impaired ability to limit its proliferation, resulting in symptomatic and disseminated disease. EBV infection – either primary or reactivated – may play an important role in the development of posttransplant lymphoproliferative disorders.

In the pediatric transplant population, viral infections often include pathogens uncommon in adults, such as varicella zoster virus (VZV), adenovirus, and respi-

ratory syncytial virus (RSV). A large proportion of pediatric patients have not been infected with the herpes viruses prior to transplantation and, therefore, are susceptible to developing primary infections from the grafted organ and/or blood products.

Anti-infective prophylaxis may reduce the morbidity associated with certain potential pathogens, and should be considered for pediatric and other high-risk patients. The judicious use of immunosuppressive drugs, including type, dosage, and duration, may limit the risk and seriousness of some opportunistic infections. It is also possible to reduce the risk of serious CMV or EBV infection by avoiding transplantation of a CMV-seropositive (donor) and/or EBV-seropositive (donor) organ into a seronegative patient.

9.6.2 Neoplasia

As a result of depressed cell-mediated immunity from immunosuppressive agents, organ transplant patients have an increased risk of developing malignancies. This risk is evidenced almost exclusively by the occurrence of lymphoproliferative disorders, squamous cell carcinomas of the skin and lip, and sarcomas. In immunosuppressed patients, T-cell cytotoxicity is impaired allowing for transformation and proliferation of EBV-infected B lymphocytes. Transformed B lymphocytes are thought to initiate oncogenesis, which ultimately culminates in the development of most posttransplant lymphoproliferative disorders. Patients – especially pediatric patients – with primary EBV infection may be at a higher risk for the development of EBV-associated lymphoproliferative disorders. Current data support an association between the development of lymphoproliferative disorders at the time of active EBV infection and muromonab-CD3 administration in pediatric liver allograft recipients.

Following the initiation of muromonab-CD3 therapy, patients should be continuously monitored for evidence of lymphoproliferative disorders through physical examination and histological evaluation of any suspect lymphoid tissue. Close surveillance is advised, as early detection with subsequent reduction of total immunosuppression may result in the regression of some of these lymphoproliferative disorders. As the potential for the development of lymphoproliferative disorders is related to the duration and extent (intensity) of total immunosuppression, physicians are advised:

- to adhere to the recommended dosage and duration of muromonab-CD3 therapy;
- to limit the number of courses of muromonab-CD3 and other anti-T-lymphocyte antibody preparations administered within a short period of time; and
- if appropriate, to reduce the dosage(s) of immunosuppressive drugs used concomitantly to the lowest level compatible with an effective therapeutic response.

A recent study examined the incidence of non-Hodgkin's lymphoma (NHL) among 45 000 kidney transplant recipients and over 7500 heart transplant recipients. This study suggested that all transplant patients, regardless of the immunosuppressive regimen employed, are at increased risk of NHL over the general population. The relative risk was highest among those receiving the most aggressive regimens. The long-term risk of neoplastic events in patients being treated with muromonab-CD3 has not been determined.

References

Abramowicz, D., Schandene, L., Goldman, M., et al. (1989) Release of tumor necrosis factor, interleukin-2, and gamma-interferon in serum after injection of OKT3 monoclonal antibody in kidney transplant recipients. *Transplantation* 47: 606–608.

Abramowicz, D., Goldman, M., Mat, O., et al. (1994) OKT3 serum levels as a guide for prophylactic therapy: a pilot study in kidney transplant recipients. *Transpl Int* 7: 258–263.

Alegre, M.-L., Gastadello, K., Abramowicz, D., et al. (1991) Evidence that pentoxifylline reduces anti-CD3 monoclonal antibody-induced cytokine release syndrome. *Transplantation* 52: 674–679.

Arenas, J.I., Laskus, T., Wilkinson, J., et al. (2003) Rejection treatment modifies HCV quasispecies evolution after transplantation. *Hepatology* 38(Suppl.1): 532.

Balk, A.H.M.M., Simoons, M.L., Jutte, N.H.P.M., et al. (1991) Sequential OKT3 and cyclosporine after heart transplantation. A randomized study with single and cyclic OKT3. *Clin Transpl* 5: 301–305.

Barr, M.L., Sanchez, J.A., Seche, L.A., et al. (1990) Anti-CD3 monoclonal antibody induction therapy. Immunological equivalence with triple-drug therapy in heart transplantation. *Circulation* 82(Suppl. IV): 291–294.

Bemelman, F.J., Buysmann, S., Surachno, J., et al. (1994) Pretreatment with divided dose of steroids strongly decreases side effects of OKT3. *Kidney Int* 46: 1674–1679.

Benvenisty, A.I., Cohen, D., Stegall, M.D., et al. (1990) Improved results using OKT3 as induction immunosuppression in renal allograft recipients with delayed graft function. *Transplantation* 49: 321–327.

Bernard, A., Boumsell, L. (1984) The clusters of differentiation (CD) defined by the First International Workshop on Human Leucocyte Differentiation Antigens. *Hum Immunol* 11(1): 1–10.

Bhat, G., Schroeder, T.J. (1997) Clinical role of immunologic monitoring during OKT3 treatment. *Transpl Proc* 29(Suppl.8): 21–26.

Bloemena, E., ten Berge, I.J.M., Surachno, J., et al. (1990) Kinetics of interleukin 6 during OKT3 treatment in renal allograft recipients. *Transplantation* 50: 330–331.

Bock, H.A., Gallati, H., Zürcher, R.M., et al. (1995) A randomized prospective trial of prophylactic immunosuppression with ATG-Fresenius versus OKT3 after renal transplantation. *Transplantation* 59: 830–840.

Broughan, T.A., Valenzuela, R., Escorcia, E., et al. (1994) Mouse antibody-coated lymphocytes during OKT3 therapy in liver transplantation. *Clin Transpl* 8: 488–491.

Broyer, M., Gagnandoux, M.-F., Guest, G., et al. (1993) Prophylactic OKT3 monoclonal antibody versus antilymphocyte globulins: a prospective, randomised study in 148 first cadaver kidney grafts. *Transplant Proc* 25: 570–571.

Caillat-Zucman, S., Blumenfield, N., Legendre, C., et al. (1981) The OKT3 immunosuppressive effect: in situ antigenic modulation of human graft-infiltrating T cells. *Transplantation* 32: 535–539.

Carey, G., Lisi, P.J., Schroeder, T.J. (1995) The incidence of antibody formation to OKT3 consequent to its use in organ

transplantation. *Transplantation* 60: 151–158.
Chatenoud, L. (1993a) Humoral immune response against OKT3. *Transplant Proc* 25(Suppl.1): 68–73.
Chatenoud, L. (1993b) OKT3-induced cytokine-release syndrome: preventive effect of anti-tumor necrosis factor monoclonal antibody. *Transplant Proc* 25(2): 47–51.
Chatenoud, L. (1994) Use of CD3 antibodies in transplantation and autoimmune disease. *Tansplant Proc* 26: 3191–3193.
Chatenoud, L. (1995) *Monoclonal antibodies in transplantation*. Medical Intelligence Unit. RG Landes Company, p. 3.
Chatenoud, L., Legendre, C., Ferran, C., et al. (1990) In vivo cell activation following OKT3 administration. Systemic cytokine release and modulation by corticosteroids. *Transplantation* 49: 697–702.
Chatenoud, L., Legendre, C., Ferran, C., et al. (1991) Corticosteroid inhibition of the OKT3-induced cytokine-related syndrome – dosage and kinetics prerequisites. *Transplantation* 51: 334–338.
Chkotua, A.B., Klein, T., Shabtai, E., et al. (2003) Kidney transplantation from living-unrelated donors: comparison of outcome with living-related and cadaveric transplants under current immunosuppressive protocols. *Urology* 62: 1002–1006.
Ciancio, G., Mattiazzi, A., Vaidya, A., et al. (2004) A prospective study with thymoglobulin/daclizumab induction, tacrolimus and steroid maintenance, comparing rapamycin with mycophenolate mofetil as adjunctive immunosuppression in simultaneous pancreas-kidney (SPK) transplantation. *J Urol* 171(4): 489–490.
Cole, E.H., Cattran, D.C., Farewell, V.T., et al. (1994) A comparison of rabbit antithymocyte serum and OKT3 as prophylaxis against renal allograft rejection. *Transplantation* 57: 60–67.
Cosimi, A.B., Jenkins, R.L., Rohrer, R.J., et al. (1990) A randomized clinical trial of prophylactic OKT3 monoclonal antibody in liver allograft recipients. *Arch Surg* 125: 781–785.
Costanzo-Nordin, M.R., Sullivan, E.J., Johnson, M.R., et al. (1990) Prospective randomized trial of OKT3 versus horse antithymocyte globulin based immunosuppressive prophylaxis in heart transplantation. *J Heart Transplant* 9: 306–315.
Costanzo-Nordin, M.R., Fisher, S.G., O'Sullivan, E.J., et al. (1993) HLA-DR incompatibility predicts heart transplant rejection independent of immunosuppressive prophylaxis. *J Heart Lung Transpl* 12: 779–789.
De Vault, Jr., G.A., Kohan, D.E., Nelson, E. W., et al. (1994) The effect of oral pentoxifylline on the cytokine release syndrome during inductive OKT3. *Transplantation* 57: 532–540.
Eason, J.D., Pasqual, M., Wee, S., et al. (1996) Evaluation of recombinant human soluble dimeric tumor necrosis factor receptor for prevention of OKT3-associated acute clinical syndrome. *Transplantation* 61(2): 224–228.
Ellenhorn, J.D.I., Woodle, E.S., Ghobreal, I., et al. (1990) Activation of human T cells in vivo following treatment of transplant recipients with OKT3. *Transplantation* 50: 608–612.
Farges, O., Ericzon, B.-G., Bresson-Hadni, S., et al. (1994) A randomized trial of OKT3-based versus cyclosporine-based immunoprophylaxis after liver-transplantation: long-term results of a European and Australian multicenter study. *Transplantation* 58: 891–898.
Ferran, C., Dy, M., Merite, S., et al. (1990) Reduction of morbidity and cytokine release in anti-CD3 MoAb-treated mice by corticosteroids. *Transplantation* 50: 642–648.
First, M.R., Schroeder, T.J., Hariharan, S. (1993) OKT-3 induced cytokine-release syndrome: renal effects (cytokine nephropathy). *Transplant Proc* 25(Suppl.1): 25–26.
Frey, D.J., Matas, A.J., Gillingham, K.J., et al. (1992) Sequential therapy – a prospective randomized trial of malg versus OKT3 for prophylactic immunosuppression in cadaver renal allograft recipients. *Transplantation* 54: 50–56.

Gaston, R.S. (1994) Cytokines and transplantation: a clinical perspective. *Transplant Sci* 4(1): 9–19.

Gaston, R.S., Deierhoi, M.H., Patterson, T., et al. (1991) OKT3 first-dose reaction: association with T-cell subsets and cytokine release. *Kidney Int* 39: 141–148.

Gebel, H.M., Lebeck, L.L., Jensik, S.C., et al. (1989a) Discordant expression of CD3 and T cell receptor antigens on lymphocytes from patients treated with OKT3. *Transplant Proc* 21: 1745–1746.

Gebel, H.M., Lebeck, L.L., Jensik, S.C., et al. (1989b) T cells from patients successfully treated with OKT3 do not react with the T-cell receptor antibody. *Hum Immunol* 26: 123–130.

Goldman, M., Gerard, C., Abramowicz, D., et al. (1992) Induction of interleukin-6 and interleukin-10 by the OKT3 monoclonal antibody: possible relevance to posttransplant lymphoproliferative disorders. *Clin Transpl* 6: 265–268.

Goumy, L., Ferran, C., Merite, S., et al. (1990) In vivo anti-CD3-driven cell activation. Cellular source of induced tumor necrosis factor, interleukin-1β, and interleukin 6. *Transplantation* 61: 83–87.

Griffith, B.P., Kormos, R.L., Armitage, J.M., et al. (1990) Comparative trial of immunoprophylaxis with RATG versus OKT3. *J Heart Transplant* 9: 301–305.

Hammond, E.A., Yowell, R.L., Greenwood, J., Hartung, L., Renlund, D., Wittwer, C. (1993) Prevention of adverse clinical outcome by monitoring of cardiac transplant patients for murine monoclonal CD3 antibody (OKT3) sensitization. *Transplantation* 55(5): 1061–1063.

Hanto, D.W., Jendrisak, M.D., So, S.K.S., et al. (1994) Induction immunosuppression with antilymphocyte globulin or OKT3 in cadaver kidney transplantation. Results of a single institution prospective randomized trial. *Transplantation* 57: 377–384.

Henell, K.R., Norman, D.J. (1993) Monitoring OKT3 treatment: pharmacodynamic and pharmacokinetic measures. *Transplant Proc* 25(Suppl.1): 83–85.

Herbelin, A., Abramowicz, D., de Groote, D., et al. (1999) CD3 antibody-induced IL-10 in renal allograft recipients: an in vivo and in vitro analysis. *Transplantation* 68(5): 616–622.

Hoffman, T., Tripathi, A.K., Lee, Y.L., et al. (1992) Stimulation of human monocytes by anti-CD3 monoclonal antibody: induction of inflammatory mediator release via immobilization of Fc receptor by adsorbed immunoglobulin and T-lymphocytes. *Inflammation* 16: 571–585.

Howard, R.J., Pfaff, W.W., Brunson, M.E., et al. (1993) Delayed graft function is associated with an increased incidence of occult rejection and results in poorer graft survival. *Transplant Proc* 25: 884.

Ippoliti, G., Negri, M., Abelli, P., et al. (1991) Preoperative prophylactic OKT3 vs RATG. A randomized clinical study in heart transplant patients. *Transplant Proc* 23: 2272–2274.

Jeyarajah, D.R., Thistlethwaite, J.R. (1993) General aspects of cytokine-release syndrome: timing and incidence of symptoms. *Transplant Proc* 25(2): 16–20.

Keever-Taylor, C.A., Craig, A., Molter, M., et al. (2001) Complement-mediated T-cell depletion of bone marrow: comparison of T10B9. 1A-31 and Muromonab-Orthoclone OKT3. *Cytotherapy* 3(6): 467–481.

Kermann, R.H., Sullivan, K., Tejani, A. (1994) Impact of HLA matching, type of crossmatch and immunosuppressive therapy on primary pediatric cadaver renal allograft survival [Abstract]. *Hum Immunol* 40(Suppl.1): 17.

Kimball, J.A., Norman, D.J., Shield, C.F., et al. (1993) OKT3 antibody response study: comparative testing of human anti-mouse antibody. *Transplant Proc* 25(Suppl.1): 74–76.

Kirklin, J.K., Bourge, R.C., White-Williams, C., et al. (1990) Prophylactic therapy for rejection after cardiac transplantation – a comparison of rabbit antithymocyte globulin and OKT3. *J Thorac Cardiovasc Surg* 99: 716–724.

Kohler, G., Milstein, C. (1976) Derivation of specific antibody producing tissue culture and tumor lines by cell fusion. *Eur J Immunol* 6: 171–178.

Kreis, H. (1993) Adverse events associated with OKT3 immunosuppression in the prevention or treatment of allograft rejection. *Clin Transpl* 7: 431–446.

Kreis, H., Legendre, C., Chatenoud, L. (1991) OKT3 in organ transplantation. *Transplant Rev* 5: 181–189.

Krensky, A.M., Weiss, A., Crabtree, G., Davis, M.M., Parham, P. (1990) T-lymphocyte-antigen interactions in transplant rejection. *N Engl J Med* 322(8): 510–517.

Kung, G., Goldstein, G., Reinherz, E.L., Schlossman, S.F. (1979) Monoclonal antibodies defining distinctive T-cell surface antigens. *Science* 206: 347.

Kung, S.C., Parikh, M., Fyfe, B., et al. (2004) Simulect induction facilitates Neoral-based steroid-free immunosuppression in primary kidney transplant recipients. *Transplant Proc* 36(Suppl.2): 475–477.

Ladowski, J.S., Dillon, T., Schatzlein, M.H., et al. (1993) Prophylaxis of heart transplant rejection with either antithymocyte globulin-, Minnesota antilymphocyte globulin-, or OKT3-based protocol. *J Cardiovasc Surg* 34: 135–140.

Latham, W.C., Cooney, R.M., Brown, K.J., et al. (1970) Preparation of purified antilymphocyte serum on an immuno-absorbent column. In proceedings of a symposium on standardization of antilymphocyte serum. 16: 171–178.

Leimenstoll, G., Zabel, P., Schroeder, P., et al. (1993) Suppression of OKT3-induced tumor necrosis factor alpha formation by pentoxifylline in renal transplant recipients. *Transplant Proc* 25: 561–563.

Lozano, M., Oppenheimer, F., Cofan, F., et al. (2001) Platelet procoagulant activity induced in vivo by muromonab CD3 infusion in uremic patients. *Thrombosis Res* 104(6): 405–411.

Macdonald, P.S., Mundy, J., Keogh, A.M., et al. (1993) A prospective randomized study of prophylactic OKT3 versus equine antithymocyte globulin after heart transplantation – increased morbidity with OKT3. *Transplantation* 55: 110–116.

McCarthy, C., Light, J.A., Aquino, A., et al. (1993) Correlation of CD3+ lymphocyte depletion with rejection and infection in renal transplants. *Transplant Proc* 25: 2477–2478.

McDiarmid, S.V., Millis, M., Terashita, G., et al. (1990) Low serum OKT3 levels correlate with failure to prevent rejection in orthotopic liver transplant patients. *Transplant Proc* 22: 1774–1776.

McDiarmid, S.V., Millis, M.J., Terasaki, P.I. (1991a) OKT3 prophylaxis in liver transplantation. *Dig Dis Sci* 36: 1418–1426.

McDiamid, S.V., Busuttil, R.W., Levy, P., et al. (1991b) The long-term outcome of OKT3 compared with cyclosporine prophylaxis after liver transplantation. *Transplantation* 52: 91–97.

Mendez, R., Abboud, H., Burdick, J., et al. (1999) Reduced intrapatient variability of cyclosporin pharmacokinetics in renal transplant recipients switched from oral Sandimmune to Neoral. *Clin Ther* 21(1): 161–171.

Menkis, A.H., Powel, A.-M., Novick, R.J., et al. (1992) A prospective randomized controlled trial of initial immunosuppression with ALG versus OKT3 in recipients of cardiac allografts. *J Heart Lung Transplant* 11: 569–576.

Mignat, C. (1997) Clinically significant drug interactions with new immunosuppressive agents. *Drug Safety* 16(4): 267–278.

Moore, C.K., O'Connell, J.B., Renlund, D.G., et al. (1991) Cardiac allograft cellular rejection during OKT3 prophylaxis in the absence of sensitization. *Transplant Proc* 23: 1055–1058.

Mühlbacher, F., Steininger, R., Längle, F., et al. (1989) OKT3 immunoprophylaxis in human liver transplantation. *Transplant Proc* 21: 2253–2254.

Muller, C., Patzke, J., Bonmann, M., et al. (1998) Alterations of protein tyrosine phosphorylation in T-cells of immunocompromised patients. *Scand J Immunol* 47(2): 101–105.

Muller, C., Bonmann, M., Heidenreich, S., et al. (1999) Tyrosine phosphorylation in peripheral T-cells of kidney transplant recipients: analyses of baseline levels and response to T-cell receptor stimulation. *Int J Mol Med* 4(2): 141–144.

Norman, D.J. (1992) Antilymphocyte antibodies in the treatment of allograft rejection: targets, mechanisms of action, monitoring, and efficacy. *Semin Nephrol* 12: 315–324.

Norman, D.J., Barry, J.M., Bennett, W.M., et al. (1991) OKT3 for induction immunosuppression in renal

transplantation: a comparative study of high versus low doses. *Transplant Proc* 23: 1052–1054.

Norman, D.J., Chatenoud, L., Cohen, D., et al. (1993) Consensus statement regarding OKT3-induced cytokine-release syndrome and human anti-mouse antibodies. *Transplant Proc* 25(Suppl.1): 89–92.

Norman, D.J., Kimball, J.A., Bennett, W.M., et al. (1994) A prospective, double-blind, randomized study of high- versus low-dose OKT3 induction immunosuppression in cadaveric renal transplantation. *Transpl Int* 7: 356–361.

O'Connell, J.B., Bristow, M.R., Hammond, E.H., et al. (1991) Antimurine antibody to OKT3 in cardiac transplantation: implications for prophylaxis and retreatment of rejection. *Transplant Proc* 23: 1157–1159.

Ohman, M., Kolb, M., Leathers, L.K., et al. (1993) Multiparameter monitoring of efficacy of OKT3-induced immune suppression in immune renal allograft recipients (abstract). *Hum Immunol* 37(Suppl.1): 95.

Pons, J.A., Bueno, F., Parilla, P., et al. (1993) Cyclosporine vs. OKT3 prophylaxis after orthotopic liver transplantation. *Transplant Proc* 25: 1949.

Raasveld, M.H.M., Bemelman, F.J., Schellekens, P.A., et al. (1993) Compliment activation during OKT3 treatment: a possible explanation for respiratory side effects. *Kidney Int* 43: 1140–1149.

Reding, R., Vraux, H., de Goyet, J. de V., et al. (1993) Monoclonal antibodies in prophylactic immunosuppression after liver transplantation. A randomized controlled trial comparing OKT3 and anti-IL-2 receptor monoclonal antibody LO-Tact-1. *Transplantation* 55: 534–541.

Reinke, P., Schwinzer, H., Hoflich, C., et al. (1997) Selective in vivo deletion of alloactivated TH1 cells by OKT3 monoclonal antibody in acute rejection. *Immunol Lett* 57(1–3): 151–153.

Renlund, D.G., O'Connell, J.B., Gilbert, E. M., et al. (1989) A prospective comparison of murine monoclonal CD-3 OKT3 antibody-based and equine antithymocyte globulin-based rejection prophylaxis in cardiac transplantation decreased rejection and less corticosteroid use with OKT3. *Transplantation* 47: 599–605.

Roitt, I.M. (1993) OKT3: immunology, production, purification and pharmacokinetics. *Clin Transplant* 7: 367–373.

Rossi, S.J., Schroeder, T.J., Hariharan, S., et al. (1993) Prevention and management of the adverse effects associated with immunosuppressive therapy. *Drug Safety* 9(2): 104–131.

Ryckman, F.C., Schroeder, T.J., Pedersen, S.H., et al. (1991) Use of monoclonal antibody immunosuppressive therapy in paediatric renal and liver transplantation. *Clin Transplant* 5: 186–190.

Sanchez, R.Q., Levy, M.F., Goldstein, R.M., et al. (2003) The changing clinical presentation of recurrent primary biliary cirrhosis after liver transplantation. *Transplantation* 76(11): 1583–1588.

Schroeder, T.J., First, M.R., Mansour, M.E., et al. (1990) Antimurine antibody formation following OKT3 therapy. *Transplantation* 49: 48–51.

Schroeder, T.J., Ryckman, F.C., Hurtubise, P.E., et al. (1991) Immunological monitoring during and following OKT3 therapy in children. *Clin Transpl* 5: 191–196.

Schroeder, T.J., Michael, A.T., First, M.R., et al. (1994) Variations in serum OKT3 concentration based upon age, sex, transplanted organ, treatment regimen, and anti-OKT3 antibody status. *Ther Drug Monit* 16: 361–367.

Shaefer, M.S., Stratta, R.J., Pirruccello, S.J., et al. (1990) Peripheral lymphocyte monitoring of liver transplant recipients being treated with OKT3 for rejection or induction immunosuppression (abstract). *Pharmacotherapy* 10(3): 248.

Shield, C.F., Kahana, L., Pirsh, J., et al. (1992) Use of indomethacin to minimize the adverse reactions associated with Orthoclone OKT3 treatment of kidney allograft rejection. *Transplantation* 54(1): 164–166.

Smith, S.L. (1996) Ten years of Orthoclone OKT3 (muromonab-CD3): a review. *J Transpl Coord* 6(3): 109–119.

Steininger, R., Mühlbacher, F., Hamilton, G., et al. (1991) Comparison of CyA, OKT3, and ATG immunoprophylaxis in human

liver transplantation. *Transplant Proc* 23: 2269–2271.
Steinmuller, D.R., Hayes, J.M., Novick, A.C., et al. (1991) Comparison of OKT3 with ALG for prophylaxis for patients with acute renal failure after cadaveric renal transplantation. *Transplantation* 52: 67–71.
Strong, M.L., Ueda, C.T. (1997) Effects of low and high density lipoproteins on renal cyclosporin A and cyclosporin G disposition in the isolated perfused rat kidney. *Pharm Res* 14(10): 1466–1471.
Swinnen, L.J., Fisher, R.I. (1993) OKT3 monoclonal antibodies induce interleukin-6 and interleukin-10: a possible cause of lymphoproliferative disorders associated with transplantation. *Curr Opin Nephrol Hypertens* 2: 670–678.
Tanaka, C., Kawai, R., Rowland, M. (1999) Physiologically based pharmacokinetics of cyclosporin A: reevaluation of dose-nonlinear kinetics in rats. *J Pharmacokinet Biopharm* 27(6): 597–623.
Taylor, D.O., Bristow, M.R., O'Connell, J.B., et al. (1994) A prospective, randomized comparison of cyclophosphamide and azathioprine for early rejection prophylaxis after cardiac transplantation: decreased sensitization to OKT3. *Transplantation* 58: 645–649.
Thistlethwaite, J.R., Haag, B.W., Gaber, A., et al. (1987) The use of OKT3 to treat steroid-resistant renal allograft rejection in patients receiving cyclosporine. *Transplant Proc* 19: 1901–1904.
Todd, P.A., Brogden, R.N. (1989) Muromonab CD3: a review of its pharmacologically and therapeutic potential. *Drugs* 37: 871–899.
Toyoda, M., Galfayan, K., Wachs, K., et al. (1995) Immunologic monitoring of OKT3 induction therapy in cardiac allograft recipients. *Clin Transplant* 9: 472–480.
Troppmann, C., Gillingham, K.J., Benedetti, C., et al. (1995) Delayed graft function, acute rejection, and outcome after cadaver renal transplantation: a multivariate analysis. *Transplantation* 59: 962–968.
Vallhonrat, H., Williams, W.W., Cosimi, A. B., et al. (1999) In vivo generation of C4d, Bb, iC3b, and SC5b-9 after OKT3 administration in kidney and lung transplant recipients. *Transplantation* 67(2): 153–258.
Vasquez, E.M., Pollak, R. (1997) OKT3 therapy increases cyclosporine blood levels. *Clin Transpl* 11(1): 38–41.
Vasquez, E.M., Fabrega, A.J., Pollak, R. (1995) OKT3-induced cytokine release syndrome: occurrence beyond the second dose and association with rejection therapy. *Transplant Proc* 27(1):873–874.
Vincenti, F.G., Vasconcelos, M., Birnbaum, J.L., et al. (1993) Pentoxifylline reduces the first-dose reactions following OKT3. *Transplant Proc* 25(2): 57–59.
Vosssen, A.C.T.M., Tibbe, G.J.M., Kroos, M. J., et al. (1995) Fc receptor binding of anti-CD3 monoclonal antibodies is not essential for immunosuppression, but triggers cytokine-related side effects. *Eur J Immunol* 25: 1492–1496.
Wechsler, M.E., Giardina, E.-G.V., Sciacca, R.R., et al. (1995) Increased early mortality in women undergoing cardiac transplantation. *Circulation* 91: 1029–1035.
Welter, H.F., Illner, W.-D., Schleibner, S., et al. (1990) Pilot study on induction treatment with high-dose OKT3: preliminary observations in kidney transplantation. *Transplant Proc* 22: 2272.
Wilde, M.I., Goa, L.G. (1996) Muromonab CD3. A reappraisal of its pharmacology and use as prophylaxis of solid organ transplant rejection. *Drugs* 51(5): 865–894.
Wissing, K.M., Morelon, E., Legendre, C., et al. A pilot trial of recombinant human interleukin-10 in kidney transplant recipients receiving OKT3 induction therapy. *Transplantation* 64(7): 999–1006.
Wollenek, G., Laufer, G., Laczkovics, A., et al. (1989) Comparison of a monoclonal anti-T cell antibody vs ATG as prophylaxis after heart transplantation. *Transplant Proc* 21: 2499–2501.
Woodle, E.S., Thistlethwaite, J.R., Jolliffe, L. K., Zivin, R.A., Collins, A., Adair, J.R., Bodmer, M., Athwal, D., Alegre, M.L., Bluestone J.A. (1992) Humanized OKT3 antibodies: successful transfer of immune modulating properties and idiotype expression. *J Immunol* 148(9): 2756–2763.

10
Natalizumab (Tysabri)

Sebastian Schimrigk and Ralf Gold

10.1
Introduction

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) where multiple foci of inflammation accompanied by demyelination and axonal transection cause various symptoms, including weakness, ataxia and other troublesome complications. During the early 1990s, different disease-modifying drugs (DMDs), including interferon (INF)-β1a (Avonex, BiogenIdec; Rebif, Serono), IFN-β1b (Betaseron, Schering), glatiramer acetate (Copaxone, Teva Pharmaceutical Industries) and mitoxantrone (Ralenova or Novantrone, Lederle-Wyeth), were found to be effective to a certain extent in influencing the course of MS. However, additional effective treatments are warranted to slow disease progression, reduce disability, and limit lesion evolution and irreversible tissue destruction.

During the past decade, substantial progress has been made in the understanding of some of the key mechanisms underlying the pathogenesis of neuroimmunological diseases. In parallel, the development of new biologicals is under way to improve treatment. A main focus here is set on monoclonal antibodies (mAbs), where natalizumab is the very first selective immunmodulatory drug with an orphan drug status for the treatment of MS.

Natalizumab is a recombinant humanized mAb that blocks the mechanism of endothelial adhesion of activated T cells in inflammatory diseases such as MS. Natalizumab (Elan Corp. plc.; Biogen Idec.) binds to the α4-chain of the heterodimeric VLA-4 receptor which is expressed predominantly on lymphocytes. Thus, natalizumab is an α_4-integrin antagonist in the class of selective adhesion molecule (SAM) inhibitors.

A rapid translation of *in-vitro* findings with VLA-4 blockade into experiments with experimental models for MS (experimental autoimmune encephalomyelitis; EAE) (Yednock et al. 1992) led to the first clinical investigations (Miller 2003) with natalizumab, followed by phase III studies where it finally proved to be a very effective drug to treat MS (Polmann et al. 2006). Approximately 3000 patients

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were enrolled in clinical trials evaluating the efficacy of natalizumab, not only in MS but also in Crohn's disease and rheumatoid arthritis.

Nevertheless, the clinical trials have shown that this drug has potentially serious adverse side effects. On February 28, 2005 natalizumab was taken off the market after reports of three patients with progressive multifocal leukencephalopathy (PML), two of whom unfortunately died. Two subjects were enrolled in the SENTINEL-Trail where they received combination treatment of natalizumab and IFN-beta (Avonex). The third patient participated in the Crohn's disease study. Following an outstanding and meticulous safety investigation, no further PML cases were found and natalizumab has been reintroduced into MS treatment in the US and Europe in June 2006.

10.2 Basic Principles

The adhesion of leukocytes to the microvascular endothelium is essential for their migration into inflamed tissue. This response is mediated by the interaction of adhesion molecules expressed on the cell surface of leukocytes and venular endothelial cells (Springer 1990). The very-late-antigen-4 (VLA-4; $\alpha_4\beta_1$ integrin, CD49d/CD29) (Fig. 10.1) and its counter-receptor on brain endothelium, vascular adhesion molecule-1 (VCAM-1), are members of the adhesion molecule family. Natalizumab binds the α_4-chain irrespective of its associated β-chain (β_1 or β_7) so it can also block the interaction of the $\alpha_4\beta_7$-integrin expressed on leukocytes interacting with the mucosal addressin cell adhesion molecule-1 (MadCAM-1), which is expressed on intestinal endothelium. This was the basis for studies in Crohn's disease.

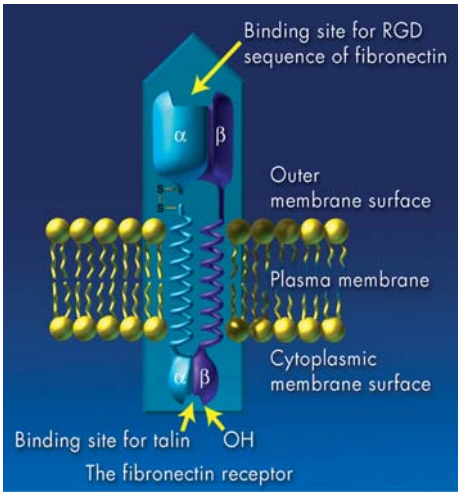

Fig. 10.1 A model of the cell-surface adhesion molecule VLA-4.

The VLA-4 receptor is a member of a large family of cell-surface adhesion molecules (integrins) that mediate both cell–cell and cell–extracellular matrix (ECM) interactions. Adhesion molecules stabilize interactions between migrating cells (e.g., lymphocytes) and their tissue standing partners. In turn, these receptors are then able to induce intracellular signal transduction. VLA-4 is built like a classic integrin molecule with a noncovalently linked α- and a β-chain which is predominantly present on T-lymphocyte membranes, but is also found on monocytes, eosinophilic granulocytes and, to a lesser degree, on neutrophils.

10.3 Mode of Action

The complex adhesion process of activated lymphocytes on inflamed brain endothelium (Fig. 10.2) is necessary for the extravasation of leukocytes through the blood–brain barrier (BBB) as a first step in the inflammation cascade of CNS tissue, as in MS (Alter et al. 2003; Baron et al. 1993). Leukocytes exhibit an initial adhesive contact to the activated endothelium which allows them to slow down and adhere to the vascular wall. This step is mediated by different selectins and integrins, and is still reversible. Activation signals provided by chemokines, matrix metalloproteinases and proinflammatory cytokines switch the integrins to a high affinity state and allow the cell to arrest and finally to transmigrate through the vascular endothelium into the brain (von Andrian and Mackay 2000). Integrins and their counter-receptors have a unique role in this multistep cascade as they modulate the process of *rolling* and *arresting* of lymphocytes.

Various modes of action of anti-VLA4 mAbs have been postulated:

- Blockade of T-cell migration by blocking adhesion to endothelial cells and interaction with ECM proteins (Gonzales-Amaro et al. 2005; Vajkoczy et al. 2001).

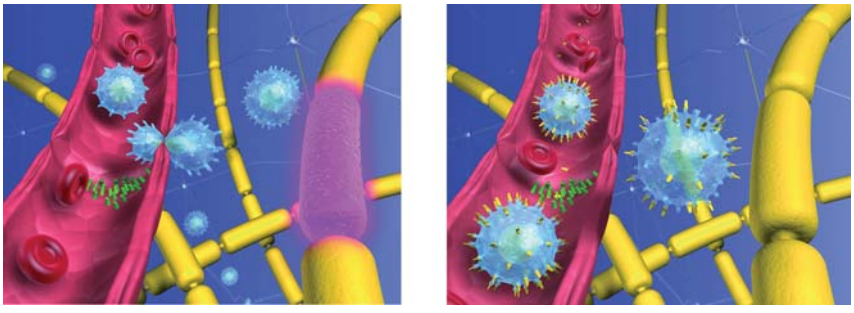

Fig. 10.2 Left: Process of extravasation of activated lymphocytes (blue) via complex binding to adhesion molecules (green). Right: blocking the adhesion process via natalizumab (yellow).

- Circulating B cells and monocytes express significant amounts of VLA-4, which highlights the possible role for VLA-4 in modulating the migration of these cell types across the BBB endothelium (Niino et al. 2006).
- Induction of apoptosis by blocking interaction of α_4-integrin-bearing leukocytes with ECM proteins such as fibronectin (Leussink et al. 2002).

A well-established hypothesis of MS pathogenesis suggests that peripherally activated T-lymphocytes migrate through the BBB and cause brain tissue destruction (Weiner 2004). VLA-4 has been found to be a most interesting target for MS treatment. During the early 1990s, Yednock discovered that the ligand pair VCAM-1 and VLA-4 was of key importance for the migration of inflammatory T cells into the brain parenchyma. Antibodies to α_4-chains of integrins reduce cellular infiltration, inhibit the development of EAE, and halt the progression of disease – or even reverse existing symptoms – by preventing inflammatory cells from crossing the BBB (von Andrian and Engelhardt 2003). Natalizumab has shown to inhibit the trafficking of leukocytes across the BBB and rapidly to reverse EAE (Kent et al. 1995; Léger et al. 1997; Yednock et al. 1992). In addition, natalizumab inhibits inflammation in experimental autoimmune neuritis (EAN) due to almost complete blockade by VLA-4 (Enders et al. 1998). The expression of VCAM-1, the major ligand for $\alpha_4\beta_1$-integrin, is increased in active CNS plaques. Blockade of this adhesion mechanism results in a reduction in proinflammatory cytokines and subsequently in a sustained decrease of inflammation (Wolinsky 2003).

10.3.1 Pharmacodynamic Profile

Natalizumab consists of the recombinant humanized α_4-integrin antibody and stabilizing salts and agents (i.e., sodium chloride, sodium dibasic phosphate heptahydrate, sodium monobasic phosphate monohydrate and polysorbate 80). The current formulation of natalizumab is provided in a buffered solution at a concentration of $20\,mg\,mL^{-1}$. The product is stable for 30 months when stored at 2 to 8 °C.

10.3.2 Pharmacokinetics

Single natalizumab doses of 1 to $6\,mg\,kg^{-1}$ produce maximal saturation (defined as >80%) of α_4-integrin receptors on the surface of lymphocytes within 24 h after administration. Approximately 90% of patients achieve over 80% saturation of α_4-integrin receptors after natalizumab doses of 3 and $6\,mg\,kg^{-1}$. Receptor saturation is maintained for 1, 3 to 4 and 6 weeks with natalizumab doses of 1, 3, and $6\,mg\,kg^{-1}$, respectively. Serum concentrations exhibit a biexponential decline,

with a relatively rapid distribution phase followed by a prolonged terminal phase (Sheremata et al. 1999). The pharmacodynamic effects and therapeutic response observed in the phase II clinical studies supported a fixed dose (300 mg) and a fixed interval (every 4 weeks) for the phase III trials.

10.4 Technology

Studies with the murine form of the antibody (AN100226m) prior to humanization had demonstrated that it was a potent inhibitor of a specific adhesion molecule important in the pathogenesis of inflammatory CNS diseases such as MS. The humanization of AN100226m was undertaken in order to reduce potential immunogenicity, to increase the *in-vivo* half-life, and to allow repeated administration for increased therapeutic benefit (Léger et al. 1997). Grafting of the complementarity-determining regions (CDRs) of AN100226 onto a human immunoglobulin (Ig) G_4 framework resulted in an antibody, natalizumab, that was approximately 99% human-derived (Rice et al. 2005; Rudick and Sandrock 2004). Natalizumab is a recombinant humanized antibody that is produced in mouse NSO murine myeloma cells and binds specifically to the α_4-subunit of the VLA-4 receptor. It is expressed at high levels on all circulating leukocytes, except for polymorphonuclear leukocytes. For principal details, see Volume I, Chapter 1.

10.5 Clinical Findings

10.5.1 Phase I Clinical Trials

Four phase I studies were completed. One study was conducted in healthy volunteers, while the other three were conducted to evaluate natalizumab in patients with either relapsing-remitting or secondary progressive MS. Different intravenous doses of natalizumab ranging between 0.03 and 6 $mg\,kg^{-1}$ were assessed. Overall, the results of the phase I trials showed natalizumab to be relatively well tolerated, with an overall adverse event profile that did not differ significantly from that of placebo (Sheremata et al. 1999, 2005).

10.5.2 Phase II Clinical Trials

Two phase II clinical trials were completed demonstrating significant reductions in inflammatory lesions, as visualized by magnet resonance imaging (MRI), and fewer patients with relapse during natalizumab treatment compared with placebo (Miller et al. 2003; Tubridy et al. 1999). The studies were conducted with

relapsing-remitting MS patients in a modern randomized, double-blind, placebo-controlled setting. Different dosage schemes and dosing time points were chosen. Pharmacokinetic and pharmacodynamic results from these studies also helped to identify the most appropriate natalizumab dose and application scheme for use in the subsequent phase III MS trials (Rudick and Sandrock 2004).

10.5.3 Phase III Clinical Trials

Two large randomized, double-blinded, placebo-controlled trials were commenced in MS patients who had experienced at least one clinical relapse during the year before entry. The AFFIRM (natalizumab versus placebo) and the SENTINEL (natalizumab plus IFN-β1a [Avonex] versus IFN-β1a plus placebo) studies were published in March 2006 (Polmann et al. 2006; Rudick et al. 2006). The level of efficacy observed in the earlier phase II trails was at least maintained in the two 2-year trials mentioned above. Overall, more than 2000 patients were included in these MS trials. In addition to the results confirmed from the phase II trial, these studies clearly showed that the progression of disability (defined as a change in the extended disability status scale, EDSS) was reduced by 42% within 2 years. The annualized rate of clinical relapse was reduced by 66% (from 0.78 to 0.27) and the number of new or enlarging brain lesions on MRI was reduced by 83%. By way of perspective, the currently used drugs, β-interferons and glatiramer acetate, diminish acute relapses by approximately 35%. Yet, only head-to-head trials of natalizumab and IFN will allow for a direct comparison (Gold et al. 2006).

Because natalizumab also blocks the interaction of the $\alpha_4\beta_7$-integrin with its ligand, MadCAM-1 (which is involved in leukocyte migration through intestinal endothelium), this treatment has also been investigated in Crohn's disease (Gordon et al. 2001). However, the results in the phase III trial have not shown any clinical significance.

Based on these 1-year phase III trial data, the FDA licensed natalizumab for reduction of relapse rate in November 2004 in the USA. Natalizumab was finally marketed under the name of Tysabri (Elan Pharmaceuticals Inc., San Francisco, CA, USA), but was suspended from the market and all clinical trials on February 28, 2005. This decision was based on two patients being diagnosed with progressive multifocal leukoencephalopathy (PML) participating in the SENTINEL trail. Retrospectively, a third patient who was enrolled in the Crohn's disease trial was shown by brain biopsy to have PML. His neurological symptoms were originally thought to be associated to an astrocytoma.

PML is a rare infection caused by the JC polyomavirus. It usually occurs in people infected with the human immunodeficiency virus (HIV), but it has also been reported in immunocompromised patients receiving prolonged treatment with immunosuppressants. The JC virus is ubiquitous (ca. 80% of the European population) and is usually acquired in childhood. The virus remains dormant in the bone marrow, kidney epithelia, and spleen, but can enter the CNS directly

during periods of viremia, such as those occurring during prolonged immunosuppression. The etiology of PML in patients treated with Tysabri is unclear. The influence of natalizumab on B-cell function and the specific manner of altering the adhesion process at the BBB, probably in synergy with β-interferon (and potentially all other immunomodulators or immunosuppressors), are possible reasons for JC virus activation. It should be noted, however, that blockade of VLA-4 also exerts widespread effects in the hematopoietic system. VLA-4 is involved in mobilizing leukocytes from bone marrow, and these effects on bone marrow may be directly relevant for the subsequent development of PML (Ransohoff 2005).

Elan pharmaceuticals and the BiogenIdec company voluntarily withdrew Tysabri from the market, and an investigation of all patients who ever received the drug was initiated. No new case of PML has since been observed (Yousry et al. 2006). An FDA advisory committee recommended that Tysabri could be reintroduced for relapsing-remitting MS as a monotherapy in June 2006 in the USA, and finally the EMEA decided to reintroduce it in July 2006 in Europe.

10.5.4
Adverse Side Effects

Apart from the above-mentioned issue, the overall tolerability of natalizumab treatment is very good. In both clinical trails most adverse side effects did not differ significantly between placebo and natalizumab. Of note, hypersensitivity reactions in more than 3.5% and anaphylactoid reactions in more than 1.4% in the natalizumab group must be mentioned. Natalizumab treatment produces increased levels of circulating white blood cells (i.e., lymphocytes, monocytes and eosinophils), although the mean values did not exceed the normal range (Miller et al. 2003). In this respect, attention must be paid to infections, cholelithiasis, urticaria (hives), irregular menstruation, and other diseases which can be associated with the application of natalizumab.

10.5.5
Neutralizing Antibodies

Persistent high-titer neutralizing anti-natalizumab antibodies (nAbs) developed in 6% of patients in the monotherapy study, and led to a loss of therapeutic efficacy and an increase in adverse side effects (Polman et al. 2006). Therefore nAbs can be evaluated for safety and efficacy after 3 to 4 months of treatment.

10.6
Indications for Tysabri

In June 2006, Tysabri received a license for the treatment of patients with relapsing forms of multiple sclerosis who:

- currently take β-interferons, for an appropriate period with ongoing relapse activity (at least one relapse in the past 12 months *and* at least nine T2 lesions *or* one gadolinium-enhancing lesion in the MRI); or
- with a high relapse rate of naive patients defined by two or more relapses during the past year with progression of disease *and* at least one gadolinium-enhancing lesion *or* significant increase of T2 lesion load compared to a previous recent MRI.

10.6.1 Clinical Applications

10.6.1.1 Preparation and Administration of Natalizumab

Aseptic technique must be used when preparing Tysabri solution for intravenous (IV) infusion. Each vial is intended for single use only. Tysabri is a colorless, clear to slightly opalescent concentrate. The vial must be inspected for particulate material prior to dilution and administration. If visible particulates are observed and/or the liquid in the vial is discolored, the vial must not be used. Tysabri must not be used beyond the expiration date stamped on the carton or vial (Fig. 10.3).

- Dose preparation: To prepare the solution, 15 mL of Tysabri concentrate is withdrawn from the vial using a sterile needle and syringe. The concentrate is then injected into 100 mL of 0.9% sodium chloride injection (USP). No other IV diluents may be used to prepare the solution. The

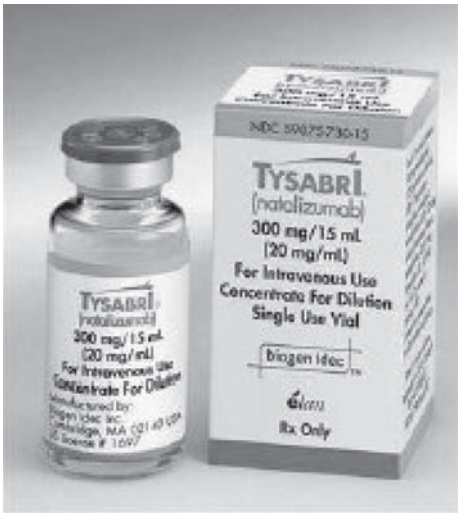

Fig. 10.3 A 15-mL vial of concentrated Tysabri 300 mg.

Tysabri solution is then gently inverted to provide complete mixing; the vial most NOT be shaken. The final solution must then be inspected visually for particulate material prior to administration.
- Infusion: Infuse Tysabri 300 mg in 100 mL 0.9% sodium chloride injection (USP) over a period of approximately 1 h. When the infusion is complete, the infusion system is flushed with 0.9% sodium chloride injection (USP).
- For safety reasons, in order to control any possible allergic reactions, the patient should remain in an outpatient setting for a further 60 min after infusion.

10.7 Outlook

The Advisory Committee of the FDA recommended a risk-minimization program with mandatory patient registration and periodic follow-up. Tysabri is available in the USA only through the Risk Management Plan, referred to as the TOUCH prescribing program.

In Europe, it is emphasized that Tysabri is administer only at centers with experience and availability of MRI. The drug may be prescribed only by neurologists, and all patients receive an "alert card". A prescribing program TYGRIS is also proposed for Europe in the near future.

In order to receive Tysabri, patients must visit their specialist doctor and also understand the risks and benefits associated with Tysabri administration.

References

Alter, A., Duddy, M., Hebert, S., Biernacki, K., Prat, A., Antel, J.P., Yong, V.W., Nuttall, R.K., Pennington, C.J., Edwards, D.R., Bar-Or, A. (2003) Determinants of human B cell migration across brain endothelial cells. *J Immunol* 170: 4497–4505.

Baron, J.L., Madri, J.A., Ruddle, N.H., Hashim, G., Janeaway, C.A. (1993) Surface expression of α4 integrin by CD4 T cells is required for their entry into brain parenchyma. *J Exp Med* 177: 57.

Enders, U., Lobb, R., Pepinsky, R.B., Hartung, H.-P., Toyka, K.V., Gold, R. (1998) The role of the very late antigen-4 (VLA-4) and its counterligand vascular cell adhesion molecule-1 (VCAM-1) in the pathogenesis of experimental autoimmune neuritis (EAN) of the Lewis rat. *Brain* 121: 1257–1266.

Gold, R., Hartung, H.P., Hohlfeld, R. (2006) Monoclonal antibodies improve therapy of relapsing multiple sclerosis. Molecular basis and clinical results of anti-VLA4 (natalizumab) therapy. *Dtsch Med Wochenschr* 131(1–2): 31–34.

Gonzalez-Amaro, R., Mittelbrunn, M., Sanchez-Madrid, F. (2005) Therapeutic anti-integrin (α4 and αL) monoclonal antibodies: two-edged swords? *Immunology* 116: 289–296.

Gordon, F.H., Lai, C.W., Hamilton, M.I., et al. (2001) A randomized placebo-controlled trial of a humanized

monoclonal antibody to α4-integrin in active Crohn's disease. *Gastroenterology* 121: 268–274.
Kent, S.J., Karlik, S.J., Cannon, C., et al. (1995) A monoclonal antibody to α4 integrin suppresses and reverses active experimental allergic encephalomyelitis. *J Neuroimmunol* 58: 1–10.
Léger, O.J., Yednock, T.A., Tanner, L., Horner, H.C., Hines, D.K., Keen, S., Saldanha, J., Jones, S.T., Fritz, L.C., Bendig, M.M. (1997) Humanization of a mouse antibody against human alpha-4 integrin: a potential therapeutic for the treatment of multiple sclerosis. *Hum Antibodies* 8(1): 3–16.
Leussink, V.I., Zettl, U.K., Jander, S., et al. (2002) Blockade of signaling via the very late antigen (VLA-4) and its counterligand vascular cell adhesion molecule-1 (VCAM-1) causes increased T cell apoptosis in experimental autoimmune neuritis. *Acta Neuropathol* 103: 131–136.
Miller, D.H., Khan, O.A., Sheremata, W.A., et al. (2003) A controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med* 348: 15–23.
Niino, M., Bodner, C., Simard, M.L., et al. (2006) Natalizumab effects on immune cell responses in multiple sclerosis. *Ann Neurol* 59(5): 748–754.
Polman, C.H., O'Connor, P.W., Havrdova, E., et al. (2006) A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med* 354: 899–910.
Ransohoff, R.M. (2005) Natalizumab and PML. *Nat Neurosci* 8: 1275.
Rice, G.P., Hartung, H.P., Calabresi, P.A. (2005) Anti-α4 integrin therapy for multiple sclerosis: mechanisms and rationale. *Neurology* 64(8): 1336–1342.
Rudick, R.A., Sandrock, A. (2004) Natalizumab: alpha 4-integrin antagonist selective adhesion molecule inhibitors for MS. *Expert Rev Neurother* 4(4): 571–580.
Rudick, R.A., Stuart, W.H., Calabresi, P.A., Confavreux, C., Galetta, S.L., Radue, E.W., Lublin, F.D., Weinstock-Guttman, B., Wynn, D.R., Lynn, F., Panzara, M.A., Sandrock, A.W. and the SENTINEL Investigators. (2006) Natalizumab plus interferon beta-1a for relapsing multiple sclerosis. *N Engl J Med* 354(9): 911–923.
Sheremata, W.A., Vollmer, T.L., Stone, L.A., Willmer-Hulme, A.J., Koller, M. (1999) A safety and pharmacokinetic study of intravenous natalizumab in patients with MS. *Neurology* 52(5): 1072–1074.
Sheremata, W.A., Minagar, A., Alexander, J.S., Vollmer, T. (2005) The role of alpha-4 integrin in the aetiology of multiple sclerosis: current knowledge and therapeutic implications. *CNS Drugs* 19(11): 909–922.
Springer, T.A. (1990) Adhesion receptors of the immune system. *Nature* 346: 425.
Tubridy, N., Behan, P.O., Capildeo, R., et al. (1999) The effect of antiα4 integrin antibody on brain lesion activity in MS. *Neurology* 53(3): 466–472.
Vajkoczy, P., Laschinger, M., Engelhardt, B. (2001) Alpha 4-integrin-VCAM-1 binding mediates G protein-independent capture of encephalitogenic T cell blasts to CNS white matter microvessels. *J Clin Invest* 108: 557–565.
von Andrian, U.H., Mackay, C.R. (2000) T-cell function and migration. *N Engl J Med* 343: 1020–1034.
von Andrian, U.H., Engelhardt, B. (2003) α_4-Integrins as therapeutic targets in autoimmune disease. *N Engl J Med* 348: 68–72.
Weiner, H.L. (2004) Multiple sclerosis is an inflammatory T-cell-mediated autoimmune disease. *Arch Neurol* 61: 1613–1615.
Wolinsky, J.S. (2003) Rational therapy for relapsing multiple sclerosis. *Lancet Neurol* 2(5): 271–272.
Yednock, T.A., Cannon, C., Fritz, L.C., Sanchez-Madrid, F., Steinman, L., Karin, N. (1992) Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha4beta1 integrin. *Nature* 356: 63–66.
Yousry, T.A., Major, E.O., Ryschkewitsch, C., et al. (2006) Evaluation of patients treated with natalizumab for progressive multifocal leukoencephalopathy. *N Engl J Med* 354: 924–933.

11
Omalizumab (Xolair)
Anti-Immunoglobulin E Treatment in Allergic Diseases

Claus Kroegel and Martin Foerster

Summary

Immunoglobulin E (IgE) plays a key role in the induction and maintenance of allergic disorders, making the molecule and its pathways an obvious target for a novel treatment approach. Omalizumab, a recombinant humanized monoclonal antibody, is the first therapeutic agent that specifically targets immunoglobulin E (IgE). Monoclonal anti-IgE Omalizumab (rhuMAb-E25; Xolair) has been approved for the treatment of moderate-to-severe persistent allergic asthma in several nations, including USA, Canada and Brazil, Australia, New Zealand, and a number of European countries.

Monoclonal antibody: Omalizumab (Xolair, Genentech) is a recombinant DNA-derived, humanized IgG1 monoclonal anti-human IgE antibody consisting of a murine mAb MAE11 directed against IgE (Fab terminus) and a human IgG frame (Fc terminus).

Indications and dosing: Omalizumab is approved for the treatment of adults and adolescents (aged > 12 years) having moderate-persistent to severe-persistent asthma with a positive skin test or *in-vitro* reactivity to perennial aeroallergens (e.g., dust mites, cats, dogs, fungi), and whose symptoms are inadequately controlled with inhaled corticosteroids. The recommended dosage of omalizumab is 150–375 mg subcutaneously (s.c.) every 2 or 4 weeks depending on pretreatment total serum IgE level and body weight.

Mode of action: Omalizumab binds to the same domain (Cε3) on the IgE molecule that interacts with the high-affinity IgE receptor (FcεRI), thereby interrupting the proinflammatory signal carried by IgE. The mode of action of rhuMAb-E25 comprises immediate (elimination of free serum IgE), short-term (down-regulation of IgE receptors on circulating basophils and dendritic cells) and long-term effects (reduction the number of tissue dwelling eosinophils and mast cells, T and B cells).

Clinical data: Several large phase III clinical trials have demonstrated that omalizumab is more effective than placebo in controlling moderate to severe allergic asthma with respect to symptom severity scores, lung function, quality of life,

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and number of exacerbations. In particular, omalizumab provides important benefits in patients with poorly controlled disease or exacerbations despite adequate therapy. In addition, the drug may aid in the treatment of other IgE-related conditions such as allergic rhinities, atopic dermatitis or food intolerance.

Adverse side effects: Omalizumab is generally well tolerated in adults and children with allergic asthma, and the incidence of adverse side effects is low. Adverse events most commonly observed are injection-site reaction, viral infection, upper respiratory tract infection, sinusitis, headache, and pharyngitis; severe side effects (anaphylaxis) are rare, and an association with malignancy is weak.

Summary: Omalizumab provides a novel second-line treatment option for moderate to severe allergic asthma and possibly for other manifestations of atopy, allowing a more individualized therapy. The available data emphasize the fundamental importance of IgE in the pathogenesis of allergic diseases.

11.1 Introduction

Worldwide, allergic diseases continue to increase, affecting more than 100 million individuals (Beasley et al. 2000; ISAAC 1998; Warner et al. 2006). The reasons underlying this worldwide epidemic of allergic diseases are not understood, but are most likely the consequence of environmental changes and improved hygiene, superimposed on a range of genetic susceptibilities.

Immunoglobulin E (IgE) is an important mediator in the pathophysiology of asthma and allergy (Kroegel 2002; Platts-Mills 2001). Approximately two-thirds of asthma is estimated to be allergic (Novak and Bieber 2003), and total IgE levels correlate with asthma rates in adults (Fig. 11.1) (Burrows et al. 1989). Atopy, which has increased over the past 30 years, does not decline with increasing age. Moreover, IgE receptors are expressed on various different cell types, further emphasizing the biological relevance of IgE. The binding of IgE to its receptors primes these cells to respond to allergen. Although the function of mast cells and basophils mainly depends on the binding of IgE, the number of IgE receptor-expressing cells is large, and includes other effector cells (eosinophils, macrophages), regulator cells (B-lymphocytic dendritic cells) as well as structural cells (epithelial cells, smooth muscle cells). Upon repeated exposure to allergens, IgE antibodies bound to FcεRI on mast cells and basophils are crosslinked, and this results in the secretion of preformed mediators (e.g., histamine, tryptase) and the generation of newly formed mediators, such as arachidonic acid metabolites (e.g., prostanoids, leukotrienes) and various cytokines. These secreted mediators mount early- and late-phase allergic reactions via multiple inflammatory effects. The early phase, which occurs within minutes of allergen exposure, is associated with increased vascular permeability, smooth muscle contraction, and local damage caused by vasoactive amines, proteases, and lipid mediators. Cytokines released during the reaction are responsible for the late-phase local inflammatory response observed between 6 and 24 h after allergen exposure. IgE is also believed

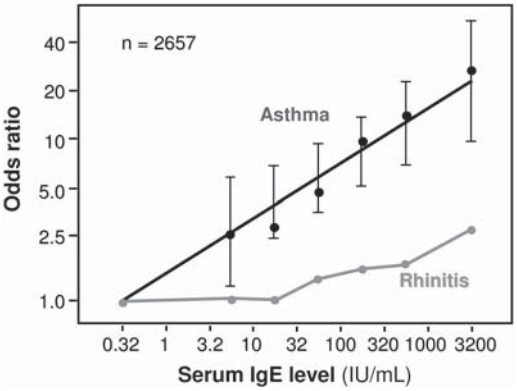

Fig. 11.1 Relationship between IgE, asthma, and rhinitis in adults. The data indicate odds ratio (OR) of having asthma at seven levels of total IgE concentrations after correction for age, gender, smoking habits, and skin-test index in a logistic analysis. The upper line represents the risk of asthma; vertical bars indicate 95% confidence intervals around the regression for each OR corresponding to a given log IgE level. The lower line represents the OR of having rhinitis at the same seven levels of serum IgE following correction of the same factors. (From Burrows et al. 1989.)

to bridge early- and late-phase allergic reactions, both directly via binding to FcεRII on eosinophils, and indirectly via activation and release of cytokines from mast cells and basophils (Fig. 11.2). Other IgE-mediated effects of relevance in asthma include IgE-dependent focusing of antigen to dendritic cells (Kehry and Yamashita 1989) and mobilization of calcium in airway smooth muscle cells (Gounni et al. 2005). Therefore, as the biologic effects of IgE are diverse and far-reaching, the inhibition of IgE can be expected to block multiple allergic mechanisms at different levels of allergic inflammation.

During recent years, several drugs have been developed aimed specifically at interfering with IgE-associated pathways, most notably humanized monoclonal antibodies (mAbs) (e.g., CGP 51901, rhuMAb-E25). Among these mAbs, the most advanced anti-IgE mAb is rhuMAb-E25 (omalizumab; Xolair; Novartis Pharmaceuticals, East Hanover, NJ, USA; Genentech Inc., South San Francisco, CA, USA). This novel drug offers a completely different treatment approach which promises several clinically relevant advantages over current treatment options. This chapter provides an overview of the immunobiology of the IgE molecule and its receptors, followed by a detailed discussion on the construction of rhuMAb-E25 and its use in the treatment of asthma and other IgE-associated diseases.

11.2
The Biology of the IgE Molecule

During the mid-1960s, Ishizaka and colleagues (Ishizaka and Ishizaka 1970) discovered a new antibody isotype, now known as IgE, thereby opening a new era

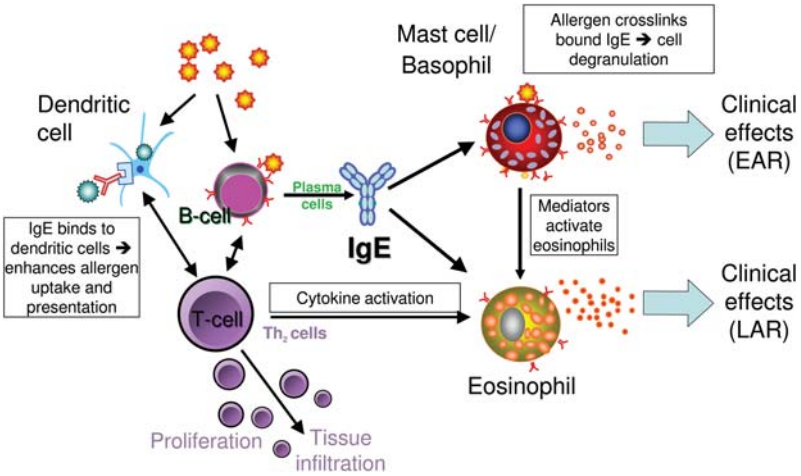

Fig. 11.2 The immunological cascade in allergy and asthma. Following inhalation, the allergen is processed by antigen-presenting cells (APCs) and presented to B cell and Th0 cells; this leads to differentiation of Th2 cells (IL-4, IL-5, IL-9, IL-13) and IgE-producing B cells (plasma cells). Repeated allergen exposure causes the immediate release of mediators from mast cells and produces the typical symptoms of acute asthma (EAR). Mediators released from eosinophils produce asthma symptoms associated with chronic allergic inflammation, in which bronchial hyperresponsiveness predominates (LAR). EAR = early allergic/asthmatic reaction; LAR = late allergic/asthmatic reaction; Th0 = uncommitted T lymphocyte (can differentiate into Th1 or Th2 lymphocytes depending on the cytokines present); Th2 = functional T lymphocyte associated with helper activity for antibody production. (Modified after Holgate et al. 2005b.)

in the pathophysiology of immunologic disorders. Since its discovery, IgE has been shown to be a key mediator in allergic diseases (Maizels 2003; Maizels and Yazdanbakhsh 2003; Yazdanbakhsh et al. 2001). In addition, IgE is associated with a number of other immunologic diseases (Fig. 11.3), where its role is less well-defined.

Like other immunoglobulins, IgE consists of two light chains and two ε-heavy chains (Fig. 11.4), but is distinguished by its ε-heavy chain constant region sequence. Unlike IgG, IgE contains four instead of three heavy chain constant domains (Cε1–Cε4) (Garman et al. 2000), with the additional domain (Cε2) replacing the hinge region (Sutton and Gould 1993). Moreover, as opposed to other immunoglobulin classes, human IgE binds specifically with both high and low affinity to receptors (FcεR) on the surface of several different cell types (Scharenberg and Kinet 1995).

The allergen-binding site on the IgE molecule is located on the variable regions of the heavy and light chains. The Fc fragment of the IgE molecule binds to two types of immunoglobulin Fcε-receptors (see below). The binding site for both FcεRI and FcεRII is located in proximity to, but on different parts of, the Cε3 domain of the IgE molecule (Garman et al. 2000; Nissim et al. 1993; Presta et al.

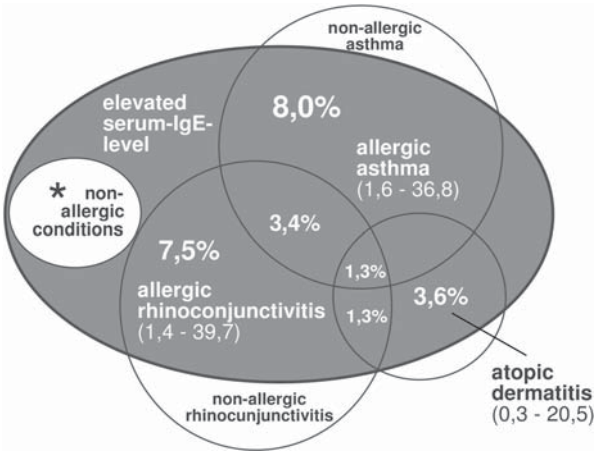

Fig. 11.3 Relationship between elevated serum IgE levels and allergic and non-allergic conditions. Large numbers refer to the prevalence of allergic diseases in children (symptoms during the past 12 months) and are based on data provided by the ISAAC Study (1998). Numbers in brackets indicate ranges. *Non-allergic diseases associated with a raised IgE level are parasitoses, AIDS, Wiskot–Aldrich syndrome, Nezelof syndrome, non-Hodgkin lymphoma, malignant tumors and the hyper-IgE-syndrome (Job syndrome).

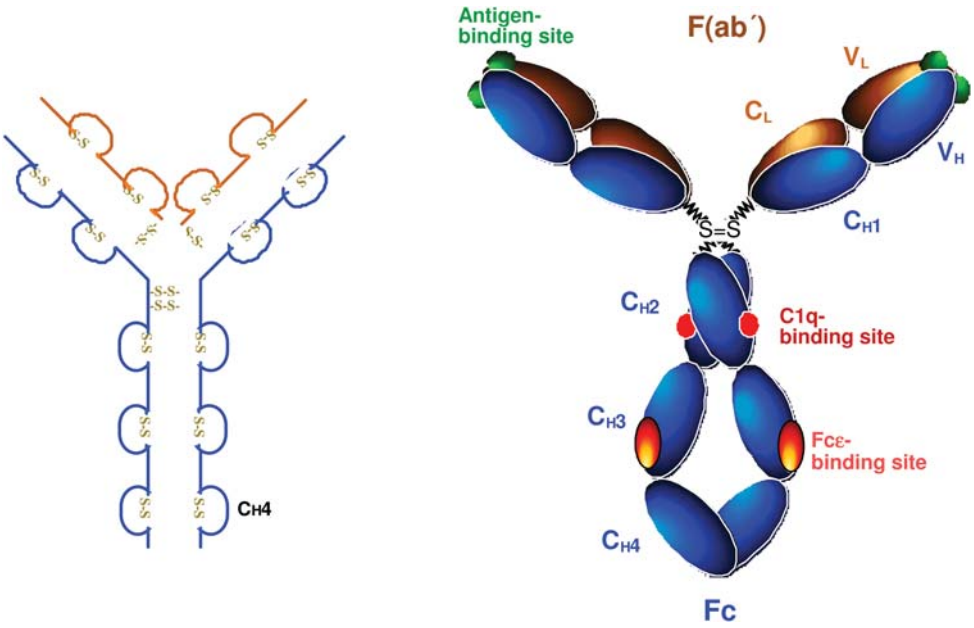

Fig. 11.4 Structure of the immunoglobulin isotype E. For details, see text.

1994; Vercelli et al. 1989). The association of the dimeric Fc fragment of the IgE antibody to its receptors has two significant consequences. First, the IgE receptor adopts the antigenic specificity of the prevalent IgE repertoire (Gaman et al. 2000). Second, a specific interaction with antigens/allergens results in crosslinking of the FcεRI, thereby initiating a signal transduction cascade and downstream intracellular effects (Turner and Kinet 1999). This process ultimately induces the release of a variety of preformed (e.g., histamine) and *de-novo* synthesized chemical mediators (e.g., leukotrienes and prostaglandins), as well as cytokines that exert their immunologic effects by interacting with specific receptors on target organs.

11.2.1 IgE Distribution and Blood Concentration

While being present in very low amounts (ng to $\mu g\,mL^{-1}$ range) in the serum of normal healthy individuals, IgE levels are significantly elevated in allergic subjects. The raised levels of specific IgE in the serum, together with a positive skin test, defines allergic sensitization (or "atopy"). However, even in highly atopic individuals, the level of plasma IgE is less than a 1000-fold that of plasma IgG (Geha et al. 2003). Indeed, in nonallergic individuals, plasma IgE levels may be 10 000- to 50 000-fold lower than that of plasma IgG. However, in contrast to IgG, most of the IgE produced is bound by FcεRI expressed by mast cells and basophils (Geha et al. 2003) and by FcεRII on various other immune cells.

11.2.2 IgE Synthesis and Regulation

IgE molecules are mainly produced by plasma cells in the mucosa-associated lymphoid tissue. The regulation of IgE production is an extremely complex process, and is controlled by various positive as well as negative factors. The major step in IgE synthesis is the regulation of the IgE class-switch recombination (Geha et al. 2003). Common to all isotype switching is the germline transcription of CH genes and the induction of activation-induced cytidine deaminase (AID) expression. IgE class-switch recombination is accomplished either through T-cell-dependent (the classic pathway) or T-cell-independent processes (the alternative pathway) (Geha et al. 2003). In addition, IgE class switching is controlled by several other mechanisms, including cytokines [interferon (IFN)-γ and interleukin (IL)-21], the engagement of surface receptors on B cells (B-cell receptor, CD45, CTLA4, and CD23), as well as transcription factors (B-cell lymphoma 6, inhibitor of DNA binding 2). A detailed review, which also provides an excellent description of the mechanisms underlying the production of IgE, is available (Geha et al. 2003).

11.3 IgE Receptors

The IgE molecule mediates immunologic its effects by coupling with two different binding sites expressed by immune cells (Table 11.1):

- the high-affinity IgE receptor (FcεRI); and
- the low-affinity IgE receptor (FcεRII or CD23).

11.3.1 FcεRI (High-Affinity IgE Receptor)

The FcεRI complex represents a high-affinity cell-surface receptor for the Fc region of antigen-specific IgE molecules. FcεRI is multimeric, and is a member of a family of related antigen/Fc receptors which show conserved structural features and similar roles in initiating intracellular signaling cascades. In humans, FcεRI controls the activation of mast cells and basophils, and participates in IgE-mediated antigen presentation. FcεRI binds to the IgE molecule with high affinity, characterized by an equilibrium dissociation constant (or binding affinity) of 10^{-9} to 10^{-10} M (Garman et al. 2000). The receptor consists of the four transmembrane polypeptides αβγ2 (Sutton and Gould 1993), and its expression is restricted to mast cells, basophils, and dendritic cells (Allam et al. 2006) (Fig. 11.5).

Table 11.1 Distribution of FcεR, its biologic role and expression in allergic disease (modified according to Nissim et al. 1991).

Cell type	*FcεR-type*	*Biological function*	*Expression in allergy*	*Regulated by IgE-binding*
Mast cells basophils	αβγ2	Synthesis and secretion of mediators and cytokines, Induction of IgE synthesis, antigen presentation	Increased	Yes
Dendritic cells	αβγ2	Antigen presentation	Increased	Yes
Langerhans cells	αγ2	Antigen presentation, priming of naive T-lymphocytes to IgE-reactive proliferation	Increased	Not known
Monocytes Macrophages	αγ2	Antigen presentation, synthesis and secretion of mediators and cytokines, enzymes and oxygen radicals	Increased	Not known
Eosinophils	αγ2	Antigen presentation?	Increased	Not known
Platelets	αγ2	Unknown	Unchanged	Not known
Epithelial cells	αγ2	Secretion of eicosanoids	Increased	Not known

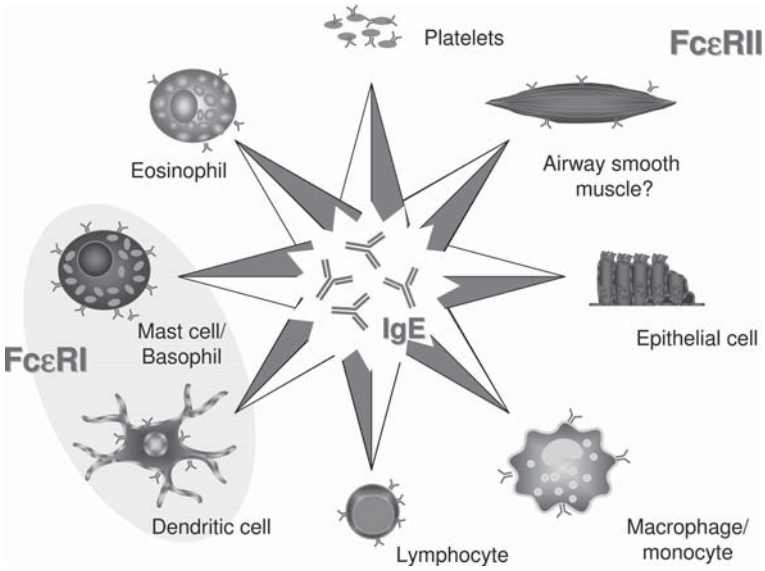

Fig. 11.5 Cells expressing the high-affinity IgE receptor FcεRI (gray shaded area) and the low-affinity IgE receptor FcεRII (the remaining cells).

FcεRI-IgE binding The extracellular portion of the α-chain enables the receptor to interact with the Cε3 of the IgE molecule (Fig. 11.6). The IgE binding site consists of two immunoglobulin-like domains (D1 and D2), and is located on the extracellular portion of the FcεRI α-chain (Turner et al. 1999). The domains are positioned at an acute angle to one another, thus creating a convex surface at the top of the molecule, and enclosing a marked cleft. One receptor binds one dimeric IgE-Fcε molecule asymmetrically through interactions at two sites (Garman et al. 1998), thereby preventing the binding of a second receptor (Turner et al. 1999). In contrast, the β- and γ-chains of the receptor are required for receptor transmembrane insertion and intracellular signal transduction (Garman et al. 2000; Turner and Kinet 1999).

FcεRI activation Whereas other receptor types (hormone or cytokine receptors) dimerize and signal after binding a well-defined ligand, the Fcε receptor types act as adaptors between highly variable antigen-binding sites and the intracellular signal transduction machinery (Garman et al. 2000). Multivalent antigens bind and crosslink IgE molecules held at the cell surface by FcεRI. Crosslinking and receptor aggregation of FcεRI induced by a multivalent antigen, is critical in triggering consecutive cellular events which eventually mount an allergic effector response in various tissues and organs. These include the release of preformed (e.g., histamine, tryptase) and newly generated mediators within minutes (e.g., leukotrienes, prostanoids) and hours [cytokines such as IL-4, IL-6, tumor necrosis

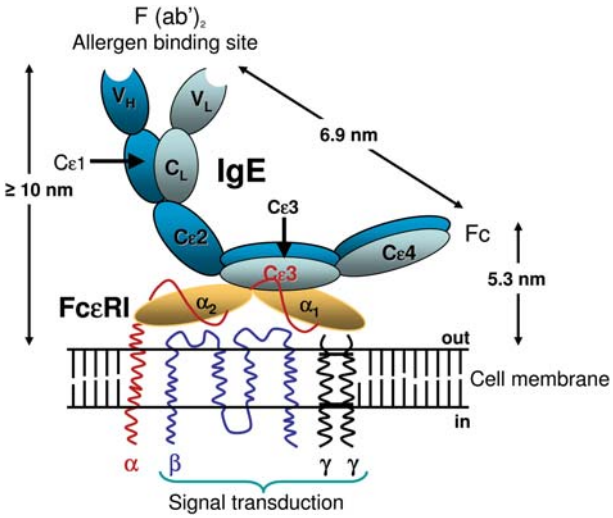

Fig. 11.6 Binding of IgE to the high-affinity receptor (FcεRI). FcεRI consists of four polypeptide chains, γβα2. The α-chain binds to five amino acids (330–335) of the Cε3 domain of Fc segment of IgE in such a manner that it lies on its side with the allergen binding site facing outwards. In contrast, FcεRII is expressed in trimeric form (γα2) on antigen-presenting cells such as monocytes and peripheral blood dendritic cells. (Modified after Holgate 1998.)

factor-alpha (TNF-α)] following repeated antigen/allergen exposure (Garman et al. 2000; Geha et al. 2003; Turner and Kinet 1999) (Fig. 11.7).

