

Reprinted from:

S. Moroney and A. Plückthun, *Modern Antibody Technology: The Impact on Drug Development*. In: *Modern Biopharmaceuticals*, J. Knäblein, Wiley-VCH Verlag, Weinheim(2005), Vol. 3, pp. 1147-1186

2

Modern Antibody Technology: The Impact on Drug Development

Simon Moroney and Andreas Plückthun

Abstract

Antibodies are now the mainstream of biopharmaceuticals. By the end of 2003, 17 marketed therapeutic antibodies generated over \$5 billion in combined annual sales, with market growth at 30%. Ten years earlier, this class of biopharmaceutical drugs was almost written off, based on disappointments experienced with the first generation of murine monoclonal antibodies. This chapter will look at how new technologies have provided solutions to problems that hampered early efforts to develop effective antibody therapeutics and transformed the market for antibody drugs. This includes the generation of fully human antibodies, their affinity maturation and the selection of antibodies to bind to particular epitopes on disease-relevant targets. The chapter will also highlight what distinguishes a therapeutic from a simple binding molecule – different modes of actions of antibodies in different molecular and cellular settings will be compared. Finally, some of the available formats of the antibody and their effect on molecular/pharmacological properties will be discussed.

Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
CDC	complement-directed cytotoxicity
CDR	complementarity-determining region
CHO	Chinese hamster ovary
CTL	cytotoxic T lymphocyte
DOX	doxorubicin
EBV	Epstein–Barr virus
FcRn	neonatal Fc receptor
GlcNAc	<i>N</i> -acetylglucosamine
GM-CSF	granulocyte macrophage colony-stimulating factor
HIV	human immunodeficiency
HLA	human leukocyte antigen
IL	interleukin
mAb	monoclonal antibody
NK	natural killer
siglec	sialic acid-binding, immunoglobulin-like lectins
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor

2.1

Introduction

The initial promise of antibody-based biopharmaceuticals has taken a long time to

be realized. The breakthrough that led to the routine generation of monoclonal antibodies (mAbs) [1] created expectations that antibodies would become a major class of drugs. That it has taken over 20 years for the potential of therapeutic antibodies to be translated into commercial success is attributable to the time needed to solve problems associated with the first generation of antibodies. While clinical efficacy and safety depend critically on the target against which the antibody is directed, as well as the exact binding epitope, the molecular properties of the therapeutic itself are equally important in a successful drug (see also Part IV, Chapter 16 and Part V, Chapter 1).

While some challenges have been largely solved, others remain. Over the next decade it is likely that additional improvements in the molecular properties of antibodies will be made, further increasing their importance as biopharmaceuticals.

The main factors that limit the clinical utility of antibodies are:

- Immunogenicity.
- Inability to reach a disease-relevant target in sufficient concentration.
- Inability to trigger a particular biological effect which translates into modification of the disease process.

Technological approaches to reduce problems in each of these areas have met with varying degrees of success and are discussed in detail in the following sections. It is useful to discuss in turn each of the molecular requirements for making a therapeutic antibody and we will begin with immunogenicity.

2.2

Immunogenicity

Early clinical applications of murine mAbs quickly encountered the problem of immunogenicity in humans. While the insight into cellular mechanisms that has been enabled by the use of mAbs in basic discovery research has been remarkable, the problems with early attempts to use antibodies in therapy cast a shadow of doubt over whether this class of molecule would ever be clinically useful. After the first clinical disappointments with mAbs in the early and mid 1980s, many gave up on their promise as therapeutic agents. The technological developments that have led to a reduction in the difficulties posed by immunogenic murine antibodies, i.e., the development of methods to make chimeric, then humanized and, finally, fully human antibodies (Fig. 2.1), count among the major success stories of the modern biotechnology era.

It is clear now that the immune reaction against the original murine format was a major factor limiting the therapeutic use of antibodies. A relatively small number of academic groups and biotechnology companies tackled these problems, and developed methods for solving them. Only within the last decade have the resulting technological solutions led to the creation of the successful class of drugs that antibodies today represent. As a result, enthusiasm for this class of drugs in the pharmaceutical industry is a very recent phenomenon.