Regulation of FcεRI expression Observations made by Malveaux and Lichtenstein in 1978 noted a correlation between cell-surface FcεRI densities on peripheral blood basophils and the serum IgE titer (Malveaux et al. 1978). More recently, several investigations (MacGlashan et al. 1997a,b; Prussin et al. 2003; Saini et al. 1999) have confirmed the relationship between serum IgE concentrations and the magnitude of FcεRI expression on basophils and mast cells (Fig. 11.8). Although the underlying mechanism is not understood, the data lend strong support to the role of total IgE concentrations in controlling the expression of FcεRI (Borkowski et al. 2001). Nevertheless, the observation that blood IgE concentrations modify the expression of FcεRI has important implications both for the immunobiology of atopy and the therapeutic approaches for allergic disease (MacGlashan 2005).

11.3.2 FcεRII (Low-Affinity IgE Receptor, CD23)

The FcεRII receptor is referred to as the low-affinity IgE receptor, although it displays only one log difference in its binding affinity compared with the

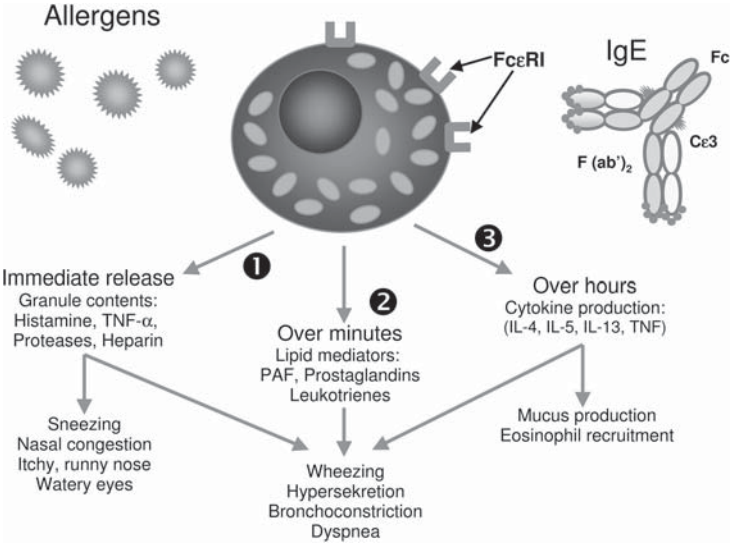

Fig. 11.7 IgE-dependent cell activation. IgE binds to high- and low-affinity receptors (FcεRI or FcεRII) on effector cells. The inflammatory cascade is initiated when IgE bound to effector cells is crosslinked by an allergen. This results in the degranulation of effector cells and the release of a comprehensive array of mediators that are linked to the pathophysiology of asthma. This in turn causes a sequential release of different types of inflammatory mediators.

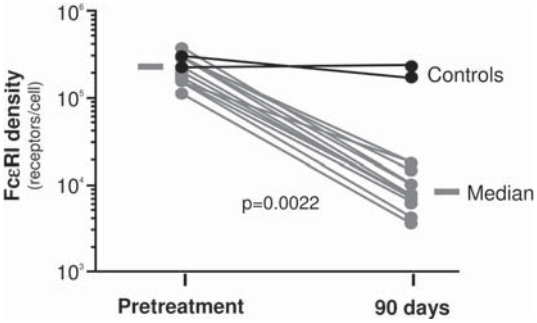

Fig. 11.8 FcεRI density on basophils prior to and 90 days after initiation of rhuMAb-E25 treatment. A series of *in-vitro* studies used circulating basophils taken from 15 subjects with perennial allergic rhinitis receiving rhuMAb-E25 for 3 months. The figure shows the results from 12 rhuMAb-E25-treated patients and two patients who acted as controls. With rhuMAb-E25 treatment, total FcεRI receptor density was markedly and significantly decreased by approximately 97% ($p = 0.0022$). The average IgE receptor numbers were reduced from approximately 240 000 to approximately 8600 receptors per cell at the 3-month measurement. (Modified after MacGlashan et al. 1997.)

high-affinity FcεRI. The equilibrium dissociation constant (binding affinity) is of $10^8\,M^{-1}$ for IgE-antigen complex and $10^7\,M^{-1}$ for IgE (Kijimoto-Ochiai 2002). In contrast to the four transmembrane polypeptides of FcεRI, the low-affinity form consists of an αγ2 trimer (Turner and Kinet 1999), and is expressed on a variety of hematopoietic cell lines, including eosinophils (Gounni et al. 1994), dendritic cells (DCs), activated macrophages, Langerhans cells, platelets, epithelial cells and possibly muscle cells (Bieber et al. 1992; Maurer et al. 1995; Novak and Bieber 2003; Wang et al. 1992) (see below; see also Fig. 11.5).

FcεRII-IgE binding Unlike other Fc receptors that are structurally classified within the immunoglobulin-like superfamily, CD23 is a 45-kDa type II transmembrane protein with an intracytoplasmic C-terminus. It is unique among Fc receptors in its homology to C-type (calcium-dependent) lectins. IgE binds to two CD23 lectin domains (D1-2 and D5-8) within the Cε3 region of the IgE molecule via protein–protein and protein–carbohydrate interactions, respectively (Henchoz et al. 1994; Kijimoto-Ochiai 2002).

FcεRII activation Activation of the trimeric FcεRII also contributes to the pathogenesis of allergic diseases (Saini and MacGlashan 2002). For instance, activation of FcεRI prevents apoptosis of monocytes by induction of the anti-apoptotic ligands *Bcl*-2 and *Bcl*-xL, and also protects from CD95 Fas-mediated apoptosis. Further, activation of FcεRII in atopic donors leads to the downstream activation of the proinflammatory transcription factor "nuclear factor κB" (Kraft et al. 2001). In the presence of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF), crosslinking FcεRI alters monocyte differentiation toward a macrophage species and away from a DC phenotype (Novak et al. 2001). Ligation of IgE to the CD23 receptor expressed on B cells leads to cell differentiation, apoptosis, and regulation of IgE synthesis (Bonnefoy et al. 1996).

FcεRII/CD23 functions CD23 has multiple functions that are controlled by a range of different ligands (Table 11.2). These include IgE (both in its secreted form and on membranes of committed B cells), CD21 (also known as complement receptor 2), CD18/CD11b and CD18/CD11c (complement receptors 3 and 4, respectively), and the vitronectin receptor. Paradoxically, CD23 engages in both the up- and down-regulation of IgE synthesis, thereby constituting a "two-way switch" in IgE homeostasis (Aubrey et al. 1992; Bonnefoy et al. 1996). When IgE binds to membrane CD23, further IgE synthesis is suppressed. In contrast, CD23-deficiency increases the level of circulating IgE by orders of magnitude. Soluble CD23 enhances IgE synthesis on binding to CD21 (Aubrey et al. 1992) and other mechanisms (Henchoz et al. 1994).

The dust mite protease Der p I cleaves CD23 close to the lectin domain, and the resulting monomeric CD23 may be a factor in the high allergenicity of dust mites (Nakamura et al. 2002). Soluble circulating CD23 fragments are found in the blood of healthy human subjects, but are commonly elevated in inflammatory or lymphoproliferative diseases, such as rheumatoid arthritis, asthma, and chronic

Table 11.2 Functional variability of CD23.

Description	*Example*	*Consequence*	*Reference*
Two forms	CD23a expressed constitutively on B cells; CD23b is expressed on monocytes, eosinophils, DCs, Langerhans cells, and platelets	Functional variability on B cells: differentiation, apoptosis, and regulation of IgE synthesis	Novak and Bieber (2003) Bonnefoy et al. (1996)
Non-IgE receptor interactions	CD21 CD11b CD11c	Variety of functions	Bonnefoy et al. (1996) Aubrey et al. (1992)
CD23 is cleaved by proteases	Soluble CD23	Proinflammatory properties, e.g., up-regulation of IgE synthesis	Herbelin et al. (1994) Henchoz et al. (1994)

lymphoblastic leukemia. It has been shown that antibodies to CD23 alleviate all three conditions, and an anti-CD23 antibody, IDEC-152 (lumiliximab), is presently undergoing clinical trials for treatment of asthma (Rosenwasser and Meng 2005).

11.4 Cell Distribution of IgE

11.4.1 Effector Cell-Associated IgE

As mentioned above, IgE is found in various tissues throughout the body. It is mainly bound to cells via either FcεRI or FcεRII on the surface of the effector (e.g., mast cell, basophils eosinophils, macrophages, and monocytes) and regulator cells (e.g., B cells and dendritic cells) (see Fig. 11.5). The complex of allergen, IgE, and FcεRI on the surface triggers a noncytotoxic, energy-dependent release of preformed, granule-associated mediators (histamine and tryptase) and the membrane-derived lipid mediators (leukotrienes, prostaglandins, and platelet-activating factor) which eventually leads to the clinical manifestation seen in allergic disease (see Fig. 11.7).

11.4.2 Antigen-Presenting Cell-Associated IgE

Antigen-presenting cells (APCs) are critical in initiating and controlling allergic inflammation. Dendritic cells and cutaneous Langerhans cells are particularly

important in asthma and atopic eczema, respectively. These present antigen to $CD4^+$ Th2 cells in a MHC class II-restricted fashion. The overproduction of GM-CSF in the airway mucosa of patients with asthma enhances antigen presentation and increases the local accumulation of macrophages (Maurer et al. 1997; Stingle and Maurer 1997). Bronchoalveolar lavage macrophages obtained from patients with asthma present allergen to $CD4^+$ T cells and stimulate the production of Th2-type cytokines, whereas alveolar macrophages from control subjects do not.

APCs facilitate antigen/allergen presentation to allergen-specific T-cells and express both FcεRI and FcεRII (or CD23) (Novak and Bieber 2003). The main role of FcεRI on APCs is antigen/allergen focusing. Multivalent allergens activate FcεRI-bound IgE on APCs, initiating a cascade of events that leads to newly synthesized MHC class II molecule for the presentation of FcεRI-targeted agents (Novak and Bieber 2003).

Dendritic cells (DCs) are potent APCs that play a significant role in promoting T-cell responses to antigens/allergens. Classically, myeloid precursor DCs (pDC1s or $CD11c^+$ cells) are high IL-12 producers that induce a Th1 response. In contrast, plasmacytoid DCs (pDC2s or $CD123^+$ cells) are low IL-12 producers that induce a Th2 response (Liu et al. 2001; Rissoan et al. 1999). However, a given DC subset has remarkable plasticity in directing different types of T-cell responses (Kalinski et al. 1999; Liu et al. 2001). Thus, the modulation of surface receptor expression may have important consequences for the development of the downstream immune sequence, including IgE-mediated type 1 hypersensitivity reactions.

11.5
Physiologic and Pathophysiologic Significance of IgE

Despite the fact that IgE was discovered more than 40 years ago, its exact biological function has not been disclosed to date. Because IgE titers are elevated in individuals suffering from helminthic infestations, IgE was originally thought to play a role in the defense against parasitic infestations (Amiri et al. 1994; Hagan et al. 1991). However, the observation that a reduction of IgE levels through anti-IgE antibody treatment of mice infected with *Schistosoma mansoni* or *Nippostrongylus brasiliensis* resulted in accelerated elimination of parasites, a decreased worm burden, and a reduction in the number of worm ova, suggested that high serum IgE levels are independent of the host defense. In fact, it is currently believed that IgE is not involved in the defense processes against parasites, and the increase in concentration may simply represent an epiphenomenon resulting from parasite-induced Th2 cell activation. Thus, to date, except for its pathogenic role in allergic inflammation, a clear biologic function cannot be assigned to IgE.

Nevertheless, because IgE is the central macromolecular mediator involved in several key positions in allergic reactions, interrupting IgE-dependent pathways appears to be a rational approach for the treatment of allergic diseases. If true, this finding has two major – but opposing – consequences. First, due to its

Table 11.3 Requirement for a Anti-IgE mAb applicable in humans (Chang et al. 1990).

- High-affinity binding to IgE,
- No binding to IgE already bound by FcεRI on mast cells and basophils,
- No binding to IgE bound by the low-affinity IgE Fc receptors (FcεRII, CD23) on various other cell types
- Binding to membrane-bound IgE (mIgE) on mIgE-expressing B cells
- Construction as a humanized mAb against IgE
- Binding of circulating IgE regardless of specificity
- Forming only small, biologically inert omalizumab:IgE complexes
- No complement activation

insignificant "physiologic" role, interrupting IgE-related allergic processes is likely to cause few adverse side effects. Second, since its natural biological function during immune reactions remains unclear, it cannot be ruled out that long-term use may eventually reveal side effects related to a so-far hidden physiologic role of IgE.

11.6 The Concept of Anti-IgE-Based Treatment

Immunoglobulin E plays a key role in the induction and maintenance of allergic disorders, and consequently the interruption of IgE-dependent pathways appears to be a rational approach for the treatment of allergic diseases (Barnes 2000; Chang et al. 1990; Davis et al. 1991, 1993). The basic idea was to engineer chimerized or humanized anti-IgE antibodies with a set of unique binding properties which could be used for the isotype-specific neutralization of IgE. However, in order to be applicable in humans, the anti-IgE mAb needed to fulfill a number of the attributes listed in Table 11.3 (Chang et al. 1990; Davis et al. 1993; Heusser and Jardieu 1997).

11.7 Construction of the Monoclonal Anti-IgE Molecule

11.7.1 Antibody Generation

Selective binding of IgE requires that the mAb binds only to free IgE and not to FcεRI-IgE (see above). Thus, the engineering of a safe and effective anti-IgE molecule necessitates identification of the binding site on human immunoglobulin E for FcεRI (Presta et al. 1994). Using a model of the IgE Fcε3 homologous to the second constant domain of IgG, homology scanning mutagenesis and replacement of individual residues were performed to determine the specific

amino acids involved in binding of human IgE to FcεRI (Nissim and Eshhar 1992). A total of six key amino acids (Arg408, Ser411, Lys414, Glu452, Arg465, and Met469) were identified. These residues are localized in three loops within the Cε3 domain, forming a ridge on the most exposed portion of the IgE molecule (Presta et al. 1994).

In order to create a novel specific inhibitor capable of blocking IgE-binding to FcεRI but lacking the capacity to stimulate degranulation of mast cells and other cell types, a strategy employing a murine mAb directed against IgE, which would bind IgE at the same site as the high-affinity receptor was developed. It was essential that this antibody lacked the unwanted side effects caused by receptor crosslinking. Thus, FcεRI would already occupy the immunoglobulin epitope preventing anti-IgE binding to cellular IgE (see Table 11.1). By virtue of binding to this epitope, the antibody would have the inherent ability to interfere with IgE responses by blocking binding of IgE to FcεRI.

Using the technique of scanning mutagenesis, a murine mAb (MAE11) directed against IgE (Saban et al. 1994; Shields et al. 1995), which had all of the properties required (see above), was identified. The antibody was selected on the basis of its ability to bind circulating IgE at the same site as the high-affinity receptor, thus blocking binding of IgE to mast cells and basophils.

In order to allow for possible chronic administration, as well as to avoid the problems of antigenicity, MAE11 was humanized with a human IgG1 framework (Fig. 11.9). The best of several humanized variants – version 25 (rhuMAb-E25) – was selected as it possessed IgE binding affinity and biological activity compared to that of the murine antibody MAE11. Only minimal changes restricted to five residues (Presta et al. 1993), corresponding to approximately 5% nonhuman residues located at the complementary-determining regions, were necessary (Fig. 11.10). Several *in-vitro* assays confirmed that rhuMAb-E25 not only prevented IgE-mediated activation of FcεRI-bearing cells but also inhibited IgE binding to human lung mast cells (Saban et al. 1994).

11.7.2
Complex Formation and Tissue Distribution

The immune complexes detected *in vitro* with IgE by both rhuMAb-E25 and a distinct monoclonal, chimeric murine-human antihuman IgE antibody were shown to be of limited size by both size-exclusion chromatography (SEC) and analytical ultracentrifugation (Fox et al. 1996; Liu et al. 1995). These studies showed that the size of rhuMAb-E25:IgE complexes formed *in vitro* were dependent on the molar ratio of rhuMAb-E25 and IgE. At molar equivalence (1 : 1 ratio), the largest complex with a molecular weight of approximately 1000 kDa predominates (Fox et al. 1996). The data are best described by a cyclic hexamer structure (Fig. 11.11), which consists of three molecules of each immunoglobulin. This cyclic structure accounts for the limited size of the complexes. At antibody or antigen excess, small heterotrimers are formed, with little detectable hexamer present at molar ratios of 3 or larger.

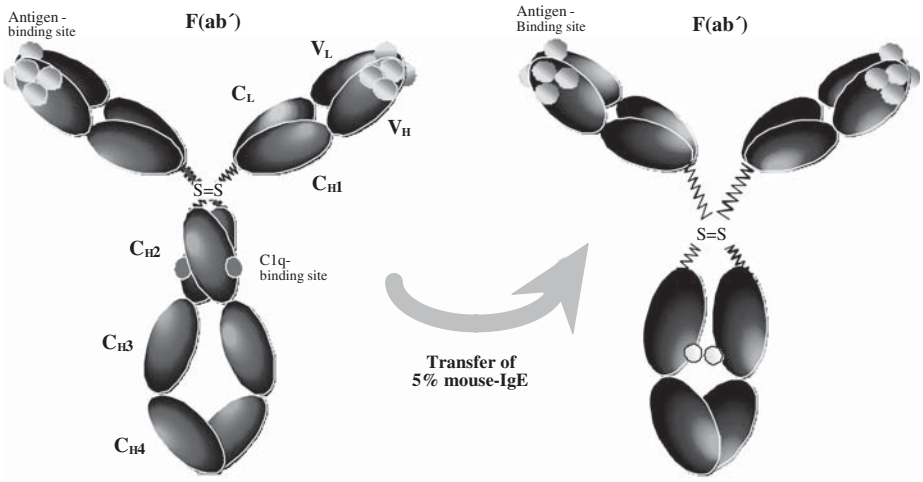

Fig. 11.9 Humanization of the monoclonal anti-IgE-antibody for omalizumab by fusion of the variable region of a mouse-anti-IgE-antibody with a human IgG1-frame. For details, see text. (Modified after Kroegel 2002.)

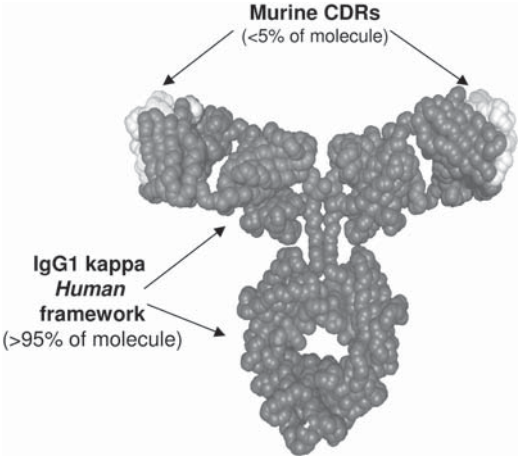

Fig. 11.10 Molecular model of rhuMAb-E25 (omalizumab). CDR = complementarity-determining region. (Adapted from Boushey 2001.)

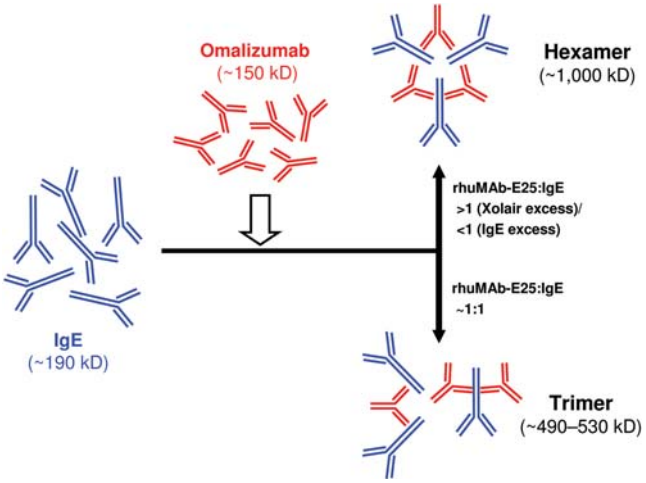

Fig. 11.11 Formation of rhuMAb-E25:IgE complexes in relation to their molar ratio.

Tissue distribution and rhuMAb-E25:IgE complex formation with IgE and rhuMAb-E25 following intravenous (IV) administration was initially studied in cynomolgus monkeys (Fox et al. 1996). ^{125}I-rhuMAb-E25 was administered as an intravenous bolus dose to wild cynomolgus monkeys that had high levels of IgE. Subsequent SEC of serum samples showed that the rhuMAb-E25:IgE complexes were of limited size and were similar to the small complexes formed *in vitro* with human IgE at antigen excess. No specific uptake of radioactivity was seen in any of the tissues collected from the animals at 1 and 96 h post administration.

Pharmacokinetic analysis revealed that both rhuMAb-E25 and rhuMAb-E25:IgE immune complexes cleared the serum compartment (Fig. 11.12), with urinary excretion as the primary route of elimination (Fox et al. 1996). Elimination of the immune complex was mediated by the interaction with FcγR (Fig. 11.13) (Lanier et al. 2003; Shields et al. 1995). Immune complex clearance was slower than IgE clearance, with a half-life of 3 weeks; therefore, although free IgE levels decrease, the total IgE level (IgE complexes with omalizumab and free IgE) is usually increased during omalizumab treatment.

11.7.3 Preclinical Results

The binding of rhuMAb-E25 to human peripheral blood basophils was assessed when basophils from 12 normal donors sensitized with ragweed-specific IgE were challenged with antigen. Only ragweed antigen induced histamine release, whereas rhuMAb-E25 failed to elicit histamine release from any donor. In other studies, the ability of rhuMAb-E25 to block IgE binding to human lung mast cells was analyzed by using strips of normal human lung perfused overnight with

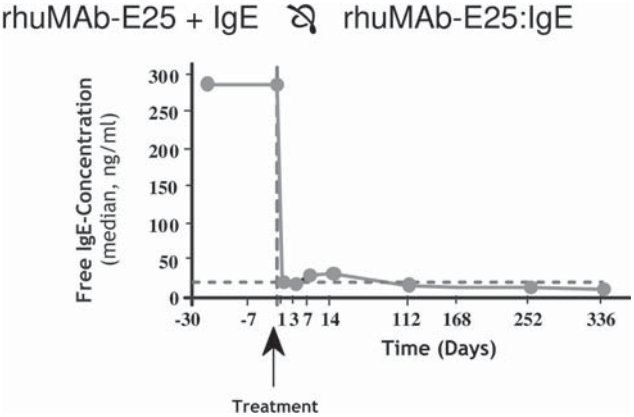

Fig. 11.12 Free serum IgE concentration prior to and following application of rhuMAb-E25. Formation of rhuMAb-E25:IgE complexes occurs within several hours and causes almost complete elimination of IgE.

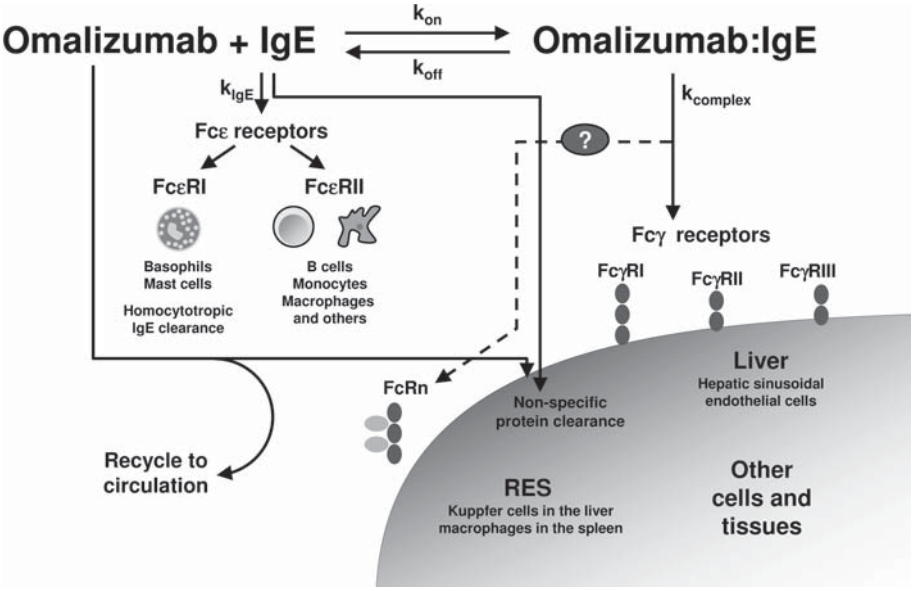

Fig. 11.13 Clearance pathways of rhuMAb-E25 (omalizumab). Clearance of omalizumab: IgE complexes occur via typical IgG pathways, including receptor-bearing cells and nonspecific mechanisms. Complexes are cleared from the body via interaction with the Fcγ receptors of platelets, leucocytes, and the reticuloendothelial system (RES).

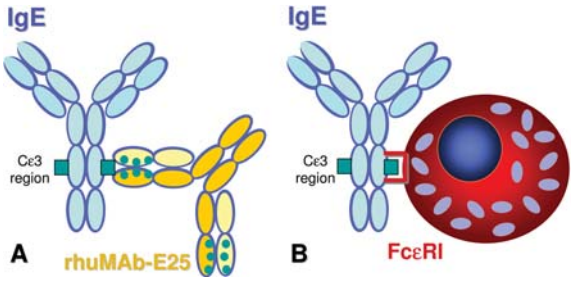

Fig. 11.14 Interaction between IgE rhuMAb-E25 and the mast cell (A) and with the humanized monoclonal anti-IgE antibody rhuMAb-E25 (omalizumab; Xolair). rhuMAb-E25 binds to the Cε3 region of the IgE molecule, which is the same part of the molecule that interacts with IgE receptors. Consequently, rhuMAb-E25 can *not* bind to receptor-bound IgE and is unlikely to generate anaphylactoid events.

ragweed-specific human IgE to sensitize the lung mast cells. Challenge with ragweed antigen induced mast cell degranulation, as measured by histamine release and muscle contraction. In contrast, rhuMAb-E25 completely inhibited this response. These data (Shields et al. 1995) confirm that rhuMAb-E25 is effective in blocking degranulation and a subsequent mediator release (Fig. 11.14).

Studies were then undertaken to examine the effects of rhuMAb-E25 *in vivo*. The ability of rhuMAb-E25 to effect IgE responses was measured in cynomolgus monkeys as rhuMAb-E25 has near-equivalent affinity for IgE purified from cynomolgus monkey serum (3×10^{-10} M) as for human IgE (1.7×10^{-10} M). To determine the ability of rhuMAb-E25 to activate cutaneous mast cells *in vivo*, 1 µg of the antibody was injected into the skin of normal cynomolgus monkeys, but failed to elicit the wheal and flare reaction indicative of mast cell degranulation. In contrast, a positive response was elicited with as little as 1 ng of a crosslinking murine mAb. Furthermore, rhuMAb-E25 failed to induce hive formation in monkey skin presensitized with 27 ng of human ragweed-specific IgE. These data were identical to the results obtained following systemic administration. Even at doses as high as 50 mg kg^{-1}, rhuMAb-E25 did not induce systemic anaphylaxis (Shields et al. 1995). Together, these preclinical studies confirmed the safety and efficacy of the designed antibody rhuMAb-E25.

11.7.4 Clinical Studies

Early studies conducted in patients with mild allergic asthma showed that the anti-IgE concept translated into demonstrable clinical effects, as shown by inhibition of allergen-induced early and late bronchoconstrictor responses (Fig. 11.15) (Boulet et al. 1997; Fahy et al. 1997). A phase II study in patients with moderate-to-severe allergic asthma reported reduced asthma exacerbations and corticosteroid requirements with the use of an intravenous formulation of omalizumab

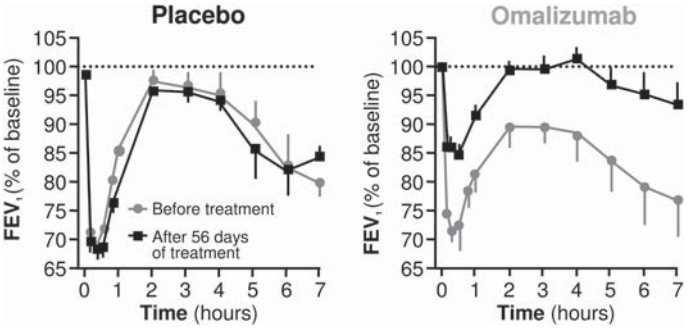

Fig. 11.15 Effect of omalizumab on the early- and late-phase asthmatic response. Right-hand panel: rhuMAb-E25 inhibits both the early asthmatic response and the late asthmatic response. Left-hand panel: placebo had no effect on either response, as would be expected. EAR = early asthmatic reaction; LAR = late asthmatic reaction. (Data from Fahy et al. 1997.)

(Milgrom et al. 1999). Subsequent phase III studies using the standard subcutaneous formulation of omalizumab were conducted in a total of 1405 children, adolescents and adults with moderate-to-severe allergic asthma and positive skin prick tests to one or more common environmental allergens (Busse et al. 2001; Milgrom et al. 2001; Soler et al. 2001). Subcutaneous injections of 150 to 375 mg omalizumab were given every 2 or 4 weeks, the dose being based on the patient's serum IgE level and bodyweight at baseline, in order to provide a dose of at least $0.016\,mg\,kg^{-1}$ omalizumab per $IU\,mL^{-1}$ IgE per 4 weeks (Hochhaus et al. 2003). Omalizumab was added on to inhaled corticosteroid (ICS) therapy for 16 weeks (steroid-stable phase) after which, during a 12-week steroid-reduction phase, the patients' doses of ICS were decreased to establish the optimal lowest dose required for an acceptable level of asthma control. The primary endpoint for the studies in adults was reduction in asthma exacerbations.

In all three studies, both the incidence and frequency of exacerbations (defined as a worsening of asthma requiring treatment with oral or intravenous corticosteroids or doubling of baseline ICS dose) were significantly reduced in the omalizumab treatment group compared with placebo, although there was also a reduction in asthma exacerbation incidence and frequency in this treatment group (Table 11.4). Patients significantly reduced their requirement for ICS, with a proportion totally withdrawing from this medication. Compared with the placebo group, a significantly greater reduction in ICS dose was achieved by the omalizumab-treated patients, and a substantially greater percentage of these were able to withdraw from ICS treatment completely. Improvements in asthma symptoms and rescue bronchodilator use were observed. Improvements in asthma-related quality of life also occurred with omalizumab treatment for both adults (Buhl et al. 2002; Finn et al. 2003) and children (Lemanske et al. 2002).

The prevention of potentially life-threatening episodes would not only provide significant benefits for patients but also reduce the overall cost of asthma care.

Table 11.4 Summary of the phase III, randomized, double-blind clinical trials comparing omalizumab with placebo in the treatment of allergic asthma.

Effect	*Omalizumab*	*Placebo*	*p-value*	*Reference*
Reduction of ICS dose	100	66.7	<0.001	Milgrom et al. (1999)
(>50%)	72.4	54.9	<0.001	Busse et al. (2001)
[%]	79	55	<0.001	Soler et al. (2001)
	74	51	0.001	Holgate et al. (2001)
Complete withdrawal	55	39	0.004	Milgrom et al. (1999)
of ICS	39.6	19.1	<0.001	Busse et al. (2001)
[%]	43	19	<0.001	Soler et al. (2001)
	21.4	15	0.198	Holgate et al. (2001)
Equivalent dose of	253	434	<0.001	Buhl et al. (2002)
inhaled BDP during	227	335	<0.001	Lanier et al. (2003)
extension phase				
[mg day^{-1}][a]				
Asthma exacerbations	0.42	2.72	<0.001	Milgrom et al. (1999)
per patient (steroid-	0.39	0.66	0.003	Busse et al. (2001)
reduction phase)[a]	0.36	0.75	<0.001	Soler et al. (2001)
Asthma exacerbations	0.28	0.54	0.006	Busse et al. (2001)
per patient (steroid-	0.28	0.66	<0.001	Soler et al. (2001)
stable phase)[a]	0.15	0.23	NS	Holgate et al. (2001)
Asthma exacerbations	0.48	1.14	<0.001	Buhl et al. (2002)
per patient	0.60	0.83	0.023	Lanier et al. (2003)
(extension phase)[a]				
Patients with ≥1 asthma	12.8	30.5	<0.001	Soler et al. (2001)
exacerbation (steroid-				
stable phase)				
[%][a]				
Subjects with ≥1 asthma	15.7	29.8	<0.001	Soler et al. (2001)
exacerbation (steroid-				
reduction phase)				
[%][a]				
Subjects with ≥1 asthma	24	40.6	<0.001	Buhl et al. (2002)
exacerbation	31.8	42.8	0.015	Lanier et al. (2003)
(extension phase)				
[%][a]				
Missed days of school[a]	0.65	1.21	0.04	Milgrom et al. (1999)

Values are median and ([a]) mean.
BDP = beclomethasone dipropionate; ICS = inhaled corticosteroids.

Therefore, lowering the rate of exacerbation is an important goal of asthma management (Masoli et al. 2004). The data from the three phase III studies were pooled and analyzed to determine the effect of omalizumab on serious exacerbations, which were measured on the basis of asthma-related emergency room visits and hospitalizations (Corren et al. 2003). Omalizumab-treated patients had

significantly fewer unscheduled outpatient visits (21.3 versus 35.5, rate ratio 0.60, $p < 0.01$) and emergency room visits (1.8 versus 3.8, rate ratio 0.47, $p = 0.05$) per 100 patient-years compared with placebo-treated patients. Importantly, hospitalizations were markedly reduced from 3.42 events per 100 patient-years for placebo treatment to 0.26 for omalizumab treatment, a rate ratio of 0.08 ($p < 0.01$).

Although the majority of asthmatic conditions can be controlled by current treatment options, at least 5% of asthma patients have severe asthma that is often inadequately controlled by ICS and long-acting β_2-agonists (LABA). These patients are at high risk of severe exacerbations and death, and have the greatest medical need among the asthmatic population. A recent double-blind, parallel group, multicenter study (INNOVATE) was conducted in patients with severe persistent asthma that was inadequately controlled despite therapy with high-dose ICS and LABA (GINA step 4 treatment) (Humbert et al. 2005). Patients were randomized to receive omalizumab or placebo as add-on therapy for 28 weeks. Omalizumab significantly reduced ($p = 0.042$) the clinically significant asthma exacerbation rate compared with the placebo group (after adjustment for imbalance in baseline exacerbation history). Omalizumab treatment also halved the severe exacerbation rate (0.24 versus 0.48, $p = 0.002$), and the incidence of emergency visits was significantly lower for omalizumab patients (0.24 versus 0.43, $p = 0.038$). The results of the INNOVATE study indicate that omalizumab is an effective add-on therapy for difficult-to-treat patients with inadequately controlled severe persistent asthma.

The efficacy of omalizumab treatment in patients with severe persistent asthma has also been demonstrated in a pooled analysis of data from seven studies (Bousquet et al. 2005). Omalizumab treatment was added to current asthma therapy and compared with placebo (five double-blind studies) or with current asthma therapy alone (two open-label studies). The studies included 4308 patients, 93% of whom had severe persistent asthma according to the GINA 2002 classification. Omalizumab treatment significantly reduced the rate of asthma exacerbations by 38% and the rate of total emergency visits by 47% ($p < 0.001$ versus control), suggesting that omalizumab may fulfill an important need in this difficult-to-treat asthma population. Subgroup analysis revealed that omalizumab was effective irrespective of age, FEV_1, gender and IgE serum concentrations (Fig. 11.16). However, young patients (aged < 18 years) with an IgE level > 150 IU mL^{-1} and a severely impaired lung function ($FEV_1 < 60\%$ predicted) appeared to show a better response to treatment.

Evaluation of the treatment by the Cochran group involved an evidence-based survey of omalizumab's utility for asthma (Walker et al. 2004). This review found that omalizumab led to a significant reduction in inhaled steroid consumption compared with placebo: –114 mg per day (95% CI –150 to –78.13, two trials). The studies seemed to show increased health of patients and decreased steroid need, although the control groups had greater than expected responses. However, the Cochran reviewers stressed that the long-term clinical significance of omalizumab-induced IgE decrease still needs to be defined.

Altogether, the above clinical studies suggest that therapy with omalizumab can have a major effect on the treatment of patients with moderate-to-severe

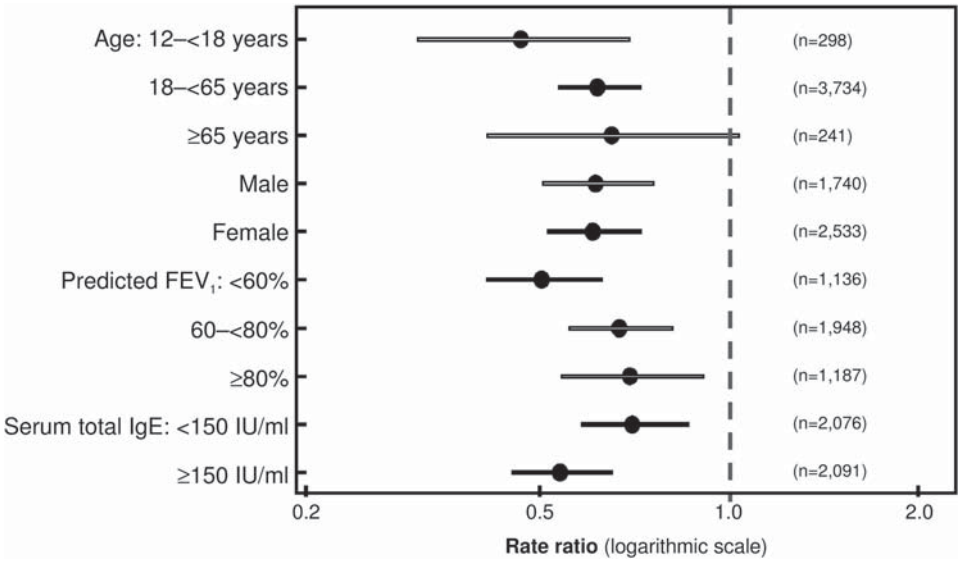

Fig. 11.16 Effect of omalizumab on exacerbation rate. A consistent reduction in asthma exacerbation rates can be observed, irrespective of baseline characteristics. (From Bousquet et al. 2005.)

Table 11.5 Summary of the beneficial effects of omalizumab in asthma.

- Reduces asthma exacerbations regardless of the type of allergic sensitization (seasonal or perennial)
- Improves asthma symptom scores
- Concomitantly improves upper airway symptoms in the case of co-existent allergic rhinitis
- Improves the quality of life of asthmatic subjects
- Steroid-sparing effect
- Reduces rescue medication

allergic asthma (Table 11.5). More importantly, omalizumab might be best used in the higher-risk patients who are at risk for serious asthma exacerbation requiring emergency department visitation, hospitalizations, or both.

11.8 Anti-Inflammatory Effects of Omalizumab

The biological effect of the anti-IgE antibody has been characterized in respect of its effect on humoral and cellular parameters, and shows actions at different levels of allergic inflammation (see Fig. 11.3).

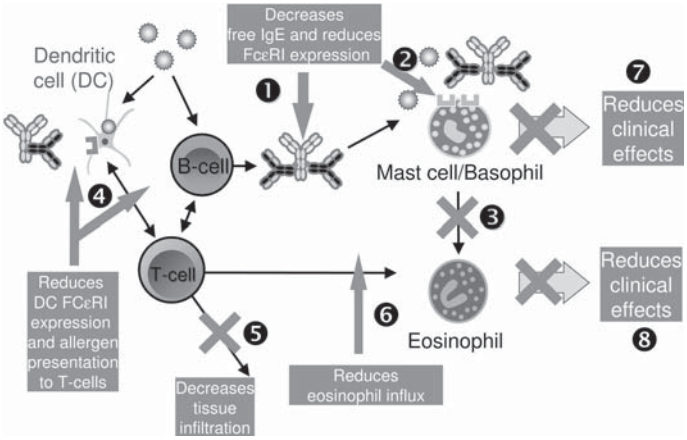

Fig. 11.17 Summary of anti-inflammatory actions of rhuMAb-E25 at different levels of allergic inflammation. ❶, decreases free IgE; ❷, reduces FcεRI expression on mast cells and basophils; ❸, blocks mast cell-mediated activation of eosinophils; ❹, reduces DC FCεRI expression on dendritic cells and allergen presentation to T cells; ❺, decreases T-cell tissue infiltration; and ❻ inhibits eosinophil tissue influx. As a consequence, the severity of both ❼ early and ❽ late asthmatic reactions are diminished.

11.8.1
Effects on Serum Free IgE Levels

The anti-IgEs under clinical evaluation and development have an association constant, K_a, for soluble IgE of approximately $10^{10}\,M^{-1}$ (Kolbinger et al. 1993), which is in the range of the affinity of the FcεRI receptor for IgE. Thus, if anti-IgE is maintained at concentrations in excess of IgE in the body, it should effectively compete with FcεRI for IgE (see Fig. 11.12). The concentrations of IgE in the blood vary widely among patients with allergy, ranging from 0.05 to 1 mg mL^{-1} found in most patients. Taking this into account, anti-IgE given in excess to the basal concentration can bind most of the IgE, leaving a minimum of free IgE available for binding to IgE Fc-receptors.

11.8.2
Effect on Cytokines

Omalizumab induces complex changes in interleukin levels, and does not decrease all Th2-related interleukins. In a study of asthmatics, circulating levels of IL-5, IL-6, IL-8, IL-10, IL-13, and s-ICAM (Noga et al. 2003) were measured before and after 16 weeks of omalizumab treatment. Anti-IgE therapy also reduced cells staining for IL-4$^+$ cells in the airways of asthmatic subjects (Djukanovic et al. 2004). The results demonstrated a significant decreased IL-13 ($p < 0.01$) and reduced IL-5 and IL-8 levels in the omalizumab group compared to baseline, whereas the concentrations of other circulating mediators were unchanged. The reasons for this differential effect of omalizumab on circulating cytokine concen-

tration are not known. However, cytokines in the peripheral blood represent a rather vague estimate of an inflammatory process which is taking place in the local inflammatory microenvironment of a defined tissue such as the airways.

11.8.3 Effects on FcεRI Cell Expression

The most readily appreciated pharmacological action of anti-IgE therapy is the indirect effect on the down-regulation of FcεRI on basophils. In earlier studies, the density of FcεRI on basophils was found to correlate strongly with the level of IgE in blood (see above). One of the clinical studies (MacGlashan et al. 1997b), in addition to *in-vitro* studies (MacGlashan et al. 1997a), show that the density of FcεRI on basophils decreased by between 95% and 99%, after anti-IgE had been administered to patients for up to 3 months. FcεRI expression on circulating basophils was significantly reduced by 99% in the presence of low serum levels of IgE during 90 days of treatment of ragweed-allergic individuals with the anti-IgE monoclonal antibody omalizumab (MacGlashan et al. 1997a). At the same time, the basophils isolated from patients after anti-IgE treatment were much less sensitive to allergen *ex-vivo* stimulation, as assessed by the degree of histamine release. In addition, dermal reagibility to allergen provocation (skin prick test) was reduced, indicating that mast cell function was also markedly decreased (MacGlashan et al. 1997a). Although the underlying mechanism for this is not completely understood, there is evidence that up-regulation is mediated by IgE interacting through FcεRI itself (MacGlashan et al. 1998).

In a phase I study, treatment with two intravenous doses of omalizumab decreased the number of FcεRI receptors from a median of 220 000 to 8300 receptors per basophil at 3 months. Receptor density was decreased to 50% of baseline by day 3, and to 97% by 90 days, further supporting this hypothesis (MacGlashan et al. 1997a). In omalizumab studies evaluating its effects on early- and late-phase allergic reactions, the decrease in FEV_1 was reduced, and the allergen concentration required to elicit bronchoconstriction was increased (Boulet et al. 1997; Fahy et al. 1997). In addition, the maximum late-phase FEV_1 was increased 60% from baseline (Fahy et al. 1997).

11.8.4 Effect on Dendritic Cell APCs

With this background, clinical studies have shown evidence that a monoclonal anti-IgE antibody, omalizumab, affects DCs and therefore might be able to modulate T-cell responses. In addition to down-regulating FcεRI expression on basophils (MacGlashan et al. 1997a), therapy with omalizumab produced a rapid reduction in surface FcεRI expression of both precursor DCs type 1 and type 2 subsets (Prussin et al. 2003). A decrease or normalization in DC1s in patients treated with omalizumab during a grass pollen season, but not in the birch pollen season, has been shown (Feuchtinger et al. 2003). These findings might be

explained by the fact that, in the absence of proinflammatory cytokines and mediators, DC1s promote the differentiation of Th2 cells. Furthermore, DC1s derived from monocytes and cultured with GM-CSF and IL-4 induce both Th1 and Th2 differentiation (Liu et al. 2001). Thus, decreased omalizumab-induced IL-4 production by mast cells during the pollen season might cause the apparent reduction in the number of myeloid DCs (Liu et al. 2001). Taken together, these findings imply that anti-IgE therapy might cause immunomodulation of T-cell responses and inhibit IgE-mediated type-I hypersensitivity.

Dendritic cells (DCs) are important in T-helper (Th) cell differentiation, with type I DCs (DC1) enhancing Th1 differentiation and type II DC (DC2) being an important factor for Th2 cell responses. DC2 cells are described as the "gatekeepers" of the immune response (Upham 2003), and IgE occupancy of DC2 FcεRI receptors is associated with greater allergen uptake and an increase in the resulting immune response (Maurer et al. 1995; Stingl and Maurer 1997). In ragweed-induced rhinitis, (Prussin et al. 2003), omalizumab treatment caused a significant decrease in basophil FcεRI expression at all time-points during the study (days 7, 14, 21, 28, 35, 42) compared with placebo ($p < 0.001$). In addition, the level of FcεRI expression on both DC1 and DC2 cells was reduced in omalizumab-treated individuals and correlated with serum levels of free IgE in patients receiving omalizumab. There also was a correlation between FcεRI expression on DC2 cells and that on DC1 cells and basophils. The maximum decrease in FcεRI expression was 73% on basophils, 52% on DC1 cells, and 83% on DC2 cells (Prussin et al. 2003). Interestingly, a significant reduction was observed as early as 7 days. These results show that the anti-IgE effects of omalizumab regulate FcεRI expression in these cell types in parallel with similar kinetics of inhibition.

11.8.5 Effect on Eosinophils

A recent study explored the effect of omalizumab on eosinophil survival induction of immunologic changes leading to eosinophil apoptosis, and also examined T-lymphocyte cytokine profiles in 19 patients with allergic asthma treated with omalizumab at a dose of at least $0.016\,mg\,kg^{-1}$ per IgE ($IU\,mL^{-1}$) every 4 weeks (Noga et al. 2006). Peripheral eosinophils and T-lymphocyte cytokine profiles were evaluated by fluorescence-activated cell sorting before treatment (baseline), at 12 weeks of treatment, and 12 weeks after discontinuation of treatment with omalizumab or placebo.

Markers of eosinophil apoptosis (Annexin V) were significantly increased in omalizumab recipients compared with placebo, whereas no changes in markers of necrosis (7-amino-actinomycin) or eosinophil activation CD69 or Fas receptor (CD95) were detected. GM-CSF$^+$ lymphocytes were reduced in omalizumab recipients compared with placebo. Fewer IL-2$^+$ and IL-13$^+$ lymphocytes were also evident in omalizumab recipients than in the placebo group. There were no significant differences in IL-5, IFN-γ, or TNF-α between the omalizumab and

placebo groups. In addition, anti-IgE therapy was associated with a decrease in sputum and tissue eosinophils accompanied by reduced $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes (Djukanovic et al. 2004). These findings provide further evidence that omalizumab has additional anti-inflammatory activity, as demonstrated by the induction of eosinophil apoptosis and down-regulation of the inflammatory cytokines IL-2 and IL-13. Whilst further studies are needed to determine the underlying mechanisms, these findings support the critical role of IgE in the regulation of inflammation in allergic asthma, namely, influencing the inflammation is key to controlling the more severe type of asthma.

11.8.6
Effects on B Cells

Experimental evidence from both *in-vitro* and *in-vivo* studies supports the ability of anti-IgE in targeting IgE-expressing B cells and in inhibiting the continual production of IgE. The idea that anti-IgE can cause these effects is that anti-IgE binds to mIgE on IgE-expressing B cells. Because mIgE represents a part of the B-cell receptor, anti-IgE may interfere B-cell signaling or even cause their lysis, as has been shown for anti-IgM or anti-IgG (Eray et al. 1994; Warner and Scott, 1991; Warner et al. 1991). However, IgE-secreting plasma cells do not express mIgE, and presumably are not affected by anti-IgE. These cells reside in the bone marrow and probably have a life span of several weeks to several months. However, since new IgE-secreting plasma cells go through mIgE-expressing B-cell stages during differentiation, if their generation is abrogated by anti-IgE treatment, the existing plasma cells will die off over a period of several weeks to months, and thus, the production of IgE will also gradually abate. In addition, memory B cells may be affected by anti-IgE. The molecular mechanisms, leading to the depletion of these cells can be explained by apoptosis and reached by the immunologic process of tolerance and/or anergy induction. If this occurs, anti-IgE may have long-term effects on the fundamental disease process. Considering its effects on B cells and DCs, there is a chance that long-term treatment with omalizumab may eventually correct the pathogenic defect underlying allergic inflammation.

11.9
Pharmacological Properties of Omalizumab

11.9.1
Pharmacodynamics

Omalizumab forms complexes with IgE at the Cε3 domain (see Fig. 11.11), the principal site of effector cell binding. IgE is thereby prevented from binding to FcεRI on various effector cells, inhibiting activation and mediator release (Easthope and Jarvis 2001; Kelly and Sorkness 2002; see Package insert, Xolair 2003). In this manner, omalizumab decreases serum-unbound IgE levels, decreases the

number of FcεRI receptors on basophils, and attenuates early- and late-phase allergic reactions (Boulet et al. 1997; Easthope et al. 2001; Fahy et al. 1997; Kelly et al. 2002; MacGlashan et al. 1997a; Package insert, Xolair 2003). Rapid, dose-dependent decreases (96–99%) in serum unbound IgE have been observed after subcutaneous administration (Package insert, Xolair 2003). A direct correlation between unbound IgE levels and the number of FcεRI receptors on circulating basophils has been shown (see above). Several large phase III clinical trials have demonstrated that omalizumab is more effective than placebo in controlling moderate to severe allergic asthma in patients who have poor disease control or exacerbations despite recommended therapy.

11.9.2 Pharmacokinetics

Omalizumab exhibits linear kinetics at doses > 0.5 mg kg^{-1} (Package insert, Xolair 2003). After subcutaneous administration, omalizumab has an average bioavailability of 62%, reaching a maximum concentration of 30.9 mg L^{-1} after 7 to 8 days. The volume of distribution is 78 mL kg^{-1}, suggesting limited distribution (i.e., plasma). In addition, an enhanced uptake into specific organs or tissues has not been observed (Easthope et al. 2001; Package insert, Xolair 2003). Omalizumab is eliminated primarily via hepatic degradation in the reticuloendothelial system and endothelial cells and, to a lesser degree, via biliary excretion (Fig. 11.13). After subcutaneous administration, the average elimination half-life of the drug is 26 days, and clearance appears to be body weight-related (i.e., doubling the body weight approximately doubles clearance). Although data regarding pharmacokinetic differences among age groups are lacking, the evaluation of small numbers of children aged between 12 and 18 years and elderly patients suggests no difference; however, the numbers of patients investigated were small (Package insert, Xolair 2003).

11.10 Adverse Effects

11.10.1 Systemic Side Effects

Overall adverse events reported for subjects treated with omalizumab compared with placebo have been similar and categorized as mild to moderate in severity. The most commonly reported effects are fatigue, arthralgias, rash, diarrhea, nausea, vomiting, dizziness, epistaxis, menorrhagia, itching, dermatitis, and hematoma [Food and Drug Administration (FDA) 2003]. Other systemic side effects included viral infection (23%), upper respiratory tract infection (20%), sinusitis (16%), headache (15%), and pharyngitis (11%). In clinical trials to date, there has been no evidence of anti-IgE–IgE immune complex disease, nor has

there been any evidence of complement activation or fixation. The platelet abnormalities seen in a small segment of monkeys has not been observed in human trials (Lanier et al. 2003).

11.10.2 Local Reactions

Injection-site reactions, including bruising, redness, warmth, and burning, were observed in 45% of the treated patients and occurred more commonly with omalizumab treatment versus placebo (12% versus 9%). Generally, these reactions developed within 1 h of injection, but their incidence decreased with continued administration.

11.10.3 Serious Adverse Effects

Most adverse effects were mild to moderate, and incidence rates were similar to those observed with placebo. However, potential safety concerns identified by the FDA in reviewing trial data on omalizumab included risks of anaphylaxis and the development of cancer.

Anaphylaxis Treatment with omalizumab is meant to prevent any risk of anaphylaxis, as the agent cannot interact with IgE already bound to cell surfaces. However, in clinical trials, three patients (<0.01%) developed anaphylaxis, characterized by urticaria and angioedema (FDA 2003). Two of the reactions were temporally associated with omalizumab administration and occurred within 2 h after the first injection. One patient (<0.1%) developed antibodies to omalizumab, and one individual developed proteinuria deemed unrelated to immune-complex hypersensitivity (Package insert, Xolair 2003; Soler et al. 2001).

Malignancies Malignant neoplasms were observed in 20 (0.5%) of 4127 omalizumab-treated patients compared with five (0.2%) of 2236 control subjects. The observed malignancies included a wide variety of predominantly epithelial or solid-organ cancers, including breast, melanoma, non-melanoma, skin, prostate, and parotid; this suggested that there was no specific association with omalizumab treatment. Moreover, the difference in malignancy rate between omalizumab-treated patients and placebo was not statistically significant. In addition, as the rate of malignancy observed in the control group was lower than expected, no unequivocal association can be deduced from the data.

11.10.4 Immune Complex Diseases

In theory, the administration of omalizumab can induce antibodies against the murine components of the drug, but no immune complex-mediated pathologic

conditions as a result of the formation of such antibodies have been observed. Although no clinical problems related to such an effect have been noted, this may represent a potential concern in specific populations. Again, further information is required on the safety profile after long-term use of omalizumab.

11.10.5 Long-Term Adverse Effects

As the majority of patients treated with omalizumab have been observed for only one year, the effect of longer use in patients who are at increased risk for cancer is not known. In particular, it is unknown whether the incidence of neoplasms is increased with long-term treatment or in high-risk patients. Therefore, omalizumab probably should not be used in patients with either a history of cancer or a strong family history of cancer until this risk relationship is better understood.

11.11 Indications

On the basis of clinical trials, the FDA approved omalizumab only for very specific indications (Table 11.6). Omalizumab is indicated for patients aged ≥12 years with moderate-to-severe persistent asthma who have a positive skin test or *in-vitro* reactivity to a perennial aeroallergen, and whose symptoms are inadequately controlled with inhaled corticosteroids. Omalizumab has not been approved for

Table 11.6 Indications for the use of omalizumab approved by the FDA (2003).

1. Second-line treatment (after first-line treatments have failed). In addition:
2. Patients must have moderate-persistent allergy-related asthma:
   - Daily symptoms
   - Daily use of inhaled short-acting beta-2-agonist
   - Exacerbations affect activity
   - Exacerbations at least twice per week, which may last for days
   - Night-time symptoms more frequently than once a week
   - Lung function of forced FEV_1 or peak expiratory flow
3. Patients must have severe-persistent allergy-related asthma:
   - Continual symptoms
   - Limited physical activity
   - Frequent exacerbations
   - Frequent night-time symptoms
   - Lung function of FEV_1 or PEF ≤60% predicted, and PEF variability >30%
4. Patients must be aged over 12 years.
5. Patients must have a positive skin test to a perennial aeroallergen (e.g., dust mite, cats, dogs, and fungi).
6. Patients must be symptomatic with inhaled corticosteroids.

children aged < 12 years, although this may change in future when relevant data are available. Omalizumab does not show any specific drug interactions, and can be used with corticosteroids and other anti-asthmatic drugs in most cases.

11.12 Contraindications

Absolute contraindications to the administration of omalizumab include a prior experience of severe hypersensitivity to omalizumab, although these reactions have only rarely been reported. Omalizumab treatment is also not indicated in patients with an IgE level $<30\,IU\,mL^{-1}$ or $>700\,IU\,mL^{-1}$, as studies proving the efficacy of the drug at these IgE concentrations are not available. Omalizumab does not alleviate asthma exacerbations acutely, and should not be used for the treatment of acute bronchospasm or status asthmaticus. The safety and efficacy of omalizumab treatment in patients with allergic conditions other than perennial aeroallergen has not been established. In addition, the clinical efficacy or safety of the use of omalizumab for the prevention of peanut or other food allergies, or for the prevention or treatment of allergic rhinitis, awaits further clinical studies (Table 11.7).

Table 11.7 Potential indications and contraindications (currently available) for the treatment of bronchial asthma and rhinitis with rhuMAb-E25 (omalizumab). Data are based on currently available information. (Modified after Kroegel et al. 2002).

Indications
• Allergic bronchial asthma
• Seasonal allergic asthma
• Perennial bronchial asthma due to unavoidable allergens (e.g., house-dust mite)
• Treatment of asthma in children/adolescent above the age of 12 years
Questionable indications
• Treatment of asthma in children under the age of 12 years
• Non-allergic, intrinsic asthma
• Food allergy
• Atopic dermatitis
• Idiopathic urticaria
• Churg–Strauss syndrome
• Specific immunotherapy
Contraindications
• Non-allergic, intrinsic bronchial asthma
• Acute asthma exacerbation
• Pregnancy
• Thrombocytopenia*

* Further studies warranted.

11.13 Preparation for Use

Omalizumab is available as a preservative-free, sterile, lyophilized powder for reconstitution with sterile water for injection. Prior to reconstitution, omalizumab should be refrigerated (2–8 °C). For single use, omalizumab is supplied in 5-mL vials designed to deliver either 150 or 75 mg on reconstitution with sterile water (not normal saline) for injection. The powder requires 15 to 30 min to dissolve; dissolution can be facilitated by constant stirring. After reconstitution, omalizumab is stable in the vial for 8 h under refrigeration, or for 4 h at room temperature. The solution should not be shaken and should be protected from direct sunlight.

The solution is viscous and must be carefully drawn up into the syringe before being administered. The injection itself may take 5 to 10 s to administer (Table 11.8). Fewer injection-site reactions are seen to occur when the solution injected is completely clear.

The baseline serum total IgE level and patient body weight are used to determine the dose (150–375 mg) and frequency (every 2 or 4 weeks) according to a standard nomogram (Table 11.9) (Package insert, Xolair 2003).

Because of the time-consuming requirements for drug preparation and the high cost of the drug, the following approach to handling should be considered when treating patients with omalizumab:

- scheduled appointments for injection of several patients; and
- preparation of the injection after the patient has arrived.

In total, a single administration of omalizumab may take more than 60 min, as a 30-min observation period is recommended following the injection.

11.14 Administration

The maximum dose per injection site is 150 mg; hence, doses exceeding 150 mg must be administered as multiple injections at different sites (Package insert,

Table 11.8 Points to be considered when administering omalizumab.

- Omalizumab 150 to 375 mg administered subcutaneously every 2 or 4 weeks.
- Dosing and frequency are determined by pretreatment serum total IgE level and body weight (Table 11.9), and should be in keeping with the FDA approved package insert.
- Doses should be adjusted for significant changes in body weight.
- Re-testing of IgE levels during treatment and up to 1 year after discontinuing treatment cannot be used as a guide for dose determination and is not medically necessary due to total serum IgE levels remaining elevated throughout this time.
- Injections are to be limited to not more than 150 mg per subcutaneous injection site, and to not more than 375 mg per day.

Table 11.9 Omalizumab dosage (in mg) and dosing schedule on baseline total serum IgE and body weight (kg) (Genentech Product Information).[a]

Baseline serum IgE [IU mL^{-1}]	*Body weight [kg]*			
	30–60	*>60–70*	*>70–90*	*>90–150*
≥30–100	150	150	150	300
>100–200	300	300	300	**225**
>200–300	300	**225**	**225**	**300**
>300–400	**225***	**225**	**300**	–
>400–500	**300**	**300**	**375**	–
>500–600	**300**	**375**	–	–
>600–700	**375**	–	–	–

* Bold values indicate dosing every 2 weeks; otherwise every 4 weeks.

a Patients whose pretreatment serum IgE levels or body weight are outside the limits of the dosing table (>30 or >700 IU mL^{-1} and <30 and >150 kg) should not be administered omalizumab.

Xolair 2003). Omalizumab is slowly absorbed after subcutaneous administration; thus, with a mean elimination half-life of 26 days, omalizumab (0.016 mg kg^{-1} IgE^{-1} [IU mL^{-1}] per 4 weeks) can be administered fortnightly or monthly. Following the injection of omalizumab, emergency medications should be available to treat severe hypersensitivity reactions, including anaphylaxis (Xolair Product Information 2004).

11.15 Clinical Dosing

The principal aim of anti-IgE mAb treatment with omalizumab is to eliminate serum free IgE levels. In early clinical trials a relationship between clinical efficacy variables and free IgE concentrations was demonstrated, with the best results obtained when serum free IgE levels were <25 ng mL^{-1} or 10.4 IU mL^{-1} (Casale et al. 1997). To achieve this goal, appropriate dosing depends on two factors (Bang and Plosker 2004):

- the subject's pretreatment serum IgE level (in IU mL^{-1}); and
- the patient's pretreatment body weight (in kg).

The minimal dosing is 0.016 mg kg^{-1} (IU mL^{-1}) IgE per 4 weeks in divided doses as necessary. On the basis of these data, the individual dosage required to reduce IgE levels to the threshold concentration mentioned above is calculated according to the nomogram given in Table 11.9. Pretreatment serum IgE levels or body

weights outside the limits of the dosing table do not qualify for treatment because: (i) the volume of the drug would be too excessive; and (ii) the expense of the drug would be too high.

11.16 Dosing Adjustments

Dosing adjustments should not be made in response to subsequent total IgE levels (Kroegel et al. 2004); however, adjustments are required for patients experiencing significant body weight changes. Dosage adjustments for patients with renal or hepatic failure are not necessary.

11.17 Precautions and Contraindications

Omalizumab is indicated for the maintenance treatment of allergic asthma, but not indicated for the treatment of acute exacerbations (e.g., asthma attack, status asthmaticus). Although omalizumab may help reduce steroid requirements, systemic or inhaled steroids should be tapered gradually to lower doses rather than be abruptly discontinued (Package insert, Xolair 2003).

11.17.1 Drug Interactions

To date, no studies evaluating the potential for drug interactions involving omalizumab have been performed. Omalizumab is neither protein-bound nor metabolized by the cytochrome P450 isoenzyme system. This suggests a low probability of drug interactions (Package insert, Xolair 2003).

11.17.2 Pregnancy and Lactation

Human data on potential harmful effects during pregnancy and lactation are not available. In animal studies, omalizumab did not demonstrate maternal toxicity, embryotoxicity, or teratogenicity during organogenesis, or adverse fetal/neonatal growth effects during late gestation, delivery, and nursing. Conversely, omalizumab (similar to IgG) may cross the placental barrier and be secreted in breast milk. The risks of absorption and harm to the fetus or nursing infant are unknown. Omalizumab is a "category B" agent, and thus should be used only if clearly needed in pregnancy. In addition, until further data become available caution should be exercised during breast-feeding (Package insert, Xolair 2003).

11.18
Monitoring of Therapy

Serum-free IgE levels will decrease to approximately 1% of baseline values within 24 h of subcutaneous administration (Casale et al. 1997). Total serum IgE levels (free plus bound) increase with treatment due to the formation of circulating omalizumab–IgE complexes. Unfortunately, serum free IgE level is currently not available only for routine measurement; therefore, the monitoring of total serum IgE levels is not useful and should not be performed in a clinical setting once therapy has been initiated (Hamilton et al. 2005). A specified commercial assay may help to optimize dosing and maximize omalizumab therapy in future.

11.19
Cost

Omalizumab is considerably more expensive than conventional asthma therapy. Omalizumab has an average wholesale price of €424 ($541) per 150-mg vial, but the individual cost of treatment is variable. Based on current dosing recommendations, monthly treatment costs range from €424 ($541) to €2547 ($3247) (Package insert; Omalizumab, Xolair 2003). The related annual costs range from €3200 ($4000) to €15 700 ($20 000), depending on the dosage (Dacus 2003), with an average of approximately €9413 ($12 000) per year (Table 11.10). This compares with approximate costs per year of €1004 ($1280) for montelukast (Singulair, Merck), €1694 ($2160) for the combination of fluticasone dipropionate and salmeterol (Advair, GlaxoSmithKline), and €502 ($680) for extended-release theophylline (Uniphyl, Purdue). In addition, visit costs may further increase as omalizumab must be administered by a physician.

Table 11.10 Doses of omalizumab injection versus annual cost.

Omalizumab dose [mg]	*Treatment interval [weeks]*	*Annual cost*	
		US$	*Euro*
150	4	3879	3043
150	2	7759	6086
300	4	7759	6086
300	2	15 518	12 173
375	2	23 277	18 259

11.20
Response to Treatment

11.20.1
Onset of Action of Anti-Immunoglobulin E Effect

The onset of action is particularly important for seasonal allergic rhinitis, when patients need to respond quickly to changing pollen seasons. A recent study attempted to measure the onset of action of omalizumab by measuring the time taken to inhibit the nasal responses to ragweed allergen (Lin et al. 2004). Omalizumab had a substantial impact on ragweed-induced reductions in nasal volume within the first 7 days to up to 12% and a further reduction to 6% until day 42. These data suggest that omalizumab acts rapidly to significantly reduce serum free-IgE levels and substantially inhibits ragweed-induced reductions in the nasal volume. Interestingly, clinical improvement was paralleled by reductions in FcεRI expression on DC1 and DC2 cells as well as basophils. It might be proposed that administering omalizumab a week before the start of the pollen season, and 4 weeks later, would protect patients from AR symptoms during the pollen season.

Despite these observations, an apparent response to treatment can take several weeks (Chang 2000). Among patients in a clinical trial who had responded to omalizumab by 16 weeks, 87% had done so by 12 weeks (Bousquet et al. 2004). These data suggest that patients should be treated for at least 12 weeks before efficacy is assessed.

11.20.2
Duration of Treatment

The optimal duration of anti-IgE therapy is unknown, but it is likely that anti-IgE has to be administered continuously in a dose-dependent fashion. Even if the generation of new IgE-producing plasma cells is blocked by anti-IgE, new IgE-expressing B cells (and, thus, new plasma cells) will be regenerated in a few weeks to a few months in the absence of anti-IgE. Thus, repeated anti-IgE dosing appears to be necessary. While patients are under anti-IgE treatment, IgE-related immune mechanisms and their manifestations are abbreviated and remain exposed to the usual allergens. If these allergens drive the immune system toward non-IgE-related responses, the disease process may be gradually attenuated. If anti-IgE can inhibit IgE-expressing memory B cells that are responsible for recurrent IgE responses, then the continuous exposure of allergens may preferentially drive the immune system towards the production of antibodies of other subclasses. If anti-IgE can indeed influence a shift in the immune response, the combination of antigen immunotherapy with anti-IgE may present another new approach for individuals suffering from severe allergies (see below).

Given that serum IgE levels and the numbers of FcεRIs increase after therapy is discontinued (Saini et al. 1999), it seems that treatment needs to be continued

for efficacy to persist, though no studies have yet been reported on the duration of effects after discontinuation. If administration is interrupted, then treatment should be resumed at the dose initially prescribed. Dosing may only need to be adjusted when substantial changes in body weight have occurred (see Table 11.9).

11.21 Non-Approved Diseases

11.21.1 Allergic Rhinitis

Omalizumab has been shown also to be useful in the treatment of allergic rhinitis, which affects a large proportion of the population (Adelroth et al. 2000; Casale et al. 1997, 1999; Plewako et al. 2002). Although often seasonal in nature, allergic rhinitis causes considerable distress to the sufferer and has a high social cost in terms of consumed healthcare resources and lost productivity. It is important to add that asthmatic patients frequently have associated rhinitis.

Several studies of omalizumab involving more than 1000 patients with severe seasonal allergic rhinitis induced by birch or ragweed pollen have been performed (Adelroth et al. 2000; Casale et al. 1997, 1999; Chervinsky et al. 2003), all of which showed a statistically significant improvement in nasal and ocular symptom severity scores, the use of rescue medication (antihistamines), and the quality of life (Table 11.11). Further studies have identified decreases in the number of inflammatory cells, including decreased eosinophil peroxidase-positive cells and decreased IgE^+-staining cells (Plewako et al. 2002). On the basis of these results it may be concluded that patients with concomitant allergic rhinitis and bronchial asthma may benefit more from treatment with omalizumab (Table 11.12) (D'Amato and Holgate 2002; D'Amato et al. 2002, 2004; Ayres et al. 2004; Bousquet et al. 2005; Holgate et al. 2005a,b).

11.21.2 Other Clinical Applications

In addition to moderate-to-severe persistent asthma (see above), a number of additional disorders relating to other organ manifestations of allergic diseases or nonallergic conditions associated with increased IgE levels may benefit from treatment with omalizumab (Table 11.13). There is clinical evidence that treatment with an anti-IgE mAb might diminish symptoms caused by peanut-induced allergic reactions in susceptible children (Leung et al. 2003), atopic dermatitis (Vigo et al. 2006), and idiopathic cold urticaria (Joshua and Boyce 2006). One study suggests that omalizumab can be used to treat healthcare workers with occupational latex allergy (Leynadier et al. 2004). In addition, there is evidence that a combination of anti-IgE with specific immunotherapy (SIT) might be

Table 11.11 Summary of the randomized, double-blind, clinical trials comparing omalizumab with placebo in the treatment of seasonal and perennial allergic rhinitis.

Parameter	*Omalizumab*	*Placebo*	*p-value*	*Reference*
Nasal symptom severity score	0.75	0.98	0.002	Casale et al. (1997)
	0.70	0.98	<0.001	Adelroth et al. (2000)
	1.0	1.4	0.001	Chervinsky et al. (2003)
Eye symptom severity score	0.41	0.67	0.001	Casale et al. (1997)
	0.43	0.54	0.031	Adelroth et al. (2000)
Use of rescue medication	0.12	0.21	0.005	Casale et al. (1997)
(mean number of tablets/day)	0.59	1.37	<0.001	Adelroth et al. (2000)
Rhinitis quality-of-life	44.1	30.6	<0.01	Adelroth et al. (2000)
scores (% of patients with significant improvement)	52	27	0.001	Chervinsky et al. (2003)
Total rhinitis quality-of-life score (five-point scale)	1.75	2.3	<0.001	Adelroth et al. (2000)
Global treatment	70.7	40.8	<0.001	Casale et al. (1997)
effectiveness (%)	59	35	0.001	Adelroth et al. (2000)
	53	34	0.001	Chervinsky et al. (2003)
Missed days of work and/or school	0.1	0.4	0.005	Casale et al. (1997)

Values shown are mean scores. Range of possible scores is 0 (no symptoms) to 3 (worst symptoms).

Table 11.12 Clinical efficacy of omalizumab in patients with allergic rhinitis and allergic asthma.

Allergic rhinitis
- Reduction in daily nasal severity score
- Reduction in the use of rescue antihistamines
- Improvement in the quality of life
- Blunting of the seasonal increase of nasal symptoms during the pollen season

Allergic asthma
- Reduction in early- and late-phase responses to allergen challenge
- Reduction in total asthma exacerbations
- Reduction in number of patients with ≥1 asthma exacerbations
- Reduction in dosage of ICS use
- More subjects achieve a complete discontinuation of corticosteroids
- Improvement in nocturnal asthma score
- Improvement in asthma quality-of-life score
- Reduction in hospitalizations and emergency department visits in high-risk asthmatic populations
- No significant change seen in FEV^1

ICS = Inhaled corticosteroid.

Table 11.13 Preliminary omalizumab therapy in non-asthmatic and non-rhinitic conditions, with beneficial effects.

Condition	*Reference*
Atopic dermatitis	Vigo et al. (2006)
Idiopathic cold urticaria	Joshua and Boyce (2006)
Protection from acute reactions after immunotherapy	Parks and Casale (2006)
Raised intensity (dose increase) of immunotherapy	Parks and Casale (2006)
Combination with allergen immunotherapy	Kuehr et al. (2002); Rolinck-Werninghaus et al. (2004)
Omalizumab in occupational allergy	Rambasek and Kavuru (2006)
Omalizumab plus immunotherapy	Parks and Casale (2006)
Food allergy (e.g., peanut)	Leung et al. (2003)
Occupational latex allergy	Leynadier et al. (2004)

beneficial. Combination therapy may permit a broader use of SIT by reducing the risk of anaphylactic side effects after SIT injections (Parks and Casale 2006). Furthermore, there is preliminary evidence that SIT in patients treated with omalizumab may improve the outcome of immunotherapy (Kuehr et al. 2002; Rolinck-Werninghaus et al. 2004). Taken together, these findings are encouraging and should prompt further studies in this regard.

11.22 Areas of Uncertainty

For the clinical trials of omalizumab, patients were enrolled with precisely defined characteristics of asthma, including sensitivity to specific perennial aeroallergens (i.e., dust mites, cockroaches, dog or cat dander). The role of omalizumab in patients with asthma who have allergies to other aeroallergens, such as molds or pollens, or who have negative allergy skin tests, has not been defined. It is also not clear to what extent omalizumab might be effective in patients with total serum IgE levels outside the trial ranges (30 to 700 IU mL^{-1} for patients aged 12 to 75 years). In addition, in clinical practice, there is considerable variability of response to omalizumab therapy. The reasons for such variability have not been established; hence, studies are needed to determine whether specific characteristics of individual patients might help to predict response.

The clinical trials performed to date have evaluated omalizumab only as adjunctive therapy with inhaled corticosteroids as compared with placebo; they have not evaluated the relative benefit of omalizumab in comparison with other available therapies, such as leukotriene-modifiers or theophylline. Also needed are comparisons with asthma therapies that are available to patients for whom low-dose inhaled corticosteroids do not control the asthma and who require step-up man-

agement (i.e., an increased dose of the corticosteroid or the addition of another medication) (National Heart, Lung, and Blood Institute 1997, 2003).

A critical point should be raised regarding the actual clinical relevance of the moderate corticosteroid-sparing effects observed in the trials, even if these reductions were significant, as well as to the substantial improvements noted in placebo groups. Given that the cost of omalizumab is substantially greater than that of conventional asthma therapy, the potential cost-effectiveness of this form of treatment will be important to assess.

The efficacy and safety of omalizumab have not been established for durations of treatment that exceed 1 year, and it is not known for how long clinical effects might persist when therapy is discontinued. As asthma is a chronic disease, long-term studies – especially in children – are needed to evaluate the effect of serum IgE suppression throughout development. To date, it cannot be excluded that adverse effects may become apparent only with follow-up into adulthood after several years. To date, only one study is available that has been performed exclusively in the pediatric age group (Hochhaus et al. 2003). Similarly, efficacy and safety studies are also needed for geriatric and non-white patients.

On the other hand, studies have indicated that the expression of FceRI on basophils might be reduced by omalizumab therapy. Thus, there may be fewer targets for the anti-FceRI antibodies with clinical improvement, which may in turn permit a reduction of the omalizumab dose required to maintain treatment success.

11.23 Outlook

The introduction of omalizumab seems likely to have a major impact in the therapy of difficult-to-treat asthma. The fact that allergic diseases often occur at more than one anatomic locality – notably the upper respiratory tract and sinuses – means that patients receiving this therapy should experience improvements in the multiple manifestations of allergy. Some 80 years after the discovery of reagin (the first anti-IgE treatment), omalizumab now represents a promising new therapeutic option for patients with allergic diseases. These studies will also encourage further investigations which focus on the selective targeting of mIgE-bearing B cells, thus inhibiting IgE synthesis before IgE production starts. The action of omalizumab on proximal processes of the allergic immune response offers the potential that anti-IgE mAbs may modulate the immune regulation and lead to a profound and long-lasting clinical improvement. As allergic rhinitis, allergic gastroenteritis, anaphylaxy and atopic dermatitis are all diseases in which IgE participates – both in immediate-hypersensitivity response and in the induction of chronic allergic inflammation – the application of omalizumab is likely to be widened in future. As subgroups of the allergic manifestations in skin and airways occur even without increased total IgE levels and in the absence of

specific IgE, the availability of anti-IgE treatment will help to establish whether there are true so-called "intrinsic" or "nonallergic" forms of the disease.

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References

Adelroth, E., Rak, S., Haahtela, T., Aasand, G., Rosenhall, L., Zetterstrom, O., Byrne, A., Champain, K., Thirwell, J., Della Gioppa, G., Sandstrom T. (2000), Recombinant humanized mAb E25, an anti-IgE mAb, in birch pollen-induced seasonal allergic rhinitis. *J Allergy Clin Immunol* 106: 253–259.

Allam, J.P., Niederhagen, B., Bücheler, M., Appel, T., Betten, H., Bieber, T., Bergé, S., Novak, N. (2006) Comparative analysis of nasal and oral mucosa dendritic cells. *Allergy* 61: 166–172.

Amiri, P., Haak-Frendscho, M., Robbins, K., Mckerrow, J.H., Stewart, T., Jardieu, P. (1994) Anti-immunoglobulin E treatment decreases worm burden and egg production in *Schistosoma mansoni*-infected normal and interferon gamma knockout mice. *J Exp Med* 180: 43–51.

Aubrey, J.P., Pochon, S., Graber, P., Jansen, K.U., Bonnefoy, J.Y. (1992) CD21 is a ligand for CD23 and regulates IgE production. *Nature* 358: 505–507.

Ayres, J.G., Higgins, B., Chilvers, E.R., Ayre, G., Blogg, M., Fox H. (2004) Efficacy and tolerability of anti-immunoglobulin E therapy with omalizumab in patients with poorly controlled (moderate-to-severe) allergic asthma. *Allergy* 59: 701–708.

Bang, L.M., Plosker, G.L. (2004) Omalizumab: a review of its use in the management of allergic asthma. *Treat Respir Med*. 3: 183–199.

Barnes, P.J. (2000) Anti-IgE therapy in asthma: rationale and therapeutic potential. *Int Arch Allergy Immunol* 123: 196–204.

Beasley, R., Crane, J., Lai, C.K., Pearce, N. (2000) Prevalence and etiology of asthma. *J Allergy Clin Immunol* 105(Suppl.): S466–S472.

Bieber, T., de la Salle, H., Wollenberg, A., Hakimi, J., Chizzonite, R., Ring, J., et al. (1992) Human epidermal Langerhans cells express the high affinity receptor for immunoglobulin E (FcεRI). *J Exp Med* 175: 1285–1290.

Bonnefoy, J.Y., Plater-Zyberk, C., Lecoanet-Henchoz, S., Gauchat, J.F., Aubry, J.P., Graber, P. (1996) A new role for CD23 in inflammation. *Immunol Today* 17: 418–420.

Borkowski, T.A., Jouvin, M.H., Lin, S.Y., Kinet, J.P. (2001) Minimal requirements for IgE-mediated regulation of surface FcεRI. *J Immunol* 167: 1290–1296.

Boulet, L.P., Chapman, K.R., Cote, J., Kalra, S., Bhagat, R., Swystun, V.A., Laviolette, M., Cleland, L.D., Deschesnes, F., Su, J.Q., DeVault, A., Fick, R.B., Cockcroft, D.W. (1997) Inhibitory effects of an anti-IgE antibody E25 on allergen-induced early asthmatic response. *Am J Respir Crit Care Med* 155: 1835–1840.

Boushey, H.A. (2001) Experiences with monoclonal antibody therapy for allergic asthma. J Allergy Clin Immunol 108: S77–S83.

Bousquet, J., Wenzel, S., Holgate, S., Lumry, W., Freeman, P., Fox, H. (2004) Predicting response to omalizumab, an anti-IgE

antibody, in patients with allergic asthma. *Chest* 125: 1378–1386.
Bousquet, J., Cabrera, P., Berkman, N., et al. (2005) The effect of treatment with omalizumab, an anti-IgE antibody, on asthma exacerbations and emergency medical visits in patients with severe persistent asthma. *Allergy* 60: 302–308.
Burrows, B., Martinez, F.D., Halonen, M., Barbee, R.A., Cline M.G. (1989) Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med* 320: 271–277.
Busse, W., Coren, J., Lanier, B.Q., McAlary, M., Fowler-Taylor, A., Della Cioppa, G., van As, N., Gupta, N. (2001) Omalizumab, anti-IgE recombinant humanized monoclonal antibody, for the treatment of severe allergic asthma. *J Allergy Clin Immunol* 108: 184–190.
Buhl, R., Hanf, G., Soler, M., Bensch, G., Wolfe, J., Everhard, F., et al. (2002) The anti-IgE antibody omalizumab improves asthma-related quality of life in patients with allergic asthma. *Eur Respir J* 20: 1088–1094.
Casale, T.B., Bernstein, I.L., Busse, W.W., LaForce, C.R., Tinkelman, D.G., Stoltz, R.R., Dockhorn, R.J., Reimann, J., Su, J.Q., Fick, R.B., Adelman, D.C. (1997) Use of an anti-IgE humanized monoclonal antibody in ragweed-induced allergic rhinitis. *J Allergy Clin Immunol* 100: 110–121.
Casale, T., Condemi, J., Miller, S., Fickm, R.B., Jr., McAlarym, M., Fowler-Taylor, A., Gupta N. (1999) rhuMAb-E25 in the treatment of seasonal allergic rhinitis (SAR). *Ann Allergy Asthma Immunol* 82: 75–82.
Chang, T.W. (2000) The pharmacological basis of anti-IgE therapy. *Nat Biotechnol* 18: 157–163.
Chang, T.W., Davis, F.M., Sun, N.C., Sun, C.R., Macglashan, D.W., Jr., Hamilton, R.G. (1990) Monoclonal antibodies specific for human IgE-producing B cells: a potential therapeutic for IgE-mediated allergic diseases. *Biotechnology (NY)* 8: 122–126.
Chervinsky, P., Casale, T., Townley, R., et al. (2003) Omalizumab, an anti-IgE antibody, in the treatment of adults and adolescents with perennial allergic rhinitis. Ann Allergy Asthma Immunol 91: 1607.
Corren, J., Casale, T., Deniz, Y., Ashby, M. (2003) Omalizumab, a recombinant humanized antiIgE antibody, reduces asthma-related emergency room visits and hospitalizations in patients with allergic asthma. *J Allergy Clin Immunol* 111: 87–90.
Dacus, J.J. (2003) Disease of the month: asthma. *PEC Update* 3: 4–6.
D'Amato, G., Holgate, S. (2002) *The Impact of Air Pollution on the Respiratory Health. Monograph of the European Respiratory Society*. Sheffield, UK.
D'Amato, G., Oldani, V., Donner, C.F. (2002) Anti-IgE monoclonal antibody: a new approach to the treatment of allergic respiratory diseases. *Arch Chest Dis* 59: 29–33.
D'Amato, G., Liccardi, G., Noschese, P., Salzillo, A., D'Amato, M., Cazzola, M. (2004) Anti-IgE Monoclonal antibody (Omalizumab) in the treatment of atopic asthma and allergic respiratory diseases. *Curr Drug Targets – Inflammation Allergy* 3: 227–231.
Davis, F.M., Gossett, L.A., Chang, T.W. (1991) An epitope on membrane-bound but not secreted IgE: implications in isotype-specific regulation. *Biotechnology (NY)* 9: 53–56.
Davis, F.M., Gossett, L.A., Pinkston, K.L., Liou, R.S., Sun, L.K., Kim, Y.W., Chang, N.T., Chang, T.W., Wagner, K., Bews, J., et al. (1993) Can anti-IgE be used to treat allergy? *Semin Immunopathol* 15: 51–73.
Djukanovic, R., Wilson, S.J., Kraft, M., Jarjour, N.N., Steel, M., Chung, K.F., Bao, W., Fowler-Taylor, A., Matthews, J., Busse, W.W., Holgate, S.T., Fahy, J.V. (2004) Effects of treatment with anti-immunoglobulin E antibody omalizumab on airway inflammation in allergic asthma. *Am J Respir Crit Care Med* 170: 583–593.
Easthope, S., Jarvis, B. (2001) Omalizumab. *Drugs* 61: 253–260.
Eray, M., Tuomikoski, T., Wu, H., Nordstrom, T., Andersson, L.C., Knuutila, S., et al. (1994) Cross-linking of surface IgG induces apoptosis in a bcl-2 expressing human follicular lymphoma line of mature B cell phenotype. *Int Immunol* 6: 1817–1827.
Fahy, J.V., Fleming, H.E., Wong, H.H., Liu, J.T., Su, J.Q., Reimann, J., Fick, R.B.,

Boushey, H.A. (1997) The effect of an anti-IgE monoclonal antibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects. *Am J Respir Crit Care Med* 155: 1828–1834.

Feuchtinger, T., Bartz, H., von Berg, A., Riedinger, F., Brauburger, J., Stengle, S., et al. (2003) Treatment with omalizumab normalizes the number of myeloid dendritic cells during the grass pollen season. *J Allergy Clin Immunol* 111: 428–430.

Finn, A., Gross, G., van Bavel, J., et al. (2003) Omalizumab improves asthma-related quality of life in patients with severe allergic asthma. *J Allergy Clin Immunol* 111: 278–284.

Food and Drug Administration, Center for Biologics Evaluation and Research. (2003) BLASTN 103976/0, review of clinical safety data: original BLS submitted on June 2, 2000 and response to complete review letter submitted on December 18, 2002. Rockville, Md.: Department of Health and Human Services.

Fox, J.A., Hotaling, T.E., Struble, C., Ruppel, J., Bates, D.J., Schoenhoff, M.B. (1996) Tissue distribution and complex formation with IgE of an anti-IgE antibody after intravenous administration in cynomolgus monkeys. *J Pharmacol Exp Ther* 279: 1000–1008.

Garman, S.C., Kinet, J.P., Jardetzky, T.S. (1998) Crystal structure of the human high-affinity IgE receptor. *Cell* 95: 951–961.

Garman, S.C., Wurzburg, B.A., Tarchevskaya, S.S., Kinet, J.P., Jardetzky, T. S. (2000) Structure of the Fc fragment of human IgE bound to its high-affinity receptor FcepsilonRI alpha. *Nature* 406: 259–266.

Geha, R.S., Jabara, H.H., Brodeur, S.R. (2003) The regulation of immunoglobulin E class-switch recombination. *Nat Rev Immunol* 3: 721–732.

GINA – Global Initiative for Asthma. (1995, updated 2002, 2003). *Global strategy for asthma management and prevention*. NIH publication number 02-3659.

Gounni, A.S., Lamkhioued, B., Delaporte, E., Dubost, A., Kinet, J.P., Capron, A., et al. (1994) The high-affinity IgE receptor on eosinophils: from allergy to parasites or from parasites to allergy? *J Allergy Clin Immunol* 94: 1214–1216.

Gounni, A.S., Wellemans, V., Yang, J., Bellesort, F., Kassiri, K., Gangloff, S., et al. (2005) Human airway smooth muscle cells express the high affinity receptor for IgE (Fc epsilon RI): a critical role of Fc epsilon RI in human airway smooth muscle cell function, *J Immunol* 175: 2613–2621.

Hagan, P., Blumenthal, U.J., Dunn, D., Simpson, A.J., Wilkins, H.A. (1991) Human IgE, IgG4 and resistance to reinfection with Schistosoma haematobium. *Nature* 349: 243–245.

Hamilton, R.G., Marcotte, G.V., Saini, S.S. (2005) Immunological methods for quantifying free and total serum IgE levels in allergy patients receiving omalizumab (Xolair) therapy. *J Immunol Methods* 303: 81–91.

Herbelin, A., Elhadad, S., Ouaaz, F., de Groote, D., Descamps-Latscha, B. (1994) Soluble CD23 potentiates interleukin-1-induced secretion of interleukin-6 and interleukin-1 receptor antagonist by human monocytes. *Eur J Immunol* 24: 1869–1873.

Henchoz, S., Gauchat, J.F., Aubry, J.P., Graber, P., Pochon, S., Bonnefoy, J.Y. (1994) Stimulation of human IgE production by a subset of anti-CD21 monoclonal antibodies: requirement of a co-signal to modulate epsilon transcripts. *Immunology* 81: 285–290.

Heusser, C., Jardieu, P. (1997) Therapeutic potential of anti-IgE antibodies. *Curr Opin Immunol* 9: 805–813.

Hochhaus, G., Brookman, L., Fox, H., et al. (2003) Pharmacodynamics of omalizumab: implications for optimized dosing strategies and clinical efficacy in the treatment of allergic asthma. *Curr Med Res Opin* 19: 491–498.

Holgate, S.T. (1998) Asthma and allergy – disorders of civilization? *Q J Med* 91: 171–184.

Holgate, S.T., Bousquet, J., Wenzel, S., Fox, H., Liu, J., Castellsague, J. (2001) Efficacy of omalizumab, an anti-immunoglobulin E antibody, in patients with allergic asthma at high risk of serious asthma-related morbidity and mortality. *Curr Med Res Opin* 17: 233–240.

Holgate, S.T., Casale, T., Wenzel, S., Bousquet, J., Deniz, Y., Reisner, C. (2005a) The anti-inflammatory effects of omalizumab confirm the central role of IgE in allergic inflammation. *J Allergy Clin Immunol* 115: 459–465.

Holgate, S.T., Djukanovic, R., Casale, T., Bousquet, J. (2005b) Anti-immunoglobulin E treatment with omalizumab in allergic diseases: an update on anti-inflammatory activity and clinical efficacy. *Clin Exp Allergy* 35: 408–416.

Humbert, M., Beaskey, R., Ayres, J., Slavin, R., Hebert, J., Bousquet, J., Beeh, K.M., Ramos, S., Canonica, G.W., Hedgecock, S., Fox, H., Blogg, M., Surrey, K. (2005) Benefits of omalizumab as add-on therapy in patients with severe persistent asthma who are inadequately controlled despite best available therapy (GINA 2002 step 4 treatment): INNOVATE. *Allergy* 60: 309–316.

ISAAC-Steering Committee. (1998) Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. *Lancet* 351: 1225–1232.

Ishizaka, K., Ishizaka, T. (1970) Biological function of IgE antibodies and mechanisms of reaginic hypersensitivity. *Clin Exp Immunol* 6: 25–31.

Joshua, A., Boyce, J.A. (2006) Successful treatment of cold-induced urticaria/anaphylaxis with anti-IgE. *J Allergy Clin Immunol* 117: 1415–1418.

Kalinski, P., Hilkens, C.M., Wierenga, E.A., Kapsenberg, M.L. (1999) T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today* 20: 561–567.

Kehry, M.R., Yamashita, L.C. (1989) Low-affinity IgE receptor (CD23) function on mouse B cells: role in IgE-dependent antigen focusing. *Proc Natl Acad Sci USA* 86: 7556–7560.

Kelly, H.W., Sorkness, C.A. (2002) Asthma. In: Dipiro, J.T., Talbert, R.L., Yee, G.C., Matzke, G.R., Wells, B.G., Posey, L.M. (Eds.), *Pharmacotherapy: a pathophysiologic approach*. 5th edn. New York: McGraw-Hill, pp. 475–510.

Kijimoto-Ochiai, S. (2002) CD23 (the low-affinity IgE receptor) as a C-type lectin: a multidomain and multifunctional molecule. *Cell Mol Life Sci* 59: 648–664.

Kolbinger, F., Saldanha, J., Hardman, N., Bendig, M.M. (1993) Humanization of a mouse anti-human IgE antibody: a potential therapeutic for IgE-mediated allergies. *Protein Eng* 6: 971–980.

Kraft, S., Katoh, N., Novak, N., Koch, S., Bieber, T. (2001) Unexpected functions of FcepsilonRI on antigen-presenting cells. *Int Arch Allergy Immunol* 124: 35–37.

Kroegel, C. (2002) *Bronchial asthma. Pathogenic basis, diagnostic and therapy*. 2nd edn. Thieme, Stuttgart, New York.

Kroegel, C., Mock, B., Reißig, A., Hengst, U., Förster, M., Machnik, A., Henzgen, M. (2002) Antibodies in allergic diseases. Selective inhibition of IgE-mediated reactions by humanized IgE-anti-IgE-antibodies. *Arzneimitteltherapie* 20: 226–237.

Kroegel, C., Reissig, A., Henzgen, M. (2004) Therapeutic dosing of omalizumab = 20x[IgE]: is this equation correct or is enough too less? *Pneumologie* 58: 543–545.

Kuehr, J., Brauburger, J., Zielen, S., Schauer, U., Kamin, W., et al. (2002) Efficacy of combination treatment with anti-IgE plus specific immunotherapy in polysensitized children and adolescents with seasonal allergic rhinitis. *J Allergy Clin Immunol* 109: 274–280.

Lanier, B.Q., Corren, J., Lumry, W., Liu, J., Fowler-Taylor, A., Gupta, N. (2003) Omalizumab is effective in the long-term control of severe allergic asthma. *Ann Allergy Asthma Immunol* 91: 154–159.

Lemanske, R.F., Nayak, A., McAlary, M., Everhard, F., Fowler-Taylor, A., Gupta, N. (2002) Omalizumab improves asthma-related quality of life in children with allergic asthma. *Pediatrics* 110: E55.

Leung, D.Y., Sampson, H.A., Yunginger, J.W., Burks, A.W., Schneider, L.C., Wortel, C.H., et al. (2003) Effect of anti-IgE therapy in patients with peanut allergy. *N Engl J Med* 348: 986–993.

Leynadier, F., Doudou, O., Gaouar, H., Le Gros, V., Bourdeix, I., Guyomarch-Cocco, L., Trunet, P. (2004) Effect of omalizumab in health care workers with occupational latex allergy. *J Allergy Clin Immunol* 113: 360–361.

Lin, H., Boesel, K.M., Griffith, D.T., et al. (2004) Omalizumab rapidly decreases nasal allergic response and FceRI on basophils. *J Allergy Clin Immunol* 113: 297–302.

Liu, J., Lester, P., Builder, S., Shire, S.J. (1995) Characterization of complex formation by humanized anti-IgE antibody and monoclonal IgE *Biochemistry* 34: 10474–10482.

Liu, Y.J., Kanzler, H., Soumelis, V., Gilliet, M. (2001) Dendritic cell lineage, plasticity and cross-regulation. *Nat Immunol* 2: 585–589.

MacGlashan, D., Jr. (2005) IgE and Fc{epsilon}RI regulation. *Ann N Y Acad Sci* 1050: 73–88.

MacGlashan, D.W., Bochner, B.S., Adelman, D.C., Jardieu, P.M., Togias, A., Lichtenstein, L.M. (1997a) Serum IgE level drives basophil and mast cell IgE receptor display. *Int Arch Allergy Immunol* 113: 45–47.

MacGlashan, D.W., Jr., Bochner, B., Adelman, D., Jardieu, P., Togias, A., McKenzie-White, J., Sterbinsky, S., Hamilton, R., Lichtenstein, L.M. (1997b) Down-regulation of FceRI expression on human basophils during in vivo treatment of atopic patients with anti-IgE antibody. *J Immunol* 158: 1438–1445.

MacGlashan, D., McKenzie-White, J., Chichester, K., et al. (1998) In vitro regulation of FceRI expression on human basophils by IgE antibody. *Blood* 91: 1633–1643.

Maizels, N. (2003) Yin outwits yang at the IgE locus. *Nat Immunol* 4: 7–8.

Maizels, R.M., Yazdanbakhsh, M. (2003) Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nat Rev Immunol* 3: 733–744.

Malveaux, F.J., Conroy, M.C., Adkinson, N.F.J., Lichtenstein, L.M. (1978) IgE receptors on human basophils: Relationship to serum IgE concentration. *J Clin Invest* 62: 176–181.

Masoli, M., Fabian, D., Holt, S., Beasley, R. (2004) Global Initiative for Asthma (GINA) Program. The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* 59: 469–478.

Maurer, D., Ebner, C., Reininger, B., Fiebiger, F., Kraft, D., Kinet, J.P., et al. (1995) The high affinity IgE receptor (FcεRI) mediates IgE-dependent allergen presentation. *J Immunol* 154: 6285–6290.

Maurer, D., Ebner, C., Reininger, B., Petzelbauer, P., Fiebiger, E., Stingl, G. (1997) Mechanisms of Fc epsilon RI-IgE-facilitated allergen presentation by dendritic cells. *Adv Exp Med Biol* 417: 175–178.

Milgrom, H., Fick, R.B., Jr., Su, J.Q., Reimann, J.D., Bush, R.K., Watrous, M.L., et al. (1999) Treatment of allergic asthma with monoclonal anti-IgE antibody. *N Engl J Med* 341: 1966–1973.

Milgrom, H., Berger, W., Nayak, A., Gupta, N., Pollard, S., McAlary, M., Fowler-Taylor, A., Rohane, P. (2001) Treatment of childhood asthma with anti-immunoglobulin E antibody (omalizumab). *Pediatrics* 108: 36–45.

Nakamura, T., Kloetzer, W.S., Brams, P., Hariharan, K., Chamat, S., Cao, X., LaBarre, M.J., Chinn, P.C., Morena, R.A., Shestowsky, W.S., Li, Y.P., Chen, A., Reff, M.E. (2002) In vitro IgE inhibition in B cells by anti-CD23 monoclonal antibodies is functionally dependent on the immunoglobulin Fc domain. *Int J Immunopharmacol* 22: 131–141.

National Heart, Lung, and Blood Institute. (1997) National Asthma Education and Prevention Program Expert Panel Report 2: guidelines for the diagnosis and management of asthma. Bethesda, Md.: National Institutes of Health (Publication no. 97-4051).

National Heart, Lung, and Blood Institute. (2003) Education and Prevention Program Expert Panel Report: guidelines for the diagnosis and management of asthma – update on selected topics 2002. Bethesda, Md.: National Institutes of Health (Publication no. 02-5074).

Nissim, A., Eshhar, Z. (1992) The human mast cell receptor binding site maps to the third constant domain of immunoglobulin E. *Mol Immunol* 29: 1065–1072.

Nissim, A., Schwarzbaum, S., Siraganian, R., Eshhar, Z. (1993) Fine specificity of the IgE interaction with the low and high affinity Fce receptor. *J Immunol* 150: 1365–1374.

Noga, O., Hanf, G., Kunkel, G. (2003) Immunological changes in allergic asthmatics following treatment with omalizumab. *Int Arch Allergy Immunol* 131: 46–52.

Noga, O., Hanf, G., Brachmann, I., Klucken, A.C., Kleine-Tebbe, J., Rosseau, S., Kunkel, G., Suttorp, N., Seybold, J. (2006) Effect of omalizumab treatment on peripheral eosinophil and T-lymphocyte function in patients with allergic asthma. *J Allergy Clin Immunol* 117: 1493–1499.

Novak, N., Bieber T. (2003) Allergic and nonallergic forms of atopic diseases. *Allergy Clin Immunol* 112: 252–262.

Novak, N., Bieber, T., Katoh N. (2001) Engagement of FcεRI on human monocytes induces the production of IL-10 and prevents their differentiation in dendritic cells. *J Immunol* 167: 797–804.

Omalizumab (Xolair): an anti-IgE antibody for asthma. (2003) *Med Lett Drugs Ther* 45: 67–68.

Package insert. (2003) Xolair (omalizumab). East Hanover, NJ: Aventis, June 2003.

Parks, K.W., Casale, T.B. (2006) Anti–immunoglobulin E monoclonal antibody administered with immunotherapy. *Allergy Asthma Proc* 27(2 Suppl.1): S33–S36.

Platts-Mills, T.A. (2001) The role of immunoglobulin E in allergy and asthma. *Am J Respir Crit Care Med* 164(8 Pt 2): S1–S5.

Plewako, H., Arvidsson, M., Petruson, K., Oancca, I., Holmberg, K., Adelroth, E.A., Gustafsson, H., Sandstrom, T., Rak, S. (2002) The effect of omalizumab on nasal allergic inflammation, *J Allergy Clin Immunol* 110: 68–71.

Presta, L.G., Lahr, S.J., Shields, R.L., Porter, J.P., Gorman, C.M., Fendly, B.M., Jardieu, P.M. (1993) Humanization of an antibody directed against IgE. *J Immunol* 151: 2623–2632.

Presta, L.G., Shields, R.L., O'Connell, L., Lahr, S., Porter, J., Gorman, C., et al. (1994) The binding site on human immunoglobulin E for its high affinity receptor. *J Biol Chem* 269: 26368–26373.

Prussin, C., Griffith, D.T., Boesel, K.M., Lin, H., Foste, B., Casale, T.D. (2003) Omalizumab treatment downregulates dendritic cell FceRI expression, *J Allergy Clin Immunol* 112: 1147–1154.

Rambasek, T., Kavuru, M.S. (2006) Omalizumab dosing via the recommended card versus use of the published formula. *J Allergy Clin Immunol* 117: 708–709.

Rissoan, M.C., Soumelis, V., Kadowaki, N., Grouard, G., Briere, F., de Waal Malefyt, R., et al. (1999) Reciprocal control of T helper cell and dendritic cell differentiation. *Science* 283: 1183–1186.

Rolinck-Werninghaus, C., Hamelmann, E., Keil, T., Kulig, M., Koetz, K., Gerstner, B., et al. (2004) The co-seasonal application of anti-IgE after preseasonal specific immunotherapy decreases ocular and nasal symptom scores and rescue medication use in grass pollen allergic children. *Allergy* 59: 973–979.

Rosenwasser, L.J., Meng, J. (2005) Anti-CD23. *Clin Rev Allergy Immunol* 29: 61–72.

Saban, R., Haak-Frendscho, M., Zine, M., Ridgway, J., Gorman, C., Presta, L., Bjorling, D., Saban, M., Jardieu, P. (1994) Human FceRI-IgG and humanized anti-IgE monoclonal antibody MaE11 block passive sensitization of human and rhesus monkey lung. *J Allergy Clin Immunol* 94: 836–843.

Saini, S.S., MacGlashan, D. (2002) How IgE upregulates the allergic response. *Curr Opin Immunol* 14: 694–697.

Saini, S.S., MacGlashan, D.W., Jr., Sterbinsky, S.A., et al. (1999) Down-regulation of human basophil IgE and FcεRI surface densities and mediator release by anti-IgE-infusions is reversible in vitro and in vivo. *J Immunol* 162: 5624–5630.

Scharenberg, A.M., Kinet, J.P. (1995) Early events in mast cell signal transduction. *Chem Immunol* 61: 72–87.

Shields, R.L., Whether, W.R., Zioncheck, K., O'Connell, L., Fendly, B., Presta, L.G., Thomas, D., Saban, R., Jardieu, P. (1995) Inhibition of allergic reactions with antibodies to IgE. *Int Arch Allergy Immunol* 107: 308–312.

Soler, M., Matz, J., Townley, R., Buhl, R., O'Brien, J., Fox, H., Thirlwell, J., Gupta, N., Della Cioppa, G. (2001) The anti-IgE antibody omalizumab reduces exacerbations and steroid requirement in allergic asthmatics, *Eur Respir J* 18: 254–261.

Sutton, B.J., Gould, H.J. (1993) The human IgE network. *Nature* 366: 421–428.
Stingl, G., Maurer, D. (1997) IgE-mediated allergen presentation via FceRI on antigen-presenting cells. *Int Arch Allergy Immunol* 113: 24–29.
Turner, H., Kinet, J.P. (1999) Signalling through the high-affinity IgE receptor FcεRI. *Nature* 402(Suppl.): B24–B30.
Upham, J.W. (2003) The role of dendritic cells in immune regulation and allergic airway inflammation. *Respirology* 8: 1408–1418.
Vercelli, D., Helm, B., Marsh, P., Padlan, E., Geha, R.S., Gould, H. (1989) The B-cell binding site on human immunoglobulin E. *Nature* 338: 649–651.
Vigo, P.G., Girgis, K.R., Pfuetze, B.L., Critchlow, M.E., Fisher, J., Hussain, I. (2006) Efficacy of anti-IgE therapy in patients with atopic dermatitis. *J Am Acad Dermatol* 55: 168–170.
Walker, S., Monteil, M., Phelan, K., Lasserson, T.J., Walters, E.H. (2004) Anti-IgE for chronic asthma in adults and children. *Cochrane Database Syst Rev* 3: CD003559.
Wang, B., Rieger, A., Kilgus, O., Ochiai, K., Maurer, D., Fodinger, D., et al. (1992) Epidermal Langerhans cells from normal human skin bind monomeric IgE via FcεRI. *J Exp Med* 175: 1353–1365.
Warner, G.L., Scott, D.W. (1991) A polyclonal model for B cell tolerance. I. Fc-dependent and Fc-independent induction of nonresponsiveness by pretreatment of normal splenic B cells with anti-Ig. *J Immunol* 146: 2185–2191.
Warner, G.L., Gaur, A., Scott, D.W. (1991) A polyclonal model for B-cell tolerance. II. Linkage between signaling of B-cell egress from G0, class II upregulation and unresponsiveness. *Cell Immunol* 138: 404–412.
Warner, J.O., Kaliner, M.A., Crisci, C.D., Del Giacco, S., Frew, A.J., Liu, G.H., Maspero, J., Moon, H.B., Nakagawa, T., Potter, P.C., Rosenwasser, L.J., Singh, A.B., Valovirta, E., Van Cauwenberge, P. (2006) World Allergy Organization Specialty and Training Council. Allergy practice worldwide: a report by the World Allergy Organization Specialty and Training Council. *Int Arch Allergy Immunol* 139: 166–174.
Yazdanbakhsh, M., van den Biggelaar, A., Maizels, R.M. (2001) Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. *Trends Immunol* 22: 372–377.

Websites

Xolair product labeling. Genentech, Inc. Accessed January, 2004, at: http://www.gene.com/gene/common/inc/pi/xolair.jsp.
Food and Drug Administration Web site. Patient safety news: first biologic for allergy-related asthma. Available at: www.accessdata.gov/scripts/cdrh/cfdocs. Accessed November 15, 2004

12
Palivizumab (Synagis)

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12.1
Nature, Role in Disease, and Biology of the Target

12.1.1
Respiratory Syncytial Virus (RSV)-Induced Disease and RSV Epidemiology

Respiratory syncytial virus (RSV) infection is the single most common cause of hospitalization for acute respiratory disease among infants [39,81,82], and an important etiology of lower respiratory disease in young children and the elderly [28,69]. By the end of the second year of life, most children have experienced at least one RSV infection, and repeat infections with RSV are common. Approximately 1 to 3% of all healthy term infants are admitted to hospital for lower respiratory tract illness (LRTI) due to primary RSV infection [14,69,82], and this admission rate can rise to and even exceed 10% in high-risk populations. Risk factors for severe RSV disease include chronic lung disease of infancy/bronchopulmonary dysplasia (CLD/BPD), birth at less than 36 weeks of gestation, clinically significant congenital heart disease (CHD), and severe immunosuppression [2,29]. Of all children admitted to hospital for RSV acute LRTI, more than half do not have any of the above-mentioned risk factors, and most of these previously healthy term infants are aged less than 6 months [26,60]. Long-term sequelae associated with RSV LRTI include recurrent wheeze and an increased risk to develop an asthma phenotype [48,71].

Although RSV may be isolated from patients year-round, RSV epidemics of LRTI among infants and young children usually occur during late fall, winter, and early spring in countries with temperate climates. In tropical or semitropical climates, RSV epidemics appear equally as regular, but the pattern of seasonality is more complex and seems to depend on crowding, humidity, and temperature variables [76]. Primary RSV infection will usually lead to clinical disease that starts as an upper respiratory tract illness (URTI) which may subsequently progress to LRTI, commonly presenting as bronchiolitis and/or pneumonia. Less frequently, RSV LRTI will present as laryngotracheobronchitis (croup), bronchitis,

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or with episodes of apnea (in very young and/or premature infants). Otitis media is another frequent sequela of RSV disease. The treatment of bronchiolitis is mostly supportive in nature, and includes adequate hydration, positioning, and respiratory support. Although widely used, pharmacological interventions offer limited benefit or none at all. There is limited evidence suggesting that nebulized epinephrine, as well as inhaled bronchodilators, may offer short-term benefits for bronchiolitis in outpatients, although evidence to support their use in inpatients is lacking [36,43]. No clinically significant improvement is associated with the systemic administration of glucocorticoids [57], and the use of ribavirin as an antiviral in RSV disease is controversial and limited to patients in need of mechanical ventilation or severely immunosuppressed patients [7,80].

12.1.2 The Target of the Antibody: The RSV Virion

RSV is an enveloped virus with a nonsegmented negative-sense RNA genome, a member of the Paramyxovirus family within the Mononegavirus order [21]. Its 15.2-kb genome consists of 10 genes that are transcribed sequentially from the 3′ end to the 5′ end by a virally encoded polymerase, and are translated into 11 protein species (Fig. 12.1).

The viral polymerase consists of the large polymerase protein L, the phosphoprotein P, and the transcription elongation factor M2-1. A second open reading frame (ORF) of the M2 gene encodes the M2-2 protein, a small protein that appears to mediate a shift from transcription to RNA replication during the infectious cycle [13]. The two nonstructural proteins NS-1 and NS-2 are not incorporated into the virion, but are important virulence factors that interfere with the host's interferon response [75]. The internal matrix protein M plays an important role during virus assembly, and the viral envelope is derived from the plasma membrane of the host cell as the virus buds from the apical surface of respiratory epithelial cells. The envelope contains three transmembrane surface glycoproteins – namely, the attachment protein G, the fusion protein F, and the small hydrophobic protein SH. The G and F glycoproteins are protective antigens that induce infectivity-neutralizing antibodies. Using monoclonal antibodies (mAbs), two antigenic subgroups (A and B) can be distinguished. While there is considerable divergence in G-protein sequence between the subgroup A and subgroup B viruses (44% amino acid identity with regard to the ectodomain [21]), the F protein sequence is more conserved (91% amino acid identity with regard to the ectodomain [51]), and antibodies directed against the F protein are generally cross-reactive between the two subgroups. The RSV F protein mediates fusion between the viral envelope and the host cell plasma membrane, thereby releasing the viral nucleocapsid into the cytoplasm. Later in the replicative cycle, F protein is expressed on the apical surface of epithelial cells and cell-to-cell fusion may occur, giving rise to multinucleated syncytia. The F protein is a type 1 glycoprotein that is synthesized as a precursor protein (F0) and activated through cleavage by a host protease into disulfide-linked subunits (F1 and F2). Cleavage releases

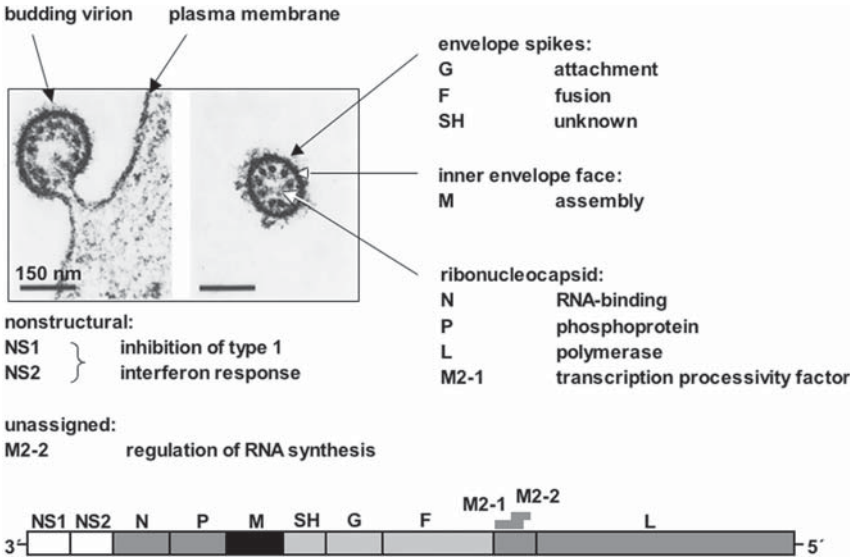

Fig. 12.1 Respiratory syncytial virus (RSV) virion, proteins and genome. Upper: Electron micrograph depicting a budding and a free RSV virion. The location of structural viral proteins is indicated by arrows. G = attachment glycoprotein; F = fusion glycoprotein; SH = small hydrophobic protein; M = matrix protein; N = nucleocapsid protein; P = phosphoprotein; L = large polymerase protein; M2-1 = transcription processivity factor; M2-2 = transcription/replication regulator protein. NS1 and NS2 = nonstructural proteins. Lower: Schematic drawing of the organization of the negative sense single-stranded RNA genome (not to scale). Graph courtesy of Peter Collins; electron micrograph by Tony Kalica, Laboratory of Infectious Diseases, NIAID, NIH.

a hydrophobic "fusion peptide" at the N-terminus of the F1 subunit that is thought to be directly involved in target membrane destabilization and membrane fusion. The protein forms homotrimers in the viral envelope that are stabilized by alpha-helical coiled coils [21].

12.1.3 Correlates of Protection from Disease

RSV infection does not induce sterilizing immunity – that is, re-infection, even with the same RSV strain, can and does occur. However, some protection is induced because subsequent RSV infection generally does not lead to severe disease in otherwise healthy subjects, and is only rarely a reason for hospitalization. RSV-neutralizing antibodies directed against the F or G glycoproteins are necessary and sufficient for protection from severe disease. Cell-mediated immune responses can also provide protection from disease, but this protection is not very durable [23]. The protective mechanism is not prevention of RSV infection but rather a restriction of virus replication. Maternally acquired IgG can protect the

newborn infant from disease, and infants born to mothers with high RSV-neutralizing antibody titers are less likely to develop severe RSV disease during their first 6 months of life [54]. The quantitative aspects of passively acquired RSV-neutralizing antibody on RSV replication in the respiratory tract were initially determined in the cotton rat model of RSV pathogenesis [62]. In this animal model, a serum-neutralizing antibody titer of 1:380 or greater leads to complete or almost complete suppression of RSV replication in lung tissue (Fig. 12.2). While the effect of passively transferred IgG is impressive in the lungs, it is less obvious in the upper respiratory tract (URT), due to limited transudation of serum IgG to the URT mucosal surface. Antibodies can prevent infection if present in sufficiently high titer at the site of infection, but generally act to restrict virus replication.

Based on these data, a polyclonal human antibody preparation (RSV-IVIG; RespiGam, Medimmune) was prepared from multiple donors who were selected

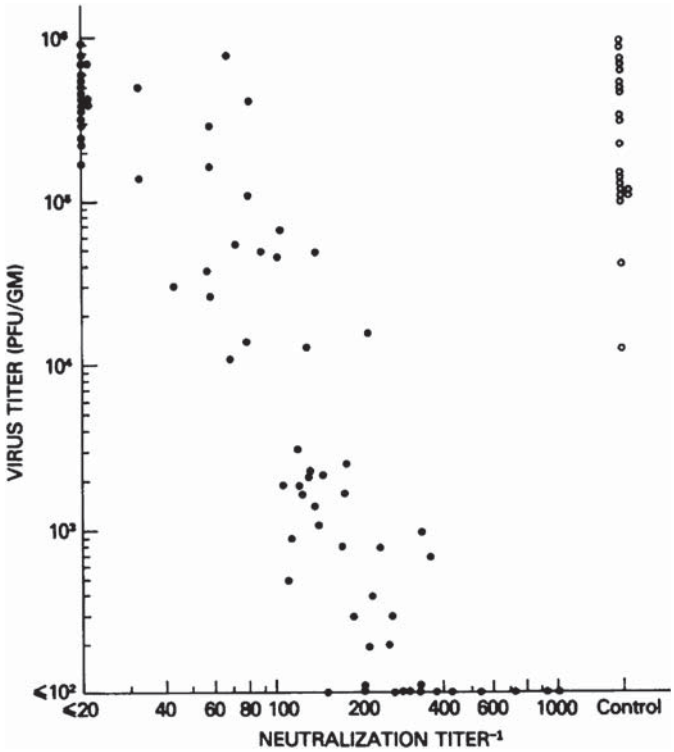

Fig. 12.2 Relationship of pulmonary RSV titer and serum RSV-neutralizing antibody titer in cotton rats receiving either RSV immune serum or control serum intraperitoneally 24 h before an intranasal challenge with 10^4 PFU of RSV. Lung titers were determined 4 days post infection. (Reprinted with permission from Ref. [62].)

for a high serum titer of RSV-neutralizing antibodies. When administered prophylactically to high-risk children at a dose of 15 mL kg^{-1} body weight, RSV-IVIG was shown to decrease RSV LRTI, RSV disease severity and RSV-related hospitalization. RSV-IVIG did not, however, prevent RSV infection [4,35]. In order to be effective, the preparation had to be administered intravenously at monthly intervals during the RSV season. The most concerning serious adverse event (SAE) associated with RSV-IVIG administration was an increased mortality in children with CHD, possibly due to volume overload and/or increased viscosity associated with RSV-IVIG administration. RSV-IVIG was licensed by the FDA in 1996 for the prevention of serious lower respiratory tract infection caused by RSV in children under 24 months of age with bronchopulmonary dysplasia (BPD), or a history of premature birth (≤35 weeks' gestation). RSV-IVIG has been replaced by palivizumab (Synagis, Medimmune) and is no longer available for use.

12.2
Origin, Engineering, and Humanization of the Antibody

A detailed analysis of antigenic sites and neutralization epitopes of the RSV F glycoprotein – one of the two major envelope glycoproteins of RSV that induce a neutralizing antibody response – was performed in the Laboratory of Infectious Diseases (LID) at the National Institutes of Allergy and Infectious Diseases, National Institutes of Health. Beeler and Coelingh used a set of 18 RSV-neutralizing murine mAbs against the F protein of RSV subgroup A strain A2 for this analysis [11]. Eleven of these mAbs were generated at LID by immunizing BALB/c mice intranasally with the A2 strain of RSV, and boosting intraperitoneally 3 weeks later with a recombinant vaccinia virus expressing the RSV F protein. Four weeks later, mice were boosted once again with an intravenous injection of sucrose gradient-purified RSV. Splenic lymphocytes were then fused with NS-1 murine myeloma cells following the procedure of Kohler and Milstein [44].

Competitive binding assays identified three nonoverlapping antigenic sites (A, B, and C) and one bridging site (AB). Thirteen mAb-resistant mutants (MARMs) were selected, and the neutralization patterns of the mAbs with these MARMs and with clinical RSV isolates identified 16 epitopes on the F protein. Cross-neutralization studies using 23 clinical isolates (18 subgroup A and five subgroup B, isolated between 1956 and 1985) and 18 mAbs revealed that antigenic sites A and C were highly conserved, while antigenic site B was highly variable [11]. Each of nine virus-neutralizing mAbs directed against antigenic site A inhibited cell-to-cell fusion very efficiently, which suggested that the binding site might be close to the active site responsible for fusion-from-within. One of these murine antibodies directed against antigenic site A, mAb1129, was selected for humanization and clinical evaluation.

The development of the humanized version of mAb1129 was described in detail by Johnson et al. and revisited by Wu et al. [41,85]. The variable segments of the heavy chain (VH) and the light chain (VL) were assembled *de novo* using PCR

and site-directed mutagenesis [45]. The human K102 sequence [12] with J-kappa-4 (NCBI gi:220083) was used as a template for the VL framework regions, and the human Cor and Cess sequences were used for VH FR1 and FR2 to FR4, respectively [61,78]. Complementarity-determining regions (CDRs) were defined using the coordinates of the known crystal structure of the MCPC603 antibody. The DNA sequences of the relevant CDRs were obtained from murine mAb1129 B cells and introduced into the DNA encoding the variable (Fab) and constant (Fc) regions of a human IgG1 molecule. With the exception of four amino acids in the VL CDR1, all CDRs were left as in mAb1129 (see Fig. 12.3). VL amino acid residues 24 through 27 (KCQL in the murine VL CDR1, underlined in Fig. 12.3) were substituted by the sequence SASS (neither murine nor human), due to a synthesis error during the humanization process [85].

Amino acids A105 in VH FR4 and L104 in VL FR4 (bold in Fig. 12.3) were not humanized, assuming (based on the coordinates of the MCPC603 antibody) that this would help maintain the structural integrity of the binding site [41]. Recently performed affinity maturation studies of palivizumab demonstrated that these two murine amino acid residues did not contribute significantly to the antibody's affinity, and they were humanized in a second-generation antibody to yield fully human germline sequences, thereby minimizing the potential immunogenicity of the antibody [85]. A model of palivizumab Fab indicating amino acid positions that affect the rate constant of association and the rate constant of dissociation (K_{on} and K_{off}) is shown in Fig. 12.4.

The prototype humanized mAb (MEDI-493, palivizumab) was initially expressed transiently in COS-1 cells (African green monkey kidney cells) using

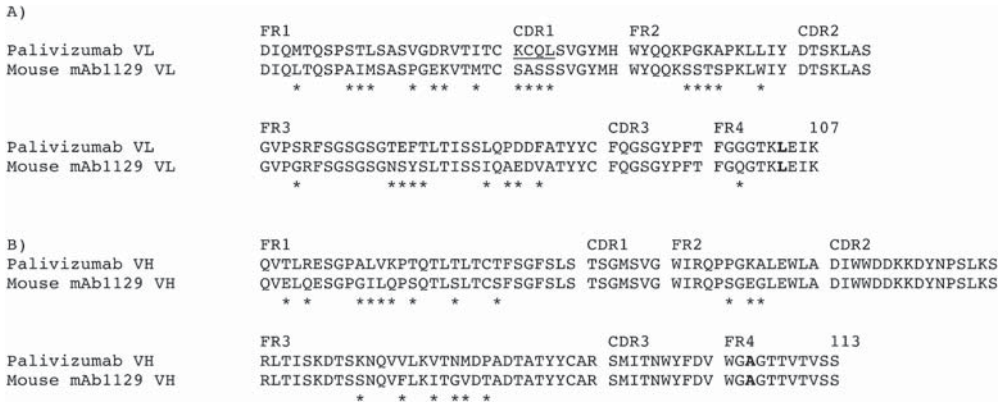

Fig. 12.3 Sequence comparison between palivizumab and mouse mAb1129 variable regions. Framework regions (FR) were humanized as described in the text; substitutions are indicated by asterisks. The human K102 sequence was used for VL (A), and the Cor and CESS sequences were used for VH FR1 and FR2-4 (B). Complementarity-determining regions (CDRs) were conserved as in mAb1129, with the exception of CDR1 which contains four random substitutions that are neither murine nor human (underlined). Amino acids L104 (VL FR4, in bold) and A105 (VH FR4, in bold) were not humanized to stabilize the binding site. (Modified from Ref. [41].)

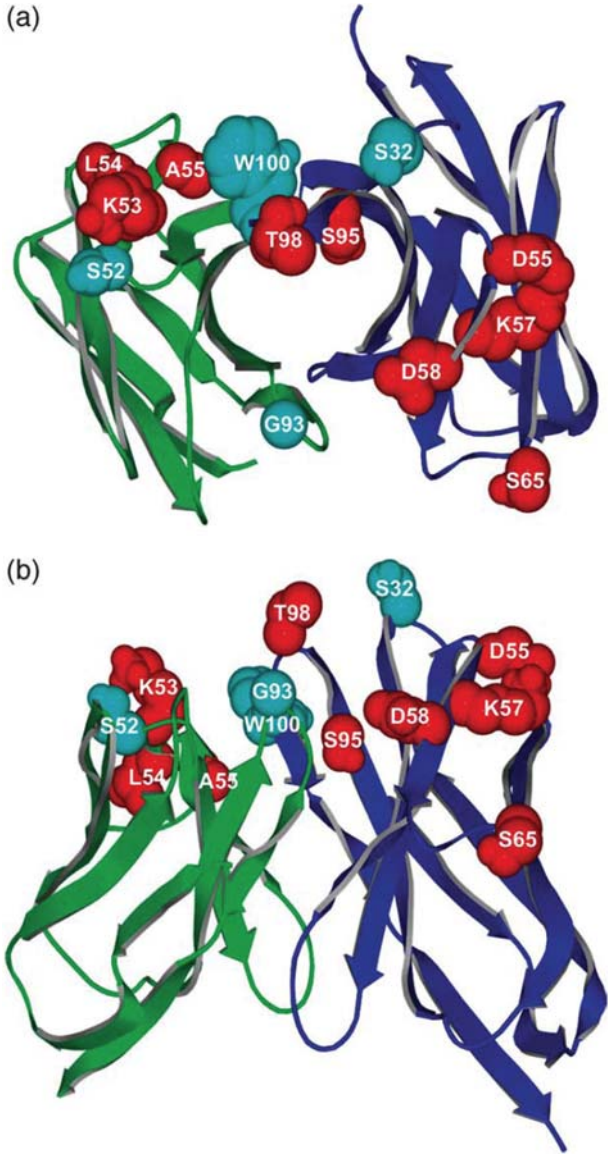

Fig. 12.4 Model of the palivizumab V region with amino acid positions that enhance antibody binding to (K_{on} positions, in red) or decrease antibody dissociation from (K_{off} positions, in blue) the RSV F protein. The heavy chain is depicted in blue, the light chain in green. It should be noted that all four positions that yielded improvements in K_{off} could also be mutated to improve K_{on}. (a) Top view (THE antigen-binding region is facing the reader). (b) Side view. The coordinates of the crystal structure of the palivizumab Fab were provided by Dr. B.C. Braden, Department of Natural Sciences, Bowie State University, Bowie, MD 20715, USA. (Reprinted with permission from Ref. [85].)

a cytomegalovirus (CMV) immediate-early promoter. Stable mouse myeloma cell lines (NSO) expressing MEDI-493 were then generated as described [10].

12.3 Mechanism of Action and Preclinical Results

Palivizumab neutralizes RSV infectivity by binding to the ectodomain of the fusion glycoprotein of RSV subgroups A and B. It also inhibits cell-to-cell fusion of RSV-infected cells. Preclinical studies of palivizumab were conducted to determine the kinetic parameters of antibody binding and to elucidate the mechanism of action. Binding properties were examined using the Biacore real-time biosensor system. Immunoaffinity-purified F protein from RSV strain A2 was used to coat the Biacore chip, and binding and dissociation were measured at antibody concentrations ranging from 12 to 400 nM. This analysis determined the rate constant of association (K_{on}) at $3 \times 10^5\,M^{-1}s^{-1}$ and the dissociation constant (K_{off}) at $4.3 \times 10^{-4}\,s^{-1}$ [41].

Three different biological assays were used to evaluate the *in-vitro* neutralizing activity of palivizumab [41]. First, in a classical plaque-reduction assay, serial dilutions of palivizumab were preincubated with 50 plaque-forming units of RSV A strain Long and RSV B strain 18537, and then placed onto HEp-2 monolayers for 1 h to allow infection of the cells. The virus–antibody suspension was then removed from the cells and cells were overlaid with methyl cellulose to prevent release of the virus into a liquid medium and spread via the liquid phase to other cells. After incubation for 5 to 6 days, the virus-induced plaques were enumerated. In this assay the effective concentration 50 (EC_{50}) – that is, 50% plaque reduction – for palivizumab was determined to be approximately $2\,\mu g\,mL^{-1}$.

In two additional *in-vitro* assays, RSV replication was quantified by measuring the amount of RSV F protein expression in 96-well plates using an RSV F protein-specific enzyme-linked immunosorbent assay (ELISA). RSV was either preincubated with antibody and HEp-2 cells were then added (microneutralization assay), or monolayers were infected first and antibody was added 4 h later, after removing the virus suspension (fusion-inhibition assay). The EC_{50} for palivizumab was calculated as $0.1\,\mu g\,mL^{-1}$ for the microneutralization assay, and at $0.2\,\mu g\,mL^{-1}$ for the fusion-inhibition assay, demonstrating that the monoclonal antibody preparation was approximately 20- to 30-fold more potent than the polyclonal preparation RSV-IVIG with regard to microneutralization and fusion-inhibition [41]. In this initial study, palivizumab was also shown to neutralize a wide spectrum of clinical isolates (34 subgroup A and 23 subgroup B isolates), although high antibody concentrations were used and EC_{50} values were not reported [41].

The *in-vivo* potency was determined in cotton rats – the same animal model that had been used previously for the evaluation of polyclonal antisera against RSV [62]. Palivizumab was administered intramuscularly (IM) or intravenously (IV) one day prior to intranasal inoculation of 10^5 plaque-forming units (PFU) of RSV subgroup A strain Long or subgroup B strain 18537, and the virus titer in

Table 12.1 Replication of RSV Long in the lungs of cotton rats following palivizumab prophylaxis (intravenous dosing).

Palivizumab dose[a] *[mg kg^{-1}]*	*N*[b]	*Serum concentration [μg mL^{-1}]*[c]	*Mean (±SE) RSV titer [log$_{10}$ PFU g^{-1} tissue]*[d]	*Mean reduction of RSV titer [log$_{10}$ PFU g^{-1} tissue]*
0	18	0	5.1 ± 0.1	0
0.312	7	2.7 ± 0.6	4.7 ± 0.1	0.4
0.625	17	5.3 ± 0.3	4.4 ± 0.1	0.7
1.25	18	10.1 ± 0.3	3.5 ± 0.2	1.6
2.5	17	28.6 ± 2.2	3.0 ± 0.2	2.1
5.0	15	55.6 ± 3.4	2.1 ± 0.1	3.0
10.0	18	117.6 ± 5.1	<2.0	>3.1

a Palivizumab was given an day −1 (i.e., 1 day before RSV challenge with 10^5 PFU intranasally).
b Data from three separate experiments were combined.
c Palivizumab serum concentration on Day 0 (i.e., the day of RSV challenge).
d Plaque-forming units of RSV g^{-1} homogenized lung tissue on Day 4 post challenge.
Modified from Ref. [41].

lung tissue was determined at 4 days after infection. For either virus, a 2-log$_{10}$ (i.e., 99%) reduction in virus titer was achieved using 2.5 mg kg^{-1} palivizumab IV or IM, corresponding to a serum concentration of approximately 30 μg mL^{-1} at the time of RSV challenge. Data for RSV Long are shown in Table 12.1 [41].

In addition to the above potency studies, a number of safety studies were conducted in mice to exclude the possibility that palivizumab might enhance viral replication or exacerbate RSV-induced lung pathology at noninhibitory antibody concentrations after primary or secondary RSV challenge. These safety concerns date back to the observation that children vaccinated with a formalin-inactivated experimental RSV vaccine during the 1960s experienced enhanced RSV disease with their first wildtype RSV infection after vaccination. One hypothesis at the time was that maternal antibodies might have been involved in enhanced disease, though it now appears that a lack of neutralizing antibodies and a Th2-biased T-cell response might have caused the increased severity of RSV disease [49]. With palivizumab, neither an increase in viral replication nor exacerbation of RSV-induced histopathology was observed after primary or secondary challenge with RSV [41].

12.4 Production, Downstream Processing, and Galenics of the Antibody

12.4.1 Production

Palivizumab is produced in a stable cell line that was developed from the NSO murine myeloma cell line. Twenty-one candidate production cell lines were

screened for growth rate and rate of antibody secretion, and a suitable cell line was selected for biological cloning and generation of an accession cell bank and a master cell bank (MCB) [63]. The MCB was extensively characterized and shown to be sterile and free of adventitious agents. A working cell bank (WCB) was generated from the MCB and shown to be safe, sterile, free of adventitious agents, and comparable to the MCB with regard to DNA profile, etc.

A stirred-tank, fed-batch system is used for the production of palivizumab from the WCB. Production is initiated by thawing a frozen vial of the WCB and expanding cells in T-flasks and in bioreactors of increasing size. When the production capacity is reached, a production bioreactor (≥10 000 L capacity) is filled, and after 18 to 22 days of incubation the bioreactor content is harvested. The initial antibody yield was reported at approximately $1\,g\,L^{-1}$ of medium, but an enhanced yield process that was approved by the FDA in 2001 increased the palivizumab yield to over $3\,g\,L^{-1}$ of medium.

12.4.2 Downstream Processing

Downstream processing includes microfiltration to remove cells and debris, and an antibody purification process which involves three-stage chromatography, acid treatment, and nanofiltration. This purification process leads to clearance of potential (viral) contaminants, and in-process validation is performed to guarantee that manufacturing is safe and results in a product of consistent quality. The bulk material is sterile-filtered once again prior to filling of the final vials.

The initial FDA approval (June 19, 1998) was granted for palivizumab manufactured by Medimmune at their Gaithersburg, Maryland, pilot facility. Supplemental approval (September 8, 1999) was then granted by the FDA for large-scale manufacture at Boehringer Ingelheim (Biberach, Germany) under contract to Medimmune, and an additional supplemental approval for Medimmune's new facility in Frederick, Maryland was granted on December 17, 1999 [63].

12.4.3 Formulations

Palivizumab (Synagis) is available in two formulations: a lyophilized powder and a liquid solution. Both formulations are for IM injection only. The newer liquid formulation was approved by the FDA in July 2004, and currently both formulations are available. It is expected that the liquid formulation will replace the lyophilized formulation as it is easier to use (reconstitution of the lyophilized powder with sterile water for IM injection takes ca. 20 min). Each 100 mg single-use vial of the lyophilized powder is formulated in 67.5 mg of mannitol, 8.7 mg histidine and 0.3 mg of glycine, and is designed to deliver 100 mg of Synagis in 1.0 mL when reconstituted with 1.0 mL of sterile water for injection. Vials containing 50 mg are also available.

The liquid solution (100 mg mL^{-1}) is supplied as a sterile, preservative-free solution which should appear clear or slightly opalescent, with a pH of 6.0. Each 100-mg single-use vial of Synagis liquid solution is formulated in 4.7 mg of histidine and 0.1 mg glycine in a volume of 1.2 mL, and is designed to deliver 100 mg of Synagis in 1.0 mL. Vials containing 50 mg are also available. Notably, the liquid formulation contains no mannitol and has an osmolality of 52 mosm kg^{-1} compared to 462 mosm kg^{-1} for the reconstituted lyophilized powder.

12.4.4
Specifications

Following general FDA suggestions regarding the use of pre-approved comparability protocols to reduce reporting requirements for changes in the manufacturing process, Medimmune conducted a comparability assessment of palivizumab lots manufactured under varying conditions. The aim of these studies was to examine whether posttranslational modifications of the antibody (deamination, oxidation or oligosaccharide variation) occurred in the manufacture of palivizumab and could potentially lead to altered mAb binding or clearance [68].

Specification ranges to be used for comparabillity testing were generated using data from biochemical and functional analyses of over 25 preclinical, clinical, and manufacturing lots. Materials manufactured for phase I through phase III clinical trials in bioreactors of increasing size (20 L, 45 L, 100 L, and 200 L) and three manufacturing consistency lots made at the same facility in 500-L bioreactors were tested with regard to binding activity, purity and microheterogeneity using methods such as F-protein-specific ELISA, size-exclusion chromatography, monosaccharide composition, oligosaccharide profile and MALDI-TOF. SDS-PAGE, isoelectric focusing, capillary electrophoresis and Western blotting results of the individual lots were also performed, and yielded comparable results. The combined data from 20-L to 500-L lots was used for product registration with the FDA. Once manufacturing was initiated at a second site, lots manufactured during the scale-up process at that site were again tested in parallel with the reference standard – that is, the material made in a 200-L bioreactor for the phase III clinical trial that led to licensure. The results of these scale-up comparability tests are shown in Table 12.2.

Cell line stability studies were conducted using palivizumab purified from cells expanded from the WCB, cells similar to the extended cell bank, and from cells that were expanded well past the extended cell bank. With the exception of the *N*-acetylglucosamine composition in material from cells expanded past the extended cell bank, all tested parameters were within the specification range, and tryptic peptide maps were consistent for all lots [68]. Studies examining the effect of time of harvest (typically 18 to 22 days after inoculation of the production bioreactor) showed that material harvested on Day 11 was comparable to that harvested on Day 18, but an early harvest on Day five resulted in an altered

Table 12.2 Comparability test results for scale-up lots of palivizumab.

Test		*Specification*	*Lot 5 (500 L)*	*Test results for three lots (bioreactor size) Lot 7 (2,000 L)*	*Lot 10 (10,000 L)*
F protein binding activity (ELISA)		75–128% of R.S.[a]	96	89	93
Size Exclusion Chromatography		1 peak >99% AUC[b]	100.0	99.9	99.8
Monosaccharide composition (%)	GlcNAc[c]	40–60	55.6	50.8	44.8
	Mannose	20–45	30.5	33.3	37.2
	Fucose	5–15	7.8	10.1	10.6
	Galactose	2–12	6.3	5.8	7.6
Oligosaccharide profile	Peak 5 to 6	0.4–1.0	0.6	0.5	0.7
	Peak 5 to 7	1.5–4.0	2.3	1.5	2.7
Mass by MALDI-TOF[d] (Da)		147,700 ± 1,000	147,178	147,178	147,470

a R.S.: reference standard.
b AUC: area under the curve.
c GlcNAc: *N*-acetylglucosamine.
d MALDI-TOF: Matrix-assisted laser desorption/ionization-time of flight mass spectrometry.
Modified from Ref. [69].

oligosaccharide profile of the material [68]. Although media glucose concentrations below the process specification ($1.2\,g\,L^{-1}$) had an impact on cell viability and product yield, there was no discernable effect on product quality [68].

12.5 Summary of Results from Clinical Studies

12.5.1 Phase III Trials

Two pivotal phase III trials were conducted to examine the safety and efficacy of palivizumab in reducing the incidence of hospitalization due to RSV disease. The first trial enrolled premature children with or without BPD/CLD, and led to the approval of palivizumab by the FDA in 1998. The second phase III trial enrolled children aged under 2 years with significant CHD, and extended the indication for prophylaxis to this high-risk group in 2003. An overview of these two trials, which are described in more detail below, is provided in Table 12.3.

12.5.1.1 Palivizumab in Premature Infants and Children with BPD

The IMpact-RSV trial evaluated the safety and efficacy of prophylaxis with palivizumab in reducing the incidence of hospitalization due to RSV disease in high-risk infants and children [2]. The trial enrolled 1502 children with prematurity and/or BPD to receive five doses of palivizumab ($15\,mg\,kg^{-1}$) or placebo during

Table 12.3 Phase III trials of palivizumab.

Trial (Reference)	*Season*	*Study participants (n)*		*RSV hospitalization rate [%]*		*Reduction of RSV hospitalization [%]*	
		Placebo	*Palivizumab*	*Placebo*	*Palivizumab*	*Absolute*	*Relative*
IMpact-RSV [2]	1996–1997	500	1002	10.6	4.8	5.8	55
CHD Trial [29]	1998–2002	648	639	9.7	5.3	4.4	45

the 1996–1997 RSV season. The trial was designed as a double-blind multicenter trial with a 2:1 randomization. A total of 139 centers in the USA, the UK, and Canada participated, and enrolled children born at ≤35 weeks' gestation and aged less than 6 months, in addition to children aged ≤24 months with BPD that required treatment within 6 months prior to enrollment. Exclusion criteria included clinically significant CHD, active or recent RSV infection, hospitalization at the time of entry that was anticipated to last more than 30 days, mechanical ventilation at the time of entry, life expectancy less than 6 months, known hepatic or renal dysfunction, seizure disorder, immunodeficiency, allergy to IgG products, and receipt of RSV-IVIG within the past 3 months or previous receipt of palivizumab, other mAbs, RSV vaccines, or other investigational agents. Patients were followed until 30 days after the last scheduled injection, for a total of 150 days. All hospitalizations were identified and children with respiratory hospitalizations were tested for RSV antigen using commercially available tests.

The primary endpoint was defined as: (i) hospitalization for a respiratory illness and positive RSV antigen test of respiratory secretions; or (ii) hospitalization for any cause, a positive RSV test, and a minimum LRTI score of 3 that was at least one point higher than that recorded at the child's last pre-illness visit. LRTI was scored as follows: 0 = no respiratory illness; 1 = URTI; 2 = mild LRI; 3 = moderate LRI; 4 = severe LRI; and 5 = mechanical ventilation. Adverse events (AEs) were reported throughout the trial, and the potential relationship to the study drug was assessed. AE severity was assessed using a toxicity table modified from the pediatric AIDS vaccine trials group toxicity table. The two treatment groups were demographically similar, and compliance was excellent, with 99% of participants receiving all five doses and 93% of study participants completing the study. RSV-related hospitalization occurred in 10.6% of placebo recipients versus 4.8% of palivizumab recipients; that is, monthly prophylaxis with palivizumab was associated with a 5.8% absolute reduction or a 55% (95% CI 38–72%) relative reduction in the primary endpoint ($p = 0.00004$) (Table 12.3). Logistic regression analysis indicated that gestational age (GA) was not a significant predictor of RSV hospitalization, and that the palivizumab effect remained statistically significant in children born at 32 to 35 weeks of gestation [2].

Amongst the secondary efficacy endpoints, a significant reduction in total days in hospital per 100 children enrolled in the trial (63 versus 36 days), in total days with increased oxygen (51 versus 30 days), and in total days with an LRI score ≥3 (47 versus 30 days), were reported. These secondary endpoints are confounded, however, in that the total number of study participants in each arm was used as denominator, and therefore the reduction in RSV hospitalization rate resulted in a reduction in total days in hospital. The mean length of stay for those patients actually hospitalized was similar in the two study groups (7.6 days versus 5.9 days for palivizumab versus placebo). RSV disease was as severe among RSV hospitalized patients who received palivizumab as it was in those who received placebo. Mean days with increased oxygen and mean days with LRI score ≥3 were also similar in the two groups. The incidence of all hospital admissions (of any cause) and of all respiratory hospital admissions (but not of non-RSV hospital admissions) was significantly reduced in the palivizumab group, thus confirming the important role that RSV disease plays in this high-risk group. Otitis media occurred at a similar frequency in the two groups.

Adverse events that were judged to be related to the study drug occurred in 11% of the palivizumab recipients, and in 10% of those receiving placebo. In addition, 2.7% and 1.8% of the children in the palivizumab and placebo groups, respectively, experienced AEs related to the injection site. Only in 0.3% of palivizumab recipients were the injections discontinued due to local AEs. As with systemic AEs, the rate of aspartate aminotransferase (AST) elevation was slightly higher in the palivizumab group (3.6% versus 1.6%), but alanine aminotransferase (ALT) elevations were not associated, and overall hepatic AEs related to the study drug occurred at similar frequencies. Five children (1.0%) in the placebo group and four (0.4%) in the palivizumab group died during the trial; none of the deaths was considered to be related to palivizumab, although the study was not powered to detect inter-group differences in mortality.

12.5.1.2 **Palivizumab in Children with Significant CHD**

The second randomized, double-blind, placebo-controlled multicenter phase III trial was conducted to evaluate the safety, tolerance, and efficacy of palivizumab in children with hemodynamically significant CHD [29]. This trial enrolled 1287 children with CHD in four consecutive seasons (1998–2002), and randomly assigned them 1 : 1 to receive five monthly intramuscular injections of 15 mg kg^{-1} palivizumab, or placebo. Among the study participants, 72% were recruited in the USA and Canada, and 28% in France, Germany, Poland, Sweden, and the UK.

Children were eligible if they were aged less than 24 months at the time of randomization, and had documented hemodynamically significant unoperated or only partially corrected CHD, as determined by the investigator. In general, patients with cyanotic CHD, with single ventricle physiology, and those with acyanotic CHD that required medical therapy, were considered to have significant CHD. Children were not eligible if they had unstable cardiac or respiratory status, including cardiac defects so severe that survival was not expected, or for which

cardiac transplantation was planned or anticipated. Additional exclusion criteria were, amongst others, noncardiac anomalies or end-organ dysfunction with anticipated survival less than 6 months, or current RSV, HIV or other acute infection [29]. Children were followed for 150 days – that is, until 30 days after the last dose of palivizumab.

All hospitalizations were identified, and RSV antigen testing of respiratory secretions was performed in all children with an acute cardiorespiratory hospitalization. The primary endpoint of the trial was the incidence of RSV hospitalization, including primary RSV hospitalizations and nosocomial RSV hospitalizations. Primary RSV hospitalization was defined as hospitalization for an acute cardiorespiratory illness in which the RSV antigen test was positive within 48 h before or after admission.

The types of cardiac lesions were similar in the two treatment groups. Within the cyanotic stratum (53% of all patients), single ventricle (hypoplastic left or right heart) and tetralogy of Fallot accounted for 21.9% and 11.4%, respectively. In the acyanotic stratum, ventricular septal defect and atrioventricular septal defect accounted for 18.0% and 7.2%, respectively, of all patients enrolled. This distribution of cardiac lesions in the study population indicates that patients with single ventricle hemodynamics – that is, those with the most severe CHD – were over-represented in this study population.

Compliance was excellent in this trial, and more than 95% of all patients completed the study. In total, 63 of 648 placebo recipients (9.7%) and 34 of 639 palivizumab recipients (5.3%) met the primary endpoint RSV hospitalization – that is, monthly palivizumab administration was associated with an absolute reduction in the RSV hospitalization rate of 4.4%, or a relative reduction by 45%. With regard to secondary endpoints, a significant reduction in the total days in hospital with RSV hospitalization (from 129 to 57 days per 100 children; 56% relative reduction) and a significant reduction in the RSV hospitalization days with increased supplemental oxygen (from 102 to 28 days per 100 children; 73% relative reduction) were reported. Again, these secondary endpoints are confounded by the reduction in RSV hospitalization rate and the use of all study children in each arm as denominator. The mean duration of RSV hospitalization was 13.3 versus 10.8 days, and the mean duration of supplemental oxygen requirement 10.4 versus 5.2 days in the placebo and palivizumab groups, respectively. Intensive care unit (ICU) admission rate, days of ICU stay, mechanical ventilation rate, and days of mechanical ventilation were all lower in the palivizumab group, but did not reach statistical significance. Heart surgery with cardiopulmonary bypass led to a 58% decrease in the serum palivizumab concentration (through dilution and bleeding), indicating that re-dosing is necessary after cardiopulmonary bypass [29].

Both phase III trials showed unequivocally that palivizumab is efficacious in reducing hospitalization for RSV disease by approximately 50% in children at high risk for severe RSV disease, including infants born at ≤35 weeks' gestation, children aged under 2 years with BPD, and children with significant CHD. Efficacy was demonstrated in infants born at <32 weeks' gestation and in infants

born at 32 to 35 weeks' gestation. Although the CHD study was not powered to permit subgroup analyses, a reduction in RSV hospitalization was observed in both the cyanotic and noncyanotic populations.

12.6 Indications and Usage

When the results of the palivizumab CHD trial had been reviewed by the FDA, the package insert for palivizumab (Syangis) was modified to include the following indications and usage instructions: "Synagis is indicated for the prevention of serious lower respiratory tract disease caused by respiratory syncytial virus (RSV) in pediatric patients at high risk of RSV disease. Safety and efficacy were established in infants with bronchopulmonary dysplasia (BPD), infants with a history of premature birth (≤35 weeks gestational age), and children with hemodynamically significant congenital heart disease (CHD)."

The recommended dose of Synagis is 15 mg kg^{-1} body weight. Patients, including those who develop an RSV infection, should continue to receive monthly doses throughout the RSV season. The first dose should be administered prior to commencement of the RSV season, and then at monthly intervals throughout the RSV season. Synagis should be administered intramuscularly using aseptic technique, preferably in the anterolateral aspect of the thigh. The gluteal muscle should not be used routinely as an injection site because of the risk of damage to the sciatic nerve. Injection volumes in excess of 1 mL should be given as a divided dose.

12.7 Clinical Reports after Approval

Palivizumab received initial approved for the prevention of RSV disease in high-risk infants in 1998 in the US, followed by approval in the European Union and in Japan in 1999 and 2002, respectively. It is now approved in over 50 countries. Following FDA approval, a considerable number of clinical studies and surveys examined the efficacy of palivizumab prophylaxis in study settings, as well as its effectiveness in field use, mostly in terms of hospitalization rates in infants receiving palivizumab. The majority of these studies were observational in nature and without concurrent controls instead, the majority of these studies compared the hospitalization rate in their palivizumab-treated population to that in a previous study, most commonly to that observed in the phase III IMpact-RSV study. As Heikkinen et al. pointed out [37], such comparisons between studies are often problematic because of differences in demographics and study design. Of particular interest in this regard is the proportion of children with BPD/CLD, as these children are at much higher risk for severe disease and hospitalization than premature infants without CLD or children with CHD [14]. In several of the studies

mentioned below, subgroup analyses were performed to address this problem (see Table 12.4); moreover, meta-analyses of the hospitalization data by subgroup have been published elsewhere [59,72].

The definition of BPD/CLD (the terms are used synonymously in this chapter) and the type of disease described by the term BPD has changed considerably since its original description by Northway et al. in 1967 [52]. Antenatal steroid therapy, postnatal surfactant therapy and advances in ventilation techniques have helped to reduce the frequency of lung injury in less immature babies, and the disease is now infrequent in infants born at ≥30 weeks' gestation or 1200 g birth weight.

Table 12.4 RSV hospitalization rate in palivizumab recipients: phase III trial and post-approval observations.

Study (Reference)	*Design*	*Season*	*N*	*CLD [%]*	*Percent RSV hospitalization rate*			*Fraction of respiratory admissions tested for RSV (% positive)*
					Overal	*CLD only*	*No CLD only*	
Impact-RSV (phase III trial)	Prospective randomized controlled trial	1996/1997	1002	50	4.8	7.9	1.8	0.95 (30)
19	Telephone survey (USA)	1998/1999	7013	n.a.	1.5	n.a.	n.a.	n.a.
74	Retrospective chart review (USA)	1998/1999	1839	22	2.3	4.0	2.1	n.a.
33	Prospective, observational, single arm (Canada, Europe)	1998/1999	565	n.a.	2.1	n.a.	n.a.	0.57 (24)
46	Prospective, observational, single arm (France)	1999/2000	516	81	7.6	9.0	3.0	0.90 (46)
55	Prospective, observational, single arm (Canada)	1999/2000	444	23	2.4	6.0	1.6	0.84 (43)
56	Prospective, observational, single arm (USA)	2000/2001	2116	24	2.9	5.8	2.1	n.a.
58	Prospective, observational, single arm (Spain)	2000/2001 2001/2002	1919	11	4.0	5.6	3.2	n.a.
50	Prospective, observational, population-based (Sweden)	2000/2001 2001/2002	390	52	4.1	n.a.	n.a.	n.a.

n.a. = not available.

A BPD workshop in 1978 defined BPD as an oxygen requirement for the first 28 days of life with radiographic changes (of the lungs), and a decade later oxygen requirement at 36 weeks' gestation (postmenstrual age) was suggested as a better predictor of long-term respiratory outcomes and became a widely used definition of BPD [70]. It is clear that for very premature infants (e.g., ≤28 weeks' gestation) the definition of BPD using 36 weeks of gestation describes a much smaller population of infants with more severe lung disease than the 28 days of age definition. A BPD workshop in 2000 suggested new definitions for mild, moderate and severe BPD [40], but this classification is not yet universally used. Many centers still use oxygen requirement at 36 weeks' gestation or at 28 days of postnatal age as BPD/CLD definitions, although often the definition is not specified in palivizumab publications.