Immunogenicity is undesirable because it can be the source of a number of safety concerns, such as hypersensitivity and allergic reactions, thrombocytopenia, anemia, etc. Very problematic with recombinant therapeutic proteins, but fortunately extremely unlikely with antibodies, are

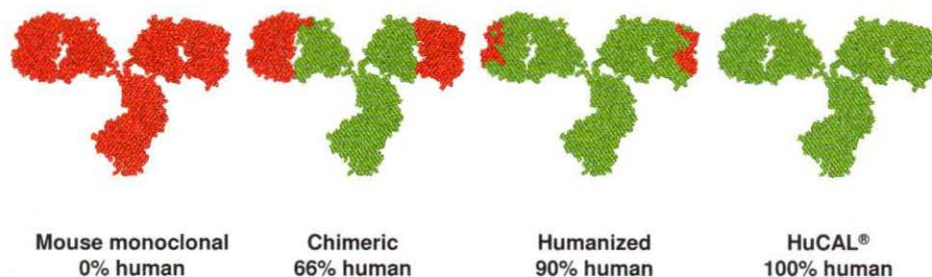


Fig. 2.1 Diagram showing the proportion of human (green) and murine (red) sequences in mouse, chimeric, humanized and human antibody structures, as exemplified by HuCAL.

cases in which the therapeutic protein elicits an immune response against one of the body's own proteins.

In addition to these safety concerns, there is still the problem of a loss of efficacy when the therapeutic molecule is removed by the immune system. This could be an issue in chronic indications, when the antibody has to be given repeatedly, as well as when the efficacy critically depends on half-life, as this may be reduced by an immune response against the therapeutic antibody. In certain other cases (for some examples, see below and Section 2.3.1), an immune response is elicited, but causes no major clinical effect.

Thus, the immunogenicity of antibodies (or any protein, for that matter) in humans is a very important parameter, but predicting it remains an inexact science. To date, no predictive scheme has emerged that can obviate the need for a clinical trial. The prediction of T cell epitopes from peptide sequence has been attempted and there are a number of websites available (<http://www.imtech.res.in/raghava/propred>, <http://mif.dfci.harvard.edu/Tools/rankpep.html> and <http://www.jenner.ac.uk/MHCPred>) [2–4] where this can be undertaken. Additionally, some antibody manufacturers test T cell epitopes experimentally in rather simple as-

says of T cell stimulation [5] by using a series of overlapping peptides covering the whole protein.

The rationale for these experiments is that the major human MHC alleles all require characteristic anchor residues in the peptides they bind. To elicit an immune reaction against a foreign protein, a T helper cell response is needed, which in turn requires that the protein is degraded to peptides and this also shows sequence specificity. A part of the protein is then presented in MHC class II on antigen-presenting cells. Peptides from the body's own proteins are also presented in MHC class II, but T cells that would recognize them do not normally exist, as they are eliminated in the thymus. In order to make a foreign protein “invisible” to T cells, none of its peptides must bind to MHC II, as T cells recognizing them will exist. Using available crystal structures and empirical data on peptide binding, profiles can be formulated for likely anchor residues [6]. While the prediction of the major antigenic epitope within a protein (or the absence of a clear hit) may be possible by exploiting the available structure and sequence information, it is still less clear whether such predictions can be extrapolated for engineering purposes. A

sufficient number of clinical trials will be required to show whether proteins can be engineered by point mutations to completely evade any MHC binding and thus T cell surveillance without losing their folding and function.

Derivatization with polyethylene glycol, or “PEGylation” (see also Part VI, Chapter 3 and Part VI, Chapter 1) [7–9], while primarily regarded as a means of increasing serum half-life of small antibody fragments (see Section 2.4.3), can also be used to decrease the immunogenicity of foreign proteins. As antibodies of fully human composition can be now obtained, PEGylation, which introduces additional manufacturing problems, might be more appropriate for modifying potential nonhuman effector domains, such as toxins (see Section 2.5.5). Nevertheless, clinical data will be needed for each individual case.

Table 2.1 summarizes data on the immunogenicity of a number of therapeutic antibodies currently on the market. As is immediately apparent from this and a wealth of data on mouse monoclonals, antibodies of more human composition are, in general, less immunogenic than those of murine origin. However, drawing firm conclusions is difficult for a number of reasons. First, immunogenicity depends on a number of factors unrelated to the molecular composition of the drug, including dose, route of administration, type of formulation and immunocompetence of the patient. Second, the strict demarcation of antibody structures into the categories “chimeric”, “humanized” or “human”, terms reflective of the way the antibodies were made, diverts attention from the key issue of sequence homology at the amino acid level. As has been pointed out [10], since mouse and human antibodies are rather homologous, the closeness of a sequence to the nearest human germline gene is perhaps a more important

determinant of immunogenicity and it may thus be advantageous to create antibodies with this property. Similarly, any human protein that has been mutated, e.g., by diversifying a region, is potentially immunogenic.