In addition to difficulties with study population demographics and varying BPD definitions, the estimation of RSV hospitalization rates in the absence of palivizumab prophylaxis can be difficult. A concurrent control group is usually unavailable as a majority of high-risk infants in high-income countries receive palivizumab, and local pretreatment surveillance data for the baseline incidence of RSV hospitalization often do not exist. This makes calculation of the attributable risk reduction for RSV-related hospitalization impossible, and as a result cost-effectiveness analyses often rely on vague assumptions. Baseline RSV hospitalization rates in the absence of palivizumab prophylaxis can differ greatly between different locations, and for many regions they are not known [25,37]. A Finnish retrospective cohort study of children born between 1991 and 2000 determined that the RSV hospitalization rate in their population of nonprophylaxis children with CLD was 12%, while for all premature infants it was 4.9%. Of 586 children who would have met the criteria for palivizumab administration, only 4.6% were hospitalized for RSV disease during the RSV season [37], compared to 10.6% in the IMpact-RSV study. As almost all hospitalized children in this study were tested for RSV on admission, this difference in hospitalization rates seems to reflect a real difference between countries, and is not likely due to under-reporting. Similarly, hospitalization rates for children with clinically significant CHD are reported to differ greatly in different settings, and a lively debate continues as to the attributable risk reduction of palivizumab prophylaxis [30].

On the other hand, the incidence of RSV LRTI and severe RSV LRTI can be similar in very different climates and different countries. A recent population-based study, for example, found almost identical RSV-attributable incidence rates for severe LRI in children aged under 1 year (15–16 per 1000 child-years) in Mozambique, South Africa and Indonesia, although the incidence of all LRTI varied more than 10-fold between these countries [64].

As palivizumab is an expensive drug and administration is invasive, there is an ongoing debate with regard to who should and who should not receive palivizumab prophylaxis. Approximately 10% of all newborns in developed countries are born prematurely (i.e., at <37 weeks' gestational age), and approximately 1% of all newborn babies are born with very low birth weight (VLBW; i.e. <1500 g, and usually <32 weeks' gestation). The prevalence of CHD in infants is approxi-

mately 0.7 to 1%, and no more than half would qualify as significant CHD. Although the efficacy of palivizumab in reducing RSV hospitalization was found to be similar in infants born at a GA <32 weeks and in those born at a GA of 32 to 35 weeks [2], the inclusion of the more mature group would multiply the cost of palivizumab prophylaxis. On the other hand, several studies have suggested that infants born at a GA of 32 to 35 weeks experience considerable morbidity with, and also subsequent to, RSV hospitalization [17,67,83].

The American Academy of Pediatrics recommends palivizumab prophylaxis for the following high-risk groups [9]:

1. Infants and children aged less than 2 years with CLD who have required medical therapy (supplemental oxygen, bronchodilator, diuretic or corticosteroid therapy) for CLD within 6 months before the anticipated start of the RSV season.
2. Infants born at 28 weeks' gestation or earlier may benefit from prophylaxis during their first RSV season, whenever that occurs during the first 12 months of life.
3. Infants born at 29 to 32 weeks' gestation may benefit most from prophylaxis up to 6 months of age.
4. Infants born between 32 and 35 weeks' of gestation, only if two additional risk factors (child care attendance, school-aged siblings, exposure to environmental air pollutants, congenital abnormalities of the airways, or severe neuromuscular disease) are present.
5. Children who are aged 24 months or younger with hemodynamically significant CHD.

Professional societies in a number of countries have published their own guidelines [1,3,5,6,18,31,32,79], but many countries include the above-mentioned groups 1, 2 and 5 which, taken together, represent less than 2% of a country's birth cohort [15]. A considerable number of reports describing the local, regional or national experience with palivizumab use after the initial FDA approval have been published. Not all can be discussed in this overview, but a selection with preference for larger and/or prospective studies are summarized here.

In the USA, an RSV Education and Compliance Helpline (REACH) program was implemented as part of a pharmacovigilance program to enhance parent education and compliance (see Table 12.4) [19,34]. Adverse event reports were collected via bimonthly telephone contacts with the parents of palivizumab recipients. The hospitalization rate for palivizumab recipients in REACH was reported as 1.5%, compared to 4.8% in the IMpact-RSV trial. A total of 2.8% of the parents reported SAEs [34]. An additional report on the REACH program determined the SAE rate among 19 958 children receiving palivizumab to be 2.5%, and the death report rate to be 3.4 per 1000, compared to a mortality rate of 4 per 1000 in the IMpact-RSV trial [22]. Except for very rare (<1 : 100 000) reports of anaphylaxis,

no new SAEs were reported in the REACH and Outcome Registry pharmacovigilance programs [22].

Nine centers in the US retrospectively reviewed the RSV hospitalization rate of their population of 1839 patients that had received palivizumab in the first season after licensure (i.e., 1998–1999) [74]. Patients included in the study had a gestational age of ≤35 weeks, were aged less than 2 years at their first injection, and had received at least one dose of palivizumab. The RSV hospitalization rate for the total population of infants receiving palivizumab was 2.3% (42 of 1839) compared to 4.8% in the IMpact-RSV trial (see Table 12.4; [74]). It should be noted, however, that this study population had less chronic lung disease than the cohort studied in the IMpact-RSV population (i.e., 22% versus 50%, respectively). The hospitalization rate for infants with CLD was 4.0% (compared to 7.9% in the IMpact-RSV) and 2.1% for infants born at ≤35 weeks' gestation without chronic lung disease (compared to 1.8% in the IMpact-RSV).

For the same season (1998–1999), a phase III/IV multicenter, single-arm, open-label study was conducted in Canada and in a number of European countries [33]. A total of 565 preterm children aged <6 months at enrollment and born at ≤35 weeks of gestation, and children with BPD aged ≤24 months at enrollment who required medical intervention during the 6-month period prior to recruitment, were enrolled and followed for 150 days for AEs and hospitalization. RSV testing was not required in this study. In total, 94% of the study participants completed the study, 14 (2.5%) discontinued for personal reasons, 1% was lost to follow-up, and 2% (11 reports) discontinued because of AEs. Among these latter 11 reports of AEs, three were considered to be possibly or probably related to palivizumab: one patient developed oxygen desaturation immediately after the third injection; another patient developed gastroenteritis; and a third patient developed abdominal and peripheral edema. Likewise, 39 patients (6.9%) reported 40 AEs that were considered related to drug administration. No SAEs were considered related to palivizumab. Two patients died from causes not related to palivizumab or RSV. A total of 51 respiratory hospitalizations was recorded, but only 29 (57%) were tested for RSV, of which seven (24%) tested positive. If it were to be assumed that 24% of all respiratory hospitalizations were due to RSV, this would translate to a 2.1% RSV hospitalization rate (see Table 12.4) [33], but this figure should be treated with caution as only half the patients admitted were tested.

A similar observational field survey (not blinded, no control group) was conducted in France in 1998/1999, prior to the approval of palivizumab in Europe [46]. A total of 516 preterm infants with a median gestational age of 28 weeks (88% born at ≤32 weeks' gestation) and a very high BPD rate of 81% (at 28 days of age) were followed (see Table 12.4) [46]. Thirty-nine infants (7.6%) were hospitalized for RSV disease, 10 required intensive care treatment, and four mechanical ventilation [46].

In the following RSV season (1999/2000), a prospective observational multicenter study (COMPOSS study) was conducted in Canada to evaluate compliance and outcomes in 444 infants born at ≤32 weeks of gestation (see Table 12.4) [55]. The study participants were aged <6 months at the onset of the RSV season or

aged <2 years and had CLD requiring oxygen therapy within the past 6 months before the onset of the season. This study cohort represented 16% of the total population who received palivizumab in Canada during that season. Compliance was good, and 77% of all doses administered were given within 30 ± 5 days. Forty hospitalizations for respiratory events were observed, including 28 admissions for LRTI, two for URTI, seven for respiratory distress due to underlying lung disease, and three for croup. Twenty-five children were admitted 28 times for LRTI, and nine of the 21 tested for RSV were positive, resulting in a 2.4% RSV hospitalization rate. This hospital admission rate was only half that reported in the IMpact-RSV study, but again it should be considered that in the IMpact-RSV study 50% of the study participants had BPD, compared to 23% in the present study. The hospitalization rate for children with BPD receiving palivizumab was similar in the two studies (6% in COMPOSS, 7.9% in IMpact-RSV). Premature infants without BPD had a hospitalization rate of 1.6%, compared to 1.8% in IMpact-RSV. However, as this was an observational study, not all RTIs were recorded and not all not RTI hospital admissions were tested for RSV.

Data on RSV hospitalization rates during the 2000/2001 RSV season in the US were reported by the Palivizumab Outcomes Registry Study Group (see Table 12.4) [56]. A total of 2116 children who received palivizumab were enrolled at 63 sites, mostly in pediatric office settings. There were no exclusion criteria; 47% of the study participants were born at 32 weeks' gestation, 45% at 32 to 35 weeks, and 8% at >35 weeks. Some 24% of the study population had CLD, and 5% had CHD. Of these patients, 14% received palivizumab for a second season (54% of these had CLD and 9% CHD). The RSV hospitalization rate was 5.8% for children with CLD, 4.3% for those with CHD, and 2.1% for premature infants without CLD (Table 12.4). The overall RSV hospitalization rate was 2.9% for the 2116 children in the registry. Almost half of all hospitalizations (and 75% of all hospitalizations in subjects receiving all injections within 35-day intervals) occurred within the first and second injection intervals, suggesting that trough serum palivizumab concentration after the first and second doses might not have been protective in some of the infants.

A prospective multicenter study enrolling infants born at ≤32 weeks' gestation and aged <6 months at the start of the RSV season was conducted in Spain during the 2000/2001 and 2001/2002 RSV seasons to assess the RSV hospitalization rate in infants receiving palivizumab (see Table 12.4) [58]. In this cohort of 1919 infants (median gestational age at birth 29 weeks, mean birth weight 1261 g, CLD in 11%), the RSV hospitalization rate was determined as 4%. A historical cohort of infants who did not receive palivizumab was derived by combining the data from two previous studies that followed infants born at ≤32 weeks of gestation and aged <6 months during the 1998/1999 and 1999/2000 RSV seasons [16]. In this control cohort (median gestational age at birth 31 weeks, mean birth weight 1426 g, 5% CLD), the RSV hospitalization rate was determined as 13%. Although it is difficult in this type of historical comparison to control for differences in RSV season severity, induced parental behavior changes that might influence exposure to RSV, and changes in a country's hospital admission poli-

cies, the 70% reduction in RSV hospitalization suggests good efficacy of palivizumab in preventing RSV hospitalization. One important difference between the two cohorts that remained unexplained was that the number of deaths in the control cohort was four times as high (13/1000) as in the palivizumab cohort (3/1000) [58].

In Sweden, a population-based, nationwide prospective study of RSV hospitalization of preterm children was conducted in the 2000/2001 and 2001/2002 RSV seasons [50]. This study was unique in covering 85% of all preterm children aged <2 years in a single country. The study aim was to assess the appropriateness of Sweden's restrictive use of palivizumab. The Swedish recommendations limited the use of palivizumab to children aged <2 years with CLD (36 weeks' GA definition) requiring active treatment during the 6 months prior to the RSV season, and to infants with a gestational age of ≤26 weeks at the start of the RSV season. Over two seasons, the RSV hospitalization rate for all 5800 children born at ≤35 weeks GA (5410 [93%] without palivizumab prophylaxis, 390 receiving palivizumab) was 3.8% (n = 218) and 5.4% (n = 97) for children born at ≤32 weeks GA. In total, 390 children were treated with palivizumab according to the Swedish recommendations, and 16 (4.1%) of those were hospitalized for RSV (see Table 12.4) [50].

In Japan, Saji et al. conducted a retrospective survey of palivizumab use in children with CHD [66]. Surveys were mailed to 476 centers using palivizumab, and 61 centers reported 108 CHD patients that were given palivizumab. Complex CHD accounted for 40% of the population, ventricular septal defect for 21%, coarctation for 11%, atrial septal defect for 9%, and hypoplastic left heart syndrome for 4%. Five of 108 (4.6%) children were hospitalized for RSV disease.

In the USA, the 2003–2004 Outcomes Registry reported for 664 children with CHD receiving palivizumab that persistent ductus arteriosus (20%), ventricular septal defect (16%) and atrial septal defect (10%) were the most common CHD indications for palivizumab prophylaxis [20].

Singleton et al. [73] compared the rate of first RSV hospitalization in eligible premature Alaska Native infants born in the Yukon Kuskokwim region at ≤35 weeks' gestation before and after the introduction of palivizumab prophylaxis. In this region of southwest Alaska, acute respiratory tract infections (RTI) are a major cause of morbidity [42]. Typically, two-thirds of all hospitalizations in children aged <3 years are due to RTI, and RSV hospitalization rates are as high as 156 per 1000 infants aged <1 year (which is much higher than rates reported in other region of North America or Europe). The observed hospitalization rate for first-time RSV disease in children born at ≤35 weeks' gestation decreased from 18/41 (44%) premature infants born between 1993 and 1996 to 9/60 (15%) premature infants born between 1998 and 2001 – that is, after the introduction of palivizumab. Although it is difficult to ascertain the palivizumab eligibility of the cohort born in 1993 to 1996, the fact that RSV hospitalization remained unchanged in term infants indicated that the decline observed in high-risk children was likely due to prophylaxis and not to decreased RSV circulation or decreased RSV testing.

12.8
Is Protective Efficacy a Function of Palivizumab Serum Concentration?

Based on the assumption that severe RSV disease is a consequence of active high-titer RSV replication in the LRT, immunoprophylaxis that restricts viral replication 100- to 1000-fold should lead to abrogation, or least amelioration, of RSV-induced LRTI. Although this assumption is not universally accepted, there is good evidence to support it, not least from studies with live-attenuated vaccines that do not cause disease in seronegative infants if their level of replication is at least 100-fold attenuated compared to wildtype RSV [84]. In cotton rats, a serum palivizumab concentration of $30\,\mu g\,mL^{-1}$ reduced the RSV titer in the LRTI approximately 100-fold (by 10^2 $TCID_{50}\,g^{-1}$ lung tissue), while a concentration of $56\,\mu g\,mL^{-1}$ led to a 1000-fold reduction in RSV titer (see Table 12.1) [41].

For the preclinical and clinical development of palivizumab it seemed reasonable, therefore, to design an IM palivizumab dosing regimen that maintained trough concentrations of at least $30\,\mu g\,mL^{-1}$ and, as a margin of safety for person-to-person variability in palivizumab pharmacokinetics, ideally a trough concentration of greater than $40\,\mu g\,mL^{-1}$ [65].

A phase I/II trial in premature infants and children found the pharmacokinetics of palivizumab to be similar to those of other human IgG1 antibodies [65]. Mean serum concentrations were $91\,\mu g\,mL^{-1}$ (range: 52 to $174\,\mu g\,mL^{-1}$) at 2 days after the first IM dose of $15\,mg\,kg^{-1}$, and $49\,\mu g\,mL^{-1}$ (range: 14 to $132\,\mu g\,mL^{-1}$) at 30 days after IM injection (Fig. 12.5). The serum half-life was calculated to be approximately 24 days. The percentages of children with trough concentrations $>40\,\mu g\,mL^{-1}$ after each of five monthly IM injections were 66, 86, 91, 96, and 95% [65].

The IMpact-RSV trial determined mean (±SE) trough concentrations in the serum of 1396 infants with a mean age of 5.7 months and a mean body weight of 4.8 kg as to be $37 \pm 1.2\,\mu g\,mL^{-1}$ prior to dose 2, with concentrations increasing to 57 ± 2.3, 68 ± 2.9, and $72 \pm 1.7\,\mu g\,mL^{-1}$ at 30 days after doses 2, 3, and 4, respectively [2].

Wu et al. reported that, in their patient population of 24 infants born at ≤30 weeks of gestation with a mean (±SD) age of 31 ± 4 days and a mean body weight of 1293 ± 236 g at study entry, only 23% of those tested had palivizumab trough concentrations $>40\,\mu g\,mL^{-1}$ at 28 days after the first dose. The mean palivizumab concentration at that time point was $32 \pm 11\,\mu g\,mL^{-1}$, with a range of 16 to $56\,\mu g\,mL^{-1}$, indicating that a significant proportion of infants might not be optimally protected. Following the report of Wu et al., data at two teaching hospitals in Italy were reviewed retrospectively [47]. Of 389 infants at risk treated with palivizumab, nine experienced breakthrough RSV infections that led to hospitalization. All nine RSV cases occurred at least 19 days after the previous palivizumab dose – five after dose 2, and four after dose 3, coinciding with the local RSV epidemic peak [47].

Singleton et al. also reported the first-time RSV hospitalization rate for protected versus unprotected days in a cohort of 335 Alaska Native palivizumab

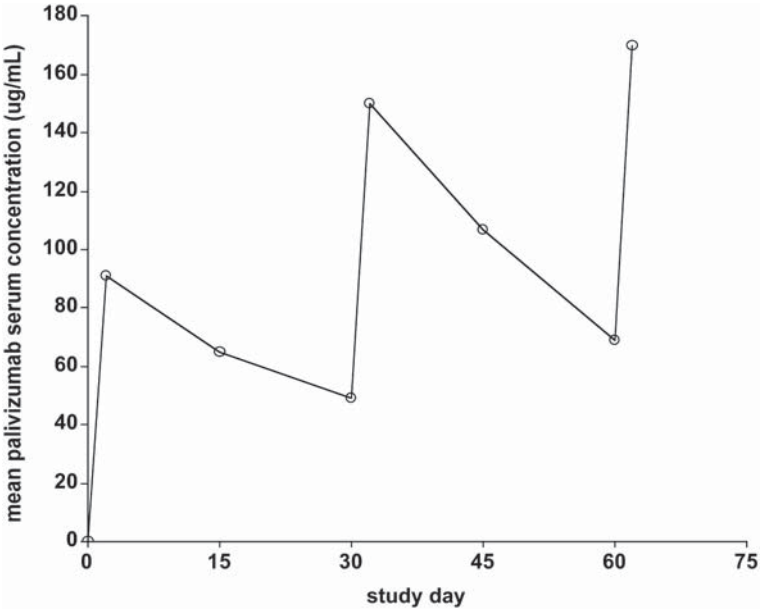

Fig. 12.5 Mean serum palivizumab concentrations in infants born at ≤35 weeks' gestation. Palivizumab (15 mg kg^{-1} body weight) was administered IM on Days 0 and 30, and then at 30-day intervals until Day 120. Data for Days 0, 2, 15, 30, 32, 45, and 60 are shown to visualize the accumulation effect that occurs with monthly palivizumab administrations. (Data from Ref. [65].)

recipients (1998–2001). Protected days were defined as days within 32 days of receiving palivizumab, while all other days within the RSV season were defined (arbitrarily) as unprotected days, based on the assumption that serum concentrations were too low to prevent RSV disease. Protection was provided for approximately two-thirds of all days in the RSV seasons. Among 69 RSV hospitalizations, 54 were first-time RSV hospitalizations, and 42 of these occurred during the RSV seasons. Twenty-one of 42 (50%) first-time RSV hospitalizations occurred on unprotected days, for a rate of 1.07 admissions per 1000 unprotected days and 0.55 per 1000 protected days – a relative risk reduction similar to that observed in the IMpact-RSV study.

Afghani et al. [8] reviewed the RSV hospitalization rate of 250 infants born at ≤35 weeks' gestation who received palivizumab in the 2003/2004 and 2004/2005 seasons at a single center. The six infants hospitalized for RSV disease were matched (1:2) with control subjects of the same gestational age and born within 2 months of the case patient. All cases and all controls had CLD. Five of the six cases were born at ≤28 weeks' gestation. In contrast to the control patients, all case patients had additional complications, such as tracheostomy, congenital anomalies, or severe intraventricular hemorrhage. There was no indication that

nonprotective palivizumab trough levels contributed to hospitalization. The mean number of palivizumab injections prior to hospitalization was 2.8, and hospitalization occurred at a mean of 15.5 days after the last injection.

Zaaijer et al. [86] used a simple pharmacokinetic model to predict palivizumab trough concentrations, and suggested an alternative dosage regimen with a shortened first interval and a dose reduction from dose 2 (15 mg kg^{-1} on day 0, followed by 10 mg kg^{-1} on days 23, 53, 83, and 113). The authors suggested that, by using this alternative regimen, mean trough levels might be maintained above 45 μg mL^{-1}, while palivizumab expenses would be reduced considerably. No clinical results using this dosing regimen have been reported to date, however.

12.9 Post-Marketing Experience with Regard to Adverse Events

During the 8-year period since FDA approval of palivizumab, over four million doses of the antibody have been administered, and today the global safety databank is of considerable size. The most common possibly related AEs reported since the approval of palivizumab are similar in frequency to those reported in the IMpact-RSV phase III trial, namely injection site reactions (2–3%), fever (1–3%), diarrhea (up to 1%), and nervousness/irritability (up to 2.5%). Ongoing pharmacovigilance programs (REACH, Outcomes Registry) are in place to detect infrequent or unexpected side effects [38]. Acute hypersensitivity reactions such as urticaria (16/100 000 exposures) and anaphylaxis (<1 : 100 000 patients) were detected as rare AEs, and the package insert was modified to include these AEs.

As palivizumab is a humanized antibody containing both human (95%) and mouse (5%) amino acid sequence, it is possible that palivizumab is immunogenic – that is, anti-palivizumab antibodies might be induced. As a follow-up to the IMpact-RSV study, the safety and immunogenicity of palivizumab administered over two consecutive seasons was evaluated in 87 children. Thirty-two placebo-recipients and 56 palivizumab recipients received five monthly doses of 15 mg kg^{-1} palivizumab in the season following the IMpact-RSV trial. Only one child (receiving palivizumab for a second season) developed an anti-palivizumab titer of greater than 1/40 (1/160 on study Day 30, falling to 1/10 on Day 120, i.e., 30 days after the fourth injection). This child did not show accelerated palivizumab clearance and did not experience any SAEs. Two of the children in this study had developed transient anti-palivizumab titers during the IMpact-RSV trial, but neither child showed reactivity upon entry or during the second season [53]. In previously conducted studies in adults, low titer anti-palivizumab antibodies could be detected in approximately one-third of adult volunteers after the first dose, but not after repeat administrations of palivizumab. These antibodies were thought to be anti-idiotypic antibodies, but they had no clinical effect and no effect on palivizumab clearance [53]. In children, low anti-palivizumab titers were observed infrequently but, in contrast to adults, they were nonspecific and directed

against the Fc portion of palivizumab and other IgGs. Again, clinical effects were not associated with an identifiable anti-palivizumab response [2,65,77].

As indicated by the manufacturer of palivizumab (see Synagis package insert), the frequency of adverse reactions that have been identified and reported during post-approval use of palivizumab is difficult to estimate as reports of these reactions are voluntary and the population is of uncertain size. Based on experience in over 400 000 patients who have received Synagis (>2 million doses), however, very rare severe acute hypersensitivity reactions were the only new SAEs detected in pharmacovigilance programs.

Severe acute hypersensitivity reactions have been reported on initial or subsequent exposure. Very rare cases of anaphylaxis (<1 case per 100 000 patients) have also been reported following re-exposure. None of the reported hypersensitivity reactions was fatal. The hypersensitivity reactions mentioned in the package insert include dyspnea, cyanosis, respiratory failure, urticaria, pruritus, angioedema, hypotonia, and unresponsiveness. The relationship between these reactions and the development of antibodies to Synagis is unknown. Limited data from post-marketing reports suggest that giving more than five doses in a single season does not increase the risk of hypersensitivity reactions.

The development of palivizumab-resistant RSV mutants is an important theoretical risk to consider. *In vitro*, palivizumab-resistant RSV can be selected in the presence of palivizumab, and these resistant mutants can replicate in cotton rats at palivizumab serum concentrations that would normally restrict RSV replication [87]. However, resistance does not seem to be a relevant issue in breakthrough RSV disease leading to RSV hospitalization. A study at eight centers in the US evaluated palivizumab binding to 371 RSV isolates – including 25 isolates from children receiving palivizumab concurrently – and found that all of them were recognized by palivizumab [24]. A total of 124 of these RSV isolates was evaluated in an RSV microneutralization test, and all of them were neutralized by palivizumab [24]. The risk of clinically significant palivizumab resistance seems therefore very low in the immunocompetent host, where RSV is eliminated within 2 weeks of infection. Even in immunosuppressed patients in whom RSV can replicate for months and cause severe disease with high mortality, palivizumab escape mutants do not seem to develop [27]. It should be noted, however, that URT secretions are commonly tested for RSV while selection-pressure for palivizumab escape mutants is higher in the LRT. Most of the RSV replication in severe disease occurs in the LRT, and palivizumab is present at higher concentration in the lungs.

12.10 Ongoing Clinical Studies and Outlook

As indicated above, Medimmune has developed a second-generation humanized RSV mAb based on palivizumab by designing and screening palivizumab mutants [85]. This second-generation mAb, motavizumab (MEDI-524, NuMax), has an improved association rate (K_{on}) and neutralizes RSV more effectively. Motavizumab is currently being studied in comparison with palivizumab in two large

clinical trials in premature infants and in infants with CHD. A third study is evaluating motavizumab versus placebo in term infants at high risk. Motavizumab is projected to enter the market in 2008/2009 if shown to be superior to palivizumab.

A large motavizumab versus palivizumab phase III study began enrollment in 2004, and is designed to compare the safety and efficacy of motavizumab with that of palivizumab for the reduction of RSV hospitalization in high-risk infants. Medically attended LRTI and otitis media are used, amongst others, as secondary endpoints. This randomized, double-blind trial enrolled approximately 6600 high-risk infants at 300 sites in 24 countries in both the Northern and the Southern hemispheres. As with the IMpact-RSV trial, the study population consists of premature infants born at ≤35 weeks GA and aged ≤6 months at randomization, as well as children with CLD aged ≤24 months who required CLD treatment during the 6-month period prior to enrollment. Five monthly IM injections of $15\,mg\,kg^{-1}$ of either motavizumab or palivizumab during the RSV season are compared for their safety and efficacy in reducing RSV hospitalization, medically attended outpatient visits for LRTI, and otitis media.

The second motavizumab versus palivizumab trial is a randomized, double-blind trial that evaluates the safety, tolerability, immunogenicity and pharmacokinetics of the two mAbs in children with CHD. Recruitment took place at approximately 160 sites in the Northern Hemisphere during the 2005–2006 RSV season. Inclusion criteria are similar to those used in the palivizumab CHD phase III trial – that is, infants with hemodynamically significant CHD aged ≤24 months at randomization. Approximately 620 patients received five monthly doses of either $15\,mg\,kg^{-1}$ motavizumab or palivizumab, and were followed until day 150 – that is, 30 days after the last injection. Hospitalization due to RSV is assessed as a secondary endpoint.

Motavizumab versus placebo is being studied in a randomized, double-blind, placebo-controlled phase III trial designed to enroll approximately 3000 healthy full-term Navajo and White Mountain Apache infants over four RSV seasons. Study participants will receive up to five monthly doses ($15\,mg\,kg^{-1}$) of motavizumab or placebo during the RSV season for two consecutive seasons. During the first year, the plans are to enroll approximately 450 infants. Similar to the data reported for Alaskan infants, full-term Native American infants are at high risk of developing severe RSV disease. As Native American children also have a high rate of wheezing and asthma, the study participants will be followed until they are aged 5 years, in order to collect data on whether reducing RSV disease during the first 2 years of life can reduce the incidence of wheezing and asthma in full-term children.

12.11 Summary

Palivizumab was the first mAb product to be approved for an infectious disease indication, and quickly developed into a very successful biopharmaceutical

product. The safety and efficacy of palivizumab in reducing severe RSV disease in high-risk infants and children was established in large and well-designed phase III trials, and its safety and effectiveness in premature children and children with CHD seems to have been confirmed in a large number of clinical reports after approval. One major problem in using palivizumab relates to its cost; RSV prophylaxis using palivizumab may be recommended in more than 5% of all infants, but this places a high financial burden on healthcare systems. In order to deal with the enormous burden of disease caused by RSV, an affordable live-attenuated vaccine would be most desirable from a public health point of view. However such a vaccine it not available at present.

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References

1 Swedish Consensus Group. (2001) Management of infections caused by respiratory syncytial virus. Scand J Infect Dis 33: 323–328.
2 The IMpact-RSV Study Group. (1998) Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. Pediatrics 102: 531–537.
3 [Recommendations for immunologic prevention of respiratory syncytial virus (RSV) infections]. (1999) Z Geburtshilfe Neonatol 203(Suppl.2): 16.
4 The PREVENT Study Group. (1997) Reduction of respiratory syncytial virus hospitalization among premature infants and infants with bronchopulmonary dysplasia using respiratory syncytial virus immune globulin prophylaxis. Pediatrics 99: 93–99.
5 American Academy of Pediatrics Committee on Infectious Diseases and Committee on Fetus and Newborn. (2003) Revised indications for the use of palivizumab and respiratory syncytial virus immune globulin intravenous for the prevention of respiratory syncytial virus infections. Pediatrics 112: 1442–1445.
6 National Advisory Committee on Immunization. (2003) Statement on the recommended use of monoclonal anti-RSV antibody (palivizumab). Can Commun Dis Rep 29: 1–15.
7 Adams, R., Christenson, J., Petersen, F., Beatty, P. (1999) Pre-emptive use of aerosolized ribavirin in the treatment of asymptomatic pediatric marrow transplant patients testing positive for RSV. Bone Marrow Transplant 24: 661–664.
8 Afghani B., Ngo, T., Zeitany R., Amin A. (2006) Premature infants who get hospitalized for respiratory syncytial virus infection despite receiving palivizumab. J Invest Med 54: S133.
9 American Academy of Pediatrics. (2006). Respiratory syncytial virus. In: Pickering, L.K., Baker, C.J., Long, S.S., McMillan, J.A. (Eds.), Red Book: 2006 Report of the Committee on Infectious

Diseases. 27th edn. American Academy of Pediatrics, Elk Grove Village, IL, USA, pp. 560–566.
10 Bebbington, C.R., Renner, G., Thomson, S., King, D., Abrams, D., Yarranton, G.T. (1992) High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. Biotechnology (New York) 10: 169–175.
11 Beeler, J.A., van Wyke Coelingh, K. (1989) Neutralization epitopes of the F glycoprotein of respiratory syncytial virus: effect of mutation upon fusion function. J Virol 63: 2941–2950.
12 Bentley, D.L., Rabbits, T.H. (1980) Human immunoglobulin variable region genes – DNA sequences of two V kappa genes and a pseudogene. Nature 288: 730–733.
13 Bermingham, A., Collins, P.L. (1999) The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. Proc Natl Acad Sci USA 96: 11259–11264.
14 Boyce, T.G., Mellen, B.G., Mitchel, E.F., Jr., Wright, P.F., Griffin, M.R. (2000) Rates of hospitalization for respiratory syncytial virus infection among children in medicaid. J Pediatr 137: 865–870.
15 Carbonell Estrany, X., Quero Jimenez, J. (2000) [Recommendations for the prevention of respiratory syncytial virus infections. Standards Committee of the Spanish Society of Neonatology. Board of Directors of the Spanish Society of Neonatology]. Ann Esp Pediatr 52: 372–374.
16 Carbonell-Estrany, X., Quero, J. (2001) Hospitalization rates for respiratory syncytial virus infection in premature infants born during two consecutive seasons. Pediatr Infect Dis J 20: 874–879.
17 Carbonell-Estrany, X., Quero, J., Bustos, G., Cotero, A., Domenech, E., Figueras-Aloy, J., Fraga, J.M., Garcia, L.G., Garcia-Alix, A., Del Rio, M.G., Krauel X., Sastre, J.B., Narbona, E., Roques, V., Hernandez, S.S., Zapatero, M. (2000) Rehospitalization because of respiratory syncytial virus infection in premature infants younger than 33 weeks of gestation: a prospective study. IRIS Study Group. Pediatr Infect Dis J 19: 592–597.
18 Chantepie, A. (2004) [Use of palivizumab for the prevention of respiratory syncytial virus infections in children with congenital heart disease. Recommendations from the French Paediatric Cardiac Society.]. Arch Pediatr 11: 1402–1405.
19 Cohen, A., Hirsch, R.L., Sorrentino, M., Top, J.R., Carlin, D., McClain, B. (1999) First year experience using palivizumab humanized monoclonal antibody for protection from RSV lower respiratory tract infection. Second World Congress of Pediatric Infectious Diseases Abstract Book. Manila, Philippines. Abstract A16.
20 Cohen, S., Boron, M., Cohen M., Rankin A., M. and the Palivizumab Outcomes Registry Study Group. (2004) Patterns of RSV prophylaxis in children with congenital heart disease (CHD): preliminary results from the 2003 to 2004 palivizumab outcomes registry. J Perinatol 24: 605.
21 Collins, P.L., Chanock, R.M., Murphy, B.R. (2001) Respiratory syncytial virus. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Strauss, S.E. (Eds.), Fields Virology. 4th edn. Lippincott Williams & Wilkins, Philadelphia. Vol. 1. pp. 1443–1486.
22 Connor, E.M., McClain, J.B., Sorrentino, M., Hirsch, R.L., Top, F.H. (2002) Safety surveillance in children treated with palivizumab (PLV). J Perinatol 22: 619.
23 Crowe, J.E., Jr., Firestone, C.Y., Murphy, B.R. (2001) Passively acquired antibodies suppress humoral but not cell-mediated immunity in mice immunized with live attenuated respiratory syncytial virus vaccines. J Immunol 167: 3910–3918.
24 DeVincenzo, J.P., Hall, C.B., Kimberlin, D.W., Sanchez, P.J., Rodriguez, W.J., Jantausch, B.A., Corey, L., Kahn, J.S., Englund, J.A., Suzich, J.A., Palmer-Hill, F.J., Branco, L., Johnson, S., Patel, N.K., Piazza, F.M. (2004) Surveillance of clinical isolates of respiratory syncytial

virus for palivizumab (Synagis)-resistant mutants. J Infect Dis 190: 975–978.
25 Duppenthaler, A., Ammann, R.A., Gorgievski-Hrisoho, M., Pfammatter, J. P., Aebi, C. (2004) Low incidence of respiratory syncytial virus hospitalisations in haemodynamically significant congenital heart disease. Arch Dis Child 89: 961.
26 Duppenthaler, A., Gorgievski-Hrisoho, M., Aebi, C. (2001) Regional impact of prophylaxis with the monoclonal antibody palivizumab on hospitalisations for respiratory syncytial virus in infants. Swiss Med Wkly 131: 146–151.
27 El Saleeby, C.M., Suzich, J., Conley, M.E., DeVincenzo, J.P. (2004) Quantitative effects of palivizumab and donor-derived T cells on chronic respiratory syncytial virus infection, lung disease, and fusion glycoprotein amino acid sequences in a patient before and after bone marrow transplantation. Clin Infect Dis 39: e17–e20.
28 Falsey, A.R., Hennessey, P.A., Formica, M.A., Cox, C., Walsh, E.E. (2005) Respiratory syncytial virus infection in elderly and high-risk adults. N Engl J Med 352: 1749–1759.
29 Feltes, T.F., Cabalka, A.K., Meissner, H.C., Piazza, F.M., Carlin, D.A., Top, F. H., Jr., Connor, E.M., Sondheimer, H.M. (2003) Palivizumab prophylaxis reduces hospitalization due to respiratory syncytial virus in young children with hemodynamically significant congenital heart disease. J Pediatr 143: 532–540.
30 Feltes, T.F., Simoes, E. (2005) Palivizumab prophylaxis in haemodynamically significant congenital heart disease. Arch Dis Child 90: 875–876.
31 Figueras Aloy, J., Quero, J., Domenech, E., Lopez Herrera, M.C., Izquierdo, I., Losada, A., Perapch, J., Sanchez-Luna, M. (2005) [Recommendations for the prevention of respiratory syncytial virus infection]. An Pediatr (Barc) 63: 357–362.
32 Forster, J. (1999) [Practice guideline by the German Society for Pediatric Infectious Diseases with respect to prevention of RSV infections through immunoglobulin administration]. Klin Padiatr 211: 476.
33 Groothuis, J.R. (2001) Safety and tolerance of palivizumab administration in a large Northern Hemisphere trial. Northern Hemisphere Expanded Access Study Group. Pediatr Infect Dis J 20: 628–630.
34 Groothuis, J.R., Nishida, H. (2002) Prevention of respiratory syncytial virus infections in high-risk infants by monoclonal antibody (palivizumab). Pediatr Int 44: 235–241.
35 Groothuis, J.R., Simoes, E.A., Levin, M.J., et al. (1993) Prophylactic administration of respiratory syncytial virus immune globulin to high-risk infants and young children. The Respiratory Syncytial Virus Immune Globulin Study Group. N Engl J Med 329: 1524–1530.
36 Hartling, L., Wiebe, N., Russell, K., Patel, H., Klassen, T.P. (2004) Epinephrine for bronchiolitis. The Cochrane Database of Systematic Reviews 2004: 1–35.
37 Heikkinen, T., Valkonen, H., Lehtonen, L., Vainionpaa, R., Ruuskanen, O. (2005) Hospital admission of high risk infants for respiratory syncytial virus infection: implications for palivizumab prophylaxis. Arch Dis Child Fetal Neonatal Ed 90: F64–F68.
38 Hudak, M. (2002) Palivizumab prophylaxis of RSV disease – results of 5097 children – the 2001 to 2002 outcomes registry. J Perinatol 22: 619.
39 Iwane, M.K., Edwards, K.M., Szilagyi, P.G., Walker, F.J., Griffin, M.R., Weinberg, G.A., Coulen, C., Poehling, K.A., Shone, L.P., Balter, S., Hall, C.B., Erdman, D.D., Wooten, K., Schwartz, B. (2004) Population-based surveillance for hospitalizations associated with respiratory syncytial virus, influenza virus, and parainfluenza viruses among young children. Pediatrics 113: 1758–1764.
40 Jobe, A.H., Bancalari, E. (2001) Bronchopulmonary dysplasia. Am J Respir Crit Care Med 163: 1723–1729.
41 Johnson, S., Oliver, C., Prince, G.A., Hemming, V.G., Pfarr, D.S., Wang, S.C.,

Dormitzer, M., O'Grady, J., Koenig, S., Tamura, J.K., Woods, R., Bansal, G., Couchenour, D., Tsao, E., Hall, W.C., Young, J.F. (1997) Development of a humanized monoclonal antibody (MEDI-493) with potent in vitro and in vivo activity against respiratory syncytial virus. J Infect Dis 176: 1215–1224.

42 Karron, R.A., Singleton, R.J., Bulkow, L., Parkinson, A., Kruse, D., DeSmet, I., Indorf, C., Petersen, K.M., Leombruno, D., Hurlburt, D., Santosham, M., Harrison, L.H. (1999) Severe respiratory syncytial virus disease in Alaska native children. RSV Alaska Study Group. J Infect Dis 180: 41–49.

43 Kellner, J.D., Ohlsson, A., Gadomski, A.M., Wang, E.E.L. (1999) Bronchodilators for bronchiolitis. The Cochrane Database of Systematic Reviews 1999: 1–18.

44 Kohler, G., Milstein, C. (1976) Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur J Immunol 6: 511–519.

45 Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad Sci USA 82: 488–492.

46 Lacaze-Masmonteil, T., Roze, J.C., Fauroux, B. (2002) Incidence of respiratory syncytial virus-related hospitalizations in high-risk children: follow-up of a national cohort of infants treated with Palivizumab as RSV prophylaxis. Pediatr Pulmonol 34: 181–188.

47 Manzoni, P., Sala, U., Gomirato, G., Coscia, A., Fabris, C. (2005) Optimal timing and dosing intervals of palivizumab in premature neonates: still some work to do. Pediatrics 115: 1439–1440.

48 Martinez, F.D. (2003) Respiratory syncytial virus bronchiolitis and the pathogenesis of childhood asthma. Pediatr Infect Dis J 22: S76–S82.

49 Murphy, B.R., Prince, G.A., Walsh, E.E., Kim, H.W., Parrott, R.H., Hemming, V. G., Rodriguez, W.J., Chanock, R.M. (1986) Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. J Clin Microbiol 24: 197–202.

50 Naver, L., Eriksson, M., Ewald, U., Linde, A., Lindroth, M., Schollin, J. (2004) Appropriate prophylaxis with restrictive palivizumab regimen in preterm children in Sweden. Acta Paediatr 93: 1470–1474.

51 Naylor, C.J., Britton, P., Cavanagh, D. (1998) The ectodomains but not the transmembrane domains of the fusion proteins of subtypes A and B avian pneumovirus are conserved to a similar extent as those of human respiratory syncytial virus. J Gen Virol 79 (Pt 6): 1393–1398.

52 Northway, W.H., Jr., Rosan, R.C., Porter, D.Y. (1967) Pulmonary disease following respirator therapy of hyaline-membrane disease. Bronchopulmonary dysplasia. N Engl J Med 276: 357–368.

53 Null, D., Jr., Pollara, B., Dennehy, P.H., Steichen, J., Sanchez, P.J., Givner, L.B., Carlin, D., Landry, B., Top, F.H., Jr., Connor, E. (2005) Safety and immunogenicity of palivizumab (Synagis) administered for two seasons. Pediatr Infect Dis J 24: 1021–1024.

54 Ogilvie, M.M., Vathenen, A.S., Radford, M., Codd, J., Key, S. (1981) Maternal antibody and respiratory syncytial virus infection in infancy. J Med Virol 7: 263–271.

55 Oh, P.I., Lanctjt, K.L., Yoon, A., Lee, D.S., Paes, B.A., Simmons, B.S., Parison, D., Manzi, P. (2002) Palivizumab prophylaxis for respiratory syncytial virus in Canada: utilization and outcomes. Pediatr Infect Dis J 21: 512–518.

56 Parnes, C., Guillermin, J., Habersang, R., et al. (2003) Palivizumab prophylaxis of respiratory syncytial virus disease in 2000–2001: results from The Palivizumab Outcomes Registry. Pediatr Pulmonol 35: 484–489.

57 Patel, H., Platt, R., Lozano, J.M., Wang, E.E.L. (2004) Glucocorticoids for acute viral bronchiolitis in infants and young children. The Cochrane Database of Systematic Reviews 2004: 1–46.

58 Pedraz, C., Carbonell-Estrany, X., Figueras-Aloy, J., Quero, J. (2003) Effect of palivizumab prophylaxis in decreasing respiratory syncytial virus hospitalizations in premature infants. Pediatr Infect Dis J 22: 823–827.

59 Pollack, P., Groothuis, J.R. (2002) Development and use of palivizumab (Synagis): a passive immunoprophylactic agent for RSV. J Infect Chemother 8: 201–206.

60 Prais, D., Danino, D., Schonfeld, T., Amir, J. (2005) Impact of palivizumab on admission to the ICU for respiratory syncytial virus bronchiolitis: a national survey. Chest 128: 2765–2771.

61 Press, E.M., Hogg, N.M. (1970) The amino acid sequences of the Fd fragments of two human gamma-1 heavy chains. Biochem J 117: 641–660.

62 Prince, G.A., Horswood, R.L., Chanock, R.M. (1985) Quantitative aspects of passive immunity to respiratory syncytial virus infection in infant cotton rats. J Virol 55: 517–520.

63 Rader, R. (2005) Biopharmaceutical Products in the U.S. and European Markets. BioPlan Associates, Inc., Rockville, MD, USA.

64 Robertson, S.E., Roca, A., Alonso, P., Simoes, E.A., Kartasasmita, C.B., Olaleye, D.O., Odaibo, G.N., Collinson, M., Venter, M., Zhu, Y., Wright, P.F. (2004) Respiratory syncytial virus infection: denominator-based studies in Indonesia, Mozambique, Nigeria and South Africa. Bull World Health Org 82: 914–922.

65 Saez-Llorens, X., Castano, E., Null, D., Steichen, J., Sanchez, P.J., Ramilo, O., Top, F.H., Jr., Connor, E. (1998) Safety and pharmacokinetics of an intramuscular humanized monoclonal antibody to respiratory syncytial virus in premature infants and infants with bronchopulmonary dysplasia. The MEDI-493 Study Group. Pediatr Infect Dis J 17: 787–791.

66 Saji, T., Nakazawa, M., Harada, K. (2005) Safety and efficacy of palivizumab prophylaxis in children with congenital heart disease. Pediatr Int 47: 397–403.

67 Sampalis, J.S. (2003) Morbidity and mortality after RSV-associated hospitalizations among premature Canadian infants. J Pediatr 143: S150–S156.

68 Schenerman, M.A., Hope, J.N., Kletke, C., Singh, J.K., Kimura, R., Tsao, E.I., Folena-Wasserman, G. (1999) Comparability testing of a humanized monoclonal antibody (Synagis) to support cell line stability, process validation, and scale-up for manufacturing. Biologicals 27: 203–215.

69 Shay, D.K., Holman, R.C., Newman, R.D., Liu, L.L., Stout, J.W., Anderson, L. J. (1999) Bronchiolitis-associated hospitalizations among US children, 1980–1996. JAMA 282: 1440–1446.

70 Shennan, A.T., Dunn, M.S., Ohlsson, A., Lennox, K., Hoskins, E.M. (1988) Abnormal pulmonary outcomes in premature infants: prediction from oxygen requirement in the neonatal period. Pediatrics 82: 527–532.

71 Sigurs, N., Gustafsson, P.M., Bjarnason, R., Lundberg, F., Schmidt, S., Sigurbergsson, F., Kjellman, B. (2005) Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. Am J Respir Crit Care Med 171: 137–141.

72 Simoes, E.A. (2002) Immunoprophylaxis of respiratory syncytial virus: global experience. Respir Res. 3(Suppl.1): S26–S33.

73 Singleton, R., Dooley, L., Bruden, D., Raelson, S., Butler, J.C. (2003) Impact of palivizumab prophylaxis on respiratory syncytial virus hospitalizations in high risk Alaska Native infants. Pediatr Infect Dis J 22: 540–545.

74 Sorrentino, M., Powers, T. (2000) Effectiveness of palivizumab: evaluation of outcomes from the 1998 to 1999 respiratory syncytial virus season. The Palivizumab Outcomes Study Group. Pediatr Infect Dis J 19: 1068–1071.

75 Spann, K.M., Tran, K.C., Collins, P.L. (2005) Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and

proinflammatory cytokines. J Virol 79: 5353–5362.

76 Stensballe, L.G., Devasundaram, J.K., Simoes, E.A. (2003) Respiratory syncytial virus epidemics: the ups and downs of a seasonal virus. Pediatr Infect Dis J 22: S21–S32.

77 Subramanian, K.N., Weisman, L.E., Rhodes, T., Ariagno, R., Sanchez, P.J., Steichen, J., Givner, L.B., Jennings, T.L., Top, F.H., Jr., Carlin, D., Connor, E. (1998) Safety, tolerance and pharmacokinetics of a humanized monoclonal antibody to respiratory syncytial virus in premature infants and infants with bronchopulmonary dysplasia. MEDI-493 Study Group. Pediatr Infect Dis J 17: 110–115.

78 Takahashi, N., Noma, T., Honjo, T. (1984) Rearranged immunoglobulin heavy chain variable region (VH) pseudogene that deletes the second complementarity-determining region. Proc Natl Acad Sci USA 81: 5194–5198.

79 Tulloh, R., Marsh, M., Blackburn, M., Casey, F., Lenney, W., Weller, P., Keeton, B.R. (2003) Recommendations for the use of palivizumab as prophylaxis against respiratory syncytial virus in infants with congenital cardiac disease. Cardiol Young 13: 420.

80 Ventre, K., Randolph, A.G. (2004) Ribavirin for respiratory syncytial virus infection of the lower respiratory tract in infants and young children. The Cochrane Database of Systematic Reviews 2004: 1–20.

81 Weigl, J.A., Puppe, W., Belke, O., Neususs, J., Bagci, F., Schmitt, H.J. (2005) Population-based incidence of severe pneumonia in children in Kiel, Germany. Klin Padiatr 217: 211–219.

82 Weigl, J.A., Puppe, W., Schmitt, H.J. (2001) Incidence of respiratory syncytial virus-positive hospitalizations in Germany. Eur J Clin Microbiol Infect Dis 20: 452–459.

83 Willson, D.F., Landrigan, C.P., Horn, S.D., Smout, R.J. (2003) Complications in infants hospitalized for bronchiolitis or respiratory syncytial virus pneumonia. J Pediatr 143: S142–S149.

84 Wright, P.F., Karron, R.A., Madhi, S.A., Treanor, J.J., King, J.C., O'Shea, A., Ikizler, M.R., Zhu, Y., Collins, P.L., Cutland, C., Randolph, V.B., Deatly, A. M., Hackell, J.G., Gruber, W.C., Murphy, B.R. (2006) The interferon antagonist NS2 protein of respiratory syncytial virus is an important virulence determinant for humans. J Infect Dis 193: 573–581.

85 Wu, H., Pfarr, D.S., Tang, Y., An, L.L., Patel, N.K., Watkins, J.D., Huse, W.D., Kiener, P.A., Young, J.F. (2005) Ultra-potent antibodies against respiratory syncytial virus: effects of binding kinetics and binding valence on viral neutralization. J Mol Biol 350: 126–144.

86 Zaaijer, H.L., Vandenbroucke-Grauls, C.M., Franssen, E.J. (2002) Optimum dosage regimen of palivizumab? Ther Drug Monit 24: 444–445.

87 Zhao, X., Chen, F.P., Megaw, A.G., Sullender, W.M. (2004) Variable resistance to palivizumab in cotton rats by respiratory syncytial virus mutants. J Infect Dis 190: 1941–1946.

13
Rituximab (Rituxan)

Michael Wenger

13.1
Introduction

Rituximab (MabThera, F. Hoffmann La-Roche; Rituxan, Genentech and Biogen Idec) – a chimeric monoclonal antibody (mAb) produced by recombinant technology – entered clinical development during the early 1990s, and subsequently received approval by American and European regulatory authorities in 1997 and 1998, respectively. In this chapter we will first discuss rituximab's production and mode of action, before focusing on its efficacy and safety in non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL). Finally, the evaluation of rituximab in new therapeutic areas including rheumatoid arthritis (RA) will be briefly described.

13.1.1
Production, Design, and Structure of Rituximab

Rituximab is a genetically engineered human/mouse chimeric mAb that is specific for the CD20 antigen on the surface of B cells (Reff et al. 1994). It is a fusion of the light- and heavy-chain variable (antigen-binding) domains of 2B8 (a murine monoclonal anti-CD20 antibody) and human kappa light-chain and gamma 1 heavy-chain constant regions (Fig. 13.1) (Boye et al. 2003; Reff et al. 1994).

Rituximab consists of two heavy chains of 451 amino acids and two light chains of 213 amino acids, with an approximate molecular weight of 145 kDa. It has a binding affinity for the CD20 antigen of approximately 8.0 nM (Reff et al. 1994).

Because the majority of the antibody is of human origin, rituximab has a low potential for immunogenicity. Indeed, no human anti-mouse immunoglobulin antibodies (HAMA) have been detected in patients treated with rituximab to date. The development of human anti-chimeric antibodies (HACA) occurs rarely (documented in <1% of rituximab-treated patients), and it is not clear whether these antibodies have any neutralizing effect (MabThera SmPC; see link on EMEA

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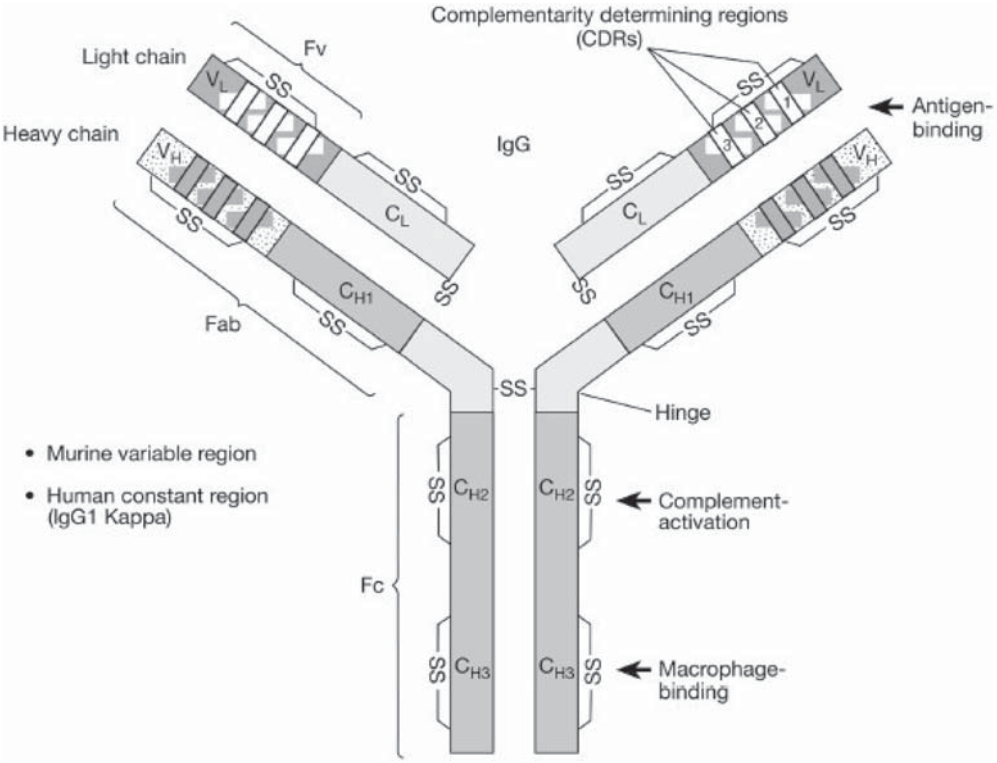

Fig. 13.1 The structure of rituximab. (Reproduced with permission from Boye et al. 2003.)

website, http://www.emea.eu.int; Rituxan datasheet, see link on Genentech website, http://www.gene.com).

Rituximab is produced by Chinese hamster ovary cells in suspension, and purified by affinity chromatography and ion-exchange chromatography. The purification process also incorporates specific viral inactivation and removal procedures. Rituximab is supplied as a sterile, clear, colorless, preservative-free liquid concentrate for intravenous (IV) infusion.

Thus, rituximab binds to the target CD20 antigen via the variable murine regions, while the remainder of the antibody interacts with human effector mechanisms to kill the target cells.

13.1.2 CD20 as a Therapeutic Target

CD20 is a transmembrane surface antigen, which is expressed only by B-cell precursors and mature B cells. This antigen appears to be involved in the regula-

tion of B lymphocyte growth and differentiation; indeed, recent data suggest that CD20 could play an important role in the influx of calcium across cell membranes, sustaining intracellular calcium concentrations and allowing the activation of B cells (Li et al. 2003).

CD20 makes an attractive target for monoclonal therapy for a number of reasons:

- It is reliably expressed on a large proportion of B cells in a number of diseases (e.g., NHL), while it is not expressed on stem cells, normal mature plasma cells or other normal tissues. Its expression is lost when normal B cells differentiate into antibody-secreting plasma cells (Nadler et al. 1981; Anderson et al. 1984).
- CD20 is present on malignant plasma cells in 20% of patients with multiple myeloma, up to 50% of patients with plasma cell leukemia, 75–100% of patients with Waldenström's macroglobulinemia, and >95% of patients with B-cell lymphoma and leukemia (Treon et al. 2003; Olszewski and Grossbard 2004).
- $CD20^+$ cells can be completely eradicated from the body without causing excessive toxicity because normal B cells will re-emerge following differentiation from stem cells, while serum immunoglobulin levels can be maintained by persisting plasma cells.
- It is not internalized after binding by antibody, and its expression is stable (Press et al. 1987). Antibodies targeting CD20 therefore remain bound to the antigen on the cell surface at a constant density determined by the level of antigen expression. The bound antibody then initiates immune processes and induces apoptosis.
- CD20 is not normally shed from the cell surface, and serum levels of the antigen are undetectable in most patients (Einfeld et al. 1988); however, soluble CD20 has been observed in some patients with CLL (Manshouri et al. 2003).

13.1.3 Mode of Action

Evidence suggests that several mechanisms may be involved in providing rituximab's therapeutic efficacy via the CD20 antigen (Fig. 13.2; Olszewski and Grossbard 2004). Because CD20 is neither shed from the cell surface nor internalized upon antibody binding, sustained binding of rituximab to the CD20 antigen

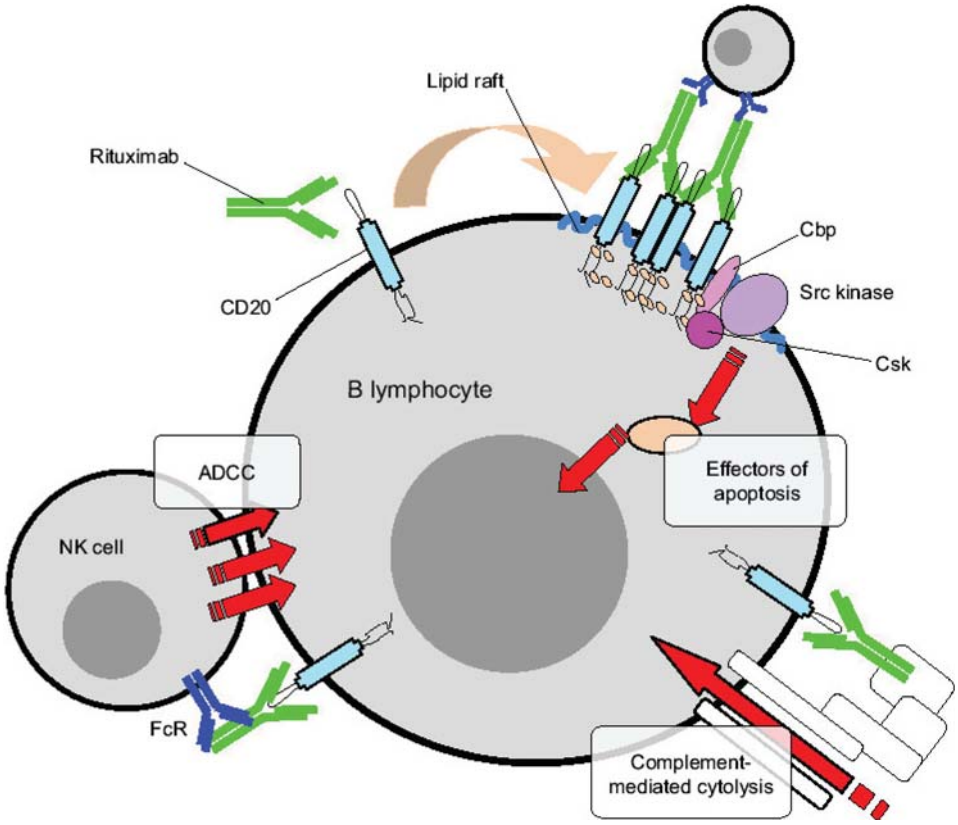

Fig. 13.2 Rituximab mechanisms of action. (Reproduced with permission from Olszewski and Grossbard 2004.)

occurs. The mechanism of cytotoxicity induced by rituximab is not fully understood, and is thought to include several mechanisms. The dominant mechanism may vary according to cellular compartment, presence or absence of concurrent chemotherapy, and type of tumor treated (Friedberg 2005).

Antibody-dependent cellular cytotoxicity (ADCC) appears to be a major *in-vivo* mechanism of rituximab. ADCC is mediated through ligation of the Fc portion of the antibody to Fc receptors (FcR) expressed by accessory cells. Complement-dependent cell lysis (CDC) occurs when rituximab binds to the complement factor C1q; it appears that translocation of CD20 into lipid rafts precedes the activation of lytic complement. The development of lipid rafts also appears to play

a key role in rituximab-induced apoptosis. Increased calcium conductivity and induction of a close proximity with Src kinases leads to caspase activation and then to apoptosis. The cellular microenvironment also appears to contribute to rituximab-induced cytotoxicity, with differential responses to rituximab occurring in lymphomas of various histologies. Finally, rituximab therapy of lymphoma cells may promote uptake and cross-presentation of lymphoma-derived peptides by antigen-presenting dendritic cells (DC), induce the maturation of DC, and allow the generation of specific cytotoxic T-cell responses – a so-called "vaccinal" effect (Friedberg 2005).

In-vitro data confirm that rituximab may potentiate the cytotoxic activity of other drugs used in the oncology setting, suggesting a synergistic effect when rituximab is used in combination with other chemotherapy agents.

Preclinical data suggesting synergy between rituximab and cytotoxic or biologic therapy includes the following:

- DHL-4 – a drug-resistant B-cell lymphoma cell line – becomes susceptible to doxorubicin and cisplatin following exposure to rituximab (Demidem et al. 1995, 1997).
- Rituximab inhibits the production of interleukin (IL)-10, which in turn down-regulates the expression of the anti-apoptotic protein, bcl-2, sensitizing B cells to the apoptotic effects of chemotherapy (Alas and Bonavida 2001).
- Fludarabine down-modulates the expression of the complement inhibitor, CD55, and increases the susceptibility of malignant B cells to lysis by rituximab (Di Gaetano et al. 2001).
- Rituximab reduces the IC_{30} and IC_{50} of cladribine, doxorubicin, mitoxantrone and bendamustine necessary for induction of apoptosis in neoplastic lymphocytes (Chow et al. 2002).

13.1.4 Preclinical Studies

The pharmacology and toxicology profiles of rituximab have been evaluated in preclinical studies in macaque cynomolgus monkeys (Reff et al. 1994). Rituximab was administered by IV injection, resulting in potent but reversible B-cell depletion, with no notable toxic effects. The maximum single dose administered was $100\,mg\,kg^{-1}$, and the highest repeat dose was $20\,mg\,kg^{-1}$ weekly for 8 weeks. There were no significant toxicological effects at any of the doses or schedules studied, and no animal died as a result of treatment with rituximab. Depletion of B cells – the intended pharmacological effect of rituximab – was observed in all animals studied, with >95% depletion after the first or second dose. B-cell

recovery occurred over a variable period of time; partial recovery occurred most commonly after 4 to 8 weeks, while recovery to baseline levels required a minimum of approximately 3 months.

13.1.5 Pharmacokinetic Studies

Extensive pharmacokinetic (PK) studies of rituximab have been carried out in patients with NHL (Onrust et al. 1999). After administration of rituximab by IV infusion at a dose of 375 mg m^{-2} once weekly for 4 weeks, peak plasma concentration (C_{max}) was 465 mg L^{-1} (Berinstein et al. 1998). Clearance of rituximab decreased following multiple infusions, from 38.2 mL h^{-1} after a single infusion to 9.2 mL h^{-1} after the fourth.

Accumulation of rituximab occurs, with the area under the concentration–time curve (AUC) increasing from 16 320 mg L^{-1} h after the first infusion to 86 125 mg L^{-1} h after the fourth (Berinstein et al. 1998). Importantly, the accumulation of rituximab is not accompanied by any increase in toxicity.

The terminal elimination half-life ($t_{1/2}$) of rituximab increased from 3.2 days after a single infusion to 8.6 days following a fourth infusion, thus confirming accumulation of the antibody (Berinstein et al. 1998). At 3 months after completion of therapy, rituximab was still detectable at a median concentration of 20.3 mg L^{-1} (range: 0.0 to 96.8 mg L^{-1}) in 63% of 116 evaluable patients, and remained detectable in 11.2% of these patients after 6 months (Berinstein et al. 1998). Ongoing studies suggest a terminal half-life of approximately 3 weeks – somewhat longer than had been previously assumed (Data on file, Roche).

Serum levels of rituximab have been shown to be dose-dependent over the dose range of 100 to 500 mg m^{-2}, given as an IV infusion (Maloney et al. 1994). An analysis of B-cell depletion showed the peripheral B lymphocyte count to be reduced by approximately 90% within 3 days of a single infusion of rituximab at a dose of 250 or 500 mg m^{-2} (Maloney et al. 1994). The clinical response to rituximab has been shown to correlate with serum concentrations of the antibody (Berinstein et al. 1998; Maloney et al. 1997a), with responders having significantly higher median serum concentrations than nonresponders throughout the 4-week treatment period ($p < 0.01$).

A study of the feasibility and efficacy of PK-based maintenance dosing of rituximab showed that serum drug levels increased appropriately after a single bolus for most patients, and were maintained for several months afterwards (Gordan et al. 2005). The majority of patients studied (28 of 29) required no more than three additional single-dose rituximab infusions in 12 months of follow-up, with monthly serum level monitoring to maintain a level at or above an arbitrarily defined threshold of 25 mg L^{-1}. A trend was noted for higher rituximab levels in patients considered to be responders (Gordan et al. 2005).

13.2
Rituximab Clinical Data in NHL and CLL

13.2.1
Overview of NHL and CLL

NHL is a heterogeneous group of malignancies originating in lymphoid tissue, with 90% being of B-cell origin. Data from Surveillance Epidemiology and End Results (SEER) reveal an age-adjusted incidence of 19.1 per 100000 men and women per year. Notably, the median age at diagnosis is 66 years, meaning that many NHL patients may be frail and/or suffering from comorbid disease (see link on SEER website, National Cancer Institute, http://seer.cancer.gov). The incidence of NHL has doubled over the past two decades in most westernized countries. Whilst improved cancer reporting and changes in disease classification may have contributed to this dramatic increase, other etiological factors are clearly important. These are thought to include congenital or acquired immunosuppression, exposure to infectious organisms (including human immunodeficiency virus, HIV), *Helicobacter pylori* and Epstein–Barr virus (EBV), as well as genetic susceptibility and exogenous factors including ultraviolet radiation, pesticides and hair dyes (Fisher and Fisher 2004). Recently, stable incidences in Western developed countries have been observed, suggesting a decline of the 1 to 2% increase in incidence observed during the 1960s to 1990s.

The cellular classification [using the World Health Organization (WHO) modification of the Revised European American Lymphoma (REAL) Classification] divides the disease into more than 20 clinicopathological entities (Pileri et al. 1998). A simplified version of the WHO Classification, together with the incidence of these types of NHL reported by the Non-Hodgkin's Lymphoma Classification Project (1998) is provided in Table 13.1. The most frequent 11 diseases occur in more than 2% of patients, and all the remaining categories occur with a frequency of less than 1%.

A clinically useful division of the cellular classification into prognostic groups is frequently used. Indolent lymphomas comprise 25 to 40% of NHLs, with follicular lymphoma (FL) being the most common type. The marginal zone lymphomas (MZL), a heterogeneous group of disorders consisting of mucosa-associated lymphoid tissue (MALT) lymphoma, nodal MZL and splenic MZL, account for approximately 8% of NHLs. Small lymphocytic lymphoma (SLL) is considered to have the same morphologic and immunophenotypic features as CLL (see below), and to constitute the same disease entity. Lymphoplasmacytic lymphoma (Waldenström's macroglobulinemia, WM) is a rare indolent lymphoma characterized primarily by the infiltration of lymphophoplasmacytic cells into bone marrow and the demonstration of an IgM monoclonal gammopathy.

Indolent NHL is typically characterized by a remitting and relapsing course, and median survival after first relapse is 4 to 5 years (Johnson et al. 1995). Transformation to an aggressive histological subtype may occur at any stage of the disease, and frequently has a fatal outcome (Yuen et al. 1995). The majority of

Table 13.1 REAL Classification of non-Hodgkin's lymphoma: subtypes occurring in at least 2% of patients in non-Hodgkin's Lymphoma Classification Project (Armitage and Weisenburger 1998).

Category	*Frequency [%]*
1 Diffuse large B-cell lymphoma (DLBCL)	31
2 Follicular lymphoma (FL)	22
3 Peripheral T-cell	6
4 Small lymphocytic lymphoma (SLL); Mantle cell lymphoma (MCL)	6
5 Marginal zone, mucosal-associated lymphoid tissue (MALT)	5
6 Primary mediastinal large B-cell (PMBCL)	2
7 Anaplastic large-T/null cell	2
8 High-grade B-cell, Burkitt-like	2
9 Lymphoblastic lymphoma	2

patients present with advanced disease, and the decision to treat is based on a number of factors including symptoms, rate of disease progression, presence of bulky disease, cytopenia or impaired end-organ function as a consequence of lymphoma, as well as patient and physician preference. Advanced-stage FL, MALT lymphoma and nodal MZL are generally managed in a similar way; treatment options include single-agent or combination chemotherapy, single-agent rituximab, rituximab in combination with chemotherapy, or high-dose chemotherapy with autologous or allogeneic stem cell support. Therapy should be individualized, taking into account the patient's age, extent of disease, comorbid conditions and the goals of therapy. Splenectomy is often the treatment of choice for splenic MZL, and the sole initial treatment for gastric MALT lymphoma confined to the gastric wall should be eradication of *H. pylori* with antibiotics (Bertoni and Zucca 2005). Treatment options for WM include single-agent or combination chemotherapy, with or without rituximab (Treon et al. 2006). SLL is managed in the same way as CLL (see below).

The majority (60–75%) of NHLs are aggressive, with the most common type being diffuse large B-cell lymphoma (DLBCL). DLBCL may be cured in a significant percentage of patients, depending on baseline tumor and patient characteristics; however, therapeutic challenges persist, especially for patients with high-risk disease (Coiffier 2005). Primary mediastinal B-cell lymphoma (PMBCL) is a DLBCL that arises in the thymus and mainly affects young adults. Burkitt's and Burkitt-like lymphoma (BL/BLL) are aggressive B-cell malignancies characterized by a rapid proliferative rate and a propensity for extranodal sites of involvement such as the gastrointestinal tract and central nervous system (CNS). Lymphoblastic lymphoma (LBL) is a rare subtype of aggressive NHL with biological features similar to those of acute lymphoblastic leukemia (ALL). In the majority

of cases, LBL shows a T-cell phenotype, and mediastinal tumors are the most frequent manifestation. Peripheral T-cell lymphomas – including precursor T-lymphoblastic lymphoma and anaplastic large-cell lymphoma T/null-cell types – represent a heterogeneous group of diseases, most of which have disappointing cure rates.

Mantle cell lymphoma (MCL) accounts for approximately 6–8% of all lymphoma diagnoses; patients usually respond well to induction therapy but have a poor median survival of only 3 to 4 years (Witzig 2005). There is no established standard of care in MCL, and patients with this disease should be treated in clinical trials wherever possible.

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in Western countries, with most cases occurring in elderly patients. The age-adjusted incidence rate according to SEER data is 3.6 cases per 100 000 men and women per year, and the median age at diagnosis is 72 years (see link on SEER website, National Cancer Institute, http://seer.cancer.gov). CLL follows a variable course, with survival ranging from months to decades. Recently, considerable progress in the identification of molecular and cellular prognostic markers has been made; in particular, the mutational profile of Ig genes and some cytogenetic abnormalities have been shown to be strongly predictive of clinical outcomes (Dighiero 2005).

13.2.2 Rituximab plus Chemotherapy Induction Therapy in Indolent NHL

For many years, patients with advanced symptomatic indolent NHL were treated either with single-agent chemotherapy (e.g., chlorambucil or cyclophosphamide) or combination chemotherapy [e.g., cyclophosphamide, vincristine, prednisone (CVP) or cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP)]. Despite the exploration of many different regimens, chemotherapy has had no major impact on survival in indolent NHL. The use of autologous stem cell transplant (ASCT) in relapsed/refractory indolent NHL may prolong disease-free survival (DFS), but its value in this setting remains uncertain due to the lack of conclusive phase III data. The use of ASCT as consolidation therapy in the first remission of advanced-stage FL indicates that improvement in time to disease progression (TTP) and even overall survival (OS) are possible, although an increase in secondary hematologic neoplasms can occur (Hiddemann 2005a). However, age and comorbidity may preclude the use of ASCT in many patients with indolent NHL.

13.2.2.1 Rituximab plus Chemotherapy in Previously Untreated Indolent NHL

Based on their different modes of action, promising *in-vitro* data and nonoverlapping toxicity profiles, rituximab and chemotherapy combinations were predicted to have additive and even synergistic efficacy in indolent NHL. This hypothesis was first examined in phase II trials and subsequently in phase III trials

(Table 13.2). The majority of investigators have examined the concurrent administration of chemotherapy and rituximab, although several groups have assessed sequential rituximab following chemotherapy. Most of the earliest trials employed four infusions of rituximab, either alone, concurrently with or sequentially after chemotherapy. As it became clear that an increase in the number of infusions of rituximab led to an increase in efficacy with a similar toxicity profile, studies using up to eight infusions of rituximab in combination with chemotherapy were initiated.

13.2.2.1.1 Concurrent Rituximab and Chemotherapy: Phase II Studies

The earliest phase II trial was initiated in 1996, and examined the safety and efficacy of six doses of rituximab together with six cycles of CHOP chemotherapy in 40 patients with indolent NHL, 31 of whom were previously untreated (Czuczman et al. 1999). All 38 evaluable patients responded, with 55% achieving a complete response (CR) using protocol-defined response criteria (Czuczman et al. 1999). Updated response rates based on the standard criteria used to assess response in lymphoma (Cheson et al. 1999) were as follows: ORR 100%; CR/unconfirmed complete response (CRu) 87%; partial response (PR) 13% (Czuczman et al. 2004). Long-term follow-up has revealed these responses to be highly durable, with the median TTP being almost 7 years. Sixteen patients remained in remission at 6 to 9 years after treatment (Czuczman et al. 2004).

A number of other phase II studies subsequently examined rituximab in combination with a variety of chemotherapeutic regimens (Table 13.2). Combining rituximab with other cyclophosphamide-based regimens [e.g., mitoxantrone and cyclophosphamide (CM) and cyclophosphamide, mitoxantrone, vincristine and prednisone (CNOP) (Economopoulos et al. 2003; Emmanouilides et al. 2003)] has resulted in high ORR and CR rates (Table 13.2), and these regimens have been well tolerated. A recently updated phase II trial conducted in Mexico randomized patients to receive either six infusions of rituximab, six cycles of CNOP or six cycles of R-CNOP (Rivas-Vera et al. 2005). There were no significant differences in the ORR and CR rates between the various arms, and median TTP and OS were not reached in all three treatment groups after a median of 24 months' follow-up. Infections were more common with R-CNOP (15%) than CNOP (6%) or rituximab alone (5%). The authors noted that their study included fewer patients with Stage IV disease than many other trials of rituximab and chemotherapy, which might explain the apparent lack of benefit of R-CNOP over CNOP alone (Rivas-Vera et al. 2005).

A study of seven infusions of rituximab plus six cycles of the purine analog fludarabine in patients with low-grade or FL enrolled 40 subjects, including 27 chemotherapy-naïve patients. An ORR of 90% (80% CR) was seen in the intention-to-treat (ITT) population, with similar outcomes observed both in chemotherapy-naïve and in relapsed patients (Czuczman et al. 2005). The median duration of response (DR), TTP and OS were not reached after a median follow-up of 44 months. Following unexpected hematological toxicities in the first 10 patients studied, a change in study design was implemented: prophylactic

Table 13.2 Trials of rituximab (R) in induction therapy regimens in previously untreated indolent non-Hodgkin's lymphoma (NHL).