That a number of the chimeric, humanized and human biopharmaceuticals in Table 2.1 are highly successful drugs proves that the movement away from murine monoclonals towards antibodies of more human composition has paid dividends in the clinic. The conclusion for drug development is that antibodies that are predominantly human in their composition are less likely to encounter problems of immunogenicity than murine antibodies.

This difference between murine antibodies and those comprising some human content is also evident in overall developmental success rates. Data from the Tufts Centre for the Study of Drug Development [11] show that the probabilities of chimeric, humanized or fully human antibodies progressing from entry into clinical trials to the market are 26, 18 and 14%, respectively, while for murine antibodies the corresponding probability is only 4.5%. Caution should be used in drawing any conclusions from the apparently higher success rates in developing chimeric over humanized and human antibodies since the sample size is limited to those 17 antibodies which had reached the market at the time the study was performed.

Although the advent of technologies that can provide fully human antibodies (see Section 2.3) would appear to have solved the problem of immunogenicity, it is to be expected that the solution to this problem is not complete. Some human antibodies are known to be immunogenic, typically through anti-idiotypic or anti-allotypic effects. For example, the fully human antibody adalimumab (Humira), which was

Table 2.1 Immunogenicity of a number of therapeutic antibodies currently on the market

Compound name	Trade name	Company	Antibody format	Antigen	Approved application	Year of first approval	Isotype	Immunogenicity ^{a)}
Antibodies affecting the immune system (inflammation, allergy, transplantation)								
Muromonab	Orthoclone OKT3	Johnson and Johnson	murine mAb	CD3	organ transplant rejection	1986	IgG2a	~ 80%
Daclizumab	Zenapax	Hoffmann La Roche	humanized mAb	CD25	kidney transplant rejection	1997	IgG1	14%
Basiliximab	Simulect	Novartis	chimeric mAb	CD25	kidney transplant rejection	1998	IgG1	< 2%
Infliximab	Remicade	Johnson and Johnson	chimeric mAb	TNF- α	rheumatoid arthritis, Crohn's disease, ankylosing spondylitis	1998, 1999	IgG1	13%
Efalizumab	Raptiva	Genentech, Serono	humanized mAb	CD11a	psoriasis	2003	IgG1	6%
Omalizumab	Xolair	Genentech, Novartis	humanized mAb	IgE	allergic asthma	2003	IgG1	< 0.1%
Adalimumab	Humira	Abbott	fully human mAb	TNF- α	rheumatoid arthritis	2003	IgG1	5%
Antibodies in oncology								
Rituximab	Rituxan	Genentech, Biogen, IDEC, Hoffmann La Roche	chimeric mAb	CD20	non-Hodgkin's lymphoma	1997	IgG1	< 1%
Trastuzumab	Herceptin	Genentech, Hoffmann La Roche	humanized mAb	HER2/ <i>neu</i>	breast cancer	1998	IgG1	< 0.1%
Gemtuzumab ozogamicin	Mylotarg	Wyeth	humanized mAb/ calicheamicin	CD33	acute myeloid leukemia	2000	IgG4	0%
Alemtuzumab	Campath-1H	Schering	humanized mAb	CD52	chronic lymphocytic leukemia	2001	IgG1	2%
Ibritumomab tiuxetan	Zevalin	Biogen, IDEC	murine mAb/ ⁹⁰ Y	CD20	non-Hodgkin's lymphoma	2002	IgG1	4%
Tositumomab	Bexxar	GSK	murine mAb/ ¹³¹ I and murine mAb	CD20	non-Hodgkin's lymphoma	2003	IgG2a	> 70%

Table 2.1 (continued)