Reference	*Regimen of R + other agents × no. of cycles*	*Evaluable patients*	*ORR*	*CR*	*PR*	*Median TTP*[a]	*Median OS*
Rituximab with concurrent chemotherapy phase II trials							
Czuczman et al. (1999, 2004)	R × 6 plus CHOP × 6	29 UT + 9 PT	100	87	13	82.3 months	
Czuczman et al. (2005)	R × 7 plus F × 6	27 UT + 13 PT	90	80	10	44+ months	44+ months
Hainsworth et al. (2005a)	R × 4 then R × 3 plus CHOP or CVP × 3 then R × 2	86 UT	93	55	38	4-year PFS: 62%	3-year OS: 95%
Martinelli et al. (2003)	R × 4 plus Chl × 6 wk, then R × 4 plus Chl × 4	15 UT + 12 PT	89	63	26		
McLaughlin et al. (2005)	R × 6 plus FND × 6	161 UT	100	88	12	4-year FFS: 70%	5-year OS: 89%
	FND × 6 then R (randomization)		96	85	11	4-year FFS: 59%	5-year OS: 86%
Economopoulos et al. (2003)	R × 6 plus CNOP × 6	42 UT	90	71	19	19.5+ months	19.5+ months
Rivas-Vera et al. (2005)	R × 6 plus CNOP × 6	144 UT	90	66	24	24+ months	24+ months
	CNOP × 6		83	63	22	24+ months	24+ months
	R × 6 (randomization)		85	49	36	24+ months	24+ months
Drapkin et al. (2003)	R × 1 then R plus P × 4 then R × 1 then R plus P × 4	43 UT + 16 PT	72	26	46	15 months	25+ months
Di Bella et al. (2005)	R plus P × 2 plus M × 1 then R × 2 plus P × 2 plus M (for total max 10 cycles)	24 UT	83	45	38	14 months	81% at 18 months

Table 13.2 *Continued*

Reference	*Regimen of R + other agents × no. of cycles*	*Evaluable patients*	*ORR*	*CR*	*PR*	*Median TTP*[a]	*Median OS*
Phase III trials							
Marcus et al. (2005);	R × 8 plus CVP × 8	162 UT	81***	41***	40	33.6 months***	3-year OS: 89%
Solal-Celigny et al. (2005)	CVP × 8 (randomization)	159 UT	57	10	47	14.5 months	3-year OS: 81%
Herold et al. (2004, 2005)	R × 8 plus MCP × 8	105 UT	92***	50***	42	2.5 year PFS:82%***	2.5 year OS: 89%**
	MCP × 8 (randomization)	96 UT	75	25	50	2.5 year PFS: 51%	2.5 year OS: 76%
Hiddemann et al. (2005b)	R × 6–8 plus CHOP × 6–8	222 UT	96*	20	76	Not reached***	2-year OS: 95%*
	CHOP × 6–8 (randomization)	205 UT	90	17	73	31 months (TTF)	2-year OS: 90%
Salles et al. (2004)	R × 6 plus CHVP-IFN × 6	184 UT	94***	76	18	2.5-year EFS: 78%**	
	CHVP-IFN × 6 (randomization)	175 UT	85	49	36	2.5-year EFS: 62%	
Chemotherapy followed by sequential rituximab phase II trials							
Cohen et al. (2002)	F plus C × 4–6, then R × 4	33 UT	88	85	3		
Emmanouilides et al. (2003)	C plus M × 2, then R plus M × 4	22 UT, 10 PT	90	72	19	30 (actuarial) months	
Gregory et al. (2002)	F plus M × 4–6, then R × 4	31 UT	97	45	52		
Jaeger et al. (2002)	CHOP × 3–8, then R × 4	41 UT	100	88	12		
Rambaldi et al. (2002, 2005)	CHOP × 6, then R × 4	77 UT	82	69	13		
Vitolo et al. (2004)	FND × 4, then R × 4	70 UT	90	83	7	3-year FFS: 50%	
Zinzani et al. (2004)	FM × 6, then R × 4[b]	72 UT	96	90	6	19+ months	19+ months
	CHOP × 6, then R × 4[b] (randomization)	68 UT	96	80	16	19+ months (PFS)	19+ months

Rituximab and immune system modulators						
McLaughlin et al. (2005)	R × 4 plus GM-CSF	14 UT + 25 PT	79	36	43	
Kimby et al. (2002)	R × 4 (induction) then R × 4	81 UT +	56	11	44	
	or R × 4 plus IFN-α-2a	45 PT	78	22	56	
	(randomization)	36	94	48	45	
		33				
Rituximab monotherapy						
Colombat et al. (2001); Solal-Celigny et al. (2004)	R × 4	49 UT	73	27	47	18 months (PFS)
Witzig et al. (2005)	R × 4	36 UT	72	36	36	2.2 years
Hainsworth et al. (2002)	R × 4, then further R × 4 at 6-month intervals for a total of 4 courses	60 UT	47	7	40	
Conconi et al. (2003)	R × 4	23 UT + 11 PT[c]	73	44	29	14.2 months (TTF)

All doses of R 375 mg m^{-2}.

a Or other parameter as indicated.

b R only given to patients with a bcl-2-positive CR or a PR after chemotherapy.

c Patients with extranodal marginal zone B-cell lymphoma of MALT type.

* $p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus comparator.

C = cyclophosphamide; Chl = chlorambucil; CR = complete response; CHOP = cyclophosphamide, doxorubicin, vincristine, prednisolone; CHVP-IFN = cyclophosphamide, doxorubicin, etoposide, prednisone, interferon-α-2a; CNOP = cyclophosphamide, mitoxantrone, vincristine, prednisolone; CLL = chronic lymphocytic leukemia; CVP = cyclophosphamide, vincristine, prednisolone; EFS = event-free survival; F = fludarabine; FFS = failure-free survival; FM = fludarabine, mitoxantrone; FND = fludarabine, mitoxantrone, dexamethasone; GM-CSF = granulocyte-macrophage colony-stimulating factor; HDMP = high-dose methyl prednisolone; M = mitoxantrone; MCP = mitoxantrone, chlorambucil, prednisolone; ORR = overall response rate; P = pentostatin; PFS = progression-free survival; PT = previously treated; PR = partial response; OS = overall survival; TTF = time to treatment failure; TTP = time to progression; UT = previously untreated.

trimethoprim/sulfamethoxazole was discontinued, the fludarabine dose was decreased by 40% in cases of prolonged cytopenia, and growth factor support was not used as prophylaxis. The hematological toxicity profile was improved in subsequently accrued patients, although grade 3/4 neutropenia was common (71%) and 24% of patients required transient growth factor support.

Pentostatin is another nucleoside analog which is less myelosuppressive than fludarabine. In a phase II trial of rituximab and pentostatin, responses were seen in 83% of previously untreated patients and 63% of pretreated patients. The median TTP was 15 months, and neutropenia was the only adverse event seen in ≥10% of patients (Drapkin et al. 2003). A further trial examined the use of these two products together with mitoxantrone (PMR) in the first-line setting. The ORR was 83% and the median response duration 10.0 months. Toxicities were increased with the PMR regimen compared with the PR regimen, with Grade 3/4 neutropenia occurring in 67% of patients. Sepsis and febrile neutropenia occurred in 8% and 17% of patients, respectively (Di Bella et al. 2005).

The safety and efficacy of rituximab in combination with chlorambucil was examined in newly diagnosed (n = 15) and relapsed/refractory (n = 14) low-grade and FL. Only one patient was withdrawn from the study due to progressive disease. No major neutropenic-related infections were observed, and no transfusion or growth factor support was required (Martinelli et al. 2003). This combination is currently being evaluated in a phase III trial by the International Extranodal Lymphoma Study Group (IELSG) in MALT lymphoma.

A phase II trial conducted between 1997 and 2002 randomized patients with previously untreated Stage IV indolent lymphoma and demonstrable bcl-2 rearrangement to either concurrent rituximab (six doses) and eight cycles of fludarabine, mitoxantrone and dexamethasone (FND) chemotherapy, or FND followed sequentially by rituximab (McLaughlin et al. 2005a). Maintenance interferon (IFN) was subsequently given in both treatment arms. There were no significant differences in ORR or CR rates between the two arms, but the concurrent regimen was associated with significantly higher molecular responses both at 6 and 12 months post therapy (89 versus 60%, $p < 0.01$ and 89 versus 68%, $p = 0.01$, respectively). Both regimens were well tolerated (McLaughlin et al. 2005a).

13.2.2.1.2 **Chemotherapy and Sequential Rituximab: Phase II Studies**

The sequential administration of fludarabine, mitoxantrone and dexamethasone (FND) and rituximab was evaluated in the US trial described above (McLaughlin et al. 2005a) and in an Italian study in elderly patients (aged >60 years) with advanced-stage FL (Vitolo et al. 2004). In this study, which assessed four cycles of FND followed by four infusions of rituximab, the ORR was 90%, with 83% CR. Patients with adverse prognostic features, such as bone marrow involvement, a poor International Prognostic Index (IPI) score and bulky disease responded as well to therapy as patients with a more favorable prognosis. The regimen was

well tolerated, with only three patients experiencing Grade 3/4 infections (Vitolo et al. 2004).

Several groups have examined the sequential administration of CHOP chemotherapy and rituximab (Table 13.2) (Jaeger et al. 2002; Zinzani et al. 2004; Rambaldi et al. 2005). Sequential rituximab improved response status, both in clinical and molecular terms in all studies. The trial conducted by Zinzani and colleagues randomized patients to either CHOP or fludarabine/mitoxantrone (FM) induction, followed in both cases by four doses of rituximab for patients who failed to achieve a bcl-2/IgH negative CR (CR–). The final CR– rate was higher in the FM arm than the CHOP arm, although no statistically significant differences in progression-free survival (PFS) or OS between treatment groups were observed (Zinzani et al. 2004). Gregory et al. (2002) have also shown that FM followed by rituximab is an effective treatment option, while the use of fludarabine and cyclophosphamide (FC) with sequential rituximab is another feasible therapeutic approach (Cohen et al. 2002).

Hainsworth and colleagues evaluated 4-weekly infusions of rituximab monotherapy followed by a short (three-cycle) course of CHOP or CVP and then two further infusions of rituximab in stage II–IV follicular lymphoma (Hainsworth et al. 2005a). Actuarial PFS at 4 years was 62%, and rates of neutropenia or fever were low (7%). A longer follow-up is required to determine whether the combination of rituximab with a shorter course of chemotherapy is as effective as longer-duration chemotherapy regimen combined with rituximab. This therapeutic approach could be particularly attractive for elderly or frail patients who tolerate chemotherapy poorly.

13.2.2.1.3 **Chemotherapy plus Rituximab: Phase III Studies**

Following the promise demonstrated by rituximab–chemotherapy combinations in phase II trials in indolent NHL, a number of prospective randomized phase III studies were initiated (Table 13.2). These studies were designed to evaluate the effect of the addition of rituximab to standard first-line chemotherapeutic regimens: CHOP (Hiddemann et al. 2005b), CVP (Marcus et al. 2005), mitoxantrone, chlorambucil, prednisolone (MCP) (Herold et al. 2004, 2005) or cyclophosphamide, doxorubicin, etoposide, prednisone plus interferon (CHVP-IFN) (Salles et al. 2004).

In the international trial evaluating the addition of eight cycles of rituximab to CVP chemotherapy, overall and complete response rates were highly significantly improved in the immunochemotherapy arm compared with the chemotherapy alone arm (81 versus 57%, $p < 0.0001$ and 41 versus 10%, $p < 0.0001$, respectively) (Marcus et al. 2005). A recently presented update after a median of 42 months' follow-up reveals that the increased response seen with R-CVP translates into highly durable and significant improvements in median TTP (33.6 versus 14.5 months, $p < 0.0001$) (Fig. 13.3), median time to next lymphoma treatment (TNLT) (46.3 versus 12.3 months, $p < 0.0001$), median DR (37.7 versus 13.5 months, $p < 0.0001$) and median DFS (44.8 versus 20.5 months, $p = 0.0005$) (Solal-Celigny et al. 2005). Median OS was not reached in both arms, but showed a strong trend

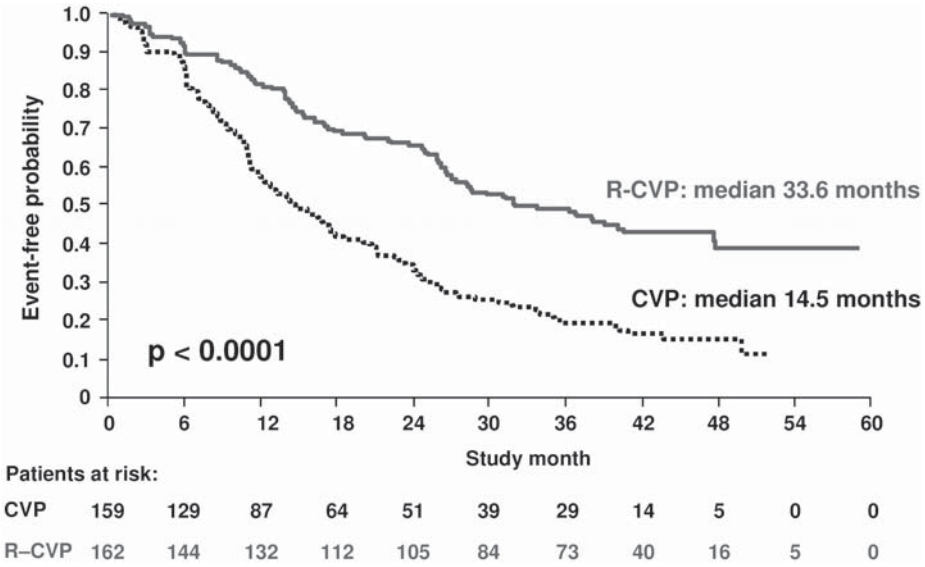

Fig. 13.3 Time to treatment progression after treatment with eight cycles of cyclophosphamide, vincristine, prednisone (CVP) chemotherapy or eight cycles of rituximab plus CVP chemotherapy in previously untreated follicular lymphoma. (Reproduced with permission from Solal-Celigny et al. 2005.)

towards an overall survival benefit for the rituximab-containing arm ($p = 0.0553$), while 3-year OS rates were estimated to be 81% in the CVP arm and 89% in the R-CVP arm. Notably, significantly more patients died due to lymphoma progression after CVP (25 deaths) than after R-CVP (12 deaths, $p = 0.02$) (Solal-Celigny et al. 2005).

A multivariate Cox regression analysis of outcomes according to baseline prognostic factors revealed that a significant improvement in outcomes was achieved with the addition of rituximab to CVP therapy, regardless of baseline patient or disease characteristics (Imrie et al. 2005). The incidence of Grade 3/4 neutropenia was higher during treatment with R-CVP (24%) than CVP (14%), but the incidence of infections and neutropenic sepsis was similar in the two groups (Marcus et al. 2005). The results of this trial led to EU regulatory approval of the R-CVP combination for first-line treatment of FL in 2004.

The German Low-Grade Lymphoma Study Group (GLSG) has conducted a randomized comparison of six or eight cycles of CHOP and R-CHOP in advanced-stage indolent FL (Hiddemann et al. 2005b). R-CHOP was superior with regard to ORR (96 versus 90%, $p = 0.011$) and TTF ($p < 0.001$). Importantly, OS was also significantly prolonged after immunochemotherapy compared with chemotherapy ($p = 0.016$). There was a 10% increase in granulocytopenia with R-CHOP compared with CHOP, but rates of infections and other therapy-associated adverse events were similar (Hiddemann et al. 2005b).

A further study conducted by Herold and colleagues in the Ostdeutsche Studiengruppe für Hämatologie und Onkologie (OSHO) evaluated the addition of eight cycles of rituximab to mitoxantrone, chlorambucil and prednisolone (MCP) chemotherapy in the first-line treatment of advanced FL (Table 13.2) (Herold et al. 2004, 2005). Once again, the addition of rituximab to chemotherapy significantly improved ORR (92 versus 75%, $p < 0.0001$) and CR rates (50 versus 25%, $p < 0.0003$) (Herold et al. 2004, 2005). After a median of 30 months' follow-up, this translated into significantly improved rates of event-free survival (EFS) (79 versus 44%, $p < 0.0001$), PFS (82 versus 51%, $p < 0.0001$) and, most importantly, OS (89 versus 76%, $p = 0.007$) (Fig. 13.4). Both R-MCP and MCP were well tolerated (Herold et al. 2004, 2005).

In the fourth randomized phase III trial, adding rituximab to the CHVP-IFN regimen led to a significant increase in ORR and CR/CRu rates (94 versus 85% and 76 versus 49%, $p < 0.0001$) (Salles et al. 2004). After a median follow-up of 30 months, the estimated 2.5-year EFS was 62% with CHVP-IFN and 78% with R-CHVP-IFN ($p = 0.003$) (Salles et al. 2004).

Taken together, these results show that rituximab has a significantly beneficial effect when added to initial chemotherapy in patients with advanced FL. Subanalyses indicate that the benefits are experienced irrespective of baseline characteristics, suggesting that an immunochemotherapy approach may overcome adverse prognostic factors in FL. Further studies are needed to establish optimal regimens for different types of patients with indolent NHL.

13.2.2.2 Rituximab plus Chemotherapy in Relapsed/Refractory Indolent NHL

Patients with relapsing/refractory indolent NHL have been traditionally treated with further courses of chemotherapy, which may include the use of non-cross-

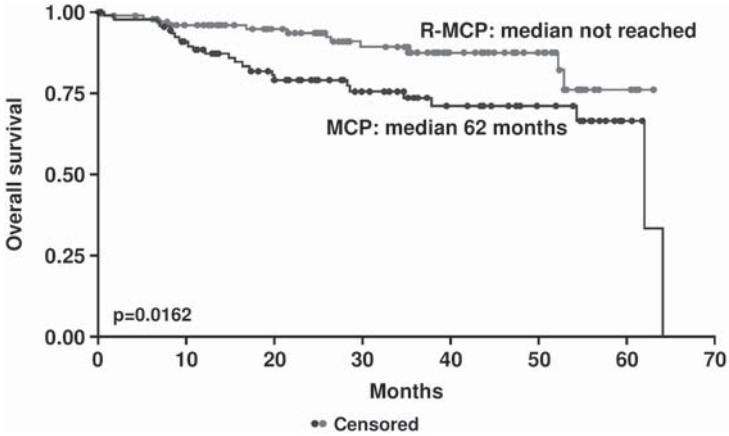

Fig. 13.4 Overall survival after treatment with eight cycles of mitoxantrone, chlorambucil, prednisolone (MCP) chemotherapy or eight cycles of rituximab plus MCP chemotherapy in previously untreated follicular lymphoma. (Reproduced with permission from Herold et al. 2004.)

resistant drugs not typically used during previous treatment. Younger, fitter patients may be eligible for ASCT and, in a small number of cases, for an allogeneic stem cell transplant (SCT). The combination of rituximab and chemotherapy is another therapeutic strategy in this setting.

13.2.2.2.1 Rituximab and Chemotherapy: Phase II Trials

As discussed above, phase II trials including patients with relapsed/refractory indolent NHL have assessed the safety and tolerability of a variety of regimens including R-CHOP (Czuczman et al. 1999, 2004), R-CNOP (Economopoulos et al. 2003), R-F (Czuczman et al. 2005), R-CM (Emmanouilides et al. 2003), R-pentostatin (Drapkin et al. 2003), and R-chlorambucil (Martinelli et al. 2003) (Table 13.2). Other investigators have also conducted phase II trial of rituximab together with chemotherapy in the relapsed setting (Table 13.3). Sacchi et al. (2003) assessed four cycles of FCR in 39 previously treated patients: ORR and CR rate according to ITT analysis were 94% and 75%, respectively and median duration of response after a median follow-up of 20 months was 26 months. There were 20 cases of Grade 3/4 neutropenia and 12 patients required treatment delays, indicating that this regimen requires careful management (Sacchi et al. 2003).

Rituximab has also been administered in combination with bendamustine (BR) (Rummel et al. 2005) and bendamustine and mitoxantrone (BMR) (Weide et al. 2004) in patients with relapsed/refractory indolent NHL (Table 13.3). These regimens are associated with high ORR and CR rates and, as expected, myelosuppression appears to be the major toxicity (Rummel et al. 2005; Weide et al. 2004). Other rituximab–chemotherapy regimens which have shown promise in phase II trials in this setting are R-CVP (Garcia-Conde et al. 2000), R-CHOP (Domingo-Domenech et al. 2002), and rituximab with vincristine and 5-day cyclophosphamide (Lazzarino et al. 2005) (Table 13.3).

13.2.2.2.2 Rituximab and Chemotherapy: Phase III Trials

In 1999, a phase III intergroup trial coordinated by the European Organization for Research and Treatment of Cancer (EORTC) Lymphoma Group was initiated by a number of groups in Europe, Canada, and Australasia. The EORTC 20891 study was designed to investigate first the effect of the addition of six doses of rituximab to six cycles of CHOP chemotherapy on the rate and quality of response in patients with relapsed FL, and second the effect of rituximab maintenance therapy (a single infusion every 3 months for 2 years) on progression-free survival (van Oers et al. 2006) (Table 13.3).

Patients who received R-CHOP induction experienced significantly improved ORR and CR rates compared with those who received CHOP induction (85 versus 72%, 29.5 versus 16%, $p < 0.0001$). This translated into significantly lengthened median PFS with R-CHOP compared with CHOP (33.1 versus 20.2 months, $p = 0.0003$, HR = 0.65) (Table 13.3). Rates of Grade 3/4 neutropenia were similar with R-CHOP and CHOP (54.7% and 48.2%, respectively). More patients on R-CHOP experienced grade 3 to 4 allergy (8 versus 0 individuals) or skin reactions

Table 13.3 Trials of rituximab (R) in induction therapy regimens in relapsed/refractory indolent NHL.

Reference	*Regimen of R + other agents × no. of cycles*	*Evaluable patients*	*ORR*	*CR*	*PR*	*Median TTP*[a]	*Median OS*
Rituximab with concurrent chemotherapy phase II trials							
Domingo-Domenech et al. (2002)	R × 6 plus CHOP × 6	16 PT	88	75	13		
Garcia-Conde et al. (2000)	R × 6 plus CVP × 6	32 PT	81	50	31		
Sacchi et al. (2003)	R × 4 plus F and C × 4	39 PT	94	75	19		
Rummel et al. (2005)	R × 1 then R plus B × 4 then R × 1	24 PT	96	71	25		
Weide et al. (2004)	R × 4 plus B and M × 4	19 PT	100	59	41	9+ months (PFS)	
Lazzarino et al. (2005)	P × 4 plus C and V × 4	29 PT	65	55	10	16.1 months (EFS)	
phase III trials							
van Oers et al. (2006)	R × 6 plus CHOP × 6	231 UT	85	29.5***	56	33.1 months***	3-year OS: 82.5%
	CHOP × 6 (randomization)	230 UT	72	16	57	20.2 months (PFS)	3-year OS: 71.9%
Forstpointner et al. (2004); Dreyling et al. (2005a)	R × 4 plus FCM × 4	67 UT	96*	39*	57	3.9 years*	4-year OS: 74%*
	FCM × 4 (randomization)		71	23	48	1.7 years (PFS)	median 3.8 years
Rituximab and immune system, modulators: phase I/II trials							
Davis et al. (2000a)	R × 4 plus IFN-α-2a	38 PT	45	11	34	8.95 months	
Sacchi et al. (2001)	R × 4 plus IFN-α	64 PT	70	33	37	19 months (DR)	3-year OS: 80%
Friedberg et al. (2002)	R × 4 plus IL-2	20 PT	55	5	50	13+ months (PFS: responders)	
Ansell et al. (2003)	R × 2 then R plus IL-2 × 2	43 PT + UT	67	25	42		
van der Kolk et al. (2003)	R × 4 plus G-CSF	26 PT	42	16	26	24 months	
Gluck et al. (2004)	R × 4 plus IL-2 daily	17 PT	29	6	24	14.9 months	
	R × 4 plus IL-2 thrice weekly	13 PT	46	38	23	16.1 months (responders)	

Table 13.3 *Continued*

Reference	*Regimen of R + other agents × no. of cycles*	*Evaluable patients*	*ORR*	*CR*	*PR*	*Median TTP*[a]	*Median OS*
Rituximab monotherapy							
Maloney et al. (1997b)	R × 4	37 PT	46	8	38	10.2 months	
McLaughlin et al. (1998)[b]	R × 4	151 PT	50	6	44	12.5 months	
Davis et al. (1999)	R × 4	28 PT[c]	43	4	39	8.1 months (responders)	
Davis et al. (2000b)	R × 4	57 PT	40	11	30	16.3 months	
Cortes-Funes et al. (2000)	R × 4	31 PT	62	23	39		
Feuring-Buske et al. (2000)	R × 4	30 PT	47	17	30	201 days	
Foran et al. (2000a)	R × 4	70 PT	46	3	43		
Igarashi et al. (2002)	R × 4	61 PT	61	23	38	245–376 days	
Martinelli et al. (2005)	R × 4	26 PT + UT[d]	77	46	31		28+ months
Piro et al. (1999)	R × 8	35 PT	60	14	46	19.4+ months (responders)	

All doses of R 375 mg m^{-2}.

a Or other parameter as indicated.

b Reanalysis according to Cheson criteria revealed ORR 56% and CR 32% (Grillo-Lopez et al. 2000).

c All patients had bulky disease (lesions >10 cm in diameter).

d All patients had histologically-proven gastric MALT lymphoma.

* $p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus comparator.

B = bendamustine; DR = duration of response; FCM = fludarabine, cyclophosphamide, mitoxantrone; G-CSF = granulocyte colony-stimulating factor; IFN = interferon; IL-12 = interleukin-12; V = vincristine. Other abbreviations as Table 13.2.

(31 versus 17), but rates of withdrawd from treatment because of taxicity were similar between groups (van Oers et al. 2006).

In a phase III trial conducted by the GLSG, a significant improvement in ORR and CR rate (96 versus 71%, p = 0.011 and 39 versus 23%, p = 0.013, respectively) occurred when rituximab was added to a regimen of fludarabine, cyclophosphamide and mitoxantrone (FCM) in patients with recurrent FL (Table 13.3) (Dreyling et al. 2005a; Forstpointner et al. 2004). Progression-free and overall survival were also significantly extended after R-FCM therapy compared with FCM therapy (median: 3.9 versus 1.7 years, p = 0.029 and 74% at 4 years versus a median of 3.8 years, p = 0.033) (Dreyling et al. 2005a).

13.2.2.3 Meta-Analysis of Rituximab and Chemotherapy in Indolent NHL

A meta-analysis to determine the effectiveness of combination chemotherapy plus rituximab versus combination chemotherapy alone, with respect to overall survival in indolent lymphoma, has been performed by the Cochrane Group (Schulz et al. 2005a). Medical databases were searched to identify randomized controlled trials of immunochemotherapy versus chemotherapy in this setting. Six eligible trials were identified as follows: rituximab plus CVP versus CVP alone in FL (Marcus et al. 2005; Solal-Celigny et al. 2005); rituximab plus MCP versus MCP alone in FL and MCL (Herold et al. 2004, 2005); rituximab plus CHOP versus CHOP alone in FL (Hiddemann et al. 2005b); rituximab plus CHOP versus CHOP alone in MCL (Lenz et al. 2005); rituximab plus FCM versus FCM alone in FL and MCL (Forstpointner et al. 2004); and rituximab plus CNOP versus CNOP alone versus rituximab alone (Rivas-Vera et al. 2005). The studies were combined and hazard ratios for overall survival were determined. The hazard ratio for all trials was 0.62 (95% CI: 0.49–0.77). For FL, the hazard ratio was 0.57 (0.43–0.77), and for MCL it was 0.60 (0.37–0.98). The authors stated that this preliminary meta-analysis demonstrated evidence for improved survival among patients with FL and MCL treated with rituximab plus chemotherapy compared with chemotherapy alone. Longer follow-up periods in existing trials and further randomized controlled trials are needed to improve the robustness of these data (Schulz et al. 2005a).

13.2.3 Induction Therapy with Rituximab plus Immune System Modulators in Indolent NHL

Not all patients are suitable for induction treatment with chemotherapy ± rituximab, particularly those who are elderly, have comorbid disease, or are unwilling to receive chemotherapy. An alternative therapeutic approach is the administration of rituximab in combination with immune system modulators, including IFN-α, interleukin (IL)-2, IL-12, and colony stimulating factors (CSFs). Immune modulators may increase the potency of rituximab by potentiation of CD20 expression, increasing Fc receptor density on effector cells or increasing numbers of effector cells (Dillman, 2003).

13.2.3.1 Rituximab plus Immune System Modulators

A regimen of four cycles of rituximab followed by an 8-week course of granulocyte-macrophage CSF (GM-CSF) was assessed in 39 evaluable patients with indolent NHL, 14 of whom were previously untreated (McLaughlin et al. 2005b). Overall and complete response rates were 79% and 36%, respectively. Tolerance of the rituximab plus GM-CSF regimen was comparable to that seen with rituximab alone. The investigators postulate that the addition of GM-CSF to rituximab enhances ADCC activity, which leads to an improved clinical response (McLaughlin et al. 2005b).

In a phase II study conducted in Denmark, Finland, Norway and Sweden, patients with symptomatic, advanced-stage CD20+ low-grade lymphoma (untreated or after first relapse) received rituximab once weekly for 4 weeks (Jurlander et al. 2004; Kimby et al. 2002). Patients with a PR or minor response (MR) were randomized to another four infusions of rituximab or to IFN-α-2a (3–4.5 MIU day^{-1}) before and during four infusions of rituximab. The ORR and CR rate in patients receiving two cycles of rituximab were 78% and 22%, respectively, and higher rates were observed in patients who received IFN-α-2a with the second cycle (94 and 48%, respectively) (Kimby et al. 2002). The frequency of MRD-negativity was 44% in patients who received a single cycle of rituximab, 66% in patients who received two cycles, and 77% in those who received two cycles of rituximab with IFN-α-2a. This trend towards a dose–response relationship was not significant due to the small number of patients in each group (Jurlander et al. 2004). The finding that one cycle of rituximab was less effective than two cycles, which in turn was less effective than two cycles plus IFN, suggests that the results with the combination regimen can be improved by dose intensification. A randomized phase III study is currently ongoing to further investigate this possibility.

13.2.3.2 Rituximab plus Immune Modulators in Relapsed/Refractory Indolent NHL

Two studies have examined the combination of rituximab and IFN-α-2a in relapsed/refractory indolent NHL (Davis et al. 2000a; Sacchi et al. 2001). Both demonstrated promising overall response rates (45% and 70%, respectively) and good tolerability (Table 13.3).

In a study of rituximab and IL-2, the ORR in 20 patients was 55%. The infusional toxicity associated with rituximab was not exacerbated by IL-2 (Friedberg et al. 2002). Gluck and colleagues (2004) found that a thrice-weekly IL-2 schedule in combination with rituximab was safe and effective. The combination of rituximab and IL-12 has also shown promising efficacy (ORR 67%, CR rate 25%), but was less well tolerated, with liver enzyme abnormalities and cytopenias being the most commonly observed toxicities (Ansell et al. 2003).

A trial of G-CSF with rituximab led to ORR and CR rates of 42% and 26%, respectively, and the median TTP was an encouraging 24 months (van der Kolk et al. 2003). The toxicity profile for the combination appeared similar to that reported for rituximab monotherapy (McLaughlin et al. 1998).

13.2.4 Induction with Rituximab Monotherapy in Indolent NHL

The earliest clinical studies of rituximab explored its use as monotherapy in relapsed/refractory indolent NHL. Following the encouraging results obtained in the relapsed setting, its efficacy and safety was subsequently examined in previously untreated patients.

13.2.4.1 Rituximab Monotherapy in Previously Untreated Indolent NHL

An early study of rituximab monotherapy as first-line treatment for indolent lymphoma enrolled patients with a low tumor burden FL (lesions ≤7 cm) (Colombat et al. 2001). Patients received four once-weekly doses of rituximab, whereby 36 of 49 evaluable patients (73%) responded, including 27% CR. In some patients, response improved over time, so that within the year following treatment the objective response rate rose to 80% with 40% CR. After 1 year, 16 of 26 patients (62%) were PCR-negative in the peripheral blood. Only two Grade 3 adverse events were reported – one case each of hypotension and hypertension. Long-term follow-up results from this trial were subsequently reported (Solal-Celigny et al. 2004). After a median follow-up of 60 months, the median PFS was 18 months and overall survival 94%.

Witzig et al. (2005) studied four once-weekly doses of rituximab in patients with previously untreated advanced Grade 1 FL. The ORR was 72% (36% CR) and the median TTP 2.2 years. Grade 3/4 adverse events occurred in 14% of patients, with 6% being considered possibly related to rituximab (one case each of neutropenia and urticaria/rash).

A further phase II study conducted by Hainsworth et al. (2002) examined the same induction schedule of rituximab in previously untreated patients with FL (61%) or SLL (39%). The ORR at 6 weeks was 47%, and only two patients had Grade 3/4 adverse events (one with chills/rigors, one with flushing/dyspnea/chest pain). Patients in this study received further maintenance courses of rituximab, and outcomes following further rituximab treatment are detailed below.

13.2.4.2 Rituximab Monotherapy in Relapsed Indolent NHL

The largest phase II study of rituximab in this setting led to the approval of rituximab for patients with relapsed/refractory indolent NHL in the US in 1997 and worldwide in 1998 (McLaughlin et al. 1998). In the analysis of 151 evaluable patients who received four once-weekly doses of rituximab, ORR was 50% with 6% CR. After a median follow-up period of 11.8 months, the projected median TTP for responders was 12.5 months. After a median follow-up period of more than 3 years, the median duration of remission was reported to be 11.2 months (McLaughlin et al. 1999). Re-evaluation of the study results following development of the Cheson criteria (Cheson et al. 1999) showed an ORR of 56% and CR of 32% (Grillo-Lopez et al. 2000). The toxicity profile demonstrated in this study has come to be recognized as characteristic for the product, and has been replicated in subsequent investigations (Kimby 2005). The majority of patients (71%)

experienced adverse events during the first infusion (usually Grade 1/2), but 55% remained free of any adverse events during infusions 2 to 4 (McLaughlin et al. 1998). The most common symptoms were transient fever, chills, nausea, asthenia, and headache. Thrombocytopenia and neutropenia occurred in less than 9% and 14% of patients, respectively, and infectious adverse events occurred in 30% (4% Grade 3).

A number of other smaller trials examined the safety and efficacy of four once-weekly doses of rituximab in relapsed/refractory NHL and are summarized in Table 13.3 (Conconi et al. 2003; Cortes-Funes et al. 2000; Davis et al. 1999, 2000b; Feuring-Buske et al. 2000; Foran et al. 2000a; Igarashi et al. 2002; Maloney et al. 1997b). Piro and colleagues (1999) examined an extended dosing schedule of eight once-weekly infusions and obtained an ORR of 60% and CR rate of 14%. Median TTP and response duration were not reached after 19.4+ and 13.4+ months, respectively. The authors reported that extending rituximab dosing neither delayed B-cell recovery, stimulated an increased incidence of HACA, nor resulted in an increased incidence of infections compared with a shorter rituximab treatment regimen.

13.2.5 Rituximab in Other Subtypes of Indolent Lymphoma

13.2.5.1 Rituximab in Marginal Zone Lymphoma

Conconi et al. (2003) investigated the clinical activity of rituximab in patients with previously untreated or relapsed MALT lymphoma. Rituximab monotherapy was effective and well tolerated, but the relapse rate (36%) was relatively high. Martinelli et al. (2005) studied rituximab in 27 patients with gastric MALT lymphoma, resistant to – or not eligible for – anti-*H. pylori* therapy. Twenty of 26 evaluable patients (77%) achieved an objective response, including 46% CR. After a median follow-up of 33 months, only two patients had relapsed, one of whom subsequently achieved a CR after treatment with rituximab and chlorambucil.

As discussed above, Martinelli et al. (2003) have also found the combination of rituximab and chlorambucil to be active and well tolerated in newly diagnosed and relapsed/refractory low-grade and FL. The study population included six patients with previously extranodal MZL. The results of the IELSG phase III trial evaluating this combination should be available in the next few years.

Bennett and co-workers (2005) performed a retrospective analysis of 11 patients with splenic MZL who received four cycles of rituximab therapy. Rituximab therapy was chosen because the patients were considered poor candidates for surgery, or had refused surgery. The instigation of rituximab resulted in prompt reduction in splenomegaly in nine of 10 patients, while a further patient with a pleural effusion demonstrated complete clearance of the effusion. Eight of the 10 responding patients had complete resolution of cytopenias. The median response duration was 21 months, and two patients who relapsed at 21 and 23 months responded to rituximab retreatment. These observations suggest that rituximab has considerable activity in splenic MZL, and could be an appropriate therapeutic

option in this disease, especially for elderly patients, or those with concomitant disease.

13.2.5.2 Rituximab in Small Lymphocytic Lymphoma

As discussed above, SLL and CLL represent different manifestations of the same disease. Treatment guidelines recommend that SLL and CLL be managed in the same way (National Comprehensive Cancer Network physician prescribing guidelines; see link on NCCN website, http://www,nccn.org). The pivotal phase II trial of rituximab in relapsed/refractory indolent NHL included 30 evaluable patients with SLL, who achieved an ORR of only 13% – markedly lower than the ORR of 60% seen in 118 evaluable patients with FL (McLaughlin et al. 1998). Another early study of four cycles of rituximab monotherapy in newly diagnosed and relapsed B-cell malignancies included 29 patients with relapsed/refractory SLL (Foran et al. 2000b). The ORR was only 14%, and the authors speculated that this might be explained by the low density of cell-surface CD20 in SLL. The use of a thrice-weekly dosing schedule of rituximab led to a somewhat higher objective response in three of seven patients with SLL (43%) (Byrd et al. 2001).

A study of rituximab in combination with cyclophosphamide and mitoxantrone included five patients with SLL/CLL (Emmanouilides et al. 2003). One patient achieved a CR and two a PR, giving an ORR of 60%. Hainsworth and colleagues assessed the use of rituximab monotherapy as maintenance therapy (four once-weekly infusions at 6-month intervals for 2 years) in patients with previously untreated CLL/SLL (Hainsworth et al. 2003a). The outcome in five patients with SLL was not reported separately. The ORR and CR rates in 44 patients were 58% and 9%, respectively, and the estimated median progression-free time was 19 months.

13.2.5.3 Rituximab in Waldenström's Macroglobulinemia

A number of investigators have studied the efficacy and safety of rituximab, either alone or in combination with other agents, in WM. In the largest study of single-agent rituximab, four once-weekly doses in 69 patients led to an ORR of 27% (Gertz et al. 2004). Extended rituximab dosing (eight infusions) has also been evaluated and ORRs of 27 to 44% were seen (Dimopoulos et al. 2002; Treon et al. 2001, 2005a). The use of rituximab in combination with chemotherapy appears to be a more effective treatment strategy in WM. For example, the use of PCR was associated with an ORR of 77% (Hensel et al. 2005), 85% of patients responded to R-CHOP therapy (Treon et al. 2005b), and the combination of rituximab, cladribine and cyclophosphamide was associated with an ORR of 94% (Weber et al. 2003). Further studies are currently under way to more fully assess the role of rituximab in this disorder.

The Third International Workshop on WM recently reported updated treatment recommendations for this disease (Treon et al. 2006). For patients with previously untreated or relapsed WM, appropriate therapeutic options include rituximab monotherapy or rituximab together with nucleoside analogs, alkylating agents or combination chemotherapeutic regimens (Treon et al. 2006).

13.2.6 Rituximab Maintenance Therapy

As discussed above, the typical course of indolent NHL is one of repeated relapses and remissions. While recent advances in NHL treatment have improved the outlook for many patients, there remain considerable unmet needs. Strategies for extending remission duration in indolent NHL without significantly increasing toxicity are eagerly sought. One approach that has the potential to do this is the use of maintenance therapy in patients who have responded to initial induction therapy.

Maintenance therapy with intermittent chemotherapy (Ezdinli et al. 1987; Stewart et al. 1988) and interferon (Rohatiner et al. 2005) has not been shown to alter the natural history of the disease. Single-agent rituximab may highly suitable for use as a maintenance therapy, as it is effective and very well tolerated. A number of investigators have evaluated different schedules of maintenance therapy in recent years (Table 13.4). Further investigation of rituximab maintenance, including its safety and efficacy and optimal duration of therapy, is currently under way in prospective randomized trials. These include the Primary RItuximab and MAintenance (PRIMA) and OSHO/GLSG trials comparing rituximab maintenance with observation after immunochemotherapy induction in previously untreated indolent NHL.

13.2.6.1 Rituximab Maintenance Therapy Following Monotherapy Induction

In a study conducted by Ghielmini and colleagues (2004), patients with previously untreated or relapsed/refractory FL who did not progress after the standard (4-week) rituximab regimen either received maintenance rituximab (single infusion at 3, 5, 7, and 9 months) or were observed without further rituximab infusions. After a median follow-up of 35 months, EFS was significantly prolonged in patients who had received rituximab maintenance therapy compared with nontreated patients (23 versus 12 months; $p = 0.024$); this effect was more pronounced in chemotherapy-naïve patients (36 versus 19 months, $p = 0.009$) (Ghielmini et al. 2004). The median duration of response was also longer after maintenance therapy than after no further treatment, both in previously untreated patients and in those with previously treated disease (NR versus 20 months, $p = 0.079$ and 25 versus 13 months, $p = 0.065$, respectively) (Table 13.4). Rituximab was well tolerated, with the incidence of late toxicity in patients evaluable beyond 1 year being only 7% in both treatment arms (Ghielmini et al. 2004).

In a phase II study of 62 patients with previously untreated but symptomatic indolent lymphoma, Hainsworth et al. (2002) investigated the impact of rituximab maintenance therapy in patients who had not progressed following four once-weekly doses of rituximab. Maintenance therapy was administered according to a schedule of four once-weekly doses at 6-monthly intervals for a maximum of four courses, or until disease progression. Following maintenance therapy, 16 of the 27 patients who had SD after induction therapy achieved a response, with ORR increasing from 47% to 73% (the CR increased from 4% to 37%)

Table 13.4 Trials of rituximab (R) maintenance therapy in patients with indolent NHL.

Reference	*Regimen of induction (I) and maintenance (M) therapy*	*Evaluable patients*	*Median follow-up*	*Key outcomes of maintenance arms R-M vs. Obs (unless otherwise stated)*		
				Median PFS[a]	*Median DR*	*Median OS*
Rituximab maintenance after rituximab monotherapy						
Ghielmini et al. (2004)	I: R × 4 M: R × 1 every 2 months for 4 doses vs. Obs	51 UT + 100 PT	35 months	23* vs. 12 months (EFS) 36** vs. 19 months (EFS for UT pts)	35+ vs. 20 months (UT pts) 25 vs. 13 months (PT pts)	
Hainsworth et al. (2002, 2003b)	I: R × 4 M: R × 4, every 6 months for 2 years	60 UT	55 months	37 months		5-year actuarial OS: 70%
Hainsworth et al. (2005b)	I: R × 4 M: R × 4, every 6 months for 2 years vs. retreatment at progression with R × 4	90 PT	41 months	31** vs. 7 months		3-year OS: 72 vs. 68%
Rituximab maintenance after chemotherapy						
Hochster et al. (2004)	I: CVP × 6–8 M: R × 4 every 6 months for 2yrs vs. Obs	120 UT (FL) 117 UT (FL)	42 months	61*** vs. 15 months		3.5 year OS: 91* vs. 75%

Table 13.4 *Continued*

Reference	*Regimen of induction (I) and maintenance (M) therapy*	*Evaluable patients*	*Median follow-up*	*Key outcomes of maintenance arms R-M vs. Obs (unless otherwise stated)*		
				Median PFS[a]	*Median DR*	*Median OS*
Rituximab maintenance after (immuno)chemotherapy						
van Oers et al. (2006)	I: CHOP × 6 vs. R × 6 + CHOP × 6 M: R × 1 every 3 months for 2 years vs. Obs	159 PT 160 PT	36 months	52*** vs. 15 months; 42*** vs. 12 months (post-CHOP induction); 52** vs. 23 months (post-R-CHOP induction)		3-year OS: 85* vs. 77%;
Forstpointner et al. (2006)	I: FCM × 4 vs. R × 4 plus FCM × 4 M: R × 4, 3 and 9 months after induction vs. Obs	176 PT (FL and MCL)	26 months		26+*** vs. 17 months; (post R-FCM induction)	

All doses of R 375 mg m^{-2}.

a Or other parameter as indicated.

* $p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus comparator.

FCM = fludarabine, cyclophosphamide, mitoxantrone; FL = follicular lymphoma; I = induction; M = maintenance; MCL = mantle cell lymphoma; MCP = mitoxantrone, chlorambucil, prednisolone; NR = not reached; Obs = observation; pts = patients. Other abbreviations as Table 13.2.

(Hainsworth et al. 2002). After a median of 55 months' follow-up, the median PFS was 37 months and the 5-year PFS and OS rates were 24% and 70%, respectively (Hainsworth et al. 2003b). Rituximab therapy was well tolerated, and rituximab maintenance was not associated with any Grade 3/4 toxicity. No cumulative or late toxicity was observed and there were no opportunistic infections (Hainsworth et al. 2002, 2003b).

In a further phase II trial, patients with relapsed or refractory indolent NHL responding to, or with stable disease after four weekly doses of rituximab therapy, were randomized to rituximab maintenance (four weekly doses every 6 months for a total of four occasions) or to retreatment with four weekly doses of rituximab at disease progression (Hainsworth et al. 2005b). The median PFS was significantly prolonged in the rituximab maintenance group compared with those receiving rituximab retreatment (31 versus 7 months, $p = 0.007$) (Table 13.4; Fig. 13.5). However, the median duration of rituximab benefit and the 3-year survival rates were similar for patients receiving rituximab maintenance and rituximab retreatment (31 versus 27 months, $p = 0.94$ and 72 versus 68%, respectively) (Hainsworth et al. 2005b). Both treatment arms were well tolerated, and there were no treatment-related hospitalizations or patient discontinuations because of therapy-related adverse effects.

13.2.6.2 Rituximab Maintenance Therapy Following Chemotherapy Induction

Extremely promising results have been obtained in an ongoing Eastern Cooperative Oncology Group (ECOG) phase III study in which patients with advanced indolent NHL responding to CVP chemotherapy were randomized to receive rituximab maintenance or observation only (Hochster et al. 2004, 2005). The majority of

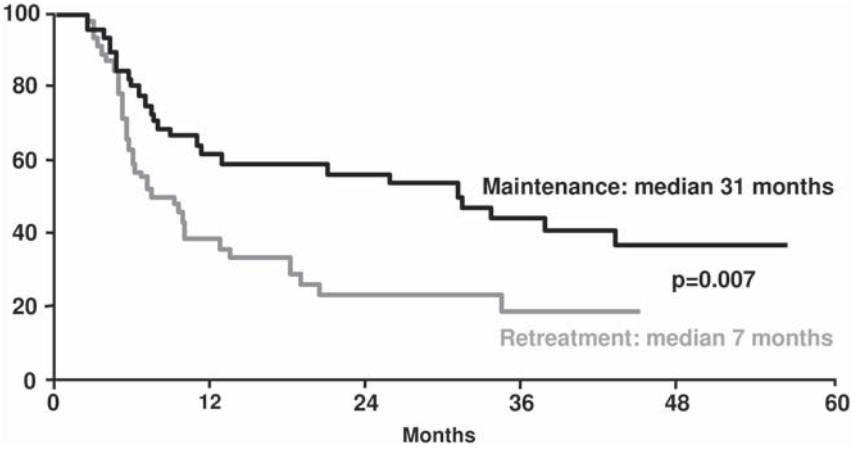

Fig. 13.5 Progression-free survival after rituximab maintenance therapy or retreatment with rituximab at progression following rituximab induction therapy for relapsed/refractory indolent NHL: the Minnie Pearl Cancer Research Network trial. (Reproduced with permission from Hainsworth et al. 2005b.)

patients studied (237/305) had FL. Patients randomized to maintenance had a significantly extended PFS (61 versus 15 months, p = 0.0000003, HR = 0.4) and OS (91 versus 75%, p = 0.03, HR = 0.5) compared to those randomized to observation. The improvements in PFS were evident regardless of FLIPI score, tumor burden or extent of residual disease (Hochster et al. 2005). Rituximab maintenance therapy was well tolerated, and did not lead to significantly higher rates of neutropenia, thrombocytopenia or infection compared with observation (Hochster et al. 2004).

13.2.6.3 Rituximab Maintenance Therapy Following Rituximab Chemotherapy Induction

As discussed above, the second primary objective of the EORTC 20891 study was to evaluate the effect of rituximab maintenance therapy (a single infusion every 3 months for 2 years) on progression-free survival (van Oers et al. 2006). Median PFS was significantly improved after maintenance therapy compared with observation (51.6 versus 15 months, $p < 0.0001$, HR = 0.4) and importantly, these improvements were seen both in patients treated with CHOP induction (42.2 versus 11.6 months, $p < 0.0001$, HR = 0.3) and R-CHOP induction (51.9 versus 23.1 months, p = 0.0043, HR = 0.54). Median OS was also significantly extended after maintenance compared with observation: 3-year OS rates 85.1 versus 77.1%, p = 0.0111, HR = 0.52. Grade 3/4 neutropenia rates were slightly higher in the maintenance arm (10.8 versus 5.4%), as were Grade 3/4 infection rates (9 versus 2.4%) (van Oers et al. 2006). This study formed the basis of the submission for approval of rituximab for maintenance therapy made to the EMEA in 2006.

The phase III GLSG trial of FCM versus R-FCM in relapsed FL discussed above also included a second randomization to rituximab maintenance (four weekly doses at 3 and 9 months post-induction) or observation only (Table 13.4). The median response duration after rituximab maintenance therapy was significantly longer than after observation only in 176 evaluable patients with FL and MCL (NR at 26 months versus 17 months, $p < 0.001$) (Table 13.4). The significant improvement in response duration for patients receiving rituximab maintenance therapy was also seen in the subgroup of FL patients who had received R-FCM induction therapy (n = 81) (NR at 26 months versus 26 months, p = 0.035) and those with MCL receiving R-FCM induction therapy (n = 47) (p = 0.049) (Table 13.4). This suggests that rituximab maintenance can benefit all patients with FL and MCL, regardless of whether or not they have received rituximab as part of their induction therapy (Forstpointner et al. 2006).

13.2.7 Rituximab Retreatment

Retreatment with rituximab – either alone or in combination with chemotherapy – appears to be an efficient treatment strategy. An early study conducted by Davis et al. (2000b) in patients with relapsed indolent NHL observed an ORR of 38% after retreatment of 58 patients with rituximab, and TTP for responders at retreat-

ment was longer than that observed after initial therapy (17.4 versus 12.4 months). Retreatment was not associated with induction of HACA or cumulative myelosuppression. Igarashi and colleagues (2001) also observed an ORR of 38% in 13 patients with relapsed indolent NHL receiving a second course of rituximab, which was well tolerated.

Lemieux et al. (2004), at the CHU-Lyon-Sud, subsequently reported the effect of a second treatment of rituximab either alone or in combination with chemotherapy in patients with a variety of lymphoma subtypes, the majority (82%) of which were indolent. The ORR was 73%, and median TTP was longer for the second treatment than the first (15.2 versus 11.3 months). The second treatment was well tolerated.

In the study of BMR in relapsed/refractory indolent NHL discussed above (Weide et al. 2004), ORR and CR rates were as good in 16 patients previously treated with rituximab as in the full study population (88 versus 94% and 56 versus 44%, respectively). Recently, Bairey and associates (2005) retrospectively evaluated rituximab retreatment in 66 patients with indolent NHL, 18 with aggressive NHL and six with MCL. The ORR to the second rituximab treatment (with or without chemotherapy) was 71%, including 39% CR. Median TTP after the second treatment was similar to that seen after the first (14 versus 12 months). In a Spanish retrospective analysis of patients with DLBCL relapsing after rituximab, the ORR to a second course of rituximab ± chemotherapy in 25 assessable patients was 92% (56% CR) (Canales et al. 2005). Nine patients received a third course of rituximab ± chemotherapy and yielded an ORR of 78% (33% CR). Adverse events following retreatment were described as not being different to those usually observed with these regimens.

As discussed above, in a phase II trial comparing rituximab maintenance (four weekly doses every 6 months for a total of four occasions) and retreatment (four weekly doses of rituximab at disease progression), median PFS was significantly prolonged in the rituximab maintenance group compared with the rituximab retreatment group (31 versus 7 months, $p = 0.007$) (Table 13.4; Fig. 13.5) (Hainsworth et al. 2005b). However, the median duration of rituximab benefit and the 3-year survival rates were similar for patients receiving rituximab maintenance and rituximab retreatment (31 versus 27 months, $p = 0.94$ and 72 versus 68%, respectively) (Hainsworth et al. 2005b).

Taken together, these observations suggest that rituximab retreatment is safe and feasible in patients with relapsed B-cell NHL. Prospective studies of rituximab retreatment are currently in progress.

13.2.8
Rituximab in Aggressive NHL

For decades, aggressive NHL was considered an incurable disease, but during the 1960s the development of anthracycline-containing combination chemotherapies meant that cure finally became an achievable goal for some patients (DeVita et al. 1975). CHOP (cyclophosphamide, doxorubicin, vincristine and predniso-

lone) has been extensively studied in randomized clinical trials, but is associated with a cure rate of only 30 to 40% in nonlocalized aggressive NHL (Fisher 1994), leaving much room for improvement.

Despite early optimism, second- and third-generation chemotherapy regimens were unable to improve on overall survival compared with CHOP; moreover, the toxicity levels and costs of these newer regimens were higher (Fisher et al. 1994). A reduction of the CHOP treatment interval from 21 to 14 days is also an option. In both elderly and young patients with good-prognosis disease, an interval reduction has been shown to result in a modest improvement in overall survival, but at the cost of increased toxicity in elderly patients (Pfreundschuh et al. 2004a,b).

High-dose therapy (HDT) followed by ASCT has been shown to improve survival compared with chemotherapy when used as front-line therapy (Milpied et al. 2004), and to consolidate complete remission after front-line chemotherapy in younger patients with poor-risk aggressive NHL (Haioun et al. 2000). While intensive chemotherapy regimens with or without ASCT may improve outcomes for younger healthier patients, the morbidity/mortality associated with this strategy makes it generally unsuitable for patients aged over 60 years (who comprise more than half of all patients with the most common form of aggressive NHL, DLBCL) (The Non-Hodgkin's Lymphoma Classification Project, 1997a,b).

The successful use of rituximab in patients with indolent NHL prompted its further evaluation in aggressive NHL. Early success in a phase II trial of R-CHOP led to the initiation of multicenter phase III trials assessing the impact of adding rituximab to CHOP/CHOP-like chemotherapy in patients with DLBCL. Rituximab has subsequently been evaluated with a number of different treatment regimens both in previously untreated (Table 13.5) and relapsed/refractory aggressive NHL (Table 13.6).

13.2.8.1 Rituximab plus Chemotherapy in Previously Untreated Aggressive NHL: Phase II Studies

An early study of R-CHOP in aggressive NHL produced extremely encouraging results (Vose et al. 2001, 2002). Patients with newly diagnosed, aggressive lymphoma were treated with six cycles of R-CHOP every 21 days. The ORR was 94%, with 61% patients achieving a CR. Even poor-prognosis patients (IPI score ≥2) had an ORR of 89% (Vose et al. 2001). These excellent results were achieved without adding significantly to the toxicity of CHOP chemotherapy. Even more encouragingly, the presence or absence of bcl-2, an anti-apoptotic protein which is often overexpressed in NHL cells and has been associated with poor prognosis in chemotherapy-treated aggressive NHL patients (Hermine et al. 1996), did not significantly affect the CR rate, and nor did patient age (Vose et al. 2001). Recently published long-term follow-up data reveal 5-year PFS and OS rates of 82% and 88%, respectively and no long-term adverse events directly related to rituximab were observed (Vose et al. 2005).

The combination of rituximab and VNCOP-B (etoposide, mitoxantrone, cyclophosphamide, vincristine, prednisolone, bleomycin) – a chemotherapy regimen developed to treat elderly patients for whom CHOP chemotherapy was not feasible

Table 13.5 Trials of rituximab (R) in previously untreated aggressive NHL.

Reference	*Regimen of R plus other agents × no. of cycles*	*Patients*	*ORR*	*CR*	*PR*	*Median PFS*[a]	*Median OS*
phase II trials							
Vose et al. (2002, 2005)	R × 6 plus CHOP × 6	33 UT[b]	94	61	33	5-year PFS: 82%	5-year OS: 88%
Rodriguez et al. (2005)	R × 6–8 plus CHOP[c] × 6–8	68 UT[d]	93	91	2		OS: 94%
Canales et al. (2005)	R × 6–8 plus CHOP/CHOP-like chemo × 6–8	80 UT	86	72	14	2-year EFS: 60%	2-year OS: 73%
Hainsworth et al. (2003c)	R × 7 plus VNCOP-B × 8	27 UT	93	37	56	21 months+	21 months+
Wilson et al. (2005)	R-DA-EPOCH + filgrastim × 6–8	60 UT 11 PT[e]	100	68	32	1.5-year PFS: 80%	1.5-year OS: 88%
Lavilla et al. (2005)	R × 6 plus CHOP-14 × 6	28 UT[g]	96	89	7		
Rigacci et al. (2006)	R × 6 plus CHOP-14 × 6	26 UT	100	77	23	DFS: 70% after 17 months	OS: 79% after 23 months
Glass et al. (2005)	R × 6 plus MegaCHOEP × 4 MegaCHOEP × 4 (historical controls)	72 UT 35 UT				3-year FFTF: 70%* 50%	3-year OS: 75% 57%
Intragumtornchai et al. (2006)	R × 7 + CHOP × 3 plus ESHAP × 4	84 UT		67	14	5-year FFS:	5-year OS:
	CHOP × 3 plus ESHAP × 4 plus HDT			44	22	61%***	61%
	CHOP × 8 (randomization)			36	24	34% 16%	43% 24%
phase III trials							
Coiffier et al. (2005);	R × 8 plus CHOP × 8	197 UT	83	76**	8	5+ years***	5+ years**
Feugier et al. (2005)	CHOP × 8 (randomization)	202 UT	69	63	6	1 year	3.1 years

Table 13.5 *Continued*

Reference	*Regimen of R plus other agents × no. of cycles*	*Patients*	*ORR*	*CR*	*PR*	*Median PFS*[a]	*Median OS*
Pfreundschuh et al. (2006)	R × 6 plus CHOP/CHOP-like chemo × 6	413 UT		86***		3-year EFS:	3-year OS:
	CHOP/CHOP-like chemo × 6 (randomization)	410 UT		68		79%*** 59%	93%*** 84%
Habermann et al. (2006)	R × 4–5 plus CHOP × 6–8	318 UT	77	32	42	3-year FFS:	3-year OS:
	CHOP × 6–8 (randomization)	314 UT	76	31	41	53%* 46%	67%* 58%
Sonneveld et al. (2005)	R × 6 plus CHOP-14 × 8	99 UT[h]		34		2-year FFS:	2-year OS:
	CHOP-14 × 8 (randomization)	98 UT[i]		30		51%** 23%	63%* 46%
Pfreundschuh et al. (2005b)	R × 8 plus CHOP-14 × 8	203 UT				70%	74% for 8 × R plus 6–8 × CHOP-14
	R × 8 plus CHOP-14 × 6	211 UT				70%	
	CHOP-14 × 8	210 UT				58%	
	CHOP-14 × 6 (randomization)	203 UT				53% (TTF)	78% for 6–8 × CHOP-14

All doses of R 375 mg m^{-2}.

a Or other parameter as indicated.

b Included 7 patients with follicular large cell lymphoma, 2 with immunoblastic lymphoma, and 2 with other aggressive lymphomas.

c Regimen contains sphingosomal vincristine in place of free vincristine.

d 56 patients had DLBCL, 4 Grade, 3 FL, 4 PTCL, 4 anaplastic large cell lymphoma, and 2 transformed indolent lymphoma.

e Included a few patients with PMBCL and aggressive B-cell lymphoma, unspecified.

f Included 7 patients with Grade 3 FL.

g Included 3 patient with Grade 3 FL.

h Included 15 patients with MCL and 6 with Grade 3 FL.

i Included 14 patients with MCL and 3 with Grade 3 FL.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus comparator.

CHOP-14 = 2-week cycles of CHOP; DFS = disease-free survival; EPOCH = etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin; ESHAP = etoposide, solumedrol, cytosine arabinoside, cisplatin; FFS = failure-free survival; FFTF = freedom from treatment failure; HDT = high-dose therapy; MegaCHOEP = cyclophosphamide, adriamycin, vincristine, etoposide, prednisone; PFS = progression-free survival; VNCOP = etoposide, mitoxantrone, cyclophosphamide, vincristine, prednisolone, bleomycin. Other abbreviations as Table 13.2.

Table 13.6 Trials of rituximab (R) in relapsed/refractory aggressive NHL.

Reference	*Regimen of R plus other agents × no. of cycles*	*Patients*	*ORR*	*CR*	*PR*	*Median PFS*[a]	*Median OS*
Trials of rituximab plus chemotherapy							
Kewalramani et al. (2004)	R × 4 plus ICE × 3	36 PT	78	53	25		
Mey et al. (2006)	R × 4 plus DHAP × 4	53 PT	62	32	30	6.7 months	8.5 months
El Gnaoui et al. (2005)	R × 4 plus GEMOX × 4	40 PT[b]	85	53	32	20 months (TTP)	
Jermann et al. (2004)	R × 4–6 plus EPOCH × 4–6	50 PT[c]	68	28	40	11.8 months (EFS)	17.9 months
Sirohi et al. (2005)	R × 4 plus GEM-P × 4	24 PT	67			1-year PFS: 51%	3-year OS: 65%
Hicks et al. (2005)	R × 8 plus ESHAP × 2–4	20 PT[d]	89			9+ months	9+ months
Venugopal et al. (2004)	R × 6 plus ESHAP × 6	14 PT[e]	64	50	14		
Trials of rituximab monotherapy							
Coiffier et al. (1998)	R × 8	45 PT +	31	9	22	105+ days (TTP)	
	R × 1 then R 500 × 7	9 UT[f]				121+ days (TTP)	
Tobinai et al. (2004)	R × 8	57 PT	37	26	11	52 days	
Rothe et al. (2004)	R × 4	21 PT	38	5	33	3.8 months (EFS)	8.6 months

All doses of R 375 g m^{-2} unless otherwise indicated.

a Or other parameter as indicated.

b 16 patients with DLBCL, 6 with FL and 2 with MCL.

c 25 patients with DLBCL, 18 with transformed large B-cell lymphoma and 7 with MCL.

d 9 patients with relapsed aggressive lymphoma, 2 with refractory aggressive lymphoma and 9 with transformed indolent lymphoma.

e Predominantly DLBCL patients.

f Includes a few patients with MCL, FL or unclassified histology.

DHAP = dexamethasone, cytarabine, cisplatin; DOC = docetaxel; EPOCH = etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin; ESHAP = etoposide, solumedrol, cytosine arabinoside, cisplatin; GEMOX = gemcitabine, oxaliplatin; GEM-P = gemcitabine, cisplatin, methylprednisolone; ICE = ifosfamide, carboplatin, etoposide; PAC = paclitaxel; T = topotecan. Other abbreviations as Table 13.2.

(Zinzani et al. 1999) – is also effective. The treatment of 41 elderly patients, the majority of whom had an age-adjusted IPI score of 2 or 3, with rituximab plus VNCOP-B resulted in an ORR of 66% and 2-year PFS and OS rates of 59% and 57%, respectively (Hainsworth et al. 2003c).

Another effective regimen for patients with aggressive NHL is dose-adjusted EPOCH (etoposide, vincristine, doxorubicin, cyclophosphamide, prednisolone) chemotherapy (DA-EPOCH). In this regimen the doses of doxorubicin, etoposide and cyclophosphamide are adjusted to achieve neutrophil nadirs of $<500\,mm^{-3}$. However, treatment with DA-EPOCH produces a significantly worse outcome for patients overexpressing bcl-2 compared with those expressing normal levels of this protein (Wilson et al. 2002, 2003).

The addition of rituximab to DA-EPOCH may overcome the adverse outcome both in DLBCL patients overexpressing bcl-2 and in those with high levels of p53 (another anti-apoptotic protein; Wilson et al. 2003). Furthermore, rituximab plus DA-EPOCH has recently been shown to be effective in all subtypes of *de-novo* DLBCL (Wilson et al. 2005), reinforcing the value of rituximab in combination with DA-EPOCH in this clinical setting.

13.2.8.2 Rituximab plus Chemotherapy in Previously Untreated DLBCL: Phase III Studies and Population Analysis

13.2.8.2.1 The GELA LNH98-5 Trial

The phase III randomized controlled study LNH98-5 conducted by the Groupe d'Etude des Lymphomes de l'Adulte (GELA), randomized 399 elderly patients (aged 60–80 years) with DLCBL to treatment either with eight cycles of CHOP or eight cycles of R-CHOP. A significant number of patients had high-risk disease; 60% of patients had IPI scores ≥2 and 65% had elevated lactate dehydrogenase (LDH) levels.

After eight cycles of treatment, the ORRs for patients treated with R-CHOP and CHOP were 83% and 69%, respectively, with significantly more R-CHOP-treated patients achieving a CR compared with those receiving CHOP alone (76 versus 63%; $p = 0.005$) (Coiffier et al. 2002). This higher CR rate translated into substantially improved long-term outcomes for patients treated with R-CHOP compared with those receiving CHOP alone (Coiffier et al. 2002; Feugier et al. 2005). With a median follow-up of 5 years, DFS, PFS (Fig. 13.6) and OS were all significantly prolonged in patients receiving R-CHOP compared with those receiving CHOP alone (Feugier et al. 2005).

Subgroup analysis demonstrates that R-CHOP confers significant benefits compared with CHOP alone regardless of age, co-morbidity or IPI score (Coiffier et al. 2002, 2003; Feugier et al. 2005). Furthermore, adding rituximab to CHOP chemotherapy appears to overcome chemotherapy failure associated with bcl-2 overexpression (Mounier et al. 2003). There was no significant difference in clinically relevant toxicity, late toxicities or the incidence of secondary tumors between patients treated with R-CHOP and those receiving CHOP (Coiffier et al. 2002; Feugier et al. 2005).

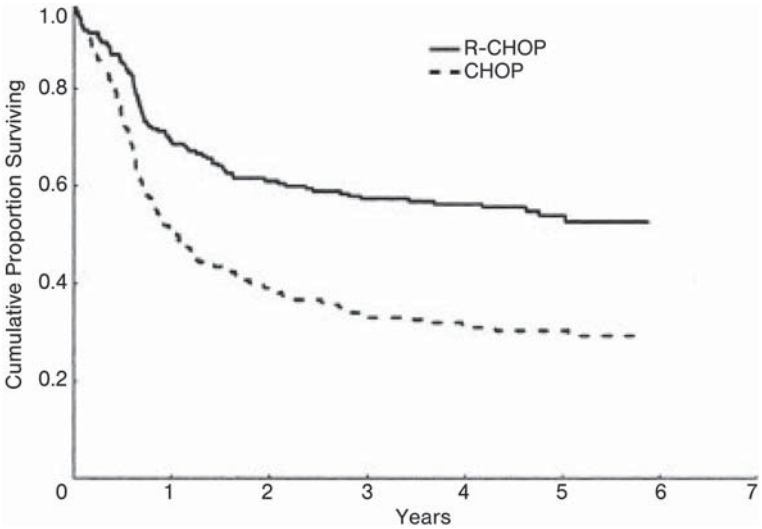

Fig. 13.6 Progression-free survival after treatment with eight cycles of CHOP chemotherapy or eight cycles of rituximab plus CHOP chemotherapy in elderly patients with previously untreated aggressive lymphoma: Groupe d'Etudes des Lymphomes de l'Adultes (GELA) LNH 98-5 trial. (Reproduced with permission from Feugier et al. 2005.)

13.2.8.2.2 **The Intergroup E4494 Trial**

Further support for the use of R-CHOP as first-line therapy in elderly patients with aggressive lymphoma in elderly patients comes from an Intergroup study performed in the US (Habermann et al. 2006). Patients aged at least 60 years were randomized to receive either R-CHOP or CHOP and were then randomized to rituximab maintenance therapy or observation only. The study was not designed to compare R-CHOP and CHOP induction therapy directly, and the ORR was similar with the two induction regimens (77 versus 76%).

It was recognized that the second randomization may have confounded interpretation of the effect of induction therapy, and secondary ("weighted") analyses were therefore performed.

Failure-free survival (FFS) at 3 years was significantly better in the R-CHOP arm (53 versus 46% for CHOP; $p = 0.04$). Moreover, when the effect of maintenance therapy was removed from analysis of the data, OS rates were also significantly improved in the R-CHOP induction group compared with the CHOP group (estimated 3-year OS, 67 versus 58%, respectively; $p = 0.05$). Furthermore, maintenance therapy with rituximab appears to provide additional benefits, with prolonged FFS rates in this arm of the study compared with the observation arm ($p = 0.009$) (Habermann et al. 2006). FFS and OS were analyzed according to the four treatment strategies assessed in the trial. The addition of rituximab to CHOP

improved the outcome of CHOP, irrespective of whether it was combined with CHOP in the induction phase or given as maintenance therapy after CHOP induction (Habermann et al. 2006).

Consistent with other studies of the addition of rituximab to chemotherapy, no significant difference in toxicity was observed between the two induction arms, with fatal infection rates of 3% and 2.5% for R-CHOP and CHOP patients, respectively (Habermann et al. 2006). Rituximab maintenance therapy was also well tolerated, although granulocytopenia was significantly higher in patients receiving maintenance rituximab compared with those being observed ($p = 0.008$).

13.2.8.2.3 The MInT Trial

The phase III, multicenter, open-label MabThera International Trial (MInT) randomized patients aged 18 to 60 years with previously untreated low-risk (IPI score ≤1) DLBCL to receive six cycles of CHOP-like chemotherapy (CHEMO) or CHOP-like chemotherapy plus rituximab (R-CHEMO) every 21 days. The chemotherapy regimens used were CHOP, CHOEP (CHOP plus etoposide), MACOP-B (methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisolone and bleomycin) and PMitCEBO (prednisolone, mitoxantrone, cyclophosphamide, etoposide, bleomycin, vincristine).

The first interim analysis of this trial took place in November 2003, and included 326 evaluable patients. At this point, the log-rank p-value for the primary endpoint of TTF was 0.0000041 after 97 events had been observed over 24 months. As this was considerably less than the critical threshold for alpha-spending for 97 events (0.00192), the Data Safety and Monitoring Committee stopped recruitment to the trial (Pfreundschuh et al. 2004c).

Data from the full-set analysis demonstrated that the 413 patients receiving R-CHEMO achieved a significantly higher CR than the 410 patients treated with CHEMO alone (Table 13.5) (Pfreundschuh et al. 2006). Moreover, significantly more CHEMO patients had progressive disease (PD) during treatment than R-CHEMO patients. As with the GELA trial, the excellent responses seen in patients receiving chemotherapy plus rituximab translated into significantly improved long-term outcomes, with a significantly longer TTF and OS for R-CHEMO patients compared with patients receiving CHEMO alone (Table 13.5; Fig. 13.7). The rate of lymphoma-associated deaths was reduced by two-thirds with R-CHEMO.

Patients benefited from the addition of rituximab to chemotherapy regardless of their IPI or bulky disease status. R-CHEMO was particularly effective in patients with an IPI score of 0 and no bulk, with a 3-year FFS of 89%, compared with 76% for patients with an IPI score of 1 and/or bulky disease ($p = 0.0162$). However, the OS rates were not significantly different between these two groups of patients, with a 3-year OS of 98% for patients with an IPI score of 0 and no bulk and 91% for patients with an IPI score of 1 and/or bulky disease ($p = 0.8$) (Pfreundschuh et al. 2006).

A secondary analysis focusing on outcomes according to chemotherapy regimen in different patient subgroups has also been presented (Pfreundschuh et al.

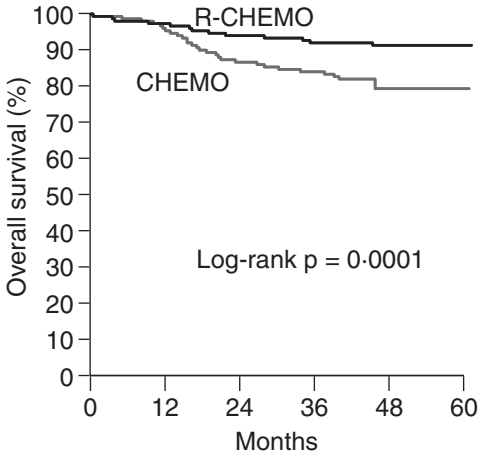

Fig. 13.7 Overall survival after treatment with six cycles of chemotherapy or six cycles of rituximab plus chemotherapy in young patients with previously untreated aggressive lymphoma: MabThera International Trial (MInT). (Reproduced with permission from Pfreundschuh et al. 2006.)

2005a). Too few patients received (R)-PMitCEBO and (R)-MACOP-B for any meaningful conclusions to be drawn. The 2-year TTF rates were significantly better after CHOEP than CHOP (65 versus 55%, $p = 0.04$), but not significantly different after R-CHOEP and R-CHOP (80 versus 82%, $p = 0.67$). This suggests that the addition of rituximab neutralizes the superiority of CHOEP over CHOP (Pfreundschuh et al. 2005a).

The benefits of adding rituximab to chemotherapy were gained without any increase in toxicity, with grade 3/4 hematological toxicity being observed in 6% to 7% of patients in each group (Pfreundschuh et al. 2006).

13.2.8.2.4 **BCCA Population Analysis**

In March 2001, the British Columbia Cancer Agency (BCCA) implemented a new provincial policy recommending that all patients with newly diagnosed advanced-stage DLBCL be treated with R-CHOP. A subsequent retrospective study demonstrated a significantly improved PFS and OS in the 152 patients treated during the 18 months after this policy change compared with the 140 patients treated during the 18 months beforehand ($p = 0.002$ and $p < 0.0001$, respectively) (Sehn et al. 2005). Multivariate analysis demonstrated that the era of treatment remained a strong predictor of both PFS ($p = 0.005$) and OS ($p < 0.001$) after controlling for age and IPI score (Sehn et al. 2005). A retrospective review of young patients with DLBCL in the Czech Lymphoma Group Registry included 90 intermediate-high/high risk (IH/H) patients treated with anthracyline-based chemotherapy (CT) and 55 IH/H patients who received rituximab plus CT (Trneny et al. 2005). The 2-year PFS and OS rates were both significantly better after R-CT compared with CT in these patients: 75.8 versus 41.8%, $p = 0.0003$ and 83.4 versus 57.0%, $p = 0.0007$, respectively (Trneny et al. 2005).

13.2.8.3 Rituximab plus Dose-Densified Chemotherapy in Previously Untreated Aggressive NHL: Phase III Studies

The multicenter prospective randomized controlled RICOVER-60 trial was designed to investigate whether an increase in the number of CHOP-14 cycles of chemotherapy administered (from six to eight) or the additional administration of eight cycles of rituximab resulted in improvements in treatment outcome in elderly patients with previously untreated DLBCL (Pfreundschuh et al. 2005b). After a median follow-up of 26 months, there was a trend towards better TTF after eight cycles of CHOP-14 compared with six cycles of CHOP-14 (58 versus 53%, $p = 0.13$). This trend was neutralized after the addition of rituximab: TTF after both six and eight cycles of R-CHOP-14 was 70%. There were no statistically significant differences in OS in the four different treatment arms. Adverse events were generally more common after eight cycles than after six cycles of therapy; the incidence of peripheral neuropathy was 14% and 8% after eight and six cycles, respectively.

The HOVON-46 study was instigated by the Nordic Lymphoma Group and the Dutch HOVON group in an effort to improve clinical outcomes in elderly patients with intermediate or high-risk NHL (Sonneveld et al. 2005). At the time of study initiation (in 2001), preliminary results from the GELA LNH98-5 trial had shown that the addition of rituximab to CHOP improved outcomes in elderly patients with aggressive NHL. It had also been shown that the use of G-CSF allowed the interval between CHOP cycles to be reduced from 3 to 2 weeks, without a significant increase in toxicity. The HOVON-46 study randomized elderly patients with previously untreated intermediate or high-risk DLBCL, MCL or Grade 3 FL to receive either six cycles of CHOP-14 chemotherapy or six cycles of rituximab in addition to six cycles of CHOP-14 (R-CHOP-14). An interim analysis after a median follow-up of 15 months showed that the R-CHOP-14-treated patients achieved significantly better FFS and OS than the CHOP-14-treated patients (2-year FFS: 51 versus 28%, $p = 0.005$; 2-year OS: 63 versus 46%, $p = 0.03$). There was no significant difference in toxicity between the two treatment arms. However, these results are immature and a final analysis will be performed in 2006.

Taken together, the results of these two trials suggest that the addition of rituximab to dose-densified CHOP chemotherapy may improve outcomes compared with dose-densified CHOP chemotherapy alone. As the follow-up period for both trials remains relatively short, a longer follow-up is needed to determine the impact of these regimens on clinical end-points, most notably survival. It is also important to note the importance of using G-CSF in order to minimize treatment-related toxicity. Further studies are currently under way to more fully assess the combination of rituximab with dose-densified CHOP chemotherapy.

13.2.8.4 Rituximab plus Chemotherapy in Relapsed Aggressive NHL

Rituximab has been combined with a number of chemotherapy regimens in the treatment of patients with relapsed or refractory aggressive lymphoma (Table 13.6). Rituximab in combination with ifosfamide, carboplatin and etoposide (R-ICE) induced very high CR rates in 34 patients with DLBCL who received this regimen prior to ASCT (Kewalramani et al. 2004). The investigators compared

the CR rates of R-ICE with those of 147 similar historical control patients who received ICE alone, and found significantly better responses with the immunochemotherapy combination (CR rates: 53% for R-ICE versus 27% for ICE; $p = 0.01$). Further follow-up is required to elucidate the effect of this combination on long-term outcomes.

Rituximab has been successfully combined with platinum-based combination chemotherapy regimens, including dexamethasone, high-dose cytarabine and cisplatin (DHAP) (Mey et al. 2006) and etoposide, solumedrol, cytarabine and cisplatin (ESHAP) (Hicks et al. 2005; Venugopal et al. 2004) in patients with relapsed or refractory aggressive lymphoma. Results suggest that the addition of rituximab to these established regimens improves efficacy compared with the chemotherapy regimen alone, without adding significantly to the toxicity of chemotherapy. Prospective phase III studies are required to fully assess the safety and efficacy of these and other immunochemotherapy combinations in relapsed/refractory aggressive NHL.

Gemcitabine-based regimens have shown promise in NHL, and the addition of rituximab to such regimens has been investigated. Rituximab in combination with gemcitabine and oxaliplatin (R-GEMOX) resulted in an ORR of 85% in a study of 40 patients with refractory or relapsed B-cell lymphoma who were ineligible for high-dose chemotherapy with ASCT. As in studies of other rituximab–combination chemotherapy regimens, the toxicity was acceptable in these patients (El Gnaoui et al. 2005).

13.2.8.5 Rituximab Monotherapy in Aggressive NHL

Although rituximab in combination with chemotherapy is widely accepted as standard therapy for the majority of patients with aggressive NHL, some patients may be unsuitable for chemotherapy because of frailty or comorbid disease, or they may be unwilling to receive such therapy. For these patients rituximab monotherapy may be an appropriate treatment option, as several studies have demonstrated anti-tumor activity and a good tolerability profile (Coiffier et al. 1998; Rothe et al. 2004; Tobinai et al. 2004) (Table 13.6).

13.2.8.6 Rituximab in Other Subtypes of Aggressive B-Cell NHL

13.2.8.6.1 Rituximab in PMBCL

The optimal management of primary mediastinal large B-cell lymphoma (PMBCL) has been widely debated. CHOP chemotherapy and more aggressive regimens such as MACOP-B and VACOP-B are widely used. At the BCCA, the addition of rituximab to CHOP chemotherapy for previously untreated PMBCL was recommended in March 2001 (Savage et al. 2006). A retrospective analysis of patients treated between 1980 and 2003 revealed 5-year OS rates of 87%, 81%, and 71% for patients treated with MACOP-B/VACOP-B ($n = 47$), R-CHOP ($n = 19$) and CHOP-like regimens ($n = 67$), respectively. Longer-term follow-up of larger numbers of patients treated with R-CHOP are required in order to assess the impact of this regimen on outcomes in PMBCL.

13.2.8.6.2 Rituximab in Burkitt's and Burkitt-Like Lymphoma or Lymphoblastic Lymphoma/Leukemia

Burkitt and Burkitt-like lymphomas represent clonal proliferations of poorly differentiated B-lineage lymphocytes. Lymphoblastic lymphoma is a rare subtype of NHL with biological features similar to those of ALL. These diseases are all highly aggressive and require intensive therapy. Multi-agent chemotherapy has typically been given in a dose-dense fashion (Kasamon and Swinnen 2004). CD20 is present on one-third of B-precursor blast cells and the majority of mature blasts, providing a rationale to explore rituximab in Burkitt's lymphoma and B-cell lymphoblastic leukemias and lymphomas (Gökbudget and Hoelzer 2004).

Thomas and colleagues (2006) have studied the addition of rituximab to hyper-CVAD in adults with newly diagnosed HIV-negative Burkitt/Burkitt-like lymphoma (n = 15) or ALL (n = 16). The 3-year OS, EFS and DFS rates were 89%, 80%, and 88%, respectively. The R-hyper-CVAD regimen was associated with a significantly lower rate of relapse than hyper-CVAD alone (7% versus 34% for all patients, $p = 0.008$; 0% versus 50% for elderly patients, $p = 0.02$). Toxicity profiles for hyper-CVAD and R-hyper-CVAD were similar (Thomas et al. 2006). Hoelzer and associates (2003), in the German Multicenter ALL Study Group (GMALL), have reported an interim analysis from a study evaluating the combination of rituximab with an established GMALL regimen in high-grade CD20-positive lymphomas. The mid-treatment ORR in 26 patients with Burkitt's lymphoma was 96%, including 60% CR, and no excess toxicity was apparent from the addition of rituximab. Further studies are currently under way to define the role of rituximab in these highly aggressive hematological malignancies.

13.2.9 Rituximab in MCL

There is currently no standard therapy for newly diagnosed or relapsed MCL. Many chemotherapy regimens including CHOP, FCM, and fractionated cyclophosphamide, vincristine, doxorubicin and dexamethasone hyper-CVAD have been shown to be highly active in producing tumor responses, but relapses typically occur. The use of HDT with ASCT and allogeneic SCT has also been extensively studied in MCL, and a randomized controlled trial has shown that for patients achieving a CR or PR after induction chemotherapy, ASCT results in a significantly better PFS compared with interferon maintenance (Dreyling et al. 2005b).

Rituximab was first studied as monotherapy in MCL, and showed some activity in this setting (Table 13.7). However, it is the combination of rituximab with chemotherapeutic regimens which has shown the greatest promise, both in previously untreated and relapsed MCL (Table 13.7).

13.2.9.1 Rituximab plus Chemotherapy in Previously Untreated MCL

A study by the GLSG compared the efficacy of CHOP alone with that of a combination of rituximab with CHOP in 122 patients with previously untreated MCL

Table 13.7 Trials of rituximab (R) in mantle cell lymphoma (MCL).

Reference	*Regimen of R plus other agents × no. of cycles*	*Evaluable patients*	*ORR*	*CR*	*PR*	*Median PFS*[a]	*Median OS*
Trials of rituximab plus chemotherapy							
Lenz et al. (2005)	R × 6 plus CHOP × 6	62 UT	94**	34***	60	21 months*	NR
	CHOP × 6 (randomization)	60 UT	75	7	68	14 months (TTF)	NR
Forstpointner et al. (2004); Dreyling et al. (2005a)	R × 4 plus FCM × 4	24 PT	58	29**	29		2.5 years*
	FCM × 4 (randomization)	24 PT	46	0	46		11 months
Howard et al. (2002)	R × 6 plus CHOP × 6	40 UT	96	48	48	16.6 months	
Romaguera et al. (2005)	R × 6–8 plus hyper-CVAD/M-A × 6–8	97 UT	97	87	10	3-year FFS = 64%	3-year OS = 82%
Romaguera et al. (2005)	R × 6–8 plus hyper-CVAD/M-A × 6–8	21 PT	95	43	52	18 months (FFS)	
Herold et al. (2004)	R × 8 plus MCP × 8	201 UT	86	42	38	19 months	
	MCP × 8 (randomization)		66	20	48	19 months	
Kahl et al. (2006)	R × 6 plus modified hyper-CVAD × 6 followed by R × 4 every 6/12 for 2 years	22 UT	77	64	14	37 months	Not reached at 37 months
Drach et al. (2005)	R × 4 plus thalidomide	18 PT	83	28	60	20.6 months	44.1 months
Trials of rituximab monotherapy							
Ghielmini et al. (2005)	Induction phase: R × 4	34 UT	27	3	24		
	Maintenance phase: R × 4 observation (randomization)	54 PT	28	2	26		
		34	41[b]	11[c]		6 months	
		27	55[b]	12[c]		12 months (EFS)	
Foran et al. (2000b)	R × 4	34 UT	38	16	22	1.2 years (median DR for all MCL patients)	
		40 PT	37	14	23		

All doses of R 375 mg m^{-2}.

a Or other parameter as indicated.

b Overall best response.

c % experiencing a CR at some time during the study.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus comparator.

DR = duration of response; EPOCH = etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin; FCM = fludarabine, cyclophosphamide, mitoxantrone; hyperCVAD = fractionated cyclophosphamide, vincristine, doxorubicin, dexamethasone; M-A = methotrexate, high-dose cytarabine; MCP = mitoxantrone, chlorambucil, prednisolone. Other abbreviations as Table 13.2.

(Lenz et al. 2005). Significant improvements were seen in overall response (94 versus 75%; p = 0.0054), CR (34 versus 7%; p = 0.00024) and median TTF (21 versus 14 months; p = 0.0131) in patients who received R-CHOP compared with patients who received CHOP alone. The authors did not notice a major difference in toxicities between the two treatment arms, and suggested that the R-CHOP combination may serve as a new baseline regimen for advanced-stage MCL (Lenz et al. 2005).

Similarly high rates of response were demonstrated in a prospective study conducted at the MD Anderson Cancer Center, which combined rituximab with a fractionated regimen of cyclophosphamide, vincristine, doxorubicin and dexamethasone (hyper-CVAD) alternating with rituximab plus high-dose methotrexate and cytarabine (Romaguera et al. 2005a). In the 97 assessable patients, a response rate of 97% was achieved, with a CR/CRu rate of 87%. Patients aged ≤65 years (n = 65) achieved significantly better outcomes than those aged >65 years (n = 32): 3-year FFS rates: 73 versus 50% (p = 0.02); 3-year OS rates: 86 versus 74% (p = 0.047). As expected, hematological toxicity was prominent and neutropenic fever occurred after 15% of treatment courses, with three deaths due to neutropenic sepsis. In addition, three patients developed and died from MDS while in remission from MCL and one patient remains alive with acute myeloid leukemia (AML). The authors concluded that the alternating R-hyper-CVAD/R-high-dose methotrexate and cytarabine regimen is an effective therapy for younger patients with previously untreated aggressive MCL. The significantly lower FFS rate seen in patients aged >65 years, and the significant hematological toxicity associated with this intense regimen, mean that it cannot be recommended as standard therapy for older patients.

Kahl and colleagues (2006) have assessed the safety and efficacy of modified hyper-CVAD followed by rituximab maintenance in previously untreated MCL. After a median follow-up of 37 months, the median PFS was 37 months, and the median OS had not been reached. As would be anticipated, the major toxicity was hematological and included 59 Grade 3/4 neutropenic events.

The phase III study of R-MCP versus MCP discussed above also included 90 patients with previously untreated MCL (Herold et al. 2004). The ORR and CR rates were higher in the R-MCP group than the MCP group (70 versus 63%, p = not significant; 32 versus 15%, p = 0.06, respectively), but the differences were not statistically significant. The median PFS was 20.5 months after R-MCP and 18.9 months after MCP (p = not significant), while the median EFS with R-MCP and MCP was 20 months and 13.9 months, respectively (p = 0.20) (Herold et al. 2004). The addition of rituximab to MCP does not appear therefore to improve outcomes as markedly in MCL patients as in FL patients.

13.2.9.2 **Rituximab plus Chemotherapy in Relapsed MCL**

There has also been much interest in studying the combination of rituximab with chemotherapy in the relapsed MCL setting. The GLSG study of R-FCM versus FCM in the relapsed setting included 50 patients with MCL. Overall and complete response rates were higher after R-FCM than FCM (58 versus 46%, p = 0.282 and

29 versus 0%, p = 0.004, respectively) (Dreyling et al. 2005a). Median overall survival was significantly extended after R-MCP compared with MCP (2.5 versus 0.9 years, p = 0.031).

The fractionated regimen of hyper-CVAD alternating with rituximab plus high-dose methotrexate and cytarabine described above has also been studied in the relapsed setting (Romaguera et al. 2005b). Preliminary results show an ORR of 95%, including 43% CR/CRu. Grade 4 neutropenia and thrombocytopenia were observed in 58% and 53% of patients, receiving rituximab plus intensive chemotherapy versus intensive chemotherapy alone, respectively.

A small study of patients with relapsed or refractory MCL using a combination of rituximab with thalidomide as salvage therapy demonstrated marked antitumor activity for this regimen (Kaufmann et al. 2004). In a recently updated analysis, the ORR was 83%, including 28% CR (Drach et al. 2005). The estimated 4-year survival was 48%. The promising results of this study have prompted the investigators to initiate a trial of rituximab in combination with CHOP and thalidomide in previously untreated patients with MCL (Drach et al. 2005).

13.2.9.3 Rituximab Monotherapy in MCL

An early phase II study conducted by Foran et al. (2000b) showed that rituximab has single-agent activity in previously untreated and relapsed MCL (Table 13.7). This was subsequently confirmed by Ghielmini and colleagues (2005). Rituximab monotherapy can thus be considered an appropriate treatment for patients unsuitable for more aggressive interventions.

13.2.10 Rituximab in CLL

CLL has traditionally been treated with alkylating agents such as chlorambucil and combination chemotherapeutic regimens such as CVP. The development of purine analogs has led to their integration into CLL therapy regimens, with fludarabine being the most widely used. However, despite improving response rates and response durations, these newer regimens are not curative and the search for more effective therapeutic options has continued.

13.2.10.1 Rituximab plus Chemotherapy in Previously Untreated CLL

A phase II study performed by the Cancer and Leukemia Group B (CALGB) examined the concurrent and sequential administration of fludarabine and rituximab in previously untreated CLL (Byrd et al. 2003). Concurrent FR was associated with higher ORR and CR rates than sequential F → R (90 versus 77% and 43 versus 28%, respectively). Infusion-related and hematological toxicity rates were higher with concurrent than sequential therapy, but infectious toxicities occurred at similar rates in the two treatment arms (Byrd et al. 2003). A retrospective comparison of outcomes with fludarabine–rituximab and fludarabine alone showed that both 2-year PFS (67 versus 45%, respectively; p < 0.0001) and 2-year OS (93 versus 81%, respectively; p = 0.0006) were significantly better in

fludarabine–rituximab recipients than fludarabine monotherapy recipients (Byrd et al. 2005). The rate of infection-related toxicities was similar in both groups. The investigators recently reported the impact of genetic prognostic factors on outcomes in trial patients for whom cryopreserved cells were available (Byrd et al. 2006). Median PFS and OS were shorter in patients with unmutated IgV_H status and high-risk interphase cytogenetics. These observations suggest that, in the future, genetic features may be used to stratify therapies, with low-risk patients being assigned to well-tolerated therapies such as fludarabine-rituximab and more intensive therapies applied to patients with a poor prognosis.

The addition of cyclophosphamide to the fludarabine–rituximab combination (FCR) induced very high response rates in previously untreated CLL patients (Keating et al. 2005a). A total of 224 patients (median age 58 years) with progressive or advanced CLL was included in the study, which demonstrated an ORR of 95% (CR 70%, nodular PR (nPR) 10% and PR 15%), the highest ever observed in a clinical trial in this setting. Two-thirds of patients showed elimination of minimal residual disease analyzed by flow cytometry. The projected 4-year TTF was estimated to be 69%. The FCR regimen was generally well tolerated, with myelosuppression and infections – as anticipated – being the most common adverse events. Minor infections were reported in 10% of treatment courses and major infections (including pneumonia and septicemia) in 2.6% of courses (Keating et al. 2005a). Extended follow-up reveals a 4-year OS rate of 83% (Keating et al. 2005b). There were three cases of AML, three of myelodysplastic syndrome (MDS), and a total of 53 second cancers occurred. There were also 25 cases of autoimmune hemolytic leukemia (AIHA) and six of red cell aplasia (RCA), indicating that patients must be carefully monitored for complications after FCR therapy (Keating et al. 2005b).

The combination of rituximab with pentostatin and cyclophosphamide (PCR) is also showing promise in CLL (Kay et al. 2006). In 64 previously untreated patients, the ORR was 91% (41% CR, 22% nPR and 28% PR). Grade 3/4 neutropenia occurred in 41% of patients, and the most common Grade 3/4 nonhematological toxicities were nausea (n = 6), vomiting (n = 4), infection (n = 6) and fever without neutropenia (n = 4). Other studies of rituximab in combination with chemotherapy in previously untreated CLL are summarized in Table 13.8.

13.2.10.2 **Rituximab plus Chemotherapy in Relapsed CLL**

Rituximab in combination with fludarabine-based regimens has also been investigated in the relapsed CLL setting. The ORR in 177 patients who received FCR was 73% (25% CR, 16% nPR and 32% PR). Almost one-third of patients achieved molecular remission in bone marrow. As in the first-line setting, myelosuppression was the most common toxicity, with Grade 3/4 neutropenia occurring in 62% of assessable treatment courses and major infections noted in 16% of treated patients (Wierda et al. 2005a). A retrospective comparison with other fludarabine-based regimens showed that statistically significantly longer estimated median overall survival was noted in patients treated with FCR compared

Table 13.8 Trials of rituximab (R) in chronic lymphocytic leukemia (CLL).

Reference	*Regimen of R plus other agents × no. of cycles*	*Evaluable patients*	*ORR*	*CR*	*PR*	*Median PFS*[a]	*Median OS*
Trials of rituximab plus chemotherapy/immunotherapy in previously untreated CLL							
Keating et al. (2005a)	R × 1 then R 500 × 5 plus FC × 6	224 UT	95	70	25	48+ months	48+ months
Byrd et al. (2003)	R × 7 plus F × 6 then R × 4	51 UT	90	47	43	2-year PFS: 70%	
	F × 6 then R × 4 (randomization)	53 UT	77	28	49	2-year PFS: 70% (estimated)	
Schulz et al. (2002)	F × 2 then R × 2 plus F × 2 then R × 2	20 UT	85	20	65		
		11 PT	90	27	63		
Savage et al. (2003)	R 125–775 × 8 plus F × 8	6 UT	100	50	50		
		4 PT	75	25	50		
Kay et al. (2006)	R × 6 plus PC × 6	64 UT	91	41	50	32.6 months	
Yunus et al. (2003)	R × 7 plus P × 6	12 UT + 22 PT	50	33	17		
Mena et al. (2005)	R × 7 plus dose-intensive P × 6	133 UT + PT[b]	42[c]	18	24	25.6 months[c]	
			34[d]			13.9 months[d]	
Ferrajoli et al. (2005)	R × 4 plus GM-CSF × 8	29 UT + 26 PT	69	9	60		
Castro et al. (2005)	R × 4 plus HDMP × 3	16 UT	88	6	81	12+ months (TTP)	
Trials of rituximab monotherapy in previously untreated CLL							
Hainsworth et al. (2003a)	R × 4, then further R every 6/12 for total of 4 courses	44 UT	58	9	49	18.6 months	
Thomas et al. (2001)	R × 8	21 UT	86	19	67		

Table 13.8 *Continued*

Reference	*Regimen of R plus other agents × no. of cycles*	*Evaluable patients*	*ORR*	*CR*	*PR*	*Median PFS*[a]	*Median OS*
Trials of rituximab plus chemotherapy/immunotherapy in relapsed/refractory CLL							
Wierda et al. (2005a)	R × 1 then R 500 × 5 plus FC × 6	177 PT	73	25	48	28 months (TTP)	42 months
Lamanna et al. (2005)	R × 5 plus PC × 6	32 PT	75	25	50	40 months (TTF)	44 months
Hensel et al. (2004)	PC × 6 then R × 9	22 PT[e]	64	5	59		
Eichhorst et al. (2005)	R × 5–7 plus CHOP × 6–8	17 PT	70				
Mavromatis et al. (2005)	R × 6 plus FG × 6	5 UT	100	20	80		
		19 PT	53	5	47		
Wierda et al. (2005b)	R 375–500 × 6 plus FCA × 6	44 PT	65	27	38	19+ months all (estimated TTP; responders)	16 months (estimated)
Faderl et al. (2005)	R × 1 then R 500 × 3 plus A	20 PT	55	30	25		
Trials of rituximab monotherapy in relapsed/refractory CLL							
Byrd et al. (2001)	R 100 × 1 then R × 11	27 PT + 6 UT	45	3	42	6 months (TTP)	
Huhn et al. (2001)	R × 4	28 PT	25	0	25	16 weeks	
Itala et al. (2002)	R × 4	24 PT	35	0	35		
O'Brien et al. (2001)	R × 1 then R 500–2250 × 3	40 PT	36	0	36	8 months (TTP)	1-yr OS: 80% (estimated)

All doses of R 375 mg m^{-2} unless otherwise indicated.

a Or other parameter as indicated.

b Mixture of NHL and CLL patients.

c ORR in CLL-UT.

d ORR in CLL-PT.

e Includes several patients with Waldenström's macroglobulinemia.

A = alemtuzumab; C = cyclophosphamide; F = fludarabine; FC = fludarabine, cyclophosphamide; FCA = fludarabine, cyclophosphamide, alemtuzumab; FG = fludarabine, genansense; HDMP = high-dose methyl prednisolone; PC = pentostatin, cyclophosphamide. Other abbreviations as Table 13.2.

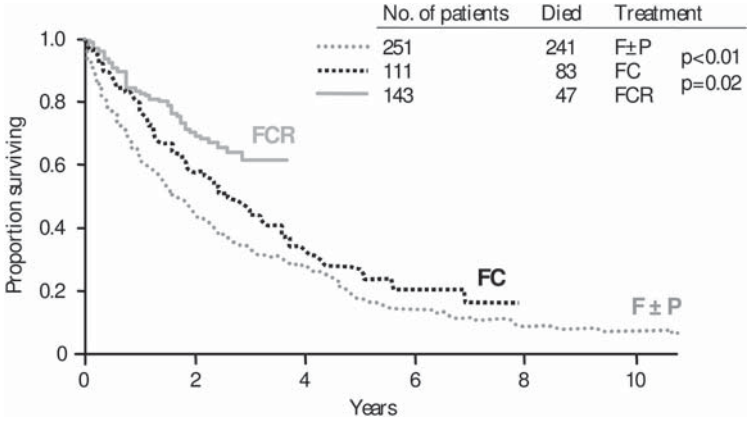

Fig. 13.8 Retrospective comparison of treatments for relapsed/refractory CLL: overall survival. (Reproduced with permission from Wierda et al. 2006.)

with patients treated with FC or fludarabine ± prednisolone (Fig. 13.8) (Wierda et al. 2006).

Wierda and colleagues (2005b) have also studied the addition of alemtuzumab to the FCR regimen in heavily pretreated patients with CLL. The ORR in 44 patients was 65%, with 27% CR and elimination of minimal residual disease by flow cytometry occurred in 92% of patients. This regimen was highly myelosuppressive, however, with nearly all patients experiencing at least one episode of Grade 3/4 neutropenia and infections occurring in 26% of patients. Faderl and associates (2005) administered rituximab together with alemtuzumab to patients with relapsed CLL. The ORR was 55%, with 30% CR. Infections occurred in 50% of patients, including cytomegalovirus (CMV) reactivation in 21%.

The PCR regimen is also under investigation in CLL patients who have previously received treatment. Lamanna et al. (2005) have reported very promising results for 32 patients with CLL and for 14 patients with other low-grade lymphoid neoplasms. The ORR was 75%, with 25% of patients achieving CR. The regimen was described as being well-tolerated, with the principal toxicity being myelosuppression (Lamanna et al. 2005).

The German CLL Study Group (GCLLSG) gave six cycles of R-CHOP to patients with fludarabine-refractory CLL or CLL with AIHA or Richter's transformation (Eichhorst et al. 2005). The ORR in 17 evaluable patients was 70%. The main toxicity was myelosuppression (59% of all documented courses), and 22% of patients developed infections. Other studies using rituximab-based regimens in relapsed/refractory CLL are summarized in Table 13.8.

13.2.10.3 **Rituximab with Immune System Modulators in CLL**

Patients with previously untreated or relapsed/refractory CLL were given four once-weekly infusions of rituximab plus thrice-weekly GM-CSF for 8 weeks

(Ferrajoli et al. 2005). The ORR in 55 patients was 69% with 9% CR, 9% nPR and 51% PR. There was a reduction in self-reported fatigue symptoms in two-thirds of the patients studied. The regimen was very well tolerated, with Grade 3/4 neutropenia and thrombocytopenia occurring in only 4% and 2% of patients, respectively. These results are very encouraging, and accrual to the study is continuing.

13.2.10.4 Rituximab Monotherapy in CLL

Rituximab monotherapy appears to have only modest efficacy in the CLL setting, possibly due to relatively low levels of CD20 expression on B cells in this disease compared with that of normal B cells or B cells in other neoplasms. Another possible reason is the presence of soluble CD20 in the plasma of CLL patients, which may inhibit the capacity of rituximab to bind to CLL B cells *in vivo* (Wierda et al. 2000c). Single-agent rituximab appears to have only modest activity in CLL (Table 13.8), but dose-densification (Byrd et al. 2001) or dose-intensification (O'Brien et al. 2001) can significantly improve response rates. The use of rituximab monotherapy as maintenance therapy (four once-weekly infusions at 6-month intervals for 2 years) has been shown to be a successful strategy in previously untreated CLL/SLL (Hainsworth et al. 2003a). At completion of therapy, ORR and CR rates were 58% and 9%, respectively and the estimated median progression-free time was 19 months.

13.2.11 Rituximab in the Transplant Setting

High-dose chemotherapy (HDT) followed by ASCT is employed in a variety of lymphomas. In order to reduce risk of relapse, *in-vitro* or *in-vivo* graft purging processes are typically employed. Rituximab has been used as an *in-vivo* purging process, being administered to patients before and/or during stem cell mobilization. Gisselbrecht and Mounier (2003) have reviewed early studies of rituximab as an *in-vivo* purging agent, which showed that rituximab effectively depletes the stem cell harvest without adversely impacting stem cell yield or engraftment or causing adverse clinical reactions.

A recently reported trial randomized patients with poor-risk FL to receive either HDT with rituximab *in-vivo* purging followed by ASCT or rituximab in combination with CHOP chemotherapy (Ladetto et al. 2005). The rate of progression or non-response was 35% in the R-CHOP arm compared with only 13% in the R-HDT + ASCT arm ($p < 0.05$). After a median of 24 months' follow-up, EFS was significantly extended in the transplant arm (66% versus 41%, $p < 0.001$). However, a longer-term follow-up is needed to determine impact on survival.

Recently, much interest has been expressed in the role of rituximab as a consolidation or maintenance therapy post-ASCT, in indolent, aggressive or MCL. Woods et al. (2005) have studied the use of two, 4-week courses of rituximab at 2 and 6 months post-ASCT in patients with recurrent FL. After a median follow-up of 4.8 years, median PFS and OS have not been reached, and 6/12 patients

achieving PCR-negativity after rituximab remained in clinical remission at a median of 4.6 years post-ASCT.

Mangel and colleagues (2004) compared outcomes in patients who received HDT with a rituximab *in-vivo* purge and ASCT followed by posttransplant rituximab maintenance with historical controls treated with conventional chemotherapy alone. Rates of PFS and OS after 3 years were significantly extended in the rituximab-ASCT arm compared with the chemotherapy arm (89 versus 29%, $p < 0.00001$ and 88 versus 65%, $p = 0.052$, respectively). The Nordic Lymphoma Group has examined the use of pre-emptive rituximab for patients with MCL relapsing after HDT with rituximab purge and ASCT (Geisler et al. 2005). Eight of 10 patients who received pre-emptive rituximab for PCR-positivity became PCR-negative, and six remained in remission for between 200 and 600 days post-rituximab. Brugger and associates (2005) have also evaluated the impact of post-transplant rituximab in patients with FL and MCL. Recently updated results with a median follow-up of 61 months showed that a median PFS had not been reached, although 5-year PFS and OS rates were higher in FL patients (90% and 100%) than MCL patients (46 and 79%).

The use of posttransplant maintenance in aggressive NHL has also yielded promising results. Patients received two courses of four once-weekly rituximab infusions after HDT and ASCT: one course started at 6 weeks post-ASCT and the second at 6 months post-ASCT (Horwitz et al. 2004). With 30 months' median follow-up, the 2-year EFS and OS rates were 83% and 88%, respectively. Neutropenia was the most common toxicity, but all cases resolved spontaneously within 7 days, or within 2 to 4 days of G-CSF administration. The LNH98-B3 GELA study has examined the impact of four once-weekly rituximab infusions given 2 months posttransplant on relapse rate in patients with poor-risk DLBCL treated with HDT and ASCT (Haioun et al. 2005). With a median follow-up of 3 years, patients randomized to receive posttransplant rituximab ($n = 139$) had a tendency towards a better EFS than patients randomized to observation only ($n = 130$) (80 versus 72%, $p = 0.10$). Rituximab was well tolerated in this setting; the only clinically relevant infections observed were two cases of varicella zoster virus infection, both of which resolved.

The use of rituximab in the transplant setting thus appears to have a beneficial effect upon outcomes, and remains under active investigation. A large trial in relapsed FL coordinated by the European Blood and Bone Marrow Transplant (EBMT) Group is assessing the impact of rituximab *in-vivo* purging and post-transplant maintenance on outcomes. The results are eagerly awaited.

13.2.12 Rituximab in Other Malignancies of B-Cell Origin

The efficacy of rituximab has been investigated on other malignancies of B-cell origin, including posttransplant lymphoproliferative disorder (PTLD), HIV-associated lymphoma, primary CNS lymphoma (PCNSL), hairy cell leukemia (HCL), and Hodgkin's disease (HD). Although the evidence available for the efficacy and

safety of rituximab in these settings is limited, the preliminary results are promising.

13.2.12.1 Rituximab in PTLD

PTLD is a life-threatening complication that occurs in patients receiving immunosuppressive therapy following organ transplantation. Following reports of the successful use of rituximab in this disease, prospective trials were initiated. Choquet et al. (2006) performed a multicenter phase II trial of four once-weekly infusions of rituximab in patients with untreated PTLD, not responding to tapering of immunosuppression. The ORR in 43 patents was 44%, and 12 patients achieved CR or CRu. The overall survival rate at 1 year was 67%. Oertel and colleagues (2005) also examined this regimen in 17 patients with PTLD. Nine patients (53%) achieved a complete remission with a mean duration of 17.8 months, and no severe adverse events were reported. A third trial in 11 patients found an ORR of 64% with six CRs, and rituximab was well tolerated (Blaes et al. 2005). These promising results have led to the instigation of new trials examining the efficacy and safety of rituximab in combination with other therapies in PTLD. The early results from a trial of sequential rituximab, CHOP and G-CSF have shown an ORR of 83%, including 62% CR, in 24 evaluable patients (Trappe et al. 2005). Grade 3/4 leucopenia occurred in 29 of 84 chemotherapy cycles applied.

13.2.12.2 Rituximab in HIV-Associated Lymphoma

HIV-associated lymphomas are a major course of morbidity and mortality in patients with HIV infection. Rituximab has been administered in combination with CHOP chemotherapy (Boue et al. 2006; Kaplan et al. 2005), cyclophosphamide, doxorubicin and etoposide (CDE) chemotherapy (Spina et al. 2005) and DA-EPOCH chemotherapy (Dunleavy et al. 2004) in this setting. The largest study published to date examined outcomes with CHOP or R-CHOP in 150 HIV-infected patients (Kaplan et al. 2005). The R-CHOP regimen was associated with longer median TTP, PFS and OS than CHOP, but these differences did not reach statistical significance because the study was underpowered to detect clinical benefit. Fewer patients died from NHL progression with R-CHOP than CHOP (14 versus 29%), but significantly more died due to treatment-related infection in the R-CHOP arm than the CHOP arm (14 versus 2%, $p = 0.035$). Most of these infectious deaths occurred in severely immunocompromised patients with a CD4+ lymphocyte count of $<50\,mm^{-3}$. A low CD4+ count is a well-recognized risk factor for febrile neutropenia and early death with cytotoxic chemotherapy, and rituximab might increase this risk. Ongoing studies will determine whether the sequential administration of chemotherapy and rituximab or the use of prophylactic antibiotics can reduce the incidence of infectious complications.

13.2.12.3 Rituximab in PCNSL

The majority of PCNSLs in immunocompetent patients are DLBCLs, and rituximab is therefore a rational treatment option. However, its potential efficacy is limited by its high molecular weight, which prevents penetration into the CNS

through an intact blood–brain barrier (Hoang-Xuan et al. 2004). Preliminary investigations of the intraventricular/intrathecal administration of rituximab – which allowed a higher concentration to be achieved in the cerebrospinal fluid (CSF) – have yielded encouraging results (Rubenstein et al. 2004; Schulz et al. 2004). For instance, Schulz and co-workers (2004) observed total clearance of malignant cells in the CSF following intraventricular rituximab, in two patients with PCNSL and in one patient with Burkitt's lymphoma with meningeal involvement. The administration of intravenous rituximab in combination with the alkylating agent temozolomide is another promising strategy (Enting et al. 2004; Wong et al. 2004). Further studies of rituximab in PCNSL are ongoing.

13.2.12.4 **Rituximab in HCL**

Hairy cell leukemia is a chronic CD20-positive B-cell malignancy which traditionally is treated with nucleoside analogs or interferon. However, many patients are refractory to or intolerant of standard therapies. Rituximab is showing promise in the treatment of this disease. Thomas et al. (2003) administered eight doses of rituximab (and a further four doses to responders who failed to achieve CR) and obtained an ORR of 80%, including 53% CR (2004). Toxicity was minimal, and no infectious events were observed. The use of chlorodeoxyadenosine followed by eight once-weekly infusions of rituximab was recently shown to eradicate minimal residual disease in all 12 patients studied, and no patients had relapsed after a median of 11 months follow-up (Ravandi-Kashani et al. 2005).

13.2.12.5 **Rituximab in HD**

Classical HD expresses CD20 on only a minority of cells, but the lymphocyte-predominant subtype of the disease (LPHD) has high CD20 expression. A phase II study conducted by the German Hodgkin Lymphoma Study Group (GHSG) assessed four once-weekly infusions of rituximab in 21 patients with relapsed LPHD (Schulz et al. 2005b). The ORR was 90% and the median TTP 31 months. The combination of rituximab and chemotherapy has been examined in classical HD. It is suggested that in these patients, rituximab may eliminate B cells from the tumor and deprive malignant lymphoid cells of important growth signals. Younes and colleagues (2005a) gave six weekly doses of rituximab and doxorubicin, bleomycin, vinblastine, dacarbazine (ABVD) chemotherapy to 72 newly diagnosed patients with classical HD. With a median follow-up of 32 months, estimated EFS and OS were 82% and 100%, respectively, In another study, the combination of gemcitabine and rituximab was evaluated in relapsed or refractory classical HD (Younes et al. 2005b); here, 13 patients (50%) responded to treatment. Studies are ongoing to further elucidate the safety and efficacy of rituximab in HD.

13.3
Rituximab in Autoimmune Disorders

The issue of the relative contributions made by T cells, B cells, cytokines and other elements to the pathogenesis of autoimmunity has been debated for many

decades. Over the past 10 years, there has been an upsurge of interest in the role of B cells and the notion that blocking them may be beneficial. Edwards and Cambridge (2001) were the first to show that B-cell depletion is a successful treatment strategy in rheumatoid arthritis (RA), and subsequently a number of groups have examined B-cell depletion in other autoimmune diseases including systemic lupus erythematosus (SLE) and autoimmune cytopenias (AC).

13.3.1 Rituximab in RA

The first evidence that rituximab might be effective against RA came from early case reports describing patients with NHL and coexisting RA. These data, coupled with evidence that B cells play a key role in RA pathogenesis, led initially to small open-label studies and later to a phase II clinical trial program. Two large phase IIb studies investigating the safety and efficacy of rituximab in RA have recently reported extremely encouraging results.

Preliminary results from the Dose-ranging Assessment iNternational Clinical Evaluation of Rituximab in rheumatoid arthritis (DANCER) study have revealed that rituximab in combination with methotrexate induced significantly better responses than methotrexate plus placebo in patients with active disease, despite previous therapy with traditional disease-modifying anti-rheumatic drugs (DMARDS) (other than methotrexate) or an anti-tumor necrosis factor (TNF) agent. (Emery et al. 2005). Efficacy was examined based on achievement of American College of Rheumatology (ACR) 20, 50, and 70 responses at 24 weeks compared with equivalent responses after methotrexate treatment (Fig. 13.9).

As its name suggests, the DANCER study was designed to investigate different dosing regimens of rituximab; $2 \times 500\,\text{mg}$ and $2 \times 1000\,\text{mg}$ doses were used (Emery et al. 2005). Although no dose–response relationship was evident with ACR20 and ACR50 responses, there was a trend towards a greater number of patients achieving an ACR70 response with the higher rituximab dose. The pattern of more patients achieving other high-hurdle endpoints (European League Against Rheumatism (EULAR) "good" response, Disease Activity Score (DAS) "remission" and "low disease") was also evident for the higher rituximab dose.

Another important finding from the DANCER study was the significant positive impact that rituximab had on patient-reported outcomes, including health-related quality of life assessments (Mease et al. 2005). This is especially significant considering the chronic, painful nature of RA.

The higher ($2 \times 1000\,\text{mg}$) dose of rituximab was studied in the Randomised Evaluation oF Long term Efficacy or rituXimab in rheumatoid arthritis (REFLEX) study, performed in patients who had active disease despite treatment with one or more anti-TNF therapies plus methotrexate (Cohen et al. 2006). The primary ITT efficacy population comprised 499 patients, including both rheumatoid factor

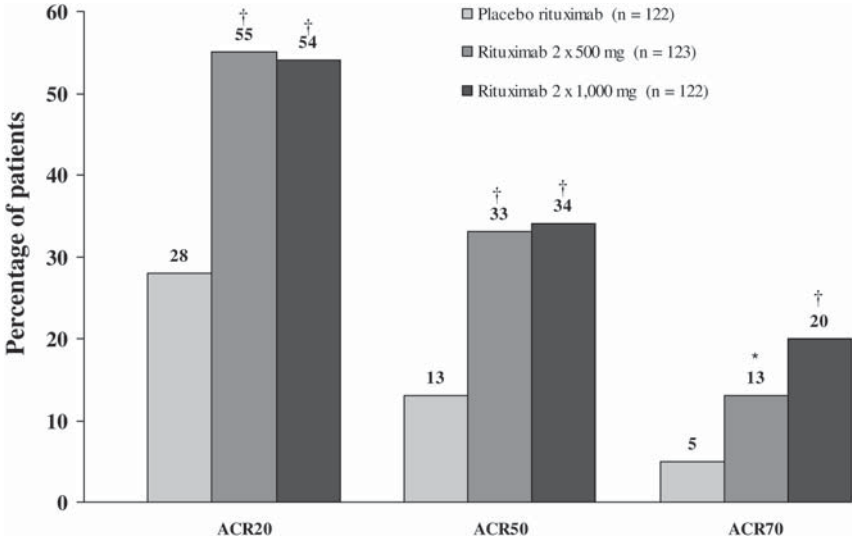

Fig. 13.9 Response rates following administration of two different doses of rituximab or placebo together with methotrexate in patients refractory to prior therapy: Dose-ranging Assessment iNternational Clinical Evaluation of Rituximab in rheumatoid arthritis (DANCER) trial.

(RF)-positive and -negative patients. Again, rituximab demonstrated a favorable efficacy profile, with significantly more rituximab-treated patients achieving ACR20, ACR50 and ACR70 responses at 24 weeks compared with those who received placebo. Symptomatic improvement in disease was again mirrored by improvements in patient-related outcomes (Keystone et al. 2005).

Preliminary data suggest that a second course of rituximab is safe and efficacious in RA (Fleischmann et al. 2005). Further studies are under way to establish the optimal dosing regimen for rituximab in RA, including time to retreatment, and to assess efficacy in earlier lines of therapy.

13.3.2 Rituximab in SLE

Recent research in SLE has led to the discovery that B cells play a key role in the pathogenesis of the disease (Anolik and Aringer 2005). As a consequence, rituximab has been used to directly target the immune cells that are abnormal in SLE, with promising results. Early studies of rituximab in this setting revealed a good tolerability profile and significant improvements in disease activity scores at 2 and 3 months, which persisted for 12 months (Looney et al. 2004). Randomized control trials of rituximab are under way in patients with SLE, and the results are eagerly awaited (Eisenberg and Looney 2005).

13.3.3 Rituximab in Autoimmune Cytopenias and Hemophilia

The autoimmune cytopenias (AC) include AIHA, pure red cell aplasia (PRCA), immune thrombocytopenia (ITP), autoimmune neutropenia (AIN), and various combinations of these disorders such as Evans' syndrome. B cells play a crucial role in the pathogenesis of these disorders, providing a rationale for the use of rituximab in their treatment. The majority of patients with AC respond to standard immunosuppressive therapy (e.g., steroids), but a proportion require second-line treatment, including cytotoxic therapy, G-CSF, or splenectomy. Some patients remain resistant even after these interventions, and the use of monoclonal antibodies has become a new approach in treating patients with severe, refractory AC. Robak (2004) has reviewed the use of rituximab in AC, and concluded that it has significant efficacy in the treatment of refractory or relapsed ITP, AIHA, and PRCA.

The treatment of acquired hemophilia typically includes steroids, chemotherapy, or intravenous immunoglobulin. Recently, interest has been shown in the use of rituximab for selective B-cell depletion in acquired hemophilia, and the results of preliminary studies have been promising (Kessler 2005). Prospective controlled studies have not yet been performed, however.

13.3.4 Rituximab in Chronic Graft-versus-Host Disease (GvHD)

Chronic GvHD is an important cause of morbidity and mortality in patients receiving allogeneic transplants. A coordinated B-cell and T-cell response occurs in at least some patients with chronic GvHD (Cutler and Antin 2006). Corticosteroids are the mainstay of therapy, but many patients are refractory to this approach. Ratanatharathorn et al. (2003) observed a sustained response in four of eight patients with chronic GvHD who received four cycles of rituximab. In a further case series, patients were permitted to receive up to eight cycles of rituximab, and five of six patients had objective responses (Canninga-van Dijk et al. 2004). Several larger series have been reported recently: Cutler and Antin (2006) obtained an ORR of 82% in 18 patients, predominantly in those with cutaneous and rheumatologic involvement, while Zaja et al. (2005) found that 64% of 25 patients with chronic GvHD with cutaneous involvement responded. Further clinical trials of rituximab in chronic GvHD are currently in progress.

13.3.5 Rituximab in Other Autoimmune Disorders

Altered development and function of B cells may be important in the pathogenesis of a range of other autoimmune disorders, including Sjögren's syndrome, antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis, and dermatomyositis. The strategy of B-cell depletion with rituximab is being investigated as

a therapeutic option in these diseases. In a phase II study in Sjögren's syndrome patients, significant improvements in subjective symptoms were achieved in 15 patients, but four developed HACAs and three had associated serum sickness (Pijpe et al. 2005). In a study of 11 patients with ANCA-associated vasculitis, four infusions of rituximab + prednisolone produced complete remission in 3 months and were well tolerated (Keogh et al. 2006). In a small pilot study in dermatomyositis, all six evaluable patients who received four once-weekly infusions of rituximab exhibited major clinical improvement, with muscle strength increasing over baseline by between 36 and 113% (Levine 2005). Maximal improvements in muscle strength occurred as early as 12 weeks after the initial infusion of rituximab. CD20+ B cells were effectively depleted in all patients by 12 weeks. Rituximab was well tolerated, with no treatment-related severe or serious adverse events during the observation period of this study. Further studies are under way in these settings.

Case reports describing the use of rituximab in a number of other autoimmune disorders have also appeared in the literature. Opsoclonus-myoclonus syndrome (OMS) is a rare serious neurological disorder with significant unmet needs. The administration of rituximab to a child with OMS led to the elimination of CSF B cells and dramatic clinical improvement (Pranzatelli et al. 2005). Myasthenia gravis, a B-cell-mediated autoimmune neuromuscular disorder, is characterized by weakness and fatigability of skeletal muscles. Rituximab has been shown to have a favorable impact on this disorder (Gajra et al. 2004). B cells are also thought to play a key role in the pathogenesis of pemphigus, and successful reports of rituximab's use have been published (Arin and Hunzelmann 2005). It should be emphasized that these data are extremely limited and do not support the routine use of rituximab in these settings. However, for refractory patients, enrolment in a clinical trial assessing the benefits of rituximab may be appropriate. Many such trials are registered on www.clinicaltrials.gov.

13.4 Summary and Conclusions

As rituximab targets the CD20 antigen on B cells, producing selective and sustained B-cell depletion, it is an attractive therapeutic option not only for B-cell malignancies but also for a range of systemic autoimmune diseases in which altered development and/or function of B cells appear to play a prominent role.

A very large body of evidence now exists to support the use of rituximab in NHL. When used in combination with a variety of chemotherapeutic regimens in indolent and aggressive NHL, rituximab increases the efficacy of these treatments. An increase in the incidence of neutropenia has been observed when rituximab is combined with some chemotherapies, but this does not appear to translate into an increased risk of infections. The combination of rituximab with CHOP chemotherapy was the first regimen in over 20 years to improve survival over CHOP chemotherapy alone, and R-CHOP is now accepted worldwide as

standard therapy for previously untreated aggressive NHL. For patients with advanced symptomatic indolent NHL, the addition of rituximab to various chemotherapies – including CHOP, CVP and MCP – also appears to improve clinical outcomes. Very encouragingly, significant improvements in overall survival have been reported with immunochemotherapy regimens in this setting.

Recently there has been much interest in the use of rituximab maintenance therapy to prolong remission in patients with previously untreated or relapsed/refractory indolent NHL who respond to induction therapy, either with chemotherapy, rituximab alone or rituximab in combination with chemotherapy. For patients with relapsed/refractory FL responding to (immuno)chemotherapy induction, the use of rituximab maintenance therapy has been shown to confer a survival advantage compared with observation only. Patients with previously untreated FL who responded to CVP induction have also been shown to have improved overall survival after maintenance therapy compared with observation.

Rituximab has also been investigated in CLL. Phase II trials of rituximab in combination with fludarabine and cyclophosphamide (FCR) have demonstrated extremely encouraging results, and the FCR regimen is currently being investigated in phase III trials. For patients with MCL – an NHL with a notoriously poor outcome – the use of rituximab in combination with chemotherapy or transplantation strategies has also been shown to improve outcomes.

Combining rituximab with immune system modulators such as cytokines provides alternative, effective options to standard chemotherapy combinations. Rituximab/cytokine combinations or rituximab monotherapy may be appropriate treatment options for patients who are unable or unwilling to receive standard chemotherapy regimens. Rituximab monotherapy may also be a suitable alternative to "watch and wait" for patients with asymptomatic indolent NHL who seek a therapeutic intervention for their disease.

The positive results obtained in hematological malignancies have prompted the study of rituximab in a number of other B-cell-associated diseases, including RA, SLE, and autoimmune cytopenias. In RA, rituximab appears to be a useful addition to the therapeutic armamentarium, inducing high rates of response in patients who are refractory to prior anti-TNF therapies.

The extensive clinical experience with rituximab has yielded a consistent safety and tolerability profile, as recently reviewed by Kimby (2005). The majority of patients receiving their first infusion of rituximab experience flu-like symptoms, while other common symptoms include headache, nausea, rash, and fatigue. Approximately 10% of patients with hematological malignancies experience more severe symptoms with their first infusion, including bronchospasm, hypoxia, and hypotension. These necessitate the interruption of the infusion and the instigation of supportive therapies as required, including oxygen, steroids, and bronchodilators. The vast majority of patients are able to receive further infusions of rituximab without experiencing severe adverse events.

Rituximab is associated with a low incidence of severe hematological adverse effects. There have been some reports of late-onset neutropenia after rituximab,

with most cases occurring in experimental studies of rituximab in the setting of transplantation. Further investigation of the mechanism of this adverse effect is required. Rarely, mucocutaneous reactions and pulmonary adverse events may occur in association with rituximab treatment. The safety and tolerability profile of rituximab in RA appears similar to that observed in oncology, although the incidence and severity of adverse events is lower. This may reflect the fact that RA patients do not experience the cytokine release or tumor lysis syndromes observed in patients with B-cell malignancies.

Rituximab has become an integral part of treatment for many patients with NHL and CLL. It is also showing great promise in a variety of autoimmune diseases, including RA and autoimmune cytopenias. A major clinical trial program continues to assess the optimal use of this therapy in patients with hematological malignancies and autoimmune disorders.

References

Alas, S., Bonavida, B. (2001) Rituximab inactivates signal transducer and activation of transcription 3 (STAT3) activity in B-non-Hodgkin's lymphoma through inhibition of the interleukin 10 autocrine/paracrine loop and results in down-regulation of Bcl-2 and sensitization to cytotoxic drugs. *Cancer Res* 61: 5137–5144.

Anderson, K.C., Bates, M.P., Slaughenhoupt, B.L., et al. (1984) Expression of human B-cell associated antigens on leukemias and lymphomas: a model of human B cell differentiation. *Blood* 63: 1424–1433.

Anolik, J.H., Aringer, M. (2005) New treatments for SLE: cell-depleting and anti-cytokine therapies. *Best Pract Res Clin Rheumatol* 19: 859–878.

Ansell, S.M. (2003) Adding cytokines to monoclonal antibody therapy: does the concurrent administration of interleukin-12 add to the efficacy of rituximab in B-cell non-Hodgkin's lymphoma? *Leuk Lymphoma* 44: 1309–1315.

Arin, M.J., Hunzelmann, N. (2005) Anti-B-cell-directed immunotherapy (rituximab) in the treatment of refractory pemphigus – an update. *Eur J Dermatol* 15: 224–230.

Armitage, J.O., Weisenburger, D.D. (1998) New approach to classifying non-Hodgkin's lymphomas: clinical features of the major histologic subtypes. Non Hodgkin's Lymphoma Classification Project. *J Clin Oncol* 16: 2780–2795.

Bairey, O., Dann, E.J., Herishanu, Y., et al. (2005) Rituximab Retreatment in B-Cell Non-Hodgkins Lymphoma Patients. Abstract 2455, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Bennett, M., Sharma, K., Yegena, S., Gavish, I., Dave, H.P., Schechter, G.P. (2005) Rituximab monotherapy for splenic marginal zone lymphoma. *Haematologica* 90: 856–858.

Berinstein, N.L., Grillo-López, A.J., White, C.A., et al. (1998) Association of serum rituximab (IDEC-C2B8) concentration and anti-tumor response in the treatment of recurrent low-grade or follicular non-Hodgkin's lymphoma. *Ann Oncol* 9: 995–1001.

Bertoni, F., Zucca, E. (2005) State-of-the-art therapeutics: marginal zone lymphoma. *J Clin Oncol* 23: 6415–6420.

Blaes, A.H., Peterson, B.A., Bartlett, N., et al. (2005) Rituximab therapy is effective for posttransplant lymphoproliferative disorders after solid organ transplantation: results of a phase II trial. *Cancer* 104: 1661–1667.

Boue, F., Gabarre, J., Gisselbrecht, C., et al. (2006) Phase II trial of CHOP plus

rituximab in patients with HIV-associated non-Hodgkin's lymphoma. *J Clin Oncol* 24: 4123–4128.

Boye, J., Elter, T., Engert, A. (2003) An overview of the current clinical use of the anti-CD20 monoclonal antibody rituximab. *Ann Oncol* 14: 520–535.

Brugger, W., Hirsch, J., Repp, R., et al. (2005) Long Term Remission in Follicular Lymphoma (FL) and Mantle Cell Lymphoma (MCL) with Rituximab Consolidation after High-Dose Chemotherapy and Autologous Stem Cell Transplantation: Extended Follow-Up of a Multicenter phase II Study. Abstract 2078, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Byrd, J.C., Murphy, T, Howard, R.S., et al (2001) Rituximab using a thrice-weekly dosing schedule in B-cell chronic lymphocytic leukemia and small lymphocytic lymphoma demonstrates clinical activity and acceptable toxicity. *J Clin Oncol* 19: 2153–2164.

Byrd, J.C., Peterson, B.L., Morrison, V.A., et al. (2003) Randomized phase 2 study of fludarabine with concurrent versus sequential treatment with rituximab in symptomatic untreated patients with B-cell chronic lymphocytic leukemia: results from Cancer and Leukemia Group B 9712 (CALGB 9712). *Blood* 101: 6–14.

Byrd, J.C., Rai, K., Peterson, B.L., et al. (2005) Addition of rituximab to fludarabine may prolong progression-free survival and overall survival in patients with previously untreated chronic lymphocytic leukemia: an updated retrospective comparative analysis of CALGB 9712 and CALGB 9011. *Blood* 105: 49–53.

Byrd, J.C., Gribben, J., Peterson, B.L., et al. (2006) Select high-risk genetic features predict earlier progression following chemoimmunotherapy with fludarabine and rituximab in chronic lymphocytic leukemia: justification for risk-adapted therapy. *J Clin Oncol* 24: 437–443.

Canales, M.A., Palacios, A., Martinez-Chamorro, C., et al. (2005) Re-Treatment with Rituximab Plus Chemotherapy in Patients with Diffuse Large-B Cell Lymphoma (DLBCL): A Spanish Multicenter Study. Abstract 2457, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Canninga-van Dijk, M.R., van der Straaten, H.M., Fijnheer, R., Sanders, C.J., van den Tweel, J.G., Verdonck, L.F. (2004) Anti-CD20 monoclonal antibody treatment in 6 patients with therapy-refractory chronic graft-versus-host disease. *Blood* 104: 2603–2606.

Castro, J.E., Bole, J.E., Prado, C.E., et al. (2005) Rituximab and high dose Methylprednisolone (HDMP) as a first line treatment for patients with chronic lymphocytic leukemia. Abstract 2969, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Cheson, B.D., Horning, S.J., Coiffier, B., et al. (1999) Report of an international workshop to standardize response criteria for non-Hodgkin's lymphoma. *J Clin Oncol* 17: 1244–1253.

Choquet, S., Leblond, V., Herbrecht, R., et al. (2006) Efficacy and safety of rituximab in B-cell post-transplant lymphoproliferative disorders: results of a prospective multicentre phase II study. *Blood* 107: 3053–3057.

Chow, K.U., Sommerlad, W.D., Boehrer, S., et al. (2002) Anti-CD20 antibody (IDEC-C2B8, rituximab) enhances efficacy of cytotoxic drugs on neoplastic lymphocytes in vitro: role of cytokines, complement, and caspases. *Haematologica* 87: 33–43.

Cohen, A., Polliack, A., Ben-Bassat, I., et al. (2002) Results of a phase II study employing a combination of fludarabine, cyclophosphamide, plus rituximab (FC + R) in relapsed follicular lymphoma patients. Abstract, updated in a poster presentation at the 44th Annual Meeting of the American Society of Hematology (ASH), 7–10 December 2002, Philadelphia, USA.

Cohen, S.B., Emery, P., Greenwald, M.W., et al. (2006) Rituximab for rheumatoid

arthritis refractory to anti-tumor necrosis factor therapy: Results of a multicenter, randomized, double-blind, placebo-controlled, phase III trial evaluating primary efficacy and safety at twenty-four weeks. *Arthritis Rheum* 54: 2793–2806.

Coiffier, B., Haioun, C., Ketterer, N., et al. (1998) Rituximab (anti-CD20 monoclonal antibody) for the treatment of patients with relapsing or refractory aggressive lymphoma: a multicenter phase II study. *Blood* 92: 1927–1932.

Coiffier, B., Lepage, E., Brière, J., et al. (2002) CHOP chemotherapy plus rituximab compared with CHOP alone in elderly patients with diffuse large-B-cell lymphoma. *N Engl J Med* 346: 235–242.

Coiffier, B., Herbrecht, R., Tilly, H., et al. (2003) GELA study comparing CHOP and R-CHOP in elderly patients with DLCL: 3-year median follow-up with an analysis according to co-morbidity factors. Abstract 2395, updated in a poster presentation at the 39th Annual Meeting of the American Society of Clinical Oncology, 29 May–2 June 2003, Chicago, USA.

Coiffier, B. (2005) State-of-the-art therapeutics: diffuse large B-cell lymphoma. *J Clin Oncol* 23: 6387–6393.

Colombat, P., Salles, G., Brousse, N., et al. (2001) Rituximab (anti-CD20 monoclonal antibody) as single first-line therapy for patients with follicular lymphoma with a low tumor burden: clinical and molecular evaluation. *Blood* 97: 101–106.

Conconi, A, Martinelli, G, Thieblemont, C, et al. (2003) Clinical activity of rituximab in extranodal marginal zone B-cell lymphoma of MALT type. *Blood* 102: 2741–2745.

Cortes-Funes, H., de la Serna, J., Flores, E., et al. (2000) Rituximab in monotherapy in patients with relapsed follicular or low grade B-cell non-Hodgkin's lymphoma: results after a 6 month follow-up cut-off. Abstract 114, updated in a poster presentation at the 36th Annual Meeting of the American Society of Clinical Oncology (ASCO), 20–23 May, 2000, New Orleans, USA.

Cutler, C., Antin, J.H. (2006) Chronic graft-versus-host disease. *Curr Opin Oncol* 18: 126–131.

Czuczman, M.S., Grillo-López, A.J., White, C.A., et al. (1999) Treatment of patients with low-grade B-cell lymphoma with the combination of chimeric anti-CD20 monoclonal antibody and CHOP chemotherapy. *J Clin Oncol* 17: 268–276.

Czuczman, M., Weaver, R., Alkuzweny, B., et al. (2004) Prolonged clinical and molecular remission in patients with low-grade or follicular non-Hodgkin's lymphoma treated with rituximab plus CHOP chemotherapy: 9-year follow-up. *J Clin Oncol* 22: 4659–4664.

Czuczman, M.S., Koryzna, A., Mohr, A., et al. (2005) Rituximab in combination with fludarabine chemotherapy in low-grade or follicular lymphoma. *J Clin Oncol* 23: 694–704.

Davis, T.A., White, C.A., Grillo-López, A.J. (1999) Single-agent monoclonal antibody efficacy in bulky non-Hodgkin's lymphoma: results of a phase II trial of rituximab. *J Clin Oncol* 17: 1851–1857.

Davis, T.A., Maloney, D.G., Grillo-López, A.J., et al. (2000a) Combination immunotherapy of relapsed or refractory low-grade or follicular non-Hodgkin's lymphoma with rituximab and interferon-α-2a. *Clin Cancer Res* 6: 2644–2652.

Davis, T.A., Grillo-López, A.J., White, C.A., et al. (2000b) Rituximab anti-CD20 monoclonal antibody therapy in non-Hodgkin's lymphoma: safety and efficacy of re-treatment. *J Clin Oncol* 18: 3135–3143.

Demidem, A., Hanna, N., Hariharan, H., et al. (1995) Chimeric anti-CD20 antibody (IDEC C2B8) is apoptotic and sensitises drug-resistant human B cell lymphomas to the cytotoxic effect of CDDP, VP-16 and toxins. *FASEB J* 9: A206.

Demidem, A., Lam, T., Alas, S., et al. (1997) Chimeric anti-CD20 (IDEC-C2B8) monoclonal antibody sensitises a B cell lymphoma cell line to cell killing by cytotoxic drugs. *Cancer Biother Radiopharm* 12: 177–186.

DeVita, V.T., Jr., Canellos, G.P., Chabner, B., et al. (1975) Advanced diffuse histiocytic lymphoma, a potentially curable disease. *Lancet* 1: 248–250.

Di Bella, N., Reynolds, C., Faragher, D., et al. (2005) An open-label pilot study of pentostatin, mitoxantrone and rituximab

in patients with previously untreated, Stage III or IV, low-grade non-Hodgkin lymphoma. *Cancer* 103: 978–984.

Di Gaetano, N., Ziao, Y., Erba, E., et al. (2001) Synergism between fludarabine and rituximab revealed in a follicular lymphoma cell line resistant to the cytotoxic activity of either drug alone. *Br J Haematol* 114: 800–809.

Dighiero, G. (2005) CLL Biology and Prognosis. *Hematology* (*Am Soc Hematol Educ Program*): 278–284.

Dillman, R.O. (2003) Treatment of low-grade B-cell lymphoma with the monoclonal antibody rituximab. *Semin Oncol* 30: 434–437.

Dimopoulos, M.A., Zervas, C., Zomas, A., et al. (2002) Treatment of Waldenström's macroglobulinemia with rituximab. *J Clin Oncol* 20: 2327–2333.

Domingo-Domenech, E., Gonzalez-Barca, E., Estany, C., et al. (2002) Combined treatment with anti-CD20 (rituximab) and CHOP in relapsed advanced-stage follicular lymphomas. *Haematologica* 87: 1229–1230.

Drach, J., Kaufmann, H., Seidl, L., et al. (2005) Updated analysis of thalidomide plus rituximab in relapsed mantle cell lymphoma. Abstract 340, updated in a poster presentation at the 9th International Congress on Malignant Lymphoma (ICML), 8–11 June 2005, Lugano, Switzerland.

Drapkin, R., Di Bella, N., Faragher, D.C., et al. (2003) Results of a phase II multicenter trial of pentostatin and rituximab in patients with low-grade B-cell non-Hodgkin's lymphoma; an effective and minimally toxic regimen. *Clin Lymphoma* 4: 169–175.

Dreyling, M.H., Forstpointner, R., Ludwig, W., et al. (2005a) Combined immuno-chemotherapy (R-FCM) results in superior remission rates and overall survival in recurrent follicular lymphoma and mantle cell lymphoma – follow-up of a prospective randomized trial of the German Low Grade Lymphoma Study Group. Abstract 6528, updated in an oral presentation at the 41st Annual Meeting of the American Society of Clinical Oncology (ASCO), May 13–17 2005, New Orleans, USA.

Dreyling, M., Lenz, G., Hoster, E., et al (2005b) Early consolidation by myeloablative radiochemotherapy followed by autologous stem cell transplantation in first remission significantly prolongs progression-free survival in mantle-cell lymphoma: results of a prospective randomized trial of the European MCL Network. *Blood* 105: 2677–2684.

Dunleavy, K., Little, R., Gea-Banacloche, J., et al. (2004) Abbreviated Treatment with Short-Course Dose-Adjusted EPOCH and Rituximab (DA-EPOCH-R) Is Highly Effective in AIDS-Related Lymphoma (ARL). Abstract 3111, updated in a poster presentation at the 45th Annual Meeting of the American Society of Hematology, 6–9 December 2003, San Diego, USA.

Economopoulos, T., Fountzilas, G., Pavlidis, N., et al. (2003) Rituximab in combination with CNOP chemotherapy in patients with previously untreated indolent non-Hodgkin's lymphoma. *Hematol J* 4: 110–115.

Edwards, J.C., Cambridge, G. (2001) Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes. *Rheumatology (Oxford)* 40: 205–211.

Eichhorst, B.F., Busch, R., Duehrsen, U., et al. (2005) CHOP Plus Rituximab (CHOP-R) in Fludarabine (F) Refractory Chronic Lymphocytic Leukemia (CLL) or CLL with Autoimmune Hemolytic Anemia (AIHA) or Richter's Transformation (RT): First Interim Analysis of a phase II Trial of the German CLL Study. Abstract 2125, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Einfeld, D.A., Brown, J.P., Valentine, M.A., et al. (1988) Molecular cloning of the human B cell CD20 receptor predicts a hydrophobic protein with multiple transmembrane domains. *EMBO J* 7: 711–717.

Eisenberg, R. and Looney, R.J. (2005) The therapeutic potential of anti-CD20 "What do B-cells do?" *Clin Immunol* 117: 207–213.

El Gnaoui, T., Dupuis, J., Joly, B., et al. (2005) Rituximab, gemcitabine and oxaliplatin (R-GEMOX): a promising

regimen for refractory/relapsed B-cell lymphoma, a single institutional pilot study. Abstract 496, 9th International Congress on Malignant Lymphoma (ICML), 8–11 June 2005, Lugano, Switzerland.

Emery, P., Filipowicz-Sosnowska, A., Szczepański, L., et al. (2005) Primary analysis of a double-blind, placebo-controlled, dose-ranging trial of rituximab, an anti-CD20 monoclonal antibody, in patients with rheumatoid arthritis receiving methotrexate (DANCER trial). *Ann Rheum Dis* 64(Suppl.3): 58.

Emmanouilides, C., Territo, M., Menco, R., et al. (2003) Mitoxantrone–cyclophosphamide–rituximab: an effective and safe combination for indolent NHL. *Hematol Oncol* 21: 99–108.

Enting, R.H., Demopoulos, A., DeAngelis, L.M., Abrey, L.E. (2004) Salvage therapy for primary CNS lymphoma with a combination of rituximab and temozolomide. *Neurology* 63: 901–903.

Ezdinli, E.Z., Harrington, D.P., Kucuk, O., et al. (1987). The effect of intensive intermittent maintenance therapy in advanced low-grade non-Hodgkin's lymphoma. *Cancer* 60: 156–160.

Faderl, S., Ferrajoli, A., Wierda, W., et al. (2005) Continuous Infusion/Subcutaneous Alemtuzumab (Campath-1H) Plus Rituximab Is Active for Patients with Relapsed/Refractory Chronic Lymphocytic Leukemia (CLL). Abstract 2963, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Ferrajoli, A., O'Brien, S.M., Faderl, S.H., et al. (2005) Rituximab Plus GM-CSF for Patients with Chronic Lymphocytic Leukemia. Abstract 721, updated in an oral presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Feugier, P., Van Hoof, A., Sebban, C., et al. (2005) Long-term results of the R-CHOP study in the treatment of elderly patients with diffuse large B-cell lymphoma: a study by the Groupe d'Étude des Lymphomes de l'Adulte. *J Clin Oncol* 23, 4117–4126

Feuring-Buske, M., Kneba, M., Unterhalt, M., et al. (2000) IDEC-C2B8 (Rituximab) anti-CD20 antibody treatment in relapsed advanced-stage follicular lymphomas: results of a phase-II study of the German Low-Grade Lymphoma Study Group. *Ann Hematol* 79: 493–500.

Fisher, R.I. (1994) Treatment of aggressive non-Hodgkin's lymphomas. Lessons from the past 10 years. *Cancer* 74: 2657–2681.

Fisher, R.I., Gaynor, E.R., Dahlberg, S., et al. (1994) A phase III comparison of CHOP versus m-BACOD versus ProMACE-CytaBOM versus MACOP-B in patients with intermediate- or high-grade non-Hodgkin's lymphoma: results of SWOG-8516 (Intergroup 0067), the National High-Priority Lymphoma Study. *Ann Oncol* 5(Suppl.2): 91–95.

Fisher, S.G., Fisher, R.I. (2004) The epidemiology of non-Hodgkin's lymphoma. *Oncogene* 23: 6524–6534.

Fleischmann, R.M., Pavelka, K., Baldassare, A., et al. (2005) Preliminary efficacy results of rituximab retreatment in patients with active rheumatoid arthritis. *Arthritis Rheum* 52(Suppl.9): S131.

Foran, J.M., Gupta, R.K., Cunningham, D., et al. (2000a) A UK multicentre phase II study of rituximab (chimaeric anti-CD20 monoclonal antibody) in patients with follicular lymphoma, with PCR monitoring of molecular response. *Br J Haematol* 109: 81–88.

Foran, J.M., Rohatiner, A.Z., Cunningham, D., et al. (2000b) European phase II study of rituximab (chimeric anti-CD20 monoclonal antibody) for patients with newly diagnosed mantle-cell lymphoma and previously treated mantle-cell lymphoma, immunocytoma, and small B-cell lymphocytic lymphoma. *J Clin Oncol* 18: 317–324.

Forstpointner, R., Dreyling, M., Repp, R., et al. (2004) The addition of rituximab to a combination of fludarabine, cyclophosphamide, mitoxantrone (FCM) significantly increases the response rate and prolongs survival as compared with FCM alone in patients with relapsed and refractory follicular and mantle cell

lymphomas: results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. *Blood* 104: 3064–3071.

Forstpointner, R., Unterhalt, M., Dreyling, M., et al. (2006) Maintenance therapy with rituximab leads to a significant prolongation of response duration after salvage therapy with a combination of rituximab, fludarabine, cyclophosphamide and mitoxantrone (R-FCM) in patients with relapsed and refractory follicular and mantle cell lymphomas—results of a prospective randomized study of the German low grade lymphoma study group (GLSG). *Blood* Aug 31; [Epub ahead of print].

Friedberg, J.W. (2005) Unique toxicities and resistance mechanisms associated with monoclonal antibody therapy. *Hematology* (*Am Soc Hematol Educ Program*) 2005: 329–334.

Friedberg, J.W., Neyberg, D., Gribben, J.G., et al. (2002) Combination immunotherapy with rituximab and interleukin 2 in patients with relapsed or refractory follicular non-Hodgkin's lymphoma. *Br J Haematol* 117: 828–834.

Gajra, A., Vajpayee, N., Grethlein, S.J. (2004) Response of myasthenia gravis to rituximab in a patient with non-Hodgkin lymphoma. *Am J Hematol* 77: 196–197.

Garcia-Conde, J.M. Conde, E., Sierra, J., et al. (2000) Rituximab (IDEC-C2B8) and CVP chemotherapy in follicular or low grade B-cell lymphoma after relapse: results after 6 months of follow-up. Abstract 96, updated in a poster presentation at the 36th Annual Meeting of the American Society of Clinical Oncology (ASCO), 20–23 May, 2000, New Orleans, USA.

Geisler, C.H., Elonen, E., Kolstad, A., et al. (2005) Nordic Mantle Cell Lymphoma (MCL) Project: Preemptive Rituximab Treatment of Molecular Relapse Following Autotransplant Can Reinduce Molecular Remission and Prolonged Disease-Free Survival. Abstract 2429, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Gertz, M.A., Rue, M., Blood, E., et al. (2004) Multicenter phase 2 trial of rituximab for Waldenström macroglobulinemia (WM): an Eastern Cooperative Oncology Group Study (E3A98). *Leuk Lymphoma* 45: 2047–2055.

Ghielmini, M., Schmitz, S.F., Cogliatti, S.B., et al. (2004) Prolonged treatment with rituximab in patients with follicular lymphoma significantly increases event-free survival and response duration compared with the standard weekly x 4 schedule. *Blood* 103: 4416–4423.

Ghielmini, M., Schmitz, S.F., Cogliatti, S.B., et al. (2005). Effect of single-agent rituximab given at the standard schedule or as prolonged treatment in patients with mantle cell lymphoma; a study of the Swiss Group for Clinical Cancer Research (SAKK). *J Clin Oncol* 23: 705–711.

Gisselbrecht, C., Mounier, N. (2003) Rituximab: enhancing outcome of autologous stem cell transplantation in non-Hodgkin's lymphoma. *Semin Oncol* 30(1 Suppl.2): 28–33.

Glass, B., Kloess, M., Reiser, M., et al. (2005) Dose Escalated CHOP + Etoposide Followed by Repetitive Autologous Stem Cell Transplantation (MegaCHOEP) with or without Rituximab for Primary Treatment of Aggressive NHL. Abstract 1492, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Gluck, W.L., Hurst, D., Yuen, A., et al. (2004) Phase I studies of interleukin (IL)-2 and rituximab in B-cell non-Hodgkin's lymphoma: IL-2 mediated natural killer cell expansion correlations with clinical response. *Clin Cancer Res* 10: 2253–2264.

Gokbuget, N., Hoelzer, D. (2004) Treatment with monoclonal antibodies in acute lymphoblastic leukemia: current knowledge and future prospects. *Ann Hematol* 83: 201–205.

Gordan, L.N., Grow, W.B., Pusateri, A., et al. (2005). Phase II trial of individualized rituximab dosing for patients with CD20-positive lymphoproliferative disorders. *J Clin Oncol* 23: 1096–1102.

Gregory, S.A., Venugopal, P., Adler, S., et al. (2002) Combined fludarabine,

mitoxantrone and rituximab achieves a high initial response as initial treatment for advanced low grade non-Hodgkin's lymphoma. Abstract, updated in a poster presentation at the 44th Annual Meeting of the American Society of Hematology (ASH), 7–10 December 2002, Philadelphia, USA.

Grillo-López, A.J., Cheson, B.D., Horning, S.J., et al. (2000) Response criteria for NHL: importance of 'normal' lymph node size and correlations with response rates. *Ann Oncol* 11: 399–408.

Habermann, T.M., Weller, E., Morrison, V.A., et al. (2006) Rituximab-CHOP versus CHOP alone or with maintenance rituximab in older patients with diffuse large B-cell lymphoma. *J Clin Oncol* 24: 3121–3217.

Habermann, T.M., Weller, E., Morrison, V.A., et al. (2004) Rituximab-CHOP versus CHOP with or without maintenance rituximab in patients 60 years of age or older with diffuse large B-cell lymphoma (DLBCL): an update. Abstract 127, updated in an oral presentation at the 46th Annual Meeting of the American Society of Hematology, 3–7 December 2004, San Diego, USA.

Hainsworth, J.D., Litchy, S., Burris, H.A., et al. (2002) Rituximab as first-line and maintenance therapy for patients with indolent non-Hodgkin's lymphoma. *J Clin Oncol* 20: 4261–4267.

Hainsworth, J.D., Litchy, S., Barton, J.H., et al. (2003a) Single agent rituximab as first-line and maintenance therapy for patients with chronic lymphocytic leukemia or small lymphocytic lymphoma. a phase II trial of the Minnie Pearl Cancer Research Network. *J Clin Oncol* 21: 1746–1751.

Hainsworth, J.D., Litchy, S., Morrissey, L., et al. (2003b) Rituximab as first-line and maintenance therapy for indolent non-Hodgkin's lymphoma (NHL): long-term follow-up of a Minnie Pearl Cancer Research Network phase II trial. Abstract 1496, updated in a poster presentation at the 45th Annual Meeting of the American Society of Hematology (ASH), 6–9 December 2003, San Diego, USA.

Hainsworth, J.D., Litchy, S., Lamb, M.R., et al. (2003c) First-line treatment with brief-duration chemotherapy plus rituximab in elderly patients with intermediate-grade non-Hodgkin's lymphoma: phase II trial. *Clin Lymphoma* 4: 36–42.

Hainsworth J.D., Litchy S., Morrissey L.H., et al. (2005a) Rituximab plus short-duration chemotherapy as first-line treatment for follicular non-Hodgkin's lymphoma: a phase II trial of the Minnie Pearl Cancer Research Network. *J Clin Oncol* 23: 1500–1506.

Hainsworth, J.D., Litchy, S., Shaffer, D.W., et al. (2005b) Maximizing therapeutic benefit of rituximab: maintenance therapy versus re-treatment at progression in patients with indolent non-Hodgkin's lymphoma – a randomized phase II trial of the Minnie Pearl Cancer Research Network. *J Clin Oncol* 23: 1088–1095.

Haioun, C., Lepage, E., Gisselbrecht, C., et al. (2000) Survival benefit of high-dose therapy in poor-risk aggressive non-Hodgkin's lymphoma: final analysis of the prospective LNH87-2 protocol – a Groupe d'Étude des Lymphomes de l'Adulte study. *J Clin Oncol* 18: 3025–3030.