Compound name	Trade name	Company	Antibody format	Antigen	Approved application	Year of first approval	Isotype	Immuno-genicity ^{a)}
Cetuximab	Erbitux	ImClone, Merck KGaA	chimeric mAb	epidermal growth factor receptor	colorectal cancer	2003	IgG1	5% of evaluable patients
Bevacizumab	Avastin	Genentech, Hoffmann La Roche	humanized mAb	VEGF	colorectal cancer	2004	IgG1	0%
Antibodies in infectious diseases Palivizumab	Synagis	Medimmune, Abbott	humanized mAb	F protein of respiratory syncytial virus	lower respiratory tract disease in infants	1998	IgG1	<1%
Antibodies in perioperative care Abciximab	ReoPro	Centocor, Eli Lilly	chimeric Fab	GPIIb/IIIa	percutaneous coronary inter-vention	1995	Fab	6%
Antibodies in central nervous system diseases Natalizumab	Tysabri	Biogen Idec/Elan	humanized mAb	VLA4	multiple sclerosis	2004	IgG4	6–10%

a) Percentage of patients with antigen-antibody titer. From FDA-approved product label or other FDA submissions.

approved at the end of 2002 for the treatment of rheumatoid arthritis, is immunogenic in a significant number of patients. However, this has not hampered its successful use in the clinic.

As we learn more about the precise molecular features that contribute to immunogenicity, further improvements will be made in antibody composition to reduce this effect. However, for the foreseeable future, only clinical data in humans will allow determination of the severity of the effect and whether it has a negative impact on clinical utility. As a result of the reliance on human clinical data to determine immunogenicity, we may expect further progress in this area to be slow. It remains to be seen whether the molecular understanding of this complex process will advance to a point that immunogenicity can be engineered out at the amino acid level. It is also possible that a complete evasion of the immune system will simply not be possible in all cases.

2.3 Technology

The therapeutic antibodies currently on the market were developed at a time when molecular engineering had not progressed to its current level. Thus, because of the long development times typical of pharmaceutical development, all antibodies that are now on the market are derived in some way from mouse mAbs. Nevertheless, as will be clear from the following subsections, molecular engineering has now progressed to a point that therapeutic antibodies can be obtained without having an animal-derived mAb as a starting point. The great number of antibodies and derivatives in various phases of clinical trials that are derived from libraries bear witness to this point.

2.3.1

Chimeric and Humanized Antibodies

In the early and mid 1980s, as the first murine mAbs were being tested in the clinic, it was quickly recognized that in order to avoid the problems associated with their immunogenicity, antibodies of more human composition would be needed. While various methods have been investigated, such as immortalization of human B cells by Epstein-Barr virus (EBV transformation) (see Section 2.3.3), most success was achieved by starting with a murine mAb and engineering it to have a more human composition. We now summarize the different ways of achieving this goal.

Historically, the first generation of hybrid antibodies (part mouse/part human) comprised the entire murine variable domains of the original mAb, with the remainder of the IgG (constant C_L domain, usually κ , plus C_{H1} , hinge, C_{H2} and C_{H3} domains) coming from a human antibody (Fig. 2.1). Thus, in these so-called *chimeric* antibodies, four out of 12 domains in the IgG remain of murine origin (two V_L and two V_H). In total, approximately two-thirds of the sequence is of human origin, the remaining one-third being murine.

Currently, five chimeric antibodies are approved for human therapeutic use (Table 2.1). The immunogenicity of two chimeric antibodies, abciximab (ReoPro) and infliximab (Remicade) has recently been evaluated in some detail [12]. ReoPro is a chimeric Fab fragment that binds to the $\alpha_{IIb}\beta_3$ integrin, also called platelet membrane glycoprotein GPIIb-IIIa [13]. GPIIb-IIIa is an adhesion receptor for fibrinogen and the von Willebrand factor, both of which carry multiple binding motifs for the integrin and thus mediate platelet aggregation. ReoPro is approved for use in

percutaneous coronary intervention to prevent cardiac ischemic complications. In order to exert its effect by inhibiting thrombus formation, it must block a large fraction of its target integrins. At least 6% of patients develop an immune response to the antibody, but re-administration of the Fab fragment remains possible. Infliximab is a chimeric IgG1 specific for human tumor necrosis factor (TNF)- α , and is approved for the acute treatment of the signs and symptoms of Crohn's disease (an inflammation of the small intestine), and for the chronic treatment of rheumatoid arthritis. An immune response occurs in at

least 10% of patients, although there does not seem to be a reduction in clinical efficacy.

The next improvement was *humanization* – the grafting of the complementarity-determining regions (CDRs) of a mouse antibody onto a human framework [14]. For this purpose, a human framework is chosen from the database of human genes for V_H , another for V_L (κ or λ), and an alignment of the murine and human sequences is made. In addition to the CDRs themselves, differences in the framework must be taken into account (Fig. 2.2). For example, the so-called outer loop (some-

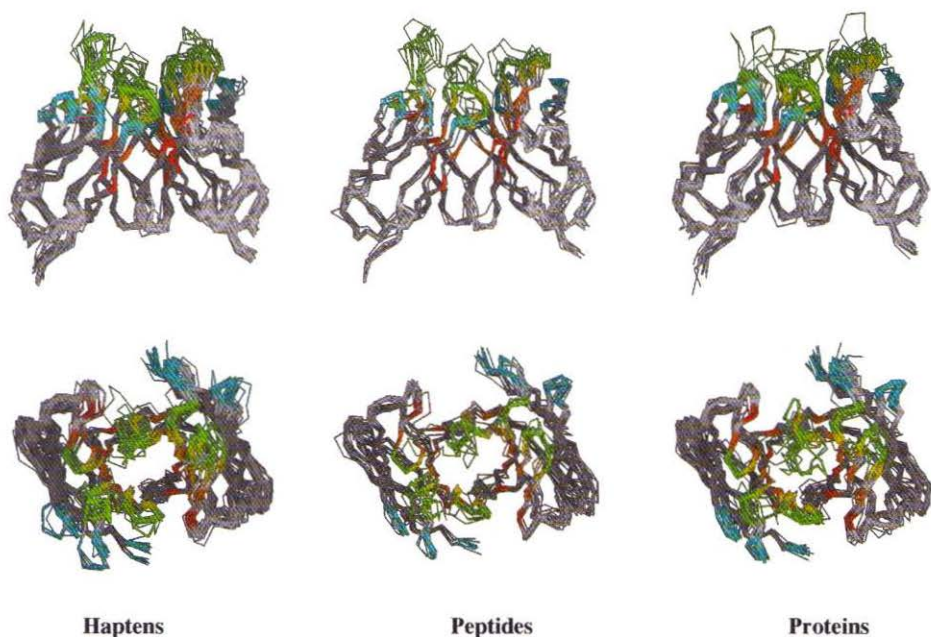


Fig. 2.2 Antibodies binding haptens, oligopeptides and oligosaccharides or proteins. Superposed crystal structures of the variable regions were sorted into three classes according to the type of antigen. Structurally variable residues within the CDRs of the antibodies are shown in green; those at the N-terminus, to the N-terminal side of CDR-1 and within the outer loop in cyan. Residues within the inner (dimer interface) β -sheet of V_L

and V_H whose side-chains contribute both to the dimer interface and to antigen binding if it reaches deep into this pocket are shown in yellow, orange and red, depending on depth. Note that these residues formally belong to the framework. The structurally least-variable residues whose C_α positions were used for the least-squares superposition of the antibody fragments are shaded gray.

times called CDR4) influences the conformation of CDR3 and a number of framework residues near the pseudo-2-fold axis (relating V_H and V_L by a rotation) are important for the binding of hydrophobic side-chains in a cavity in the binding site [15]. Such residues, even though formally from the murine donor, frequently need to be maintained. This involvement of framework residues is also the reason why the immune system uses different variable domain subtypes to bind antigens of many shapes and compositions (Fig. 2.3).

A structural model is usually built, exploiting the great number of three-dimensional structures of antibodies available to-

day (<http://www.biochem.unizh.ch/antibody>) (over 300 structures of independent V_H , 220 independent V_K and 40 independent V_L sequences). Then a “loop-grafted” version of the antibody, carrying additional framework mutations as necessary, can be created with similar affinity to that of the original mouse monoclonal. While this goal can usually be achieved, albeit with significant effort in cloning, engineering, and assay development, the greatest limitation of this technology is that a mouse mAb with the desired specificity is needed as a starting point.