Haioun, C., Mounier, N., Emile, J.F., et al. (2005) Rituximab vs. Observation after High-Dose Consolidative First-Line Chemotherapy (HDC) with Autologous Stem Cell Transplantation in Poor Risk Diffuse Large B-Cell Lymphoma. Final Analysis of the LNH98-B3 GELA Study. Abstract 677, updated in an oral presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Hensel, M., Krasniqi, F., Villalobos, M., et al. (2004) Pentostatin, cyclophosphamide and rituximab is an active regimen with low toxicity for previously treated patients with B-cell chronic lymphocytic leukemia and Waldenström's macroglobulinemia. Abstract 6557, updated in a poster presentation at the 40th Annual Meeting of the American Society of Clinical Oncology (ASCO), 5–8 June 2004, New Orleans, USA.

Hensel, M., Villalobos, M., Kornacker, M., et al. (2005) Pentostatin/cyclophosphamide with or without rituximab: an effective regimen for patients with Waldenström's

macroglobulinemia/lymphoplasmacytic lymphoma. *Clin Lymphoma Myeloma* 6: 131–135.

Hermine, O., Haioun, C., Lepage, E., et al. (1996) Prognostic significance of bcl-2 protein expression in aggressive non-Hodgkin's lymphoma. Groupe d'Étude des Lymphomes de l'Adulte (GELA). *Blood* 87: 265–272.

Herold, M., Pasold, R., Srock, S., et al. (2004) Results of a prospective randomised open label phase II study comparing rituximab plus mitoxantrone, chlorambucil and prednisolone chemotherapy (R-MCP) versus MCP alone in untreated advanced indolent non-Hodgkin's lymphoma (NHL) and mantle cell lymphoma (MCL). Abstract 584, updated in an oral presentation at the 46th Annual Meeting of the American Society of Hematology, (ASH), 3–7 December 2004, San Diego, USA.

Herold, M. Pasold, R. Srock, S., et al. (2005) Rituximab plus mitoxantrone, chlorambucil, prednisolone (R-MCP) is superior to MCP alone in advanced indolent and follicular lymphoma – results of a phase III study (OSHO39). Abstract 60, updated in an oral presentation at the 9th International Conference on Malignant Lymphoma (ICML), June 8–11, 2005, Lugano, Switzerland.

Hicks, L., Buckstein, R., Mangel, J., et al. (2005) Rituximab increases response rate to ESHAP in relapsed, refractory, or transformed aggressive B-cell lymphoma. Abstract 490, 9th International Conference on Malignant Lymphoma (ICML), June 8–11, 2005, Lugano, Switzerland.

Hiddemann, W., Buske, C., Dreyling, M., et al. (2005a) Treatment strategies in follicular lymphomas: current status and future perspectives. *J Clin Oncol* 23: 6394–6399.

Hiddemann, W., Kneba, M., Dreyling, M., et al. (2005b) Front-line therapy with rituximab added to the combination of cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) significantly improves the outcome of patients with advanced stage follicular lymphomas as compared to CHOP alone – results of a prospective randomized study of the German Low Grade Lymphoma Study Group (GLSG). *Blood* 106: 3725–3732.

Hoang-Xuan, K., Camilleri-Broet, S., Soussain, C. (2004) Recent advances in primary CNS lymphoma. *Curr Opin Oncol* 16: 601–606.

Hochster, H.S., Weller, E., Ryan, T., et al. (2004) A phase III trial of CVP with or without maintenance rituximab in advanced indolent lymphoma (NHL). Abstract 6502, updated in an oral presentation at the 40th Annual Meeting of the American Society of Clinical Oncology (ASCO), 5–8 June 2004, New Orleans, USA.

Hochster, H.S, Weller, E., Gascoyne, R., et al. (2005) Maintenance rituximab after CVP results in superior clinical outcome in advanced follicular lymphoma (FL): results of the E1496 trial from the Eastern Cooperative Oncology Group and the Cancer and Leukemia Group B. Abstract 349, updated in an oral presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Hoelzer, D., Bauer, K-H., Giagounidis, A., et al. (2003) Short intensive chemotherapy seems successful in Burkitt NHL, Mature B-ALL and Other High-Grade B-NHL. Abstract 236, updated in an oral presentation at the 45th Annual Meeting of the American Society of Hematology, 6–9 December 2003, San Diego, USA.

Horwitz, S.M., Negrin, R.S., Blume, K.G., et al. (2004) Rituximab as adjuvant to high-dose therapy and autologous hematopoietic cell transplantation for aggressive non-Hodgkin lymphoma. *Blood* 103: 777–783.

Howard, O.M., Gribben, J.G., Neuberg, D.S., et al. (2002) Rituximab and CHOP induction therapy for newly diagnosed mantle-cell lymphoma: molecular complete responses are not predictive of progression-free survival. *J Clin Oncol* 20: 1288–1294.

Huhn, D., von Schilling, C., Wilhelm, M., et al. (2001) Rituximab therapy of patients with B-cell chronic lymphocytic leukemia. *Blood* 98: 1326–1331.

Igarashi, T., Ohtsu, T., Fujii, H., et al. (2001) Re-treatment of relapsed indolent B-cell

lymphoma with rituximab. *Int J Hematol* 73: 213–221.

Igarashi, T., Kobayashi, Y., Ogura, M., et al. (2002) Factors affecting toxicity, response and progression-free survival in relapsed patients with indolent B-cell lymphoma and mantle cell lymphoma treated with rituximab: a Japanese phase II study. *Ann Oncol* 13: 928–943.

Imrie, K., Belch, A., Pettengell, R., et al. (2005) Rituximab plus CVP chemotherapy versus CVP alone as first-line treatment for follicular lymphoma: treatment effect according to baseline prognostic factors. Abstract 6525, updated in an oral presentation at the 41st Annual Meeting of the American Society of Oncology (ASCO), 13–17 May 2005, New Orleans, USA.

Intragumtornchai, T., Bunworasate, U., Nakorn, T.N., et al. (2006) Rituximab-CHOP-ESHAP vs CHOP-ESHAP-mgh-dose therapy vs conventional CHOP chemotherapy in high-intermediate and high-risk aggressive non-Hodgkin's lymphoma. *Leuk Lymphoma* 47: 1306–1314..

Itala, M., Geisler, C.H., Kimby, E., et al. (2002) Standard-dose anti-CD20 antibody rituximab has efficacy in chronic lymphocytic leukaemia: results from a Nordic multicentre study. *Eur J Haematol* 69: 129–134.

Jaeger, G., Neumeister, P., Brezinschek, R., et al. (2002) Rituximab (anti-CD20 monoclonal antibody) as consolidation of first-line CHOP chemotherapy in patients with follicular lymphoma: a phase II study. *Eur J Haematol* 69: 21–26.

Jermann, M., Jost, L.M., Taverna, Ch., et al. (2004) Rituximab-EPOCH, an effective salvage therapy for relapsed, refractory or transformed B-cell lymphomas: results of a phase II study. *Ann Oncol* 15: 511–516.

Johnson, P.W., Rohatiner, A.Z., Whelan, J.S., et al. (1995) Patterns of survival in patients with recurrent follicular lymphoma: a 20-year study from a single center. *J Clin Oncol* 13: 140–147.

Jurlander, J., Geisler, C., Hagberg, H., et al. (2004) Long-term complete molecular remissions in untreated symptomatic follicular lymphoma treated with rituximab as single agent and in combination with interferon-alpha-2a: analysis of minimal residual disease in the randomized phase II study M39035. Abstract 1393, updated in a poster presentation at the 46th Annual Meeting of the American Society of Hematology, (ASH), 3–7 December 2004, San Diego, USA.

Kahl, B., Longo, W., Eickhoff, J., et al (2006) Maintenance rituximab following induction chemoimmunotherapy may prolong progression-free survival in mantle cell lymphoma: a pilot study from the Wisconsis Oncology Network. *Ann Oncol* 17: 1418–1423.

Kaplan, L.D., Lee, J.Y., Ambinder, R.F., et al. (2005) Rituximab does not improve clinical outcome in a randomized phase 3 trial of CHOP with or without rituximab in patients with HIV-associated non-Hodgkin lymphoma: AIDS-Malignancies Consortium Trial 010. *Blood* 106: 1538–1543.

Kasamon, Y.L., Swinnen, L.J. (2004) Treatment advances in adult Burkitt lymphoma and leukemia. *Curr Opin Oncol* 16: 429–435.

Kaufmann, H., Raderer, M., Wöhrer, S., et al. (2004) Antitumor activity of rituximab plus thalidomide in patients with relapsed/refractory mantle cell lymphoma. *Blood* 104: 2269–2271.

Kay, N.E., Geyer, S.M., Call, T.G., et al. (2006) Combination chemoimmunotherapy with pertostatin, cyclophosphomide and rituximab shows significant clinical activity with low accomponying toxicity in previously untreated B-chronic lymphocytic leukemia. *Blood* Sep 28; [Epub ahead of print].

Keating, M.J., O'Brien, S., Albitar, M., et al. (2005a) Early results of a chemoimmunotherapy regimen of fludarabine, cyclophosphamide, and rituximab as initial therapy for chronic lymphocytic leukemia. *J Clin Oncol* 23: 4079–4088.

Keating, M.J., O'Brien, S., Albitar, M., et al. (2005b) Extended Follow-Up of a Chemo-Immunotherapy Regimen FCR (Fludarabine, F; Cyclophosphamide, C; and Rituximab, R) as Initial Therapy for Chronic Lymphocytic Leukemia (CLL).

Abstract 2118, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Keogh, K.A., Ytterberg, S.R., Fervenza, F.C., et al. (2006) Rituximab for Refractory Wegener's Granulomatosis: Report of A Prospective, Open-Label Pilot Trial. *Am J Respir Crit Care Med* 173: 180–187.

Kessler, C.M. (2005) New perspectives in hemophilia treatment. *Hematology* (*Am Soc Hematol Educ Program*), 429–435.

Kewalramani, T., Zelenetz, A.D., Nimer, S. D., et al. (2004) Rituximab and ICE as second-line therapy before autologous stem cell transplantation for relapsed or primary refractory diffuse large B-cell lymphoma. *Blood* 103: 3684–3688.

Keystone, E.C., Burmester, G.R., Furie, R., et al. (2005) Improved quality of life with rituximab plus methotrexate in patients with active rheumatoid arthritis who experienced inadequate response to one or more anti-TNF-α therapies. *Arthritis Rheum* 52(Suppl.9): S141.

Kimby, E. (2005) The safety and tolerability of rituximab (MabThera). *Cancer* 31: 456–473.

Kimby, E., Geisler, C., Hagberg, H., et al. (2002) Rituximab as a single agent and in combination with interferon-alpha-2a as treatment of untreated and first relapse follicular or other low-grade lymphomas. A randomized phase II study. Abstract 289, 8th International Conference on Malignant Lymphoma (ICML), 12–15 June 2002, Lugano, Switzerland.

Ladetto, M., Ricca, I., Benedetti, F., et al. (2005) Rituximab-Supplemented High-Dose Sequential Chemotherapy (HDS) Has Superior Response Rate and Event-Free Survival (EFS) Compared to R-CHOP in Poor Risk Follicular Lymphoma (FL) at Diagnosis: Results from a Multicenter Randomized GITMO Trial. Abstract 675, updated in an oral presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Lamanna, N., Kalaycio, M., Maslak, P., et al. (2005) Pentostatin, Cyclophosphamide, and Rituximab (PCR) Has Comparable Activity but Appears To Be Better Tolerated Than Fludarabine, Cyclophosphamide, and Rituximab (FCR) in Patients with Previously Treated Chronic Lymphocytic Leukemia. Abstract 2127, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Lavilla, E., Perez Encinas, M., Romero, E., et al. (2005) Combination of CHOP-14 with rituximab for newly-diagnosed aggressive B cell lymphoma. Preliminary data of a prospective multicentric regimen. Abstract 480, 9th International Conference on Malignant Lymphoma, 8–11 Jun 2005, Lugano, Switzerland.

Lazzarino, M., Arcaini, L., Orlandi, E., et al. (2005) Immunochemotherapy with rituximab, vincristine and 5-day cyclophosphamide for heavily pretreated follicular lymphoma. *Oncology* 68: 146–153.

Lemieux, B., Bouafia, F., Thieblemont, C., et al. (2004) Second treatment with rituximab in B-cell non-Hodgkin's lymphoma: efficacy and toxicity on 41 patients treated at CHU-Lyon Sud. *Hematol J* 5: 467–471.

Lenz, G., Dreyling, M., Hoster, E., et al. (2005) Immunochemotherapy with rituximab and cyclophosphamide, doxorubicin, vincristine, and prednisone significantly improves response and time to treatment failure, but not long-term outcome in patients with previously untreated mantle cell lymphoma: results of a prospective randomized trial of the German Low Grade Lymphoma Study Group (GLSG). *J Clin Oncol* 23: 1984–1992.

Levine, T.D. (2005) Rituximab in the treatment of dermatomyositis: an open-label pilot study. *Arthritis Rheum* 52: 601–607.

Li, H., Ayer, L.M., Lytton, J., Deans, J.P. (2003) Store-operated cation entry mediated by CD20 in membrane rafts. *J Biol Chem* 278: 42427–42434.

Looney, R.J., Anolik, J.H., Campbell, D., et al. (2004) B cell depletion as a novel treatment for systemic lupus erythematosus: a phase I/II dose-

escalation trial of rituximab. *Arthritis Rheum* 50: 2580–2589.
Maloney, D.G., Lies, T.M., Czerwinski, D.K., et al. (1994) phase I clinical trial using escalating single-dose infusion of chimeric anti-CD20 monoclonal antibody (IDEC-C2B8) in patients with recurrent B-cell lymphoma. *Blood* 84: 2457–2466.
Maloney, D.G., Grillo-López, A.J., Bodkin, D.J., et al. (1997a) IDEC-C2B8: results of a phase I multiple-dose trial in patients with relapsed non-Hodgkin's lymphoma. *J Clin Oncol* 15: 3266–3274.
Maloney, D.G., Grillo-López, A.J., White, C.A., et al. (1997b) IDEC-C2B8 (rituximab) anti-CD20 monoclonal antibody in patients with relapsed low-grade non-Hodgkin's lymphoma. *Blood* 90: 2188–2195.
Mangel, J., Leitch, H.A., Connors, J.M., et al. (2004) Intensive chemotherapy and autologous stem-cell transplantation plus rituximab is superior to conventional chemotherapy for newly diagnosed advanced stage mantle-cell lymphoma: a matched pair analysis. *Ann Oncol* 15: 283–290.
Manshouri, T., Do, K.A., Wang, X., et al. (2003) Circulating CD20 is detectable in the plasma of patients with chronic lymphocytic leukaemia and is of prognostic significance. *Blood* 101: 2507–2513.
Marcus, R., Imrie, K., Belch, A., et al. (2005) CVP chemotherapy plus rituximab compared with CVP as first-line treatment for advanced follicular lymphoma. Blood 105, 1417–1423.
Martinelli, G., Laszlo, D., Bertolini, F., et al. (2003) Chlorambucil in combination with induction and maintenance rituximab is feasible and active in indolent non-Hodgkin's lymphoma. *Br J Haematol* 123: 271–277.
Martinelli, G., Laszlo, D., Ferreri, A.J., et al. (2005) Clinical activity of rituximab in gastric marginal zone non-Hodgkin's lymphoma resistant to or not eligible for anti-*Helicobacter pylori* therapy. *J Clin Oncol* 23: 1979–1983.
Mavromatis, B., Rai, K.R., Wallace, P.K., et al. (2005) Efficacy and Safety of the Combination of Genasense (Oblimersen Sodium, Bcl-2 Antisense Oligonucleotide), Fludarabine and Rituximab in Previously Treated and Untreated Subjects with Chronic Lymphocytic Leukemia. Abstract 2129, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.
McLaughlin, P., Grillo-López, A.J., Link, B.K., et al. (1998) Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. *J Clin Oncol* 16: 2825–2833.
McLaughlin, P., Hagemeister, F.B., Grillo-López, A.J. (1999) Rituximab in indolent lymphoma: the single-agent pivotal trial. *Semin Oncol* 26 (Suppl.14): 79–87.
McLaughlin, P., Rodriguez, M., Hagemeister, F., et al. (2005a) Stage IV indolent lymphoma: a randomized trial of concurrent versus sequential FND (fludarabine, mitoxantrone, dexamethasone) and rituximab, with interferon maintenance. Abstract 247, updated in a poster presentation at the 9th International Conference on Malignant Lymphoma (ICML), June 8–11 2005, Lugano, Switzerland.
McLaughlin, P., Liu, N., Poindexter, N., et al. (2005b) Rituximab plus GM-CSF (Leukine) for indolent lymphoma. Abstract 104, updated in an oral presentation at the 9th International Conference on Malignant Lymphoma (ICML), June 8–11 2005, Lugano, Switzerland.
Mease, P., Szechiński, J., Greenwald, M., et al. (2005) Improvements in patient reported outcomes over 24 weeks for rituximab with methotrexate in rheumatoid arthritis patients in phase IIb trial (DANCER). *Arthritis Rheum* 52(Suppl.9): S138.
Mena, R.M., Smith, J., Geils, G.F., et al. (2005) Enhanced Safety and Tolerability of Dose Intensive Pentostatin and Rituximab in Patients with CLL or Low-Grade B-Cell NHL. Abstract 937, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Mey, U.J., Orlopp, K.S., Flieger, D., et al. (2006) Dexamethasone, high-dose cytarabine, and cisplatin in combination with rituximab as salvage treatment for patients with relapsed or refractory aggressive non-Hodgkin's lymphoma. *Cancer Invest* 24: 593–600.

Milpied, N., Deconinck, E., Gaillard, F., et al. (2004) Initial treatment of aggressive lymphoma with high-dose chemotherapy and autologous stem-cell support. *N Engl J Med* 350: 1287–1295.

Mounier, N., Briere, J., Gisselbrecht, C., et al. (2003) Rituximab plus CHOP (R-CHOP) overcomes bcl-2-associated resistance to chemotherapy in elderly patients with diffuse large B-cell lymphoma (DLBCL). *Blood* 101: 4279–4284.

Nadler, L.M., Ritz, J., Hardy, R., et al. (1981) A unique cell surface antigen identifying lymphoid malignancies of B cell origin. *J Clin Invest* 67: 134–140.

O'Brien, S., Kantarjian, H., Thomas, D.A., et al. (2001) Rituximab dose-escalation trial in chronic lymphocytic leukemia. *J Clin Oncol* 19: 2165–2170.

Oertel, S.H., Verschuuren, E., Reinke, P., et al. (2005) Effect of anti-CD 20 antibody rituximab in patients with post-transplant lymphoproliferative disorder (PTLD). *Am J Transplant* 5: 2901–2906.

Olszewski, A.J., Grossbard, M.L. (2004) Empowering targeted therapy: lessons from rituximab. *Sci STKE* July 6 (241), pe30.

Onrust, S.V., Lamb, H.M., Barman Balfour, J.A. (1999) Rituximab. *Drugs* 58: 79–88.

Pfreundschuh, M., Trümper, L., Kloess, M., et al. (2004a) Two-weekly or 3-weekly CHOP chemotherapy with or without etoposide for the treatment of elderly patients with aggressive lymphomas: results of the NHL-B2 trial of the DSHNHL. *Blood* 104: 634–641.

Pfreundschuh, M., Trümper, L., Kloess, M., et al. (2004b) Two-weekly or 3-weekly CHOP chemotherapy with or without etoposide for the treatment of young patients with good-prognosis (normal LDH) aggressive lymphomas: results of the NHL-B1 trial of the DSHNHL. *Blood* 104: 626–633.

Pfreundschuh, M.G., Trümper, L., Ma, D., et al. (2004c) Randomised intergroup trial of first line treatment for patients ≤60 years with diffuse large B-cell non-Hodgkin's lymphoma (DLBCL) with a CHOP-like regimen with or without the anti-CD20 antibody rituximab – early stopping after the first interim analysis. Abstract 6500, updated in an oral presentation at the 40th Annual Meeting of the American Society of Clinical Oncology, 5–8 June 2004, New Orleans, USA.

Pfreundschuh, M., Trümper, L., Österborg, A., et al. (2006) CHOP-like chemotherapy plus rituximab versus CHOP-like chemotherapy alone in young patients with good-prognosis diffuse large B-cell lymphoma: a randomised controlled trial by the Mab Thera International Trial (MInT) Group. *Lancet Oncol* 7: 379–391.

Pfreundschuh, M.G., Ho, A., Wolf, M., et al. (2005a) Treatment results on CHOP-21, CHOEP-21, MACOP-B and PMitCEBO with and without rituximab in young good-prognosis patients with aggressive lymphomas: rituximab as "equalizer" in the MinT (MabThera International Trial Group) study. Abstract 6529, updated in an oral presentation at the 41st Annual Meeting of the American Society of Clinical Oncology (ASCO), 13–17 May, 2005, Orlando, Florida, USA.

Pfreundschuh, M., Kloess, M., Schmits, R., et al. (2005b) Six, Not Eight Cycles of Bi-Weekly CHOP with Rituximab (R-CHOP-14) Is the Preferred Treatment for Elderly Patients with Diffuse Large B-Cell Lymphoma (DLBCL): Results of the RICOVER-60 Trial of the German High-Grade Non-Hodgkin Lymphoma Study Group (DSHNHL). Abstract 13, updated in an oral presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Pijpe, J., van Imhoff, G.W., Spijkervet, F.K., et al. (2005) Rituximab treatment in patients with primary Sjögren's syndrome: an open-label phase II study. *Arthritis Rheum* 52: 2740–2750.

Pileri, S.A., Milani, M., Fraternali-Orcioni, G., et al. (1998) From the R.E.A.L. Classification to the upcoming WHO

scheme: a step toward universal categorization of lymphoma entities? *Ann Oncol* 9: 607–612.

Piro, L.D., White, C.A., Grillo-López, A.J., et al. (1999) Extended rituximab (anti-CD20 monoclonal antibody) therapy for relapsed or refractory low-grade or follicular non-Hodgkin's lymphoma. *Ann Oncol* 10: 655–661.

Pranzatelli, M.R., Tate, E.D., Travelstead, A. L., Longee, D. (2005) Immunologic and clinical responses to rituximab in a child with opsoclonus-myoclonus syndrome. *Pediatrics* 115: e115–119.

Press, O.W., Appelbaum, F., Ledbetter, J.A., et al. (1987) Monoclonal antibody 1F5 (anti-CD20) serotherapy of human B cell lymphomas. *Blood* 69: 584–591.

Rambaldi, A., Lazzari, M., Manzoni, C., et al. (2002) Monitoring of minimal residual disease after CHOP and rituximab in previously untreated patients with follicular lymphoma. *Blood* 99: 856–862.

Rambaldi, A., Carlotti, E., Oldani, E., et al. (2005) Quantitative PCR of bone marrow BCL2/IgH+ cells at diagnosis predicts treatment response and long-term outcome in follicular non-Hodgkin lymphoma. *Blood* 105: 3428–3433.

Ratanatharathorn V., Ayash L., Reynolds C., et al. (2003) Treatment of chronic graft-versus-host disease with anti-CD20 chimeric monoclonal antibody. *Biol Blood Marrow Transplant* 9: 505–511.

Ravandi-Kashani, F., O'Brien, S., Keating, M., et al. (2005) Complete Eradication of Minimal Residual Disease (MRD) in Patients with Hairy Cell Leukemia (HCL) after Cladribine (2CDA) Followed by an Extended Course of Rituximab. Abstract 3258, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), December 10–13, 2005, Atlanta, USA.

Reff, M.E., Carner, K., Chambers, K.S., et al. (1994) Depletions of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* 83: 435–445.

Rigacci, L., Nassi, L., Alterini, R., et al. (2006) Dose-dense CHOP plus rituximab (R-CHOP-14) for the treatment of elderly patients with high-risk diffuse large B cell lymphoma. *Acta Haematologica* 115: 22–27.

Rivas-Vera, S., Báez, E., Sobrevilla-Calvo, P., et al. (2005) Is first line single agent rituximab the best treatment for indolent non-Hodgkin lymphoma? Update of a multicentric study comparing rituximab versus CNOP versus rituximab plus CNOP. Abstract 2431, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), December 10–13, 2005, Atlanta, USA.

Robak, T. (2004) Monoclonal antibodies in the treatment of autoimmune cytopenias. *Eur J Haematol* 72: 79–88.

Rodriguez, M.A., Dang, N.H., Fayad, L., et al. (2005) Phase II Study of Sphingosomal Vincristine in CHOP+/− Rituximab for Patients with Aggressive Non-Hodgkin's Lymphoma (NHL): Promising 3 Year Follow-Up Results in Elderly Patients. Abstract 943, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December, Atlanta, USA.

Rohatiner, A.Z., Gregory, W.M., Peterson, B., et al. (2005) Meta-analysis to evaluate the role of interferon in follicular lymphoma. *J Clin Oncol* 23: 2215–2223.

Romaguera, J.E., Fayad, L., Rodriguez, M.A., et al. (2005a) High rate of durable remission after treatment of newly diagnosed aggressive mantle-cell lymphoma with rituximab plus hyper-CVAD alternating with rituximab plus high-dose methotrexate and cytarabine. *J Clin Oncol* 23: 7013–7023.

Romaguera, J.E., Fayad, L.E., Wang, M., et al. (2005b) High (95%) response rates in relapsed/refractory mantle cell lymphoma after R-HCAD alternating with R-methotrexate/cytarabine (R-M-A). Abstract 2446, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December, Atlanta, USA.

Rothe, A., Schulz, H., Elter, T., et al. (2004) Rituximab monotherapy is effective in patients with poor risk refractory aggressive non-Hodgkin's lymphoma. *Haematologica* 89: 875–876.

Rubenstein, J.L., Shen, A.,, Abrey, L., et al. (2004) Results from a phase I study of

intraventricular administration of rituximab in patients with recurrent lymphomatous meningitis. Abstract 6593, updated in a poster presentation at the 40th Annual Meeting of the American Society of Clinical Oncology (ASCO), 5–8 June 2004, New Orleans, USA.

Rummel, M.J., Al-Batran, S.E., Kim, S.-Z., et al. (2005) Bendamustine plus rituximab is effective and has a favorable toxicity profile in the treatment of mantle cell and low-grade non-Hodgkin's lymphoma. *J Clin Oncol* 23: 3383–3389.

Sacchi, S., Tucci, A., Merli, F., et al. (2003) Efficacy controls and long-term follow-up of patients (pts) treated with FC plus rituximab for relapsed follicular NHL. Abstract 4901, 45th Annual Meeting of the American Society of Hematology (ASH), 6–9 December 2003, San Diego, USA.

Sacchi, S., Federico, M., Vitolo, U., et al. (2001) Clinical activity and safety of combination immunotherapy with interferon-α2a and rituximab in patients with relapsed low grade non-Hodgkin's lymphoma. *Haematologica* 86: 951–958.

Salles, G., Foussard, C., Mounier, N., et al. (2004) Rituximab added to αIFN-CHVP improves the outcome of follicular lymphoma patients with a high tumor burden: first analysis of the GELA-GOELAMS FL-2000 randomised trial in 359 patients. Abstract 160, updated in an oral presentation at the 46th Annual Meeting of the American Society of Hematology (ASH), 3–7 December 2004, San Diego, USA.

Savage, D.G., Cohen, N.S., Hesdorffer, C.S., et al. (2003) Combined fludarabine and rituximab for low grade lymphoma and chronic lymphocytic leukemia. *Leuk Lymphoma* 44: 477–481.

Savage K.J., Al-Rajhi N., Voss N., et al. (2006) Favorable outcome of primary mediastinal large B-cell lymphoma in a single institution: the British Columbia experience. *Ann Oncol* 17: 123–130.

Schulz, H., Klein, S.K., Rehwald, U., et al. (2002) Phase 2 study of a combined immunochemotherapy using rituximab and fludarabine in patients with chronic lymphocytic leukemia. *Blood* 100: 3115–3120.

Schulz, H., Pels, H., Schmidt-Wolf, I., Zeelen, U., Germing, U., Engert, A. (2004) Intraventricular treatment of relapsed central nervous system lymphoma with the anti-CD20 antibody rituximab. *Haematologica* 89: 753–754.

Schulz, H., Skoetz, N., Bohlius, J., et al. (2005a) Does Combined Immunochemotherapy with the Monoclonal Antibody Rituximab Improve Overall Survival in the Treatment of Patients with Indolent Non-Hodgkin Lymphoma? Preliminary Results of a Comprehensive Meta-Analysis. Abstract 351, updated in an oral presentation at the 47th Annual Meeting of the American Society of Haematology (ASH), 10–13 December 2005, Atlanta, USA.

Schulz, H., Trelle, S., Reiser, M., et al. (2005b) Rituximab in Relapsed Lymphocyte Predominant Hodgkin's Disease (LPHD). Long-Term Results of a phase-II Study from the German Hodgkin Lymphoma Study Group (GHSG). Abstract 1503, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Sirohi, B., Cunningham, D., Norman, A., et al. (2005) Use of Gemcitabine, Cisplatin and Methylprednisolone (GEM-P) with or without rituximab in relapsed and refractory patients with Diffuse Large B Cell Lymphoma (DLBCL). Abstract 939, updated in a poster presentation at the 47th Annual Meeting of the American Society of Haematology (ASH), 10–13 December 2005, Atlanta, USA.

Sehn, L.H., Donaldson, J., Chhanabhai, M., et al. (2005) Introduction of combined CHOP plus rituximab therapy dramatically improved outcome of diffuse large B-cell lymphoma in British Columbia. *J Clin Oncol* 23: 5027–5033.

Solal-Celigny, P., Salles, G.A., Brousse, N., et al. (2004) Single 4-Dose Rituximab Treatment for Low-Tumor Burden Follicular Lymphoma (FL): Survival Analyses with a Follow-Up (F/Up) of at Least 5 Years. Session Type: Abstract 585 updated in an oral presentation at the 46th Annual meeting of the American Society

of Hematology (ASH), 3–7 December 2004, San Diego, USA.
Solal-Celigny, P., Imrie, I., Belch, A., et al. (2005) Mabthera (Rituximab) Plus CVP Chemotherapy for First-Line Treatment of Stage III/IV Follicular Non-Hodgkin's Lymphoma (NHL): Confirmed Efficacy with Longer Follow-Up. Abstract 350 updated in an oral presentation at the 47th Annual meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.
Sonneveld, P., van Putten, W., Holte, H., et al. (2005) Intensified CHOP with Rituximab for Intermediate or High-Risk Non-Hodgkin's Lymphoma: Interim Analysis of a Randomized phase III Trial in Elderly Patients by the Dutch HOVON and Nordic Lymphoma Groups. Abstract 16, updated in an oral presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.
Spina, M., Jaeger, U., Sparano, J.A., et al. (2005) Rituximab plus infusional cyclophosphamide, doxorubicin, and etoposide in HIV-associated non-Hodgkin lymphoma: pooled results from 3 phase 2 trials. *Blood* 105: 1891–1897.
Stewart, W.P., Crowther, D., McWilliam, L.J., et al. (1988) Maintenance chlorambucil after CVP in the management of advanced stage, low-grade histologic type non-Hodgkin's lymphoma. A randomized prospective study with an assessment of prognostic factors. *Cancer* 61: 441–447.
The Non-Hodgkin's Lymphoma Classification Project. (1997a) A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. *Blood* 89: 3909–3918.
The Non-Hodgkin's Lymphoma Classification Project. (1997b) Effect of age on the characteristics and clinical behavior of non-Hodgkin's lymphoma patients. *Ann Oncol* 8: 973–978.
Thomas, D.A., O'Brien, S., Giles, F.J., et al. (2001) Single agent rituximab in early stage chronic lymphocytic leukaemia. Abstract 1533, updated in a poster presentation at the 43rd Annual meeting of the American Society of Hematology (ASH), 7–11 December 2001, Orlando, USA.
Thomas, D.A., O'Brien, S., Bueso-Ramos, C., et al. (2003) Rituximab in relapsed or refractory hairy cell leukemia. *Blood* 102: 3906–3911.
Thomas, D.A., Faderl, S., O'Brien, S., et al. (2006) Chemoimmunotherapy with hyper-CVAD plus rituximab for the treatment of adult Burkitt and Burkitt-type lymphoma or acute lymphoblastic leukemia. *Cancer* 106: 1569–1580.
Tobinai, K., Igarashi, T., Itoh, K., et al. (2004) Japanese multicenter phase II and pharmacokinetic study of rituximab in relapsed or refractory patients with aggressive B-cell lymphoma. *Ann Oncol* 15: 821–830.
Trappe, R., Oertel, S., Choquet, S., et al. (2005) Sequential Treatment with the Anti-CD 20 Antibody Rituximab and CHOP+GCSF Chemotherapy in Patients with Post-Transplant Lymphoproliferative Disorder (PTLD): First Interim Analysis of a Multicenter phase II Study. Abstract 932, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.
Treon, S.P., Agus, D.B., Link, B., et al. (2001) CD20-directed antibody-mediated immunotherapy induces responses and facilitates hematologic recovery in patients with Waldenström's macroglobulinemia. *J Immunother* 24: 272–279.
Treon, S.P., Kelliher, A., Keele, B., et al. (2003) Expression of serotherapy target antigens in Waldenström's macroglobulinemia: therapeutic applications and considerations. *Semin Oncol* 30: 248–252.
Treon, S.P., Emmanouilides, C., Kimby, E., et al. (2005a) Extended rituximab therapy in Waldenström's macroglobulinemia. *Ann Oncol* 16: 132–138.
Treon, S.P., Hunter, Z., Barnagan, A.R. (2005b) CHOP plus rituximab therapy in Waldenström's macroglobulinemia. *Clin Lymphoma* 5: 273–277.
Treon, S.P., Gertz, M.A., Dimopoulos, M., et al. (2006) Update on treatment

recommendations from the Third International Workshop on Waldenström's Macroglobulinemia. *Blood* 107: 3442–3446.

Trneny M., Belada D., Vasova I., et al. (2005) Rituximab combination with anthracycline based chemotherapy significantly improved the outcome of young patients with diffuse large B-cell lymphoma in low as well in high risk subgroups. Abstract 2444, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Van der Kolk, L.E., Grillo-López, A.J., Baars, J.W., et al. (2003) Treatment of relapsed B-cell non-Hodgkin's lymphoma with a combination of chimeric anti-CD20 monoclonal antibodies (rituximab) and G-CSF: final report on safety and efficacy. *Leukemia* 17: 1658–1664.

van Oers, M.H., Klasa, R., Marcus, R.E., et al. (2006) Rituximab maintenance improves clinical outcome of relapsed/ resistant follicular non-Hodgkin's lymphoma, both in patients with old without rituximab during induction: results of a prospective randomized phase III intergroup trial. *Blood* 108: 3295–3301.

Venugopal, O., Gretory, S.A., Showel, J., et al. (2004) Rituximab (Rituxan) Combined with ESHAP Chemotherapy Is Highly Active in Relapsed/ Refractory Aggressive Non-Hodgkin's Lymphoma. Abstract 4636, 46th Annual Meeting of the American Society of Hematology (ASH), 3–7 December 2004, San Diego, USA.

Vitolo, U., Boccomini, C., Ladetto, M., et al. (2004) Sequential brief chemo-immunotherapy FND + rituximab in elderly patients with advanced stage follicular lymphoma (FL): high clinical and molecular remission rate associated with a prolonged failure-free survival. Abstract 1320, updated in a poster presentation at the 46th Annual Meeting of the American Society of Hematology (ASH), 4–7 December 2004, San Diego, USA.

Vose, J.M., Link, B.K., Grossbard, M.L., et al. (2001) Phase II study of rituximab in combination with CHOP chemotherapy in patients with previously untreated, aggressive non-Hodgkin's lymphoma. *J Clin Oncol* 19: 389–397.

Vose, J.M., Link, B.K., Grossbard, M.L., et al. (2002) Long term follow-up of a phase II study of rituximab in combination with CHOP chemotherapy in patients with previously untreated aggressive non-Hodgkin's lymphoma (NHL). Abstract 1396, updated in a poster presentation at the 44th American Society of Hematology Annual Meeting, 6–10 December 2002, Philadelphia, USA.

Vose, J.M., Link, B.K., Grossbard, M.L., et al. (2005) Long-term update of a phase II study of rituximab in combination with CHOP chemotherapy in patients with previously untreated, aggressive non-Hodgkin's lymphoma. *Leuk Lymphoma* 46: 1569–1573.

Weber, D.M., Dimopoulos, M.A., Delasalle, K., et al. (2003) 2-Chlorodeoxyadenosine alone and in combination for previously untreated Waldenstrom's macroglobulinemia. *Semin Oncol* 30: 243–247.

Weide, R., Heymanns, J., Thomalla, J., et al. (2004) Bendamustine/mitoxantrone/ rituximab (BMR): a very effective and well tolerated immuno-chemotherapy for relapsed and refractory indolent lymphomas – results of a multicentre phase-II-study of the German Low Grade Lymphoma Study Group (GLSG). Abstract 2482, updated in a poster presentation at the 46th Annual Meeting of the American Society of Hematology (ASH), 4–7 December 2004, San Diego, USA.

Wierda, W., O'Brien, S., Wen, S., et al. (2005a) Chemoimmunotherapy with fludarabine, cyclophosphamide, and rituximab for relapsed and refractory chronic lymphocytic leukemia. *J Clin Oncol* 23: 4070–4078.

Wierda, W.G., O'Brien, S., Faderl, S., et al. (2006) A retrospective comparison of three sequential groups of patients with recurrent/refractory chronic lymphocytic leukemia treated with fludarabine-based regimens. *Cancer* 106: 337–345.

Wierda, W., O'Brien, S., Ferrajoli, A., et al. (2005b) Salvage Therapy with Combined Cyclophosphamide (C), Fludarabine (F), Alemtuzumab (A), and Rituximab (R)

(CFAR) for Heavily Pre-Treated Patients with CLL. Abstract 719, updated in an oral presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Wierda, W.G., Kipps, T.J., Keating, M.J. (2005c) Novel immune-based treatment strategies for chronic lymphocytic leukemia. *J Clin Oncol* 23: 6325–6332.

Wilson, W.H., Grossbard, M.L., Pittaluga, S., et al. (2002). Dose-adjusted EPOCH chemotherapy for untreated large B-cell lymphomas: a pharmacodynamic approach with high efficacy. *Blood* 99: 2685–2693.

Wilson, W.H., Pittaluga, S., Gutierrez, G., et al. (2003) Dose-adjusted EPOCH-rituximab in untreated diffuse large B-cell lymphoma: Benefit of rituximab appears restricted to tumors harboring anti-apoptotic mechanisms. Abstract 356, updated in an oral presentation at the American Society of Hematology Annual Meeting, 6–9 December 2003, San Diego, USA.

Wilson, W.H., Porcu, P., Hurd, D., et al. (2005) Phase II study of dose-adjusted EPOCH-R in untreated de novo CD20+ diffuse large B-cell lymphoma – CALGB 50103. Abstract 6530, updated in an oral presentation at the 41st Annual Meeting of the American Society of Clinical Oncology, May 13–17, Orlando, Florida, USA.

Witzig, T.E. (2005) Current treatment approaches for mantle-cell lymphoma. *J Clin Oncol* 23: 6409–6414.

Witzig, T.E., Vukov, A.M., Habermann, T. M., et al. (2005) Rituximab therapy for patients with newly diagnosed, advanced-stage, follicular grade I non-Hodgkin's lymphoma: a phase II trial in the North Central Cancer Treatment Group. *J Clin Oncol* 23: 1103–1108.

Wong, E.T., Tishler, R., Barron, L., Wu, J.K. (2004) Immunochemotherapy with rituximab and temozolomide for central nervous system lymphomas. *Cancer* 101: 139–145.

Woods, A., Buckstein, R., Mangel, J., et al. (2005) Prolonged remissions in patients with recurrent follicular lymphoma treated with ASCT and rituximab as in vivo purging and consolidative immunotherapy. Abstract 404, 9th International Conference on Malignant lymphoma (ICML), 8–11 June 2005, Lugano, Switzerland.

Younes, A., McLaughlin, P., Fayad, L.E., et al. (2005a) Six Weekly Doses of Rituximab Plus ABVD for Newly Diagnosed Patients with Advanced Stage Classical Hodgkin Lymphoma: Depletion of Reactive B-Cells from the Microenvironment by Rituximab. Abstract 1499, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Younes, A., Fayad, L., Pro, B., et al. (2005b) Gemcitabine Plus Rituximab Therapy of Patients with Relapsed and Refractory Classical Hodgkin Lymphoma. Abstract 1498, updated in an oral presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Yuen, A.R., Kamel, O.W., Halpern, J., et al. (1995) Long-term survival after histologic transformation of low-grade follicular lymphoma. *J Clin Oncol* 13: 1726–1733.

Yunus, F., George, S., Smith, J., et al. (2003) Phase II Multicenter Trial of Pentostatin and Rituximab in Patients with Previously Treated and Untreated Chronic Lymphocytic Leukemia (CLL). Abstract 5168, 45th Annual Meeting of the American Society of Hematology (ASH), 6–9 December 2003, San Diego, USA.

Zinzani, P.L., Storti, S., Zaccaria, A., et al. (1999) Elderly aggressive-histology non-Hodgkin's lymphoma: first-line VNCOP-B regimen experience on 350 patients. *Blood* 94: 33–38

Zinzani P.L., Pulsoni A., Perrotti A., et al. (2004) Fludarabine plus mitoxantrone with and without rituximab versus CHOP with and without rituximab as front-line treatment for patients with follicular lymphoma. *J Clin Oncol* 22: 2654–2661.

Zaja, F.F., Bacigalupo, A.A., Bosi, A.A., et al. (2005) Rituximab may allow to improve multi-organ involvement in patients with cGVHD. Abstract 3100, updated in a poster presentation at the 47th Annual Meeting of the American Society of Hematology (ASH), 10–13 December 2005, Atlanta, USA.

Websites

Surveillance Epidemiology and End Results (SEER), National Cancer Institute. Cancer stat fact sheets: non-Hodgkin's lymphoma. http://seer.cancer.gov/statfacts/html/nhl.html (accessed January 2006)

Surveillance Epidemiology and End Results (SEER), National Cancer Institute. Cancer stat fact sheets: chronic lymphocytic leukemia. http://seer.cancer.gov/statfacts/html/clyl.html (accessed January 2006)

European Medicines Agency (EMEA). MabThera Summary of Product Characteristics (SmPC). Available from URL: http://www.emea.eu.int/humandocs/Humans/EPAR/mabthera/mabthera.htm (accessed January 2006)

Genentech, Inc. Rituxan prescribing information. http://www.gene.com/gene/products/information/pdf/rituxan-prescribing.pdf (accessed January 2006)

National Comprehensive Cancer Network Physician Prescribing Guidelines. Non-Hodgkin's lymphoma, v1 2006 http://www.nccn.org/professionals/physician_gls/PDF/nhl.pdf (accessed February 2006)

Clinicaltrials.gov. A service of the US National Institutes of Health http://www.clinicaltrials.gov/ (accessed February 2006)

14
Trastuzumab (Herceptin)
A Treatment for HER2-Positive Breast Cancer

Paul Ellis

14.1
Introduction

Worldwide, it is estimated that more than 1 million women have breast cancer, of whom around two-fifths will die from the disease (Parkin et al. 2005). The progression of breast cancer can be separated into five stages depending on the size of tumor, the involvement of lymph nodes, and the spread of tumor cells to other tissues/organs. Stages 0 to 2 are classified as early, stage 3 as locally advanced, and stage 4 as advanced or metastatic breast cancer (MBC). Human epidermal growth factor receptor 2 (HER2) is overexpressed in approximately 20 to 30% of breast cancers (Owens et al. 2004; Penault-Llorca et al. 2005; Ross et al. 2004; Slamon et al. 1987, 1989) and is associated with aggressive tumor behavior and poor prognosis (Ménard et al. 2001; Press et al. 1997; Slamon et al. 1987). The HER2 status of a tumor can be assessed using immunohistochemistry (IHC), which provides a score of 0 to 3+ based on HER2 protein expression (IHC 3+ indicating HER2 overexpression), or by DNA-based methodologies such as fluorescence *in-situ* hybridization (FISH) or chromogenic *in-situ* hybridization, which measure HER2 gene amplification. The term "HER2-positive" is used to describe a tumor that has HER2 protein overexpression and/or HER2 gene amplification.

Trastuzumab (Herceptin) is a biologically engineered, humanized immunoglobulin-1, anti-HER2 monoclonal antibody (mAb) developed by Genentech/Roche that is directed against the extracellular domain of HER2 (Carter et al. 1992). Antibody production uses a genetically engineered Chinese hamster ovary (CHO) cell line grown on a commercial scale. The DNA coding sequence of trastuzumab is inserted into these cells using standardized recombinant techniques. The antibody is secreted into the culture medium by the cells, and is purified using standard chromatographic and filtration methods. Trastuzumab is 95% human and 5% murine, and is supplied as a lyophilized powder to be

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reconstituted with bacteriostatic or sterile water for either multidose or single-dose vials.

Trastuzumab is the first commercially available mAb-based therapy for the treatment of breast cancer, and is currently available in more than 90 countries worldwide. It has been shown to markedly inhibit the growth of breast tumors (Baselga et al. 1998; Pegram et al. 1999; Pietras et al. 1998). Although the exact mechanisms of this effect are not yet clear, recent studies have suggested a number of possible mechanisms by which trastuzumab exerts its anti-tumor effects. A high infiltration of leukocytes and natural killer (NK) cells has been noted in HER2-positive patients following trastuzumab treatment (Arnould et al. 2006; Gennari et al. 2004), suggesting that immune cells are recruited by trastuzumab to attack the tumor cells by antibody-dependent cellular cytotoxicity. When combined with standard chemotherapy agents, trastuzumab has been found to inhibit the anti-apoptotic effect of HER2 overexpression, and thereby trigger apoptosis (Kim et al. 2005). Other possible mechanisms of action for trastuzumab are decreasing HER2 phosphorylation and downstream signaling (Ekerljung et al. 2006), interacting with other signaling pathways (Holbro et al. 2003; Le et al. 2006), HER2 receptor down-modulation and internalization from the cell membrane (Klapper et al. 2000), and the inhibition of constitutive HER2 cleavage/shedding mediated by metalloproteinase which prevents homodimerization of HER2 derivatives (Molina et al. 2001).

Approval was granted by the US Food and Drug Administration (FDA) in September 1998, and by the European Medicines Agency in September 2000, for the use of trastuzumab in women with HER2-positive MBC. The initial license was based on data from two pivotal trials, one using trastuzumab as second-/third-line monotherapy (see Section 14.2.1) (Cobleigh et al. 1999), and a second using first-line trastuzumab plus chemotherapy (the H0648g trial, including paclitaxel for patients relapsing after prior adjuvant anthracycline chemotherapy; see Section 14.2.1) (Slamon et al. 2001).

In 2006, the European Commission approved the use of trastuzumab for the adjuvant treatment of HER2-positive early breast cancer (EBC), based on results from the international HERA (HERceptin Adjuvant) study (see Section 14.3). Trastuzumab given after standard chemotherapy was found significantly to reduce the risk of cancer remission by 46%, and to be well tolerated compared with chemotherapy alone (Piccart-Gebhart et al. 2005). This was supported by the results of three other major global and US studies: the North Central Cancer Treatment Group (NCCTG) N9831 trial, the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-31 trial and the Breast Cancer International Research Group (BCIRG) 006 trial (see Section 14.3).

14.2
Metastatic Breast Cancer

14.2.1
Trastuzumab Monotherapy

The efficacy and safety of trastuzumab (4 mg kg^{-1} body weight loading dose followed by 2 mg kg^{-1} weekly) was studied in a multicenter, open-label, single-arm trial comprising 222 women with HER2-positive (IHC 2+/3+) MBC that had progressed after one or two chemotherapy regimens (Cobleigh et al. 1999). Two or more lines of therapy had previously been given to 68% of patients; 94% had received anthracyclines and 67% taxanes. In the intent-to-treat population, there were eight complete and 26 partial responses, giving an objective response rate of 15%. The median duration of response was 9.1 months, which was substantially longer than the 5.2 months achieved with the chemotherapy regimens used previously by these women. Median survival was 13 months (Fig. 14.1), and median time to progression was 3.1 months. These results highlight the clinical benefits of trastuzumab as second- or third-line monotherapy in HER2-positive MBC. Patients with IHC 3+ and/or FISH-positive disease derived the most clinical benefit from trastuzumab in this study (Cobleigh et al. 1999).

After promising results with trastuzumab as second-/third-line monotherapy, the efficacy and safety of trastuzumab as first-line monotherapy (4 mg kg^{-1} loading dose followed by 2 mg kg^{-1} weekly, or 8 mg kg^{-1} loading dose followed by 4 mg kg^{-1}

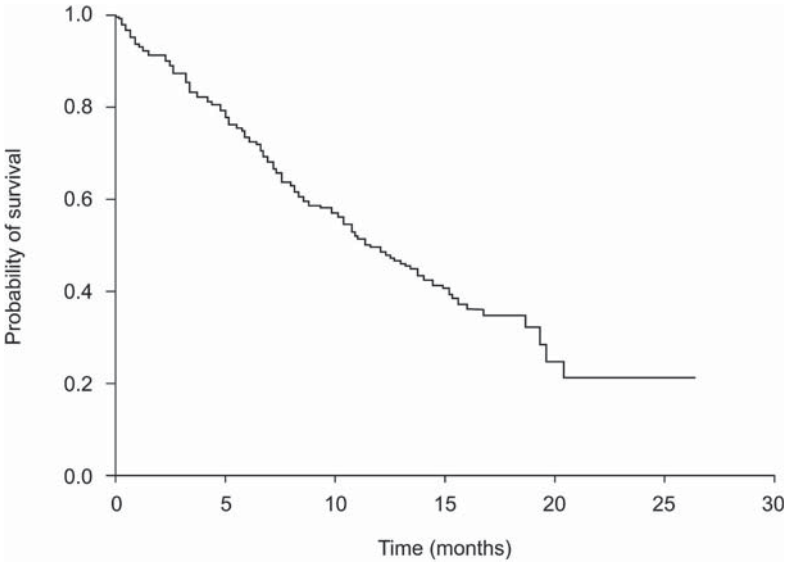

Fig. 14.1 Survival with trastuzumab as second-/third-line monotherapy given weekly (Cobleigh et al. 1999).

weekly) was investigated in a phase II multicenter trial involving 114 patients with previously untreated HER2-positive (IHC 2+/3+) MBC (Vogel et al. 2002). The objective response rate was 26%, with seven complete and 23 partial responses. For patients with IHC 3+ tumors (n = 111), the response rate was 35%; however, no response was seen in IHC 2+ patients. Clinical benefit (complete, partial or minor response, or stable disease for longer than 6 months) rates of 48% and 7% were observed in IHC 3+ and 2+ patients, respectively. Response rates in 108 assessable patients with and without HER2 gene amplification by FISH were 34% and 7%, and clinical benefit rates were 48% and 10%, respectively. At 12-month follow-up or later, 57% of patients with objective response and 51% with clinical benefit had not progressed. The median overall survival was 24.4 months. There was no clear evidence of any dose-dependent relationship for response or survival. As was also seen in the Cobleigh study (Cobleigh et al. 1999), IHC 3+ and/or FISH-positive patients had the greatest clinical benefit in this study (Vogel et al. 2002).

Ideally, treatment should be convenient for the patient, and this can be achieved with less-frequent dosing, which may also have a positive health-economic impact because of reduced hospital and clinic visits. Thus, in addition to the standard weekly regimen, a 3-weekly regimen of trastuzumab monotherapy was investigated. The efficacy, safety and pharmacokinetics of 3-weekly trastuzumab ($8\,mg\,kg^{-1}$ loading dose followed by $6\,mg\,kg^{-1}$) were assessed in a phase II single-arm trial comprising 105 patients with previously untreated HER2-positive MBC (Baselga et al. 2005). The median time to progression was 3.4 months, and median duration of response 10.1 months. The overall response rate was 19% and the clinical benefit rate 33%. In patients with measurable, centrally confirmed HER2-positive disease (per-protocol subset), these rates were even higher: 23% response rate and 36% clinical benefit rate. After a further 12 months of follow-up, the overall response rate was 24% and the clinical benefit rate remained at 36% in the per-protocol subset. These data are within the range observed in other trials of weekly trastuzumab plus paclitaxel, and indicate that the 3-weekly regimen is as potentially efficacious as the weekly regimen when used in combination with paclitaxel. Furthermore, the 3-weekly schedule did not compromise the safety of trastuzumab in women with HER2-positive MBC.

14.2.2 Trastuzumab in Combination with Taxanes

Pivotal randomized combination trials of trastuzumab have demonstrated that trastuzumab plus a taxane (paclitaxel or docetaxel) is associated with superior clinical benefit compared with a taxane alone. In a randomized, multicenter, phase III trial comprising 469 women with previously untreated HER2-positive (IHC 2+/3+) MBC (Slamon et al. 2001; Eiermann and International Herceptin Study Group, 2001), patients who had previously received anthracyclines in the adjuvant setting were randomized to receive paclitaxel ($175\,mg\,m^{-2}$, 3-weekly) either alone (n = 96) or with trastuzumab ($4\,mg\,kg^{-1}$ loading dose followed by

2 mg kg^{-1} weekly; n = 92) (Slamon et al. 2001). All other patients were randomized to receive anthracycline (doxorubicin 60 mg m^{-2} or epirubicin 75 mg m^{-2}) plus cyclophosphamide (600 mg m^{-2}) either alone (n = 138) or with trastuzumab (4 mg kg^{-1} loading dose followed by 2 mg kg^{-1} weekly; n = 143).

At 30-month median follow-up, the addition of trastuzumab to paclitaxel improved all clinical endpoints compared with paclitaxel alone. The improvement in median overall survival from 18.4 to 22.1 months (18 to 25 months in IHC 3+ patients) with addition of trastuzumab is particularly notable because of the aggressive nature of HER2-positive disease and the poor prognosis of these patients. Furthermore, the crossover design of the study, in which 73% of patients subsequently received trastuzumab after progressing on single-agent paclitaxel, biased against observing a survival advantage (Slamon et al. 2001). In a similar pattern to that seen in the weekly monotherapy trials (Vogel et al. 2002), the trastuzumab-paclitaxel combination provided the greatest clinical benefit in patients with IHC 3+ and/or FISH-positive disease (Baselga, 2001; Slamon et al. 2001).

The combination of trastuzumab and docetaxel was investigated in the pivotal randomized phase II trial (M77001) comprising 186 patients with previously untreated MBC (Extra et al. 2005; Marty et al. 2005). Patients received 3-weekly docetaxel (100 mg m^{-2}) with or without weekly trastuzumab (4 mg kg^{-1} loading dose followed by 2 mg kg^{-1}). Patients in the docetaxel-alone arm could cross over to receive trastuzumab on disease progression. At 24-month follow-up, the addition of trastuzumab to docetaxel significantly improved all clinical outcomes investigated (Table 14.1), including an increase in median overall survival from 22.7 to 31.2 months (treatment benefit of 8.5 months; p = 0.0325) (Fig. 14.2). Twice as many patients who received trastuzumab plus docetaxel lived 3 years or longer compared to those given docetaxel only. Furthermore, patients who crossed over from docetaxel monotherapy to receive trastuzumab on progression (57%) appeared to survive longer than those who did not receive trastuzumab (Marty et al. 2005).

Table 14.1 Clinical outcomes with trastuzumab plus docetaxel, and docetaxel alone (Marty et al. 2005).

	Trastuzumab + docetaxel (n = 92)	***Docetaxel alone (n = 94)***	***p-value***
ORR, %	61.0	34.0	0.0002
Median DoR [months]	11.7	5.7	0.0009
Median TTP [months]	11.7	6.1	0.0001
Median OS[a] [months]	31.2	22.7	0.0325

a Kaplan-Meier estimates.
DoR = duration of response; ORR = overall response rate; OS = overall survival; TTP = time to progression.

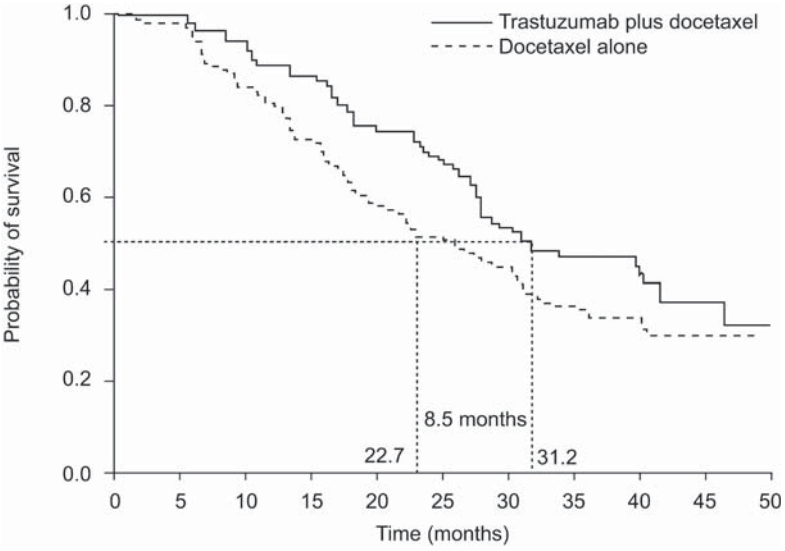

Fig. 14.2 Overall survival with trastuzumab plus docetaxel and docetaxel alone (Extra et al. 2005; Marty et al. 2005).

In addition to the pivotal trials, several phase II MBC trials have confirmed that the combination of trastuzumab plus a taxane improves clinical outcomes compared with taxane alone. In these trials, response rates for trastuzumab given weekly or 3-weekly in combination with paclitaxel or docetaxel ranged from 36 to 81% and from 44 to 75% for paclitaxel and docetaxel combinations, respectively.

14.2.3 Trastuzumab in Combination with other Standard Chemotherapy

Trials have been reported that investigated trastuzumab in combination with several chemotherapeutic agents commonly used in the treatment of MBC. Phase II trials of trastuzumab plus vinorelbine as first- or subsequent-line therapy showed objective response rates ranging from 43% to 85% (Bayo et al. 2004; Bernardo et al. 2004; Burstein et al. 2001, 2003, 2006; Chan et al. 2005; de Wit et al. 2004; Glogowska et al. 2004; Jahanzeb et al. 2002; Papaldo et al. 2006), indicating that this combination is highly active in the treatment of MBC. The combination of trastuzumab with gemcitabine in patients with HER2-positive MBC has also been reported as effective, with objective response rates of 36 to 40% in phase II trials (Christodoulou et al. 2003; O'Shaughnessy et al. 2004; Stemmler et al. 2005). A phase II study of the 5-fluorouracil prodrug capecitabine plus trastuzumab in patients with pretreated MBC achieved an objective response rate of 60% (Schaller et al. 2005), and this was mirrored in a phase II study of

first-line trastuzumab-capecitabine therapy (objective response rate, 63%; n = 43) (Xu et al. 2006).

Combinations of trastuzumab with each of the aforementioned agents were generally well tolerated.

14.2.4 Trastuzumab in Triple Combination

Trastuzumab-containing triple combinations (e.g., trastuzumab/carboplatin/paclitaxel, trastuzumab/cisplatin/docetaxel, trastuzumab/epirubicin/docetaxel, trastuzumab/vinorelbine/docetaxel, or trastuzumab/gemcitabine/paclitaxel) can produce high response rates and are generally feasible if overlapping toxicity profiles are avoided. A phase II randomized trial of trastuzumab plus docetaxel with or without capecitabine in patients with HER2-positive metastatic or locally advanced breast cancer (CHAT) is currently in progress. An initial safety analysis of 110 patients reported an incidence of adverse events similar to that seen in trials of trastuzumab plus docetaxel or trastuzumab plus capecitabine (Wardley et al. 2005). The randomized phase III trial BCIRG 007 investigated the treatment of HER2-positive MBC with trastuzumab either in combination with docetaxel, or with both docetaxel and carboplatin (Forbes et al. 2006). Although addition of the platinum compound carboplatin did not improve clinical outcome, this combination remains a valid therapeutic option and is widely used in the United States.

Both regimens were well tolerated, but differed in their toxicity profiles. In patients treated with trastuzumab and docetaxel significant increases in sensory neuropathy, myalgia, skin and nail changes were reported. In triple combination therapy patients, the incidence of thrombocytopenia was significantly higher, and there was an increase in nausea and vomiting episodes.

14.2.5 Trastuzumab in Combination with Hormonal Therapies

Trastuzumab is still being assessed in combination with hormonal therapies, including tamoxifen, and the aromatase inhibitors letrozole, anastrozole (the TAnDEM trial) and exemestane (the eLEcTRA trial). Several pivotal trials using trastuzumab either as a single agent or combined with chemotherapy drugs have shown that hormone-receptor status does not affect the efficacy of trastuzumab (Marty et al. 2005).

14.3 Early Breast Cancer

Primary systemic therapy (PST) – also referred to as “neoadjuvant therapy” – is being used increasingly in women with EBC to reduce tumor size, thereby

providing more opportunity for breast-conserving surgery, and to decrease the number of positive nodes. Several PST studies have evaluated trastuzumab treatment for 12 weeks before surgery and up to 40 weeks after surgery, provided that the patient is responding or has stable disease.

The overall response rate (70–95%) and the pathological complete response rate (7–42%) in these studies compare favorably with results from phase III and/or randomized trials of PST anthracyclines and/or taxane-containing regimens in patients with unselected (HER2-positive or negative) breast cancer (Bear et al. 2003; Evans et al. 2002; Perez and Rodeheffer 2004; Vinholes et al. 2001). Furthermore, a phase II study evaluated the efficacy of a triple regimen of trastuzumab, docetaxel and cisplatin PST for HER2-positive breast cancer patients with untreated locally advanced disease (Hurley et al. 2006). Progression-free and overall survival were reported to have significantly improved for these patients following treatment with this triple combination.

Based on excellent data in MBC and the promising results in the PST trial, there was a clear rationale to investigate trastuzumab in the adjuvant setting. Four major randomized, multicenter trials examined different adjuvant trastuzumab-based treatment options, both sequential to and concomitant with chemotherapy: HERA (Piccart-Gebhart et al. 2005), NSABP B-31 (Romond et al. 2005), NCCTG N9831 (Romond et al. 2005), and BCIRG 006 (Slamon et al. 2005) (Table 14.2). Results from a smaller trial of HER2-positive women in Finland (FinHer) have also been made available (Joensuu et al. 2006). More than 14 000 women with EBC were enrolled into these five trials. All patients were required to have HER2-positive invasive breast cancer and to have undergone either lumpectomy or mastectomy in order to be included. The regimens of chemotherapy and trastuzumab treatment differed between the trials and are outlined in Fig. 14.3.

In the HERA trial, patients completed a regimen of chemotherapy, with or without radiotherapy, and were randomized to 3-weekly trastuzumab or observation only over 1 to 2 years (Piccart-Gebhart 2005). A significant disease-free survival improvement of 6.3% ($p < 0.0001$) (Fig. 14.4) and an overall survival benefit of 2.7% ($p = 0.0115$) (Fig. 14.5) were reported at 3 years following treatment with trastuzumab. All patient subgroups showed a significant treatment effect.

A joint analysis of the NSABP B-31 and NCCTG N9831 trials in North America assessed the standard adjuvant chemotherapy regimen of doxorubicin plus cyclophosphamide (AC), followed initially with paclitaxel with or without 1 year of trastuzumab (Romond et al. 2005). Significantly longer disease-free survival was observed among those patients who were treated with trastuzumab compared with those who had not received trastuzumab. A significant improvement in overall survival was also noted at 2 years' median follow-up.

The BCIRG 006 trial compared the three treatment regimens of AC followed by docetaxel (as reference treatment), AC followed by docetaxel plus trastuzumab, and an anthracycline-free regimen of docetaxel in combination with carboplatin and trastuzumab (Slamon et al. 2005). The two trastuzumab-containing regimens were associated with significantly improved disease-free survival compared with that not including trastuzumab. Patients receiving trastuzumab with

Table 14.2 Summary of patient characteristics from the adjuvant trastuzumab early breast cancer (EBC) trials (Joensuu et al. 2006; Piccart-Gebhart et al. 2005; Romond et al. 2005; Slamon et al. 2005).

	HERA (n = 5090)	***NSABP B-31 and NCCTG N9831 combined analysis (n = 5535)***	***BCIRG 006 (n = 3222)***	***FinHer (n = 232)***[a]
Age <50 years [%]	51	51	52	51
Node-negative disease [%]	32[b]	5.7	29[c]	16[d]
Grade III tumors [%]	60	69	n.a.	65
Taxane-based chemotherapy [%]	26	100	100	50
Planned endocrine therapy [%]	46	52	54 (with ER+ and/or PgR+ tumors)	
Normal cardiac function [%]	At completion of locoregional therapy and chemotherapy	At completion of doxorubicin + cyclophosphamide	After surgery	After surgery

a HER2-positive subgroup.
b Only if tumor >1 cm.
c Only if other concomitant risk factors.
d Only if tumor >2 cm and PgR-negative.
ER = estrogen receptor; PgR = progesterone receptor; n.a. = not available.

docetaxel had a 51% reduced risk of relapse compared with those receiving only docetaxel. A 39% decrease in risk was seen in patients receiving docetaxel plus carboplatin and trastuzumab.

The small randomized FinHer trial aimed to compare docetaxel and vinorelbine as part of a chemotherapy regimen in 1010 patients with EBC (Joensuu et al. 2006). Patients were tested for HER2 status, and those shown to be positive by IHC (3+ or 2+; n = 232) were further randomized either to receive (n = 116) or not receive (n = 116) trastuzumab over 9 weeks. At 3 years' follow-up, patients who were given trastuzumab had better recurrence-free survival than those who did not.

Overall, these trials indicate that trastuzumab adjuvant therapy can decrease the risk of recurrence by approximately 50% for women with EBC. HERA and the joint analysis of NSABP B-31 and NCCTG N9831 revealed a significant improvement in overall survival after 1 year of adjuvant trastuzumab treatment (a 33% reduction in the risk of death). This is of significance because the ultimate aim of all adjuvant trials is to improve the overall survival of patients, potentially leading to more patients being cured. The results from analysis after 1 to 3 years' follow-up indicate a disease-free survival benefit for trastuzumab treatment in all

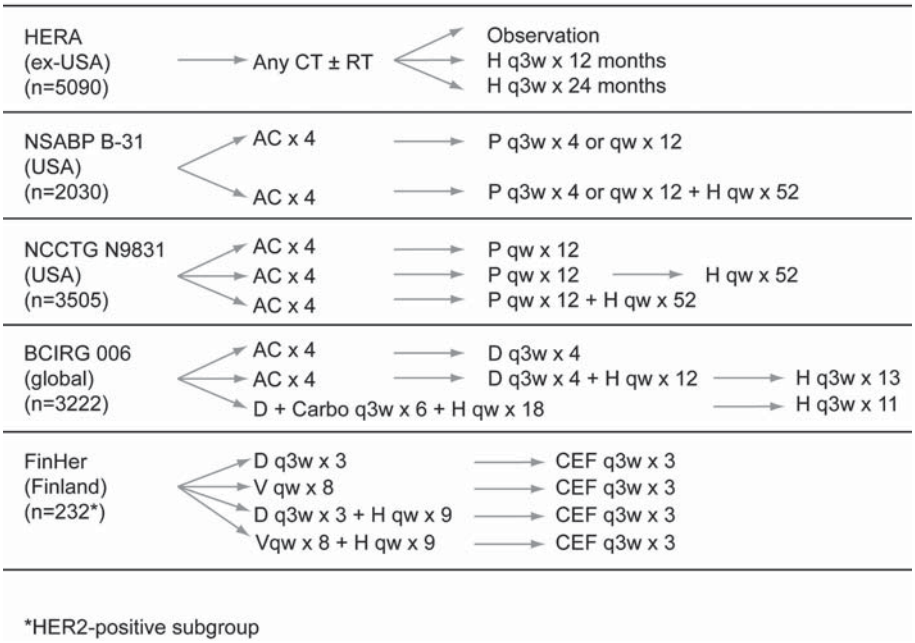

Fig. 14.3 Study designs of trastuzumab EBC trials: HERA, NSABP B-31, NCCTG N9831, BCIRG 006 and FinHer (Joensuu et al. 2006; Piccart-Gebhart et al. 2005; Romond et al. 2005; Slamon et al. 2005).

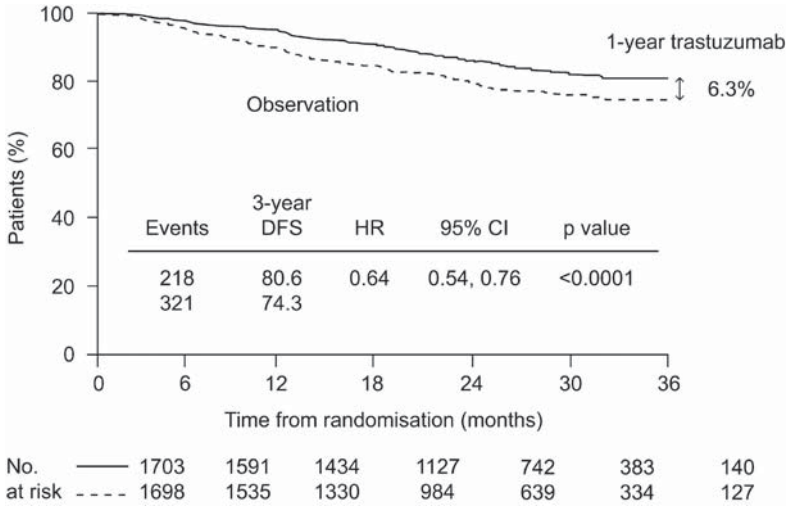

Fig. 14.4 Disease-free survival progression of patients in the HERA trial at 2-year median follow-up (Piccart-Gebhart 2005).

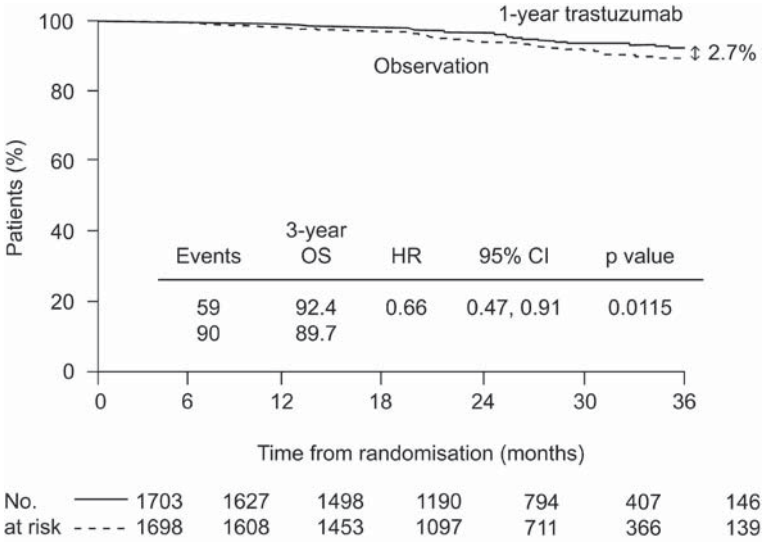

Fig. 14.5 Overall survival progression of patients in the HERA trial at 2-year median follow-up (Piccart-Gebhart 2005).

five EBC trials (Fig. 14.6). Following the success of these trials, trastuzumab has recently gained approval from the European Commission for use as an adjuvant treatment for HER2-positive EBC.

14.4 Trastuzumab Treatment in other Tumor Types

In addition to breast cancer, overexpression of HER2 has been seen in a number of other tumor types, including ovarian, bladder, salivary gland, endometrial, esophageal, gastric, pancreatic and non-small-cell lung (NSCLC).

A large randomized phase III trial was conducted in NSCLC (Gatzemeier et al. 2004). Unfortunately, the majority of patients recruited into this trial were not HER2-positive according to the standards in place today, and tumors were diagnosed as IHC 2+. Currently, knowledge regarding the definition of HER2 positivity has improved, and IHC 2+ cases are now classified as equivocal, requiring further analysis by ISH. Cases identified as IHC 2+ and ISH negative are regarded as HER2-negative. This helps to explain why the trial failed its endpoints.

ToGA, a phase III, open-label, randomized, multicenter study, is an ongoing clinical development program to evaluate the efficacy, safety, pharmacokinetics and effect on quality of life of first-line fluoropyrimidine and cisplatin with or without trastuzumab in 374 patients with HER2-positive advanced gastric cancer. Patient recruitment began in September 2005, with treatment given until progression. Recruitment for this study is currently ongoing.

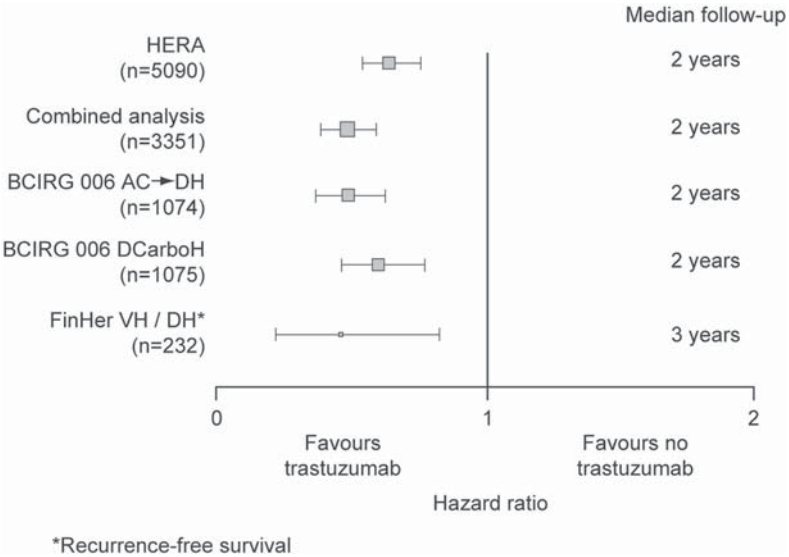

Fig. 14.6 Summary of disease-free survival in the five pivotal EBC trials (Joensuu et al. 2006; Piccart-Gebhart et al. 2005; Romond et al. 2005; Slamon et al. 2005).

14.5 Safety

Trastuzumab monotherapy or combination therapy is generally well tolerated, with only mild to moderate adverse side effects; the majority of patients are not expected to experience grade 3 or 4 adverse events. Cardiac events and serious infusion-related reactions are the most clinically important side effects; however, these occur relatively infrequently and are readily manageable with standard therapeutic interventions (see Sections 14.5.1 and 14.5.2). Trastuzumab monotherapy is not associated with cumulative toxicity (Cook-Bruns 2001), and does not induce the adverse events such as alopecia, myelosuppression or mucositis that are common with chemotherapy (Cobleigh et al. 1999; Vogel et al. 2002). Also, routine use of anti-emetics is not required with trastuzumab. The decision to continue trastuzumab therapy in any patients who experience adverse events will depend on the outcome of individual benefit:risk assessment.

In two monotherapy trials, the most commonly reported adverse events were mild to moderate infusion-related reactions, which occurred in around 40% of patients (Vogel et al. 2002). These reactions were mostly associated with the first infusion, did not lead to infusion interruption, and resolved with standard treatment. Hematological toxicities frequently seen with chemotherapy, such as neutropenia, anemia and thrombocytopenia, were uncommon following trastuzumab monotherapy (Cobleigh et al. 1999; Vogel et al. 2002), and severe adverse events were rare.

As with trastuzumab monotherapy, the most common adverse events in the pivotal phase III combination trial were mild infusion-related reactions (fever and chills) (Slamon et al. 2001). Trastuzumab does not generally appear to exacerbate the toxicity of chemotherapy (Slamon et al. 2001; Marty et al. 2005), apart from increases in anemia and leucopenia when used in combination with paclitaxel or anthracycline plus cyclophosphamide (Slamon et al. 2001). These events generally resolved with standard treatments.

A similar pattern of adverse events was observed more recently in a randomized phase II pivotal trial (M77001) in which 188 HER2-positive (IHC 3+) MBC patients were given 3-weekly docetaxel ($100\,mg\,m^{-2}$) with or without weekly trastuzumab ($2\,mg\,kg^{-1}$) until disease progression (Marty et al. 2005). There was little difference in safety profile between the two treatment groups, although some nonhematological toxicities usually seen with docetaxel were more frequently reported within the combination group. A higher incidence of adverse events was reported in combination therapy patients; however, fewer patients from this group were withdrawn from the trial due to serious adverse events.

Trastuzumab is an immunoglobulin and is, therefore, secreted into human milk. Hence, breastfeeding is not recommended for the duration of trastuzumab therapy and for 6 months after the last dose of trastuzumab.

14.5.1 Cardiac Adverse Events

Trastuzumab has been associated with an increased risk of cardiac events (Perez and Rodeheffer 2004; Seidman et al. 2002; Slamon et al. 2001; Suter et al. 2004), and careful cardiac monitoring is recommended for all patients undergoing treatment. Clinical trial data showed that these events were more common in patients who received concomitant anthracyclines or who had previously received anthracyclines (Seidman et al. 2002).

In the pivotal clinical trial conducted by Slamon and colleagues, cardiac events were reported for 27% of patients who received trastuzumab in combination with anthracyclines and cyclophosphamide compared with 8% who received only anthracyclines and cyclophosphamide (Seidman et al. 2002; Slamon et al. 2001). In women with prior anthracycline therapy, cardiac events were reported for 13% of patients who received trastuzumab plus paclitaxel compared with 1% who received only paclitaxel (Seidman et al. 2002). Most trastuzumab-treated patients who developed cardiac dysfunction were symptomatic (75%) and the majority improved with standard treatment for congestive heart failure (79%) (Seidman et al. 2002). A Cardiac Task Force analyzed the cardiac-event data from this trial and concluded that: ". . . in most cases the cardiac events observed may reflect an exacerbation of anthracycline-induced cardiotoxicity; symptomatic heart failure was associated with concomitant or previous anthracycline use; and the cardiac events are generally reversible and can usually be managed with standard medical treatment." (Suter et al. 2004).

It is recommended that all patients are assessed for cardiac disease risk factors before receiving trastuzumab in order to identify those susceptible to cardiac events. Evaluation of the left ventricular ejection fraction (LVEF), an indicator of cardiac function, is also recommended before and during trastuzumab therapy (Suter et al. 2004). In 2006, an expert group met to discuss the prevalence of cardiac events and to prepare some recommendations for clinicians to prevent and monitor such events. The three proposed algorithms (Figs. 14.7–14.9) are applicable only to those patients receiving trastuzumab for 12 months. It is possible that these recommendations may alter when 24-month data from the HERA trial become available. The first algorithm (Fig. 14.7) outlines the recommended cardiac criteria to be considered when determining the appropriate course of trastuzumab therapy. A number of recommendations were made concerning asymptomatic LVEF decline during adjuvant trastuzumab treatment (Fig. 14.8), and the occurrence of New York Heart Association (NYHA) class II or III/IV cardiac events during adjuvant trastuzumab (Fig. 14.9).

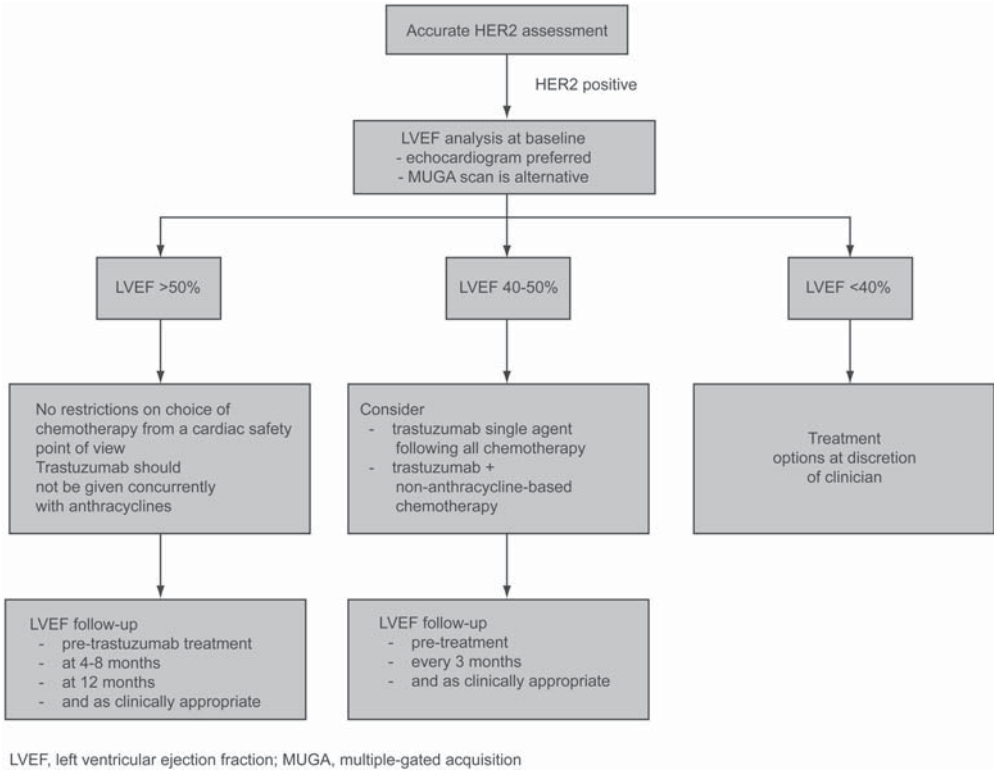

Fig. 14.7 Cardiac criteria for initiating adjuvant trastuzumab. LVEF = left ventricular ejection fraction; MUGA = multiple-gated acquisition.

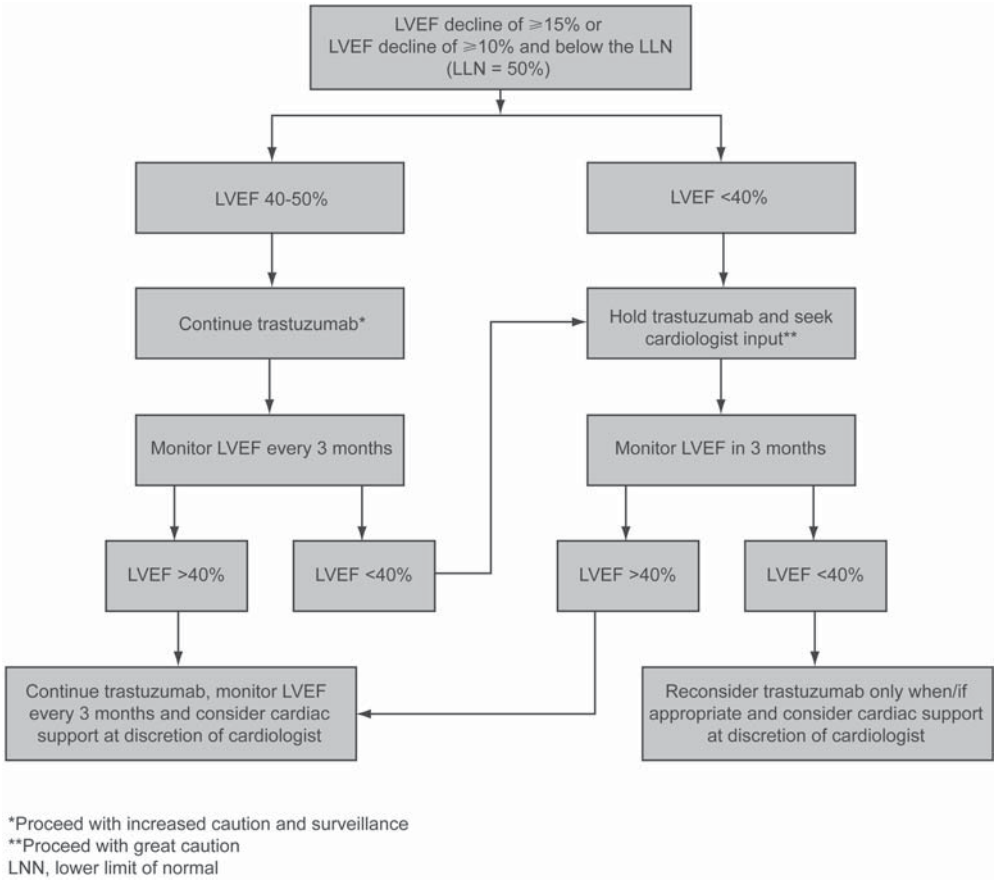

Fig. 14.8 Treatment choices for asymptomatic declines in LVEF occurring during adjuvant trastuzumab therapy.

Prospective cardiac monitoring was included in trastuzumab trials conducted after the pivotal combination trial. An analysis of data pooled from six phase II and III trastuzumab trials in HER2-positive MBC comprising a total of 629 patients (418 of whom received trastuzumab) showed that the incidence of clinically significant cardiac events (congestive heart failure) in patients who received trastuzumab was only 2.7% (Marty et al. 2003). This incidence is much lower than that seen in the pivotal trial of trastuzumab plus paclitaxel (12%) (Slamon et al. 2001), and is a consequence of stringent cardiac eligibility criteria and regular cardiac monitoring.

The increased risk of cardiac events observed with trastuzumab when given in combination with anthracyclines has prevented filing and approval of this treatment for use in HER2-positive MBC. However, new anthracyclines with improved cardiac safety (epirubicin and liposomal doxorubicin) have been investigated to address this situation.

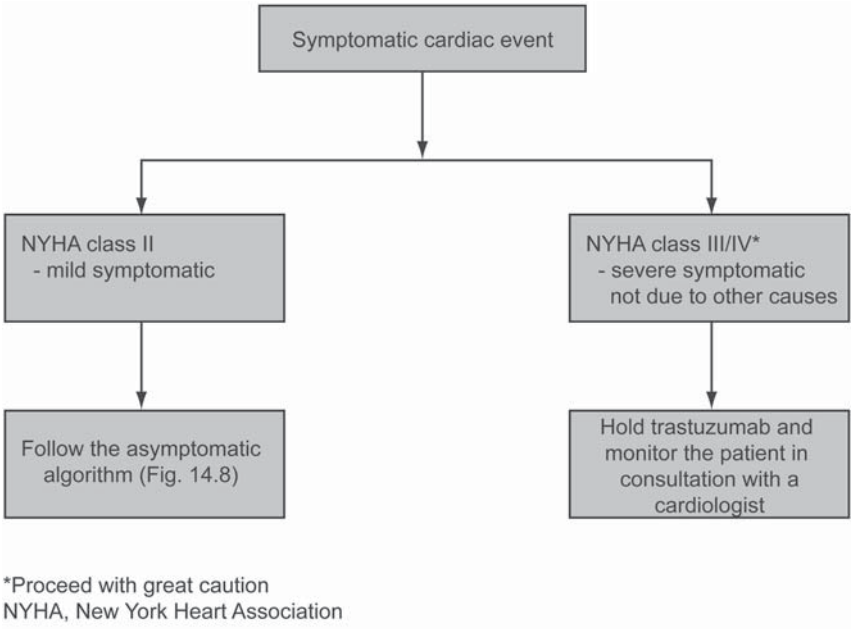

Fig. 14.9 Treatment choices based on symptomatic cardiac events occurring during adjuvant trastuzumab therapy. NYHA = New York Heart Association.

The HERCULES multicenter phase II trial examined the cardiac safety of trastuzumab plus epirubicin-cyclophosphamide (n = 25), compared with epirubicin-cyclophosphamide alone (n = 24), in patients with MBC. Only two patients in the triple-combination drug arm experienced a cardiac event, compared with one in the epirubicin-cyclophosphamide arm (Langer et al. 2003).

Patients in the four major adjuvant EBC trials (HERA, NSABP B-31, NCCTG N9831 and BCIRG 007) showed similar and acceptable levels of cardiac events. A small overall increase of 0.6 to 3.3% in the incidence of congestive heart failure was seen, the majority of which improved with treatment.

14.5.2 Infusion-related Reactions

Infusion-related reactions in the pivotal trastuzumab clinical trials were common but generally mild, and occurred mostly with the first infusion; severe cases were rare (Slamon et al. 2001; Vogel et al. 2002). Initial post-marketing surveillance, however, has revealed more serious trastuzumab-associated infusion-related reactions, which presented as fatal complications in some cases (Cook-Bruns 2001). The incidence of infusion-related deaths (death within 24 h of infusion) has been

reported as 0.04% (9 of 25 000 patients; cut-off March 2000) and that of infusion-related events overall as 0.3% (74 of 25 000 patients). No new concerns have arisen since, and a further analysis is not indicated. Most of the nine patients with fatal infusion-related reactions had significant pre-existing pulmonary compromise secondary to advanced malignancy, and several were receiving supportive oxygen therapy at the time of their first trastuzumab infusion.

Serious infusion-related reactions are characterized by respiratory symptoms, such as dyspnea, bronchospasm and respiratory distress, and may be accompanied by an anaphylactoid reaction with hypotension and rash. These symptoms generally appear within 2 h following the start of the first trastuzumab infusion, and can be managed through cessation, treatment with antihistamines, corticosteroids and β-antagonists, and administration of oxygen. Further infusions of trastuzumab to patients who experienced serious reactions have not been associated with any recurrence. Patients who are at risk of developing severe infusion-related reactions can be identified before the initiation of treatment. Overall, therefore, serious infusion-related events associated with trastuzumab occur infrequently and do not impact on the favorable benefit : risk profile of this drug.

14.5.3 Age Considerations

In general, older age is associated with poorer clinical outcome in breast cancer; however, for patients with MBC, the addition of trastuzumab to chemotherapy (paclitaxel or anthracycline-based) has been shown to increase overall response rate and survival compared with chemotherapy alone, irrespective of patient age (Fyfe et al. 2001). The overall response rate increased from 33% to 52% in patients aged less than 60 years, and from 28% to 44% in patients aged more than 60 years. Corresponding figures for survival are from 23 to 26 months and from 14 to 19 months, respectively. These clinical benefits indicate that trastuzumab combination therapy should be considered for patients, whatever their age. The safety of trastuzumab in patients aged less than 18 years has not been established.

14.5.4 Patient Considerations

Trastuzumab is not recommended for use in patients with known hypersensitivity to murine proteins, trastuzumab, or any of its excipients. Patients with severe dyspnea at rest due to complications of advanced malignancy, or those requiring supplementary oxygen, are also not recommended to receive trastuzumab because of the possibility of serious infusion-related reactions. Patients with symptomatic heart failure, history of hypertension or coronary artery disease, or LVEF < 50% should undergo a careful benefit : risk assessment before they receive treatment.

14.6 Dosing/Scheduling

Two dosing schedules are labeled and used for trastuzumab. Based upon an initial assumption of a half-life of 5.8 days, a weekly schedule was developed and licensed for MBC: 4 mg kg^{-1} loading dose followed by 2 mg kg^{-1} weekly.

A retrospective analysis of pooled pharmacokinetic data using population pharmacokinetics indicated a mean half-life of 28.5 days. Subsequently, a 3-weekly schedule was developed with the same total doses as the weekly schedule: 8 mg kg^{-1} loading dose followed by 6 mg kg^{-1} 3-weekly from Week 3 onwards.

Since the 3-weekly dosage had been used in the adjuvant trial program, this schedule was licensed by the European Agency for the Evaluation of Medicinal Products (EMEA) for the treatment of patients with HER2-positive EBC following surgery, chemotherapy (neoadjuvant or adjuvant) and radiotherapy (if applicable).

14.7 Conclusions

Trastuzumab has become a backbone therapy for patients with HER2-positive breast cancer. It has been shown to provide benefits with respect to all clinical outcomes, but the survival benefits achieved with trastuzumab in MBC and EBC are the most impressive. In both settings, clinical trials provide level 1 evidence for improvement of overall survival.

At the same time, trastuzumab in general adds little to the toxicities of respective combination partners. The adverse events which have caused the most concern are cardiac events. Specific inclusion/exclusion criteria and cardiac monitoring have helped to control the incidence of cardiac events to an acceptable level. Overall, trastuzumab is characterized by an excellent benefit : risk ratio.

References

Arnould, L., Gelly, M., Penault-Llorca, F., Benoit, L., Bonnetain, F., Migeon, C., et al. (2006) Trastuzumab-based treatment of HER2-positive breast cancer: an antibody-dependent cellular cytotoxicity mechanism? *Br J Cancer* 94: 259–267.

Baselga, J. (2001) Herceptin alone or in combination with chemotherapy in the treatment of HER2-positive metastatic breast cancer: pivotal trials. *Oncology* 61(Suppl.2): 14–21.

Baselga, J., Norton, L., Albanell, J., Kim, Y.-M., and Mendelsohn, J. (1998) Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/*neu* overexpressing human breast cancer xenografts. *Cancer Res* 58: 2825–2831.

Baselga, J., Carbonell, X., Castañeda-Soto, N.-J., Clemens, M., Green, M., Harvey, V., et al. (2005) Phase II study of efficacy, safety, and pharmacokinetics of trastuzumab monotherapy administered on a 3-weekly schedule. *J Clin Oncol* 23: 2162–2171.

Bayo, J., Mayordomo, J.I., Sanchez-Rovira, P., Perez-Carrion, R., Illaramendi, J., Gonzalez Flores, E., et al. (2004) Trastuzumab and vinorelbine combination in the treatment of Her2 positive metastatic breast cancer. *Proc Am Soc Clin Oncol* 23: 67, abstract 763.

Bear, H.D., Anderson, S., Brown, A., Smith, R., Mamounas, E.P., Fisher, B., et al. (2003) The effect on tumor response of adding sequential preoperative docetaxel to preoperative doxorubicin and cyclophosphamide: preliminary results from National Surgical Adjuvant Breast and Bowel Project Protocol B-27. *J Clin Oncol* 21: 4165–4174.

Bernardo, G., Palumbo, R., Bernardo, A., Villani, G., Melazzini, M., Poggi, G., et al. (2004) Final results of phase II study of weekly trastuzumab and vinorelbine in chemonaive patients with HER2-overexpressing metastatic breast cancer. *Proc Am Soc Clin Oncol* 23: 59, abstract 731.

Burstein, H.J., Kuter, I., Campos, S.M., Gelman, R.S., Tribou, L., Parker, L.M., et al. (2001) Clinical activity of trastuzumab and vinorelbine in women with HER2-overexpressing metastatic breast cancer. *J Clin Oncol* 19: 2722–2730.

Burstein, H.J., Harris, L.N., Marcom, P.K., Lambert-Falls, R., Havlin, K., Overmoyer, B., et al. (2003) Trastuzumab and vinorelbine as first-line therapy for HER2-overexpressing metastatic breast cancer: multicenter phase II trial with clinical outcomes, analysis of serum tumor markers as predictive factors, and cardiac surveillance algorithm. *J Clin Oncol* 21: 2889–2895.

Burstein, H.J., Keshaviah, A., Baron, A., Hart, R., Lambert-Falls, R., Marcom, P.K., et al. (2006) Trastuzumab and vinorelbine or taxane chemotherapy for HER2+ metastatic breast cancer: The TRAVIOTA study. *J Clin Oncol* (Meeting Abstracts) 24: 40s, abstract 650.

Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B.B., Henner, D., Wong, W.L.T., et al. (1992) Humanization of an anti-p185^{HER2} antibody for human cancer therapy. *Proc Natl Acad Sci USA* 89: 4285–4289.

Chan, A., Petruzelka, L., Untch, M., Martin, M., Gil, M., Guillem Porta, V., et al. (2005) Long term survival of vinorelbine (N) and trastuzumab (H) as first line therapy for HER2-positive metastatic breast cancer patients (HER2 + MBC) (pts). *J Clin Oncol* (Meeting Abstracts) 23: 25s, abstract 587.

Christodoulou, C., Fountzilas, G., Razi, E., Tsavdaridis, D., Karina, M., Lambropoulos, S., et al. (2003) Gemcitabine and trastuzumab combination as salvage treatment in patients with HER 2-positive metastatic breast cancer. *Proc Am Soc Clin Oncol* 22: 42, abstract 166.

Cobleigh, M.A., Vogel, C.L., Tripathy, D., Robert, N.J., Scholl, S., Fehrenbacher, L., et al. (1999) Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 17: 2639–2648.

Cook-Bruns, N. (2001) Retrospective analysis of the safety of Herceptin immunotherapy in metastatic breast cancer. *Oncology* 61(Suppl.2): 58–66.

de Wit, M., Becker, K., Thomssen, C., Harbeck, N., Hoffmann, R., Villena, C., et al. (2004) Vinorelbine and trastuzumab as first line therapy in patients with HER2-positive metastatic breast cancer – interim analysis of a prospective, open-label, multicentre phase II trial. *Ann Oncol* 15(Suppl.3): 37, abstract 138P.

Eiermann, W., International Herceptin Study Group (2001) Trastuzumab combined with chemotherapy for the treatment of HER2-positive metastatic breast cancer: pivotal trial data. *Ann Oncol* 12(Suppl.1): S57–S62.

Ekerljung, L., Steffen, A.C., Carlsson, J., Lennartsson, J. (2006) Effects of HER2-binding affibody molecules on intracellular signaling pathways. *Tumour Biol* 27: 201–210.

Evans, T., Gould, A., Foster, E., Crown, J.P., Leonard, R., Mansi, J.L. (2002) Phase III randomised trial of adriamycin (A) and docetaxel (D) versus A and cyclophosphamide (C) as primary medical therapy (PMT) in women with breast cancer: an ACCOG study. *Proc Am Soc Clin Oncol* 21: 35a, abstract 136.

Extra, J.-M., Cognetti, F., Maraninchi, D., Snyder, R., Mauriac, L., Tubiana-Hulin, M., et al. (2005) Long-term survival demonstrated with trastuzumab plus docetaxel: 24-month data from a randomised trial (M77001) in HER2-positive metastatic breast cancer. *J Clin Oncol* 23: 17s, abstract 555.

Forbes, J.F., Kennedy, J., Pienkowski, T., Valero, V., Eiermann, W., von Minckwitz, G., et al. (2006) BCIRG 007: randomized phase III trial of trastuzumab plus docetaxel with or without carboplatin first line in HER2 positive metastatic breast cancer (MBC): main time to progression (TTP) analysis. *J Clin Oncol* 24: 7s, abstract LBA516.

Fyfe, G.A., Mass, R., Murphy, M., Slamon, D. (2001) Survival benefit of trastuzumab (Herceptin) and chemotherapy in older (age >60) patients. *Proc Am Soc Clin Oncol* 20: 48a, abstract 189.

Gatzemeier, U., Groth, G., Butts, C., Van Zandwijk, N., Shepherd, F., Ardizzoni, A., et al. (2004) Randomized phase II trial of gemcitabine–cisplatin with or without trastuzumab in HER2-positive non-small-cell lung cancer. *Ann Oncol* 15: 19–27.

Gennari, R., Menard, S., Fagnoni, F., Ponchio, L., Scelsi, M., Tagliabue, E., et al. (2004) Pilot study of the mechanism of action of preoperative trastuzumab in patients with primary operable breast tumors overexpressing HER2. *Clin Cancer Res* 10: 5650–5655.

Glogowska, I., Sienkiewicz-Kozlowska, R., Bauer-Kosinska, B., Jaczewska, S., Pienkowski, T. (2004) Trastuzumab (T) plus vinorelbine (VNR) as first combination in Her-2 overexpressing patients with metastatic breast cancer. *Proc Am Soc Clin Oncol* 23: 235, abstract 3165.

Holbro, T., Beerli, R.R., Maurer, F., Koziczak, M., Barbas III, C.F., Hynes, N.E. (2003) The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci USA* 100: 8933–8938.

Hurley, J., Doliny, P., Reis, I., Silva, O., Gomez-Fernandez, C., Velez, P., et al. (2006) Docetaxel, cisplatin, and trastuzumab as primary systemic therapy for human epidermal growth factor receptor 2–positive locally advanced breast cancer. *J Clin Oncol* 24: 1831–1838.

Jahanzeb, M., Mortimer, J.E., Yunus, F., Irwin, D.H., Speyer, J., Koletsky, A.J., et al. (2002) Phase II trial of weekly vinorelbine and trastuzumab as first-line therapy in patients with $HER2^+$ metastatic breast cancer. *Oncologist* 7: 410–417.

Joensuu, H., Kellokumpu-Lehtinen, P.-L., Bono, P., Alanko, T., Kataja, V., Asola, R., et al. (2006) Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. *N Engl J Med* 354: 809–820.

Kim, C., Bryant, J., Horne, Z., Geyer, C.E., Wickerham, D.L., Wolmark, N., et al. (2005) Trastuzumab sensitivity of breast cancer with co-amplification of HER2 and cMYC suggest pro-apoptotic function of dysregulated cMYC in vivo. Presented at San Antonio Breast Cancer Symposium, abstract 46.

Klapper, L.N., Waterman, H., Sela, M., Yarden, Y. (2000) Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. *Cancer Res* 60: 3384–3388.

Langer, B., Muscholl, M., Pauschinger, M., Thomssen, Ch., Eidtmann, H., Untch, M., et al. (2003) A prospective study of NT-pro brain natriuretic peptide (NT-proBNP) and troponin T (TnT) in the HERCULES (M77003) study (epirubicin + cyclophosphamide ± Herceptin) in patients with metastatic breast cancer (MBC). Poster 223 presented at the 26th Annual San Antonio Breast Cancer Symposium, San Antonio, TX, USA, December 3–6, 2003.

Le, X.F., Bedrosian, I., Mao, W., Murray, M., Lu, Z., Keyomarsi, K., et al. (2006) Anti-HER2 antibody trastuzumab inhibits CDK2-mediated NPAT and histone H4 expression via the PI3K pathway. *Cell Cycle* 5: 1654–1661.

Marty, M., Baselga, J., Gatzemeier, U., Leyland-Jones, B., Suter, T., Klingelschmitt, G., et al. (2003) Pooled analysis of six trials of trastuzumab (Herceptin): exploratory analysis of changes in left ventricular ejection fraction (LVEF) as a surrogate for clinical cardiac

events. *Breast Cancer Res Treat* 82(Suppl.1): S48, abstract 218.

Marty, M., Cognetti, F., Maraninchi, D., Snyder, R., Mauriac, L., Tubiana-Hulin, M., et al. (2005) Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: the M77001 study group. *J Clin Oncol* 23: 4265–4274.

Ménard, S., Fortis, S., Castiglioni, F., Agresti, R., Balsari, A. (2001) HER2 as a prognostic factor in breast cancer. *Oncology* 61(Suppl.2): 67–72.

Molina, M.A., Codony-Servat, J., Albanell, J., Rojo, F., Arribas, J., Baselga, J. (2001) Trastuzumab (Herceptin), a humanized anti-HER2 receptor monoclonal antibody, inhibits basal and activated HER2 ectodomain cleavage in breast cancer cells. *Cancer Res* 61: 4744–4749.

O'Shaughnessy, J.A., Vukelja, S., Marsland, T., Kimmel, G., Ratnam, S., Pippen, J.E. (2004) Phase II study of trastuzumab plus gemcitabine in chemotherapy-pretreated patients with metastatic breast cancer. *Clin Breast Cancer* 5: 142–147.

Owens, M.A., Horten, B.C., Da Silva, M.M. (2004) HER2 amplification ratios by fluorescence in situ hybridization and correlation with immunohistochemistry in a cohort of 6556 breast cancer tissues. *Clin Breast Cancer* 5: 63–69.

Papaldo, P., Fabi, A., Ferretti, G., Mottolese, M., Cianciulli, A.M., Di Cocco, B., et al. (2006) A phase II study on metastatic breast cancer patients treated with weekly vinorelbine with or without trastuzumab according to HER2 expression: changing the natural history of HER2-positive disease. *Ann Oncol* 17: 630–636.

Parkin, D.M., Bray, F., Ferlay, J., Pisani, P. (2005) Global cancer statistics, 2002. *CA Cancer J Clin* 55: 74–108.

Pegram, M., Hsu, S., Lewis, G., Pietras, R., Beryt, M., Sliwkowski, M., et al. (1999) Inhibitory effects of combinations of HER-2/*neu* antibody and chemotherapeutic agents used for treatment of human breast cancers. *Oncogene* 18: 2241–2251.

Penault-Llorca, F., Vincent-Salomon, A., Mathieu, M.C., Trillet-Lenoir, V., Khayat, D., Marty, M., et al. (2005) Incidence and implications of HER2 and hormonal receptor overexpression in newly diagnosed metastatic breast cancer (MBC). *J Clin Oncol* (Meeting Abstracts) 23: 69s, abstract 764.

Perez, E.A., Rodeheffer, R. (2004) Clinical cardiac tolerability of trastuzumab. *J Clin Oncol* 22: 322–329.

Piccart-Gebhart, M.J. (2005) First results of the HERA trial. A randomized three-arm multi-centre comparison of: 1 year Herceptin, 2 years Herceptin or no Herceptin in women with HER-2 positive primary breast cancer who have completed adjuvant chemotherapy. Slide presentation at the 41st ASCO Annual Meeting, Orlando, FL, USA, May 13–17, 2005. http://www.asco.org/ac/1,1003,_12-002511-00_18-0034-00_19-005816-00_21-001,00.asp. 2005. 7-7-2005.

Piccart-Gebhart, M.J., Procter, M., Leyland-Jones, B., Goldhirsch, A., Untch, M., Smith, I., et al. (2005) Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med* 353: 1659–1672.

Pietras, R.J., Pegram, M.D., Finn, R.S., Maneval, D.A., Slamon, D.J. (1998) Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA-reactive drugs. *Oncogene* 17: 2235–2249.

Press, M.F., Bernstein, L., Thomas, P.A., Meisner, L.F., Zhou, J.Y., Ma, Y., et al. (1997) HER-2/neu gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas. *J Clin Oncol* 15: 2894–2904.

Romond, E.H., Perez, E.A., Bryant, J., Suman, V.J., Geyer Jr., C.E., Davidson, N. E., et al. (2005) Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med* 353: 1673–1684.

Ross, J.S., Fletcher, J.A., Bloom, K.J., Linette, G.P., Stec, J., Symmans, W.F., et al. (2004) Targeted therapy in breast cancer. The HER-2/*neu* gene and protein. *Mol Cell Proteomics* 3: 379–398.

Schaller, G., Bangemann, N., Weber, J., Kleine-Tebbe, A., Beisler, G.K., Conrad, B.,

et al. (2005) Efficacy and safety of trastuzumab plus capecitabine in a German multicentre phase II study of pretreated metastatic breast cancer. *J Clin Oncol* (Meeting Abstracts) 23: 57s, abstract 717.

Seidman, A., Hudis, C., Pierri, M.K., Shak, S., Paton, V., Ashby, M., et al. (2002) Cardiac dysfunction in the trastuzumab clinical trials experience. *J Clin Oncol* 20: 1215–1221.

Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., McGuire, W.L. (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science* 235: 177–182.

Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., et al. (1989) Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 244: 707–712.

Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., et al. (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 344: 783–792.

Slamon, D., Eiermann, W., Robert, N., Pienkowski, T., Martin, M., Pawlicki, M., et al. (2005) Phase III randomized trial comparing doxorubicin and cyclophosphamide followed by docetaxel (AC→T) with doxorubicin and cyclophosphamide followed by docetaxel and trastuzumab (AC →TH) with docetaxel, carboplatin and trastuzumab (TCH) in HER2 positive early breast cancer patients: BCIRG 006 study. *Breast Cancer Res Treat* 94(Suppl.1): S5, abstract 1.

Stemmler, H.J., Kahlert, S., Brudler, O., Beha, M., Müller, S., Stauch, B., et al. (2005) High efficacy of gemcitabine and cisplatin plus trastuzumab in patients with HER2-overexpressing metastatic breast cancer: a phase II study. *Clin Oncol (R Coll Radiol)* 17: 630–635.

Suter, T.M., Cook-Bruns, N., Barton, C. (2004) Cardiotoxicity associated with trastuzumab (Herceptin) therapy in the treatment of metastatic breast cancer. *Breast* 13: 173–183.

Vinholes, J., Bouzid, K., Salas, F., Mickiewicz, E., Valdivia, S., Ostapenko, V., et al. (2001) Preliminary results of a multicentre phase III trial of taxotere and doxorubicin (AT) versus 5-fluorouracil, doxorubicin and cyclophosphamide (FAC) in patients (pts) with unresectable locally advanced breast cancer (ULABC). *Proc Am Soc Clin Oncol* 20: 26a, abstract 101.

Vogel, C.L., Cobleigh, M.A., Tripathy, D., Gutheil, J.C., Harris, L.N., Fehrenbacher, L., et al. (2002) Efficacy and safety of trastuzumab as a single agent in first-line treatment of *HER2*-overexpressing metastatic breast cancer. *J Clin Oncol* 20: 719–726.

Wardley, A., Antón-Torres, A., Otero Reyes, D., Jassem, J., Toache, L.M.Z., Alcedo, J.C., et al. (2005) CHAT – an open-label, randomised, Phase II study of trastuzumab plus docetaxel with or without capecitabine in patients with advanced and/or metastatic HER2-positive breast cancer: second interim safety analysis. Poster 6094 presented at the 28th Annual San Antonio Breast Cancer Symposium, San Antonio, TX, USA, 8-11 December, 2005.

Xu, L., Song, S., Zhu, J., Luo, R., Li, L., Jiao, S., et al. (2006) A phase II trial of trastuzumab (H) + capecitabine (X) as first-line treatment in patients (pts) with HER2-positive metastatic breast cancer (MBC). *J Clin Oncol* (Meeting Abstracts) 24: 577s (abstract 10615).

15
Abciximab, Arcitumomab, Basiliximab, Capromab, Cotara, Daclizumab, Edrecolomab, Ibritumomab, Igovomab, Nofetumomab, Satumomab, Sulesomab, Tositumomab, and Votumumab

Christian Menzel and Stefan Dübel

Summary

This chapter offers an overview of all of the approved antibodies which are not described in separate chapters in this handbook. This includes antibodies for immunotherapy and *in-vivo* imaging which were approved but later withdrawn, or the marketing of which has been stopped. Interestingly, among the latter is a majority of the approved *in-vivo* imaging agents using radionuclides. Here, a development is seen from the pure diagnostic tool towards the combined imaging and treatment regime of Bexxar or Zevalin. Further information on immunotargeted radionuclides can be found in Chapter 1 of Volume II (Immunoscintigraphy and Radioimmunotherapy).

Note: The data presented in this chapter represent opinions compiled from public domain sources. Despite some parts of this chapter citing the drug leaflets, they are not to be construed as recommendations for treatments. Full prescribing information must be consulted for any of the drugs or procedures discussed herein.

15.1
Abciximab (Reopro)

Abciximab is the Fab fragment of a chimeric mouse/human antibody, 7E3. Reopro is produced by continuous perfusion culture of the antibody 7E3 by mammalian cells and subsequent papain digestion to obtain the Fab fragment. Reopro binds to the platelet surface protein GPIIb/IIIa receptor, a member of the integrin family and the major factor involved in platelet aggregation. By inhibiting the binding of GPIIb/IIIa receptor to a number of serum proteins – such as von

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Willebrand factor, fibrinogen, and other adhesion proteins – Abciximab inhibits the platelet aggregation.

Abciximab is FDA approved as an adjunct to percutaneous coronary intervention for the prevention of cardiac ischemic complications in patients undergoing percutaneous coronary intervention, or in patients with unstable angina not responding to conventional medical therapy when percutaneous coronary intervention is planned within 24 h.

Information Sources/References

http://www.fda.gov.
http://www.reopro.com.
Drug leaflet.
Oster, Z.H., Srivastava, S.C., Som, P., Meinken, G.E., Scudder, L.E., Yamamoto, K., Atkins, H.L., Brill, A.B., Coller, B.S. (1985) Thrombus radioimmunoscintigraphy: an approach using monoclonal antiplatelet antibody. *Proc Natl Acad Sci USA* 82: 3465–3468.
Faulds, D., Sorkin, E.M. (1994) Abciximab (c7E3 Fab). A review of its pharmacology and therapeutic potential in ischaemic heart disease. *Drugs* 48: 583–598.
Schneider, D.J., Aggarwal, A. (2004) Development of glycoprotein IIb-IIIa antagonists: translation of pharmacodynamic effects into clinical benefit. *Expert Rev Cardiovasc Ther* 2: 903–913.

15.2
Arcitumomab (CEA-Scan)

The targeting component of CEA-Scan (Arcitumomab) consists of an antibody Fab fragment generated from a murine IgG_1, called IMMU-4. Arcitumomab is further covalently labeled with the radioactive metastable technetium isotope ^{99m}Tc (half-life 6.01 h) via exposed sulfhydryl (SH)-groups. These are chemically generated by reduction of the disulfide bonds linking the two Fab fragments in the Fab_2 fragments generated by pepsin digest from the IMMU-4 IgG preparation produced from murine ascites.

Arcitumomab binds specifically to carcinoembryonic antigen (CEA, CD66e, *CEACAM5*), a protein expressed by colorectal and many other tumors, but which is also present in certain inflammatory conditions such as Crohn's disease or inflammatory bowel disease. CEA can be shed, and its serum level is usually determined for the prognosis of patients with recurrent disease after having undergone resection of colorectal cancer. Arcitumomab exhibits no cross-reactivity to any genetically distinct CEA variants or surface granulocyte nonspecific cross-reacting antigen (NCA). Even though shedding occurs, the antibody still detects cell membrane-located CEA. Elevated CEA levels of up to 2000 ng mL^{-1} led to 50% antibody complexation with serum CEA, whereas no complexation was observed with plasma levels below 250 ng mL^{-1}.

CEA-Scan was reconstituted with ^{99m}Tc shortly before application, with imaging normally taking place between 2 and 5 h after injection, using a standard nuclear

camera. Delayed imaging might interfere with kidney, gallbladder and intestinal activity. Pharmacokinetic studies revealed a terminal half-life time after intravenous injection of CEA-Scan of approximately 14 h.

Four clinical trials were conducted to determine the imaging efficacy and safety of CEA-Scan. Joint application of CEA-Scan and standard detection methods such as computed tomography (CT) scanning significantly increased the surgical confirmation of scan-identified lesions as cancer. The positive prediction value (PPV) rose to from 83% for CEA-Scan and 86% from CT scanning alone, to 97% by complementary analysis.

Due to heterologous protein administration, the development of human anti-mouse antibodies (HAMA) may evolve, but has been reported in less than 1% of the patients. Observed adverse side effects included transient eosinophilia, fever, urticaria, and headache. No long-term studies of possible mutagenic or cancerogenic effects from the ionizing radiation from ^{99m}Tc have yet been carried out.

CEA-Scan was approved in 1996 by FDA for radioimmunoscintigraphy. The indication for CEA-Scan is solely in patients with histologically demonstrated carcinomas of the colon or rectum to evaluate the presence, location and extent of recurrence and/or metastases. The technique also provided additional information to standard noninvasive imaging techniques such as CT scanning or ultrasonography. CEA-Scan is also indicated in cases of suspected recurrence or metastasis of colon or rectum carcinomas due to rising serum levels of CEA if standard detection methods fail. However, CEA-Scan is indicated neither for differential diagnosis of colorectal carcinomas nor as a screening tool.

Previously, CEA-Scan was marketed by Immunomedics Inc. in Europe and USA, until 2005 when the product was withdrawn from EU market for commercial reasons. It is also no longer marketed in the USA.

Information Sources/References

http://www.fda.gov.
http://www.emea.eu.int.
http://www.dpgonline2.com/pkginserts/ceascan.pdf (CEA-Scan label).
Goldenberg, D.M., Nabi, H.A. (1999) Breast cancer imaging with radiolabeled antibodies. *Semin Nucl Med* 29: 41–48.
Swayne, L.C., Goldenberg, D.M., Diehl, W. L., Macaulay, R.D., Derby, L.A., Trivino, J.Z. (1991) SPECT anti-CEA monoclonal antibody detection of occult colorectal carcinoma metastases. *Clin Nucl Med* 16: 849–852.

15.3 Basiliximab (Simulect)

Basiliximab, a recombinant mouse/human chimeric IgG_1k, binds specifically to the interleukin (IL)-2 receptor alpha chain (IL-2Ra, or Tac or CD25) on the surface of activated T lymphocytes. The production of basiliximab is achieved in a mouse myeloma cell line transformed with two plasmids encoding the mouse heavy and light chain variable region genes genetically fused to human heavy and light

chain constant region genes. Interestingly, the V-region genes of the chimeric antibody are derived from Hybridoma RFT5, a cell line originally identified by its capability to secrete an IgG_1 which, *in vitro*, can kill a Hodgkin's lymphoma cell line (L450) when chemically coupled to deglycosylated ricin A-chain to form an immunotoxin (Engert et al. 1991). Immunotoxins based on this antibody underwent further evaluation for cancer treatment, including single chain antibody fusion proteins with a *Pseudomonas* exotoxin A derivative (see Chapter 3, Volume II).

Basiliximab acts as an inhibitor of IL-2 receptor function, by binding to its alpha subunit with a sub-nanomolar affinity ($10^{10}\,M^{-1}$). The expression of this subunit is found exclusively on activated T cells. Binding of the antibody to this receptor prevents the immunostimulatory signaling induced by IL-2 molecules. In a double-blind, randomized, placebo-controlled study with 340 patients for the prophylaxis of acute renal transplant rejection in adults, when used in combination with a triple immunosuppressive regimen (cyclosporine, corticosteroids and azathioprine), basiliximab reduced acute rejection (at 0–6 months) from 35% to 21%.

Basiliximab is approved for the prophylaxis of acute organ rejection in patients undergoing renal transplantation when used as part of an immunosuppressive regimen that includes cyclosporine and corticosteroids. An extension of indications has been attempted in a number of studies, with some controversial results (for a review, see Buhaescu et al. 2005). Basiliximab is the active ingredient of Simulect, marketed by Novartis.

References

Buhaescu, I., Segall, L., Goldsmith, D., Covic, A. (2005) New immunosuppressive therapies in renal transplantation: monoclonal antibodies. *J Nephrol* 18(5): 529–536.

Engert, A., Martin, G., Amlot, P., Wijdenes, J., Diehl, V., Thorpe, P. (1991) Immunotoxins constructed with anti-CD25 monoclonal antibodies and deglycosylated ricin A-chain have potent anti-tumour effects against human Hodgkin cells *in vitro* and solid Hodgkin tumours in mice. *Int J Cancer* 49(3): 450–456.

Sources

http://www.fda.gov.

Nashan, B., Moore, R., Amlot, P., Schmidt, A.-G., Abeywickrama, K., Soulillou, J.-P. (1997) Randomised trial of basiliximab versus placebo for control of acute cellular rejection in renal allograft recipients. CHIB 201 International Study Group. *Lancet* 350: 1193–1198.

Ponticelli, C., Yussim, A., Cambi, V., Legendre, C., Rizzo, G., Salvadori, M., Kahn, D., Kashi, H., Salmela, K., Fricke, L., Heemann, U., Garcia-Martinez, J., Lechler, R., Prestele, H., Girault, D. (2001) Simulect Phase IV Study Group. A randomized, double-blind trial of basiliximab immunoprophylaxis plus triple therapy in kidney transplant recipients. *Transplantation* 72(7): 1261–1267.

15.4 Capromab Pendetide (ProstaScint)

The key ingredient of ProstaScint is a radiolabeled murine IgG_1 kappa monoclonal antibody (mAb) called CYT-351 (designation by Cytogen) or 7E11-C5 (designation by Horoszewicz et al., 1987). The antibody conjugated with a tripeptide linker-chelator (GYK-DTPA) is called Capromab Pendetide, which binds a radioactive isotope of indium (^{111}In, half-life 2.8 days) via the chelator. The murine IgG is produced by a hybridoma cell line derived from a fusion of spleen cells of mice, immunized with whole cells and membrane extracts from human prostate adenocarcinoma cells, with myeloma cells. 7E11-C5 binds specifically to the prostate-specific membrane antigen (PSMA). PSMA, which is present on normal and neoplastic prostate epithelial cells, is a glycosylated membrane protein (MW ~ 100 kDa). The antibody was found to be most reactive on malignant primary and metastatic prostate cells, whereas benign prostatic hypertrophy and normal prostate cells reacted to a lesser extent. No cross-reactivity was found for other carcinoma, lymphoma, sarcoma or melanoma cell lines.

ProstaScint is injected intravenously as a single dose of 0.5 mg. Single photon emission computed tomography (SPECT) imaging follows 30 min after injection, not for determination of prostate cancer cells but for background staining of the vascular and pelvic anatomical structure. The actual imaging takes place 72 to 120 h later. Pharmacokinetic studies revealed an elimination half-life for ProstaScint of approximately 67 h.

Clinical studies revealed an accuracy of ProstaScint from 55% to 70%. However, the PPV for patients with low risk for lymph node metastases or recurrence of disease is below 20%. The PPV rises to 75% when ^{111}In-Capromab Pendetide was administered in scans with patients at very high risk of recurrence or nodal metastases.

Mild adverse side effects were reported in 4% of the patients, ranging from liver toxicity (hyperbilirubinemia), hypo- and hypertension, and hypersensitivity. However, HAMA development was reported after single injection in 1% of the patients leading to a high level of anti-mouse antibodies (100 ng mL^{-1}) and 4% of patients with levels of ~8 ng mL^{-1}. Therefore, repeated administration is not recommended.

ProstaScint was approved by the FDA in 1996 for radioimmunoscintigraphy. ^{111}In-labeled Capromab Pendetide is indicated for radiodiagnostic imaging in patients, with biopsy-proven prostate cancer, who are at high risk of pelvic lymph node metastasis, recurrent and/or metastatic prostate cancer, and also in patients after prostatectomy with a high risk of occult metastatic disease. ProstaScint allows the detection, staging and follow-up of prostate adenocarcinomas. It is also found to improve the diagnosis of nodal metastasis and prostate carcinomas when used in conjunction with other diagnostic methods for nodal metastasis. The radioimmunoconjugate is manufactured and distributed by Cytogen Corp. in the USA.

Information Sources/References

http://www.fda.gov (Label; SBA).
Horoszewicz, J.S., Kawinski, E., Murphy, G.P. (1987) Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients. *Anticancer Res* 7: 927–935.
Seo, Y., Franc, B.L., Hawkins, R.A., Wong, K.H., Hasegawa, B.H. (2006) Progress in SPECT/CT imaging of prostate cancer. *Technol Cancer Res Treat* 5: 329–336.

15.5
^{131}I-chTNT-1/B (Cotara)

The antibody is a chimeric mAb labeled with the radioactive iodine isotope ^{131}I (half-life 8.02 days). Synonyms are Tumor Necrosis Therapy-1 (TNT-1) and ^{131}I-chTNT-1/B. This agent targets double-stranded (ds) DNA and DNA/histone H1 complexes in the necrotic inner core of solid tumors. Cotara is based on the Tumor Necrosis Therapy concept, where the antibody is injected into the bloodstream or directly into the brain in order to bind to the inner core of necrotic tumors. Rapidly growing cancer cells start degenerating in the inner core of a tumor, with their cell membrane becoming porous. This facilitates the access of antibodies to the intracellular space and, in the case of Cotara, to bind ds-DNA. Upon radiation, neighboring viable cells are destroyed, thereby increasing the necrotic area of the malignant tumor.

The clinical trial jointly conducted with the brain cancer consortium "New Approaches to Brain Tumor Therapy" (NABTT) is evaluating the safety and efficacy of a single infusion of Cotara for treatment of patients with first or second recurrence of glioblastoma multiforme (GBM). The designated indication for Cotara is for two types of brain tumor in patients either with GBM (an extremely deadly and the most frequent form of brain cancer with a 1-year survival rate below 30%) or anaplastic astrocytoma [1,2]. The drug was granted orphan drug status in the USA in 1999 for the treatment of GBM and anaplastic astrocytoma, and two years later in the EU. Currently, Cotara is manufactured and marketed by Peregrine Pharmaceuticals Inc., and received approval from the FDA in 2003 for a clinical phase III study. In 2003, the Chinese company MediPharm Biotech Co Ltd. received marketing approval for licensed ^{131}I-chTNT-1/B in China from the State Food and Drug Administration (SFDA) of China. The approved indication is for treatment of refractory advanced lung cancer (stages II and IV), with the regimen occurring in two doses given between 2 and 4 weeks apart.

Currently, Cotara is produced in NS0 murine myeloma cells. The radiolabeled antibody is administered either intravenously or intratumorally with additional potassium iodine to avoid thyroid uptake of any radioactive iodine. According to MediPharm's published clinical data of 107 enrolled patients, 3.7% achieved complete remission and 30.8% partial remission [3]. Side effects included an adverse effect on the formation of mature hematopoietic cells in the bone marrow.

Neutrophil, platelet and hemoglobin toxicity was also observed to a lesser extent when Cotara was administered intratumorally. To date, however, no development of HAMA has been detected.

The approval of Cotara in China made it the third approved radiolabeled antibody for human radioimmunotherapy. The other two antibodies for radiotherapy are Bexxar and Zevalin for the treatment of human malignant B-cell non-Hodgkin lymphoma (see below).

Information Sources/References

http://www.fda.gov.

1 Central Brain Tumor Registry of the US (CBTRUS) data, 1998–2002.

2 http://www.peregrineinc.com.

3 Chen, S., Yu, L., Jiang, C., Zhao, Y., Sun, D., Li, S., Liao, G., Chen, Y., Fu, Q., Tao, Q., Ye, D., Hu, P., Khawli, L.A., Taylor, C.R., Epstein, A.L., Ju, D.W. (2005) Pivotal study of iodine-131-labeled chimeric tumor necrosis treatment radioimmunotherapy in patients with advanced lung cancer. *J Clin Oncol* 3: 1538–1547.

Street, H.H., Goris, M.L., Fisher, G.A., Wessels, B.W., Cho, C., Hernandez, C., Zhu, H.J., Zhang, Y., Nangiana, J.S., Shan, J.S., Roberts, K., Knox, S.J. (2006) Phase I study of 131I-chimeric(ch) TNT-1/B monoclonal antibody for the treatment of advanced colon cancer. *Cancer Biother Radiopharm* J21: 243–256.

Shapiro, W.R., Carpenter, S.P., Roberts, K., Shan, J.S. (2006) (131)I-chTNT-1/B mAb: tumour necrosis therapy for malignant astrocytic glioma. *Expert Opin Biol Ther* 6: 539–545.

15.6 Daclizumab (Zenapax)

Daclizumab (Zenapax) is a humanized IgG_1 mAb that binds specifically to the alpha subunit (~55 alpha, CD25, or Tat subunit) of the human high-affinity interleukin-2 (IL-2) receptor that is expressed on the surface of activated lymphocytes. Daclizumab is a composite of human and murine antibody sequences. The human sequences were derived from the constant domains of human IgG_1 and the variable framework regions of the Eu myeloma antibody. The murine sequences were derived from the complementarity-determining regions of a murine anti-Tat antibody. Daclizumab immunosuppressive function results from its binding to an IL-2 receptor. In particular, it binds with high affinity to the Tat subunit of the high-affinity IL-2 receptor complex expressed on activated (but not resting) lymphocytes, and inhibits IL-2 binding. The administration of Zenapax inhibits IL-2-mediated activation of lymphocytes, a critical pathway in the cellular immune response involved in allograft rejection.

Zenapax is indicated for the prophylaxis of acute organ rejection in patients receiving renal transplants. It is typically used as part of an immunosuppressive regimen that includes cyclosporine and corticosteroids. It is further under evaluation for the treatment of multiple sclerosis (study NCT00071838).

Information Sources/References

Drug leaflet.
http://www.clinicaltrials.gov.
http://www.rochetransplant.com.
Swiatecka-Urban, A. (2003) Anti-interleukin-2 receptor antibodies for the prevention of rejection in pediatric renal transplant patients: current status. *Paediatr Drugs* 5: 699–671.
Church, A.C. (2003) Clinical advances in therapies targeting the interleukin-2 receptor. *Q J Med* 96: 91–102
Carswell, C.I., Plosker, G.L., Wagstaff, A.J. (2001) Daclizumab: a review of its use in the management of organ transplantation. *BioDrugs* 15: 745–773.

15.7
Edrecolomab (Panorex 17-1A)

Edrecolomab is a murine monoclonal IgG_{2a} antibody to tumor-associated epithelial cell adhesion molecule (EpCAM, or CO17-1A) antigen. Edrecolomab (marketing name Panorex) was the first mAb to be approved for cancer therapy (1994 in Germany, for the treatment of Dukes' C colorectal cancer). It attaches to EpCAM, a human cell-surface glycoprotein that is found on normal epithelial cells and on a number of tumor cells found in colon and breast carcinomas, and a vide variety of other tumors. The loss of the Ep-CAM CO17-1A epitope expression predicts survival in patients with gastric cancer. The antibody's mode of action is believed to rely both on antibody-dependent cell-mediated cytotoxicity (ADCC) and the induction of a host anti-idiotypic response, as a survival advantage was reported for edrecolomab-treated patients who developed anti-anti-idiotypic antibodies compared with those who did not.

Following the approval of edrecolomab, a significant number of studies were conducted with the agent; in particular, several smaller studies were performed where edrecolomab did not demonstrate consistent benefit either in monotherapy or in combination with other anticancer agents. In particular, the results of a large randomized multi-center study showed that edrecolomab monotherapy was associated with significantly shorter overall survival and disease-free survival compared to that with 5-fluorouracil (5-FU) and folinic acid for the treatment of stage III colon cancer. Moreover, when it was added to 5-FU and folinic acid it did not result in a superior outcome to treatment with 5-FU and folinic acid alone. At present, the role of edrecolomab in the management of colorectal cancer remains uncertain, as the clinical studies were halted and marketing suspended by the distributor during the summer of 2000.

Information Sources/References

NCI Thesaurus, 2004_11_17.
Glaxo Wellcome Press Release, July 2000.
http://clinicaltrials.gov.
Wils, J.A. (2001) Therapy strategies for colorectal cancer: state of the art and beyond 5-FU/leucovorin. In: *Oncology*

Biotherapeutics. Cancer Communications Limited. ISSN 1470-7217. pp. 3–22.
Adkins, J.C., Spencer, C.M. (1998) Edrecolomab (monoclonal antibody 17-1A). *Drugs* 56: 619–626.
Mellstedt, H., Frodin, J.E., Biberfeld, P., Fagerberg, J., Giscombe, R., Hernandez, A., Masucci, G., Li, S.L., Steinitz, M. (1991) Patients treated with a monoclonal antibody (ab1) to the colorectal carcinoma antigen 17-1A develop a cellular response (DTH) to the 'internal image of the antigen' (ab2). *Int J Cancer* 30: 344–349.
Lobuglio, A.F., Saleh, M., Peterson, L., Wheeler, R., Carrano, R., Huster, W., Khazaeli, M.B. (1986) Phase I clinical trial of CO17-1A monoclonal antibody. *Hybridoma* 5(Suppl.1): S1171–S1123.

15.8
Gemtuzumab Ozogamicin (Mylotarg)

Gemtuzumab is a recombinant humanized IgG_4, kappa antibody (hP67) that binds specifically to the CD33 antigen, a sialic acid-dependent adhesion protein found on the surface of leukemic blasts and immature normal cells of myelomonocytic lineage, but not on normal hematopoietic stem cells. CD33 is expressed on the surface of leukemic blasts in more than 80% of patients with acute myeloid leukemia (AML). CD33 is also expressed on normal and leukemic myeloid colony-forming cells, including leukemic clonogenic precursors, but it is not expressed on pluripotent hematopoietic stem cells or on nonhematopoietic cells. For clinical use, Gemtuzumab is conjugated to the cytotoxic antitumor antibiotic, *N*-acetyl-gamma calicheamicin (ozogamicin). This antibody is conjugated to *N*-acetyl-gamma calicheamicin via a bifunctional linker, to form the drug available as Mylotarg. *N*-Acetyl-gamma calicheamicin is a low molecular-weight chemical compound produced by the bacterium, *Micromonospora echinospora* ssp. *calichensis*. The antibody portion of Mylotarg, the anti-CD33 hP67.6 antibody, is produced by mammalian cell suspension culture using a myeloma NS0 cell line. Mylotarg contains amino acid sequences of which approximately 98.3% are of human origin. The constant region and framework regions contain human sequences, while the complementarity-determining regions are derived from a murine antibody (p67.6) that binds to CD33. Gemtuzumab ozogamicin has approximately 50% of the antibody loaded with 4–6 moles calicheamicin per mole antibody, which results in a molecular mass of 151 to 153 kDa. The remaining 50% of the antibody is not linked to the calicheamicin derivative. Binding of Mylotarg to CD33 results in the formation of a complex that is internalized. Upon internalization, the calicheamicin derivative is released in the lysosomes of the tumor cell. The released calicheamicin derivative binds to DNA in the minor groove, resulting in DNA double strand breaks and cell death.

Mylotarg is indicated for the treatment of patients with CD33-positive AML in first relapse who are aged ≥60 years and are not considered candidates for other cytotoxic chemotherapy.

Information Sources/References

Drug leaflet.
http://www.wyeth.com.
Giles, F., Estey, E., O'Brien, S. (2003) Gemtuzumab ozogamicin in the treatment of acute myeloid leukemia. *Cancer* 98: 2095–2104.
Tsimberidou, A.M., Giles, F.J., Estey, E., O'Brien, S., Keating, M.J., Kantarjian, H. M. (2006) The role of gemtuzumab ozogamicin in acute leukaemia therapy. *Br J Haematol* 132: 398–409.
Fenton, C., Perry, C.M. (2006) Spotlight on gemtuzumab ozogamicin in acute myeloid leukaemia. *BioDrugs* 20: 137–139.
Press, O.W., Shan, D., Howell-Clark, J., Eary, J., Appelbaum, F.R., Matthews, D., King, D.J., Haines, A.M., Hamann, P., Hinman, L., Shochat, D., Bernstein, I.D. (1996) Comparative metabolism and retention of iodine-125, yttrium-90, and indium-111 radioimmunoconjugates by cancer cells. *Cancer Res* 56: 2123–2129.

15.9
Ibritumomab (Ibritumomab Tiuxetan, Zevalin)

Zevalin (ibritumomab tiuxetan) is an immunoconjugate resulting from a thiourea covalent bond between the mouse mAb ibritumomab (clone: IDEC-2B8) and the linker-chelator tiuxetan [*N*-[2-bis(carboxymethyl)amino]-3-(*p*-isothiocyanatophenyl)-propyl]-[*N*-[2-bis(carboxymethyl)amino]-2-(methyl)-ethyl]glycine. This linker-chelator provides a high-affinity, conformationally restricted binding site for a radioactive isotope of indium (^{111}In, half-life 2.8 days) or a radioactive isotope of yttrium, ^{90}Y (half-life 64.1 h). The indium- and yttrium-labeled antibodies are also named IDEC-In2B8 and IDEC-Y2B8, respectively, in some publications. The chimerized version of the antibody used in the Zevalin preparation is antibody IDEC-C2B8 [1] (rituximab; see Chapter 13), which is also part of the treatment regime with Zevalin.

Ibritumomab is a murine IgG_1 kappa mAb directed against the CD20 antigen, a nonglycosylated phosphoprotein on the cell surface of normal and malignant B lymphocytes. The antibody is produced in Chinese hamster ovary (CHO) cells. It is thought to induce apoptosis, as this activity was observed in CD20-positive B-cell lines *in vitro*, in addition to the effect of the radionuclides in the therapeutic regimen, which affect the neighboring cells (bystander effect) by inducing highly reactive radicals that damage a variety of biomolecules.

Zevalin is FDA approved for the treatment of relapsed or refractory low-grade, indolent non-Hodgkin's lymphoma (NHL), including patients with rituximab-refractory follicular NHL. The treatment regimen is complex and requires the administration of two different doses in two steps: An infusion of rituximab (see Chapter 13) preceding ^{111}In- Zevalin (emitting gamma for imaging) by not more than 4 h is followed 7 to 9 days later by a second infusion of rituximab followed by ^{90}Y Zevalin within 4 h of completion of the rituximab infusion. Whole-body gamma camera images are required at 48 to 72 h following infusion of the ^{111}In-labeled ibritumomab to confirm correct biodistribution before the therapeutic dose labeled with ^{90}Y can be given. This is necessary to detect those patients who

show an excessive uptake of the antibody by the reticuloendothelial system (resulting in significant accumulation of radioactivity in the liver, spleen, and bone marrow) or other normal organs. Patients with these altered biodistributions cannot be treated with the therapeutic yttrium conjugate.

Clinical studies are either ongoing or have been conducted for the use of ^{90}Y-ibritumomab tiuxetan to treat mantle cell lymphoma (MCL), relapsed or refractory diffuse large B-cell lymphoma (DLBCL), and as conditioning regimen for stem cell transplantation and novel combination regimens.

Information Sources/References

Drug leaflet.
http://www.zevalin.com.
http://www.fda.gov.
1 Reff, M.E., Carner, K., Chambers, K.S., Chinn, P.C., Leonard, J.E., Raab, R., Newman, R.A., Hanna, N., Anderson, D. R. (1994) Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. *Blood* 83 435–445.
Cheung, M.C., Haynes, A.E., Stevens, A., Meyer, R.M., Imrie, K. and Members of the Hematology Disease Site Group of the Cancer Care Ontario Program in Evidence-Based Care. (2006) Yttrium 90 ibritumomab tiuxetan in lymphoma. *Leuk Lymphoma* 47: 967–977.

15.10 Igovomab (Indimacis-125)

Indimacis 125 consists of an indium-radiolabeled $(Fab)_2$ fragment from a murine monoclonal IgG_1 specific for the cancer antigen 125 (CA125). The antibody (Igovomab; clone name OC125) is produced in a murine hybridoma cell line. The $(Fab)_2$ fragment is generated by proteolytic cleavage with pepsin from the complete IgG, and becomes covalently linked to the chelating agent diethylenetriamine penta-acetic acid (DTPA). Prior to application, labeling must be carried out with a radioactive isotope of indium (^{111}In, half-life 2.8 days) via the chelator DTPA.

The mucin CA125 (*MUC16*; 2353 kDa) as an oncofetal protein is present on more than 90% of all ovarian serous adenocarcinomas. Due to low level of CA125 in early stages of cancer, Indimacis is unsuitable for routine screening.

Indimacis was used for radioimmunoscintigraphic imaging of ovarian cancer in cases of relapse with increased serum levels of CA125 detected, but a lack of confirmation by ultrasound or CT scanning. In these cases, Indimacis scintigraphy revealed 50% of cases as being positive for relapse. The radiolabeled antibody was given intravenously and did not produce any severe side effects such as allergic reactions. Indimacis 125 was approved in the EU in 1996. Indimacis-125 was manufactured and marketed by CIS Bio International/Schering until 1999, when it was withdrawn by the company.

Information Sources/References

Spada, S., Walsh, G. (2005) *Approved Biopharmaceutical Products*. CRC Press, Boca Raton, USA.
Uttenreuther-Fischer, M.M., Feistel, H., Wolf, F., Jäger, W. (1997) Distribution of radiolabelled anti-CA125 monoclonal antibody OC125-F(ab)2-fragment following resection guided by antibodies (REGAJ) in ovarian cancer patients. *J Clin Lab Anal* 11: 94–103.

15.11
Nofetumomab (Verluma)

Verluma was marketed as a kit to label the CD20-specific antibody nofetumomab with the radioactive metastable technetium isotope ^{99m}Tc (half-life 6.01 h). It was approved by the FDA in 1996. The radiolabeled antibody was used for radioimmunoscintigraphy of tumors, especially small cell lung cancer (SCLC) metastases. Nofetumomab was a murine Fab derived from a monoclonal IgG_{2b} antibody (NR-LU-10) produced from a hybridoma cell line. The Fab fragment is generated by proteolytic cleavage of whole IgG by papain. The labeling is performed by complexing the radioisotope with a phenothioate ligand, which is subsequently linked to the Fab fragment.

The serum half-life of Verluma was 1.5 h, and the elimination half-life 10.5 h. Imaging was carried out at between 14 and 17 h after injection. In the clinical trial involving 89 patients with confirmed SCLC, Tc-labeled Verluma accurately determined whether the disease was extensive or limited on 82% of occasions. If the test indicated extensive disease, the result was true in 94% of the patients. However, if the test indicated limited disease, it was less valuable as a diagnostic aid, failing to image tumors in some body organs in approximately 23% of patients. Because of these false negative readings, additional standard diagnostic tests, such as a bone or CT scan or a bone marrow biopsy should be performed when limited disease is found.

Unspecific accumulation of ^{99m}Tc-Nofetumomab at nontumor sites such as the organs of excretion (e.g., gallbladder, intestine, kidneys, urinary bladder), regions of inflammation and areas of recent surgery were observed. Further tests (e.g., CT examinations, bone scan) were necessary to exclude extensive-stage disease in cases of imaging interpretation for limited-stage disease. Mild adverse reactions such as fever and skin rash were reported.

The clinical indication of Verluma was radiodiagnostic detection of extensive-stage disease in patients with biopsy-confirmed and untreated SCLC.

Verluma was developed and manufactured by Dr. Karl Thomae GmbH (affiliated to Boehringer Ingelheim Pharma KG). Marketing was carried out by NeoRx Corp. and DuPont Merck in the USA. Verluma is no longer on the market after DuPont Merck terminated its licensed distribution in 1999.

Information Sources/References

http://www.fda.gov.
Breitz, H.B., Tyler, A., Bjorn, M.J., Lesley, T., Weiden, P.L. (1997) Clinical experience with Tc-99m nofetumomab merpentan (Verluma) radioimmunoscintigraphy. *Clin Nucl Med* 22: 615–620.
Spada, S., Walsh, G. (2005) *Approved Biopharmaceutical Products*. CRC Press, Boca Raton, USA.

15.12
Satumomab (OncoScint/Oncorad: B72.3n)

OncoScint was the first FDA-approved mAb for tumor imaging [1], passing regulatory processes in 1992. OncoScint consists of a murine monoclonal IgG_1 kappa antibody (satumomab, MAb B72.3) conjugated with a tripeptide linker chelator module (GYK-DTPA). Antibody production was achieved by cultivation of a murine hybridoma cell line in an airlift bioreactor. The antibody linker conjugate is loaded prior to application with the radioisotope indium (^{111}In, half-life 2.8 days). Satumomab binds specifically to the tumor-associated antigen TAG-72, which is highly glycosylated. TAG-72 is expressed on 94% of colorectal and almost all common epithelial ovarian carcinomas, but it is also frequently associated with non-small cell lung, pancreatic, gastric and other carcinomas [2].

The elimination half-life of OncoScint is 56 h. Clinical studies revealed a PPV of 68% for patients with ovarian adenocarcinoma, and of 70% in patients with colorectal cancer. Adverse effects observed were, to a low extent, fever and in less-frequent cases allergic reaction including skin rash, hypo-, and hypertension. Due to the mouse origin of the antibody, development of human anti-mouse antibodies (HAMA) was detected in more than 55% of the patients after a single dose injection.

The approved indication for OncoScint is to determine the location, extent and follow-up of confirmed colorectal and ovarian carcinomas, especially in cases for presurgical imaging, providing complementary information when used with CT scan.

OncoScint was distributed by Cytogen in the USA, but is no longer commercially available.

Information Sources/References

1 Bohdiewicz, P.J. (1998) Indium-111 satumomab pendetide: the first FDA-approved monoclonal antibody for tumor imaging. J Nucl Med Technol 26: 155–163.
2 Thor, A., Ohuchi, N., Szpak, C.A., Johnston, W.W., Schlom, J. (1986) Distribution of oncofetal antigen tumor-associated glycoprotein-72 defined by monoclonal antibody B72.3. *Cancer Res* 46: 3118–3124.

15.13 Sulesomab (LeukoScan, MN-3)

The targeting component of LeukoScan is the Fab fragment of the murine IgG Sulesomab (anti-NCA-90, IMMU-MN3). Sulesomab binds the carcinoembryonic antigen (CEA) and the surface granulocyte nonspecific cross-reacting antigen (NCA90). The mAb is produced in murine ascites fluid. Antibodies were produced by ascites technology from a hybridoma cell line derived from a fusion of lymphocytes isolated from CEA-immunized mice with the SP2/0 mouse myeloma cell line. The full IgG is proteolytically cleaved by pepsin, generating a Fab_2 which is further chemically reduced resulting in a mixture of Fab-SH, Fab_2, heavy and light chains. The antibody is labeled with the radioactive metastable technetium isotope ^{99m}Tc (half-life 6.01 h) shortly before administering the radioimmunoconjugate.

The antigen NCA90 is present on almost all neutrophils. Being highly motile, neutrophils are rapidly found at points of infection and inflammation, whereby they become a suitable target to visualize infection/inflammation.

Radioimmunoscintigraphy is performed between 1 and 8 h after the injection of LeukoScan, using a standard nuclear camera for planar imaging or SPECT.

Clinical studies of efficacy and safety revealed a significantly increased sensitivity and accuracy of LeukoScan when compared to conventional imaging with *in-vitro*-labeled autologous white blood cells (WBC). The sensitivity of LeukoScan was 88% compared to 72% in WBC, and accuracy 76.6% compared to 70.9%, whereas specificity decreased.

In clinical safety evaluation involving over 350 patients, no incidents of HAMA development were detected. However, HAMA titers must be determined before repeated administration of LeukoScan. No animal studies were performed to determine the carcinogenic and mutagenic potential of LeukoScan due to the low-energy gamma radiation of ^{99m}Tc.

LeukoScan's approved indication is diagnostic imaging to determine the location and extent of infection/inflammation in the bone of patients with probable osteomyelitis, as well as in patients with diabetic foot ulcers. When a bone scan is positive and imaging with LeukoScan negative, then infection is unlikely. When a bone scan is negative, imaging with LeukoScan may rarely show a positive response, and this may indicate early osteomyelitis.

The potential for LeukoScan to identify inflammatory bowel disease, pelvic inflammatory disease, fever of unknown origin, subacute endocarditis and acute, atypical appendicitis was demonstrated in recent years, suggesting possible future indications.

LeukoScan is manufactured by Eli Lilly Pharma and marketed by Immunomedics GmbH in Europe and Australia. It was approved in the EU in 1997, but is not approved by the FDA.

Information Sources/References

http://www.emea.eu.int.
Gratz, S., Schipper, M.L., Dorner, J., Hoffken, H., Becker, W., Kaiser, J.W., Behe, M., Behr, T.M. (2003) LeukoScan for imaging infection in different clinical settings: a retrospective evaluation and extended review of the literature. *Clin Nucl Med* 28: 267–276.

15.14
Tositumomab; Iodine ^{131}I Tositumomab (Bexxar)

Bexxar is one of two FDA-approved radioimmunotherapy agents; approval was received in 2003. Bexxar is composed of a mixture of a radiolabeled and an unlabeled version of the mouse mAb tositumomab. The radioactive iodine isotope ^{131}I (half-life 8.02 days) is covalently bound to the monoclonal murine IgG_2 lambda antibody (initially inconsistently both referred to as B1 or anti-B1 in the literature). Tositumomab is produced from a murine hybridoma cell line by Boehringer Ingelheim Pharma KG. The antibody recognizes an epitope located on the extracellular domain of CD20 (also known as human B-lymphocyte-restricted differentiation antigen; synonyms Bp35 or B1) present on B cells, including non-Hodgkin B-cell lymphoma (NHL). According to the National Cancer Institute, NHL was the sixth leading cause for cancer-related deaths in the USA in 2003, with an estimated 18 840 deaths and 59 000 new cases in 2006 [1]. CD20 is a transmembrane phosphoprotein which is present on pre-B lymphocytes as well as mature B lymphocytes; it is also expressed on more than 90% of B-cell NHLs. Neither shedding nor internalization of CD20 occurs. The mechanism of action of Bexxar, which is responsible for a sustained depletion of transformed and nontransformed CD20-positive lymphocytes, is assumed to be complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and ionizing radiation from the radioisotope. All of these mechanisms trigger cell death, including induction of apoptosis leading to the killing of malignant B-cell lymphocytes [2].

The therapeutic regimen is applied in a two-step approach. In the first step, nonlabeled tositumomab for improved biodistribution of radiolabeled antibody is administered, followed by an ^{131}I-tositumomab infusion to determine optimal dosing. The second (therapeutic) step follows 7 to 14 days after the dosimetric step.

Two multicenter studies were conducted with 40 patients. In study 1, patients did not respond to rituximab or progressive disease after rituximab treatment. Study 2 comprised 60 patients who were refractory to chemotherapy. The overall response rate ranged from 68% (Study 1) to 47% (Study 2), with a median duration of response of between 12 and 18 months, and a pathologically and clinically complete responses of 33% and 20%, respectively. The most common adverse side reactions observed were cytopenias (neutropenia, thrombocytopenia and

anemia) as well as secondary leukemia were the most frequently observed events in clinical trials (a total of 230 patients). The most common nonhematologic side effects were fever, weakness infections and allergic reactions such as bronchospasm and angioedema. Further associated risks of Bexxar include hypothyroidism, HAMA formation and (due to the ionizing radiation) not only secondary leukemia but also solid tumors and myelodysplasia.

The indication of Bexxar is not for the initial treatment of CD20-positive NHL but only for the treatment of patients with CD20-positive, follicular NHL, with and without transformation, whose disease is refractory to rituximab and has relapsed following chemotherapy. Bexxar is manufactured and marketed by GlaxoSmithKline in the USA.

Information Sources/References

http://www.bexxar.com.
http://www.fda.gov.
1 http://www.cancer.gov.
2 Cardarelli, P.M., Quinn, M., Buckman, D., Fang, Y., Colcher, D., King, D.J., Bebbington, C., Yarranton, G. (2002) Binding to CD20 by Anti-B1 Antibody or F(ab')2 is sufficient for induction of apoptosis in B-cell lines. *Cancer Immunol Immunother* 51: 15–24.

Nowakowski, G.S., Witzig, T.E. (2006) Radioimmunotherapy for B-cell non-Hodgkin lymphoma. *Clin Adv Hematol Oncol* 4: 225–231.

Press, O.W., Eary, J.F., Appelbaum, F.R., Martin, P.J., Badger, C.C., Nelp, W.B., Glenn, S., Butchko, G., Fisher, D., Porter, B., Matthews, D.C., Fisher, L.D., Bernstein. I.D. (1993) Radiolabeled-antibody therapy of B-cell lymphoma with autologous bone marrow support. *N Engl J Med* 329: 1219–1224.

15.15
Votumumab (Humaspect)

Votumumab is a human IgG_{3k} (clone 88BV59H21-2V67-66, in the literature frequently referred to as 88BV59) derived from a monoclonal lymphoblastoid cell line, which has been immortalized by infection with Epstein–Barr virus (EBV). The antibody binds to a cytokeratin tumor-associated antigen, CTA 16.88, which is found in non-necrotic areas of most epithelial-derived tumors including carcinomas of the colon, pancreas, breast, ovary, and lung. Conjugated to the radioactive metastable technetium isotope ^{99m}Tc (half-life 6.01 h), the agent was approved in 1998 for the detection of carcinoma of the colon or rectum.

Marketing authorization in Europe was granted in September 1998, for use in the treatment of patients with histologically proven carcinoma of the colon or rectum for imaging of recurrence or metastases. This authorization expired in September 2003 when the supplier opted not to renew it.

Information Sources/References

Wolff, B.G., Bolton, J., Baum, R., Chetanneau, A., Pecking, A., Serafini, A. N., Fischman, A.J., Hoover, H.C., Jr., Klein, J.L., Wynant, G.E., Subramanian, R., Goroff, D.K., Hanna, M.G. (1998) Radioimmunoscintigraphy of recurrent, metastatic, or occult colorectal cancer with technetium Tc 99m 88BV59H21-2V67-66 (HumaSPECT-Tc), a totally human monoclonal antibody. Patient management benefit from a phase III multicenter study. *Dis Colon Rectum* 41: 953–962.

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