A related approach to humanization, i.e., antibody resurfacing [16–18], relies on

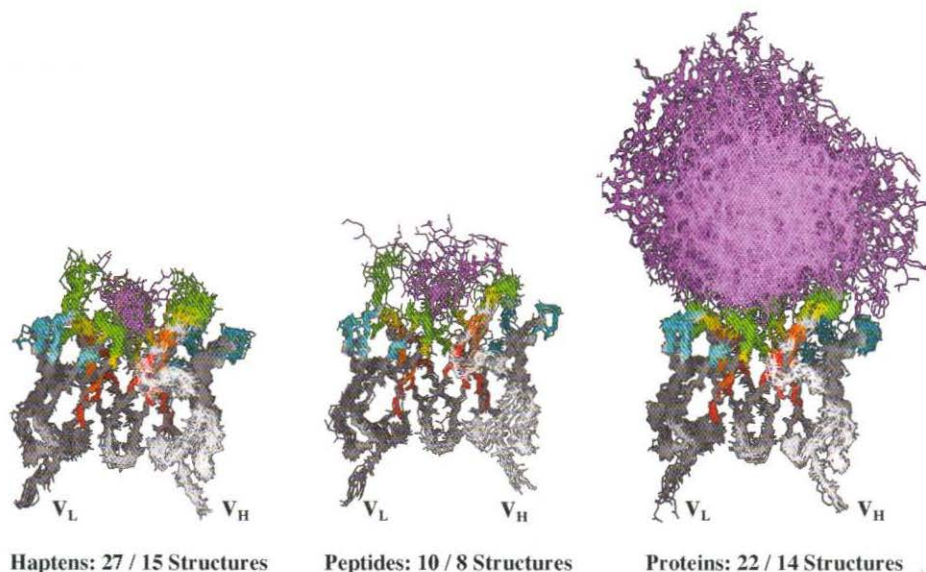


Fig. 2.3 Complexes of antibodies binding haptens, oligopeptides and oligosaccharides or proteins. The antigens are colored pink; parts of the antibody are colored as in Fig. 2.2. It can be seen that there is extensive binding to residues which formally belong to the framework, either in the dimer interface region for hapten binders or binders of peptides (which frequently use a side-chain in a hapten-like binding mode), and to the outer loop in protein binders. Hapten binders commonly form a deep, funnel-shaped binding pocket en-

larged by a long CDR-L1 and open CDR-H3 conformation, while protein binders preferentially utilize a relatively flat antigen binding surface characterized by a short CDR-L1 and a closed CDR-H3 conformation. This is one of the main reasons why the immune system uses different frameworks, rather than different CDRs all on the same framework. It also highlights the points to consider in loop grafting. More details can be found elsewhere [15].

making point mutations in surface residues of the murine antibody, converting amino acids to those found in human frameworks. This process requires alignment of the sequences of the original murine antibody with various human congeners that fulfill requirements of sequence compatibility with the antibody being modified. One such antibody is now in a phase I clinical trial [19, 20].

The first humanized antibody to enter the clinic was alemtuzumab (CAMPATH-1H) which is directed against CD52, a glycosylphosphatidylinositol-anchored glycoprotein with unknown function, present on lymphocytes. CD52 is abundantly expressed on B and T cells, macrophages, monocytes, and eosinophils. Alemtuzumab is used for the treatment of non-Hodgkin's lymphoma [21] and was approved in 2001 as first-line treatment for chronic lymphocytic leukemia. It has been proposed [22] that alemtuzumab inhibits the growth of B and T cells by cross-linking of CD52. It has also been suggested that the antibody works entirely through its effector function, using both antibody-dependent cellular cytotoxicity (ADCC) and complement-directed cytotoxicity (CDC) [23]. Alemtuzumab has also been tested as an immunosuppressive reagent in transplantation and autoimmune diseases. When the humanized antibody was administered i.v., no immune response was elicited (but infusion site reactions were observed), while a s.c. injection did elicit an anti-idiotypic immune response in two of 32 patients [24].

Immunogenicity data for some other humanized antibodies are summarized in Table 2.1.

2.3.2

The Limitations Imposed by Immunization

The use of immunization to generate antibodies in animals is one of the oldest techniques in biology. The generation of mAbs [1] uses an elegant cellular cloning technique to obtain (usually murine) antibodies with single specificities, but still must start with an immunized animal. Chimerization and humanization are modern molecular engineering methods, but they also require immunization to provide a mAb as a starting point.

Even adalimumab (Humira), a fully human antibody directed against TNF- α , was obtained using a pre-existing mouse monoclonal as a starting point and a process termed "guided selection" [25]. In this approach, one chain of the murine antibody, in recombinant form, was used in phage display together with a library of human antibodies encoding the other chain, followed by the reciprocal experiment, thereby generating an "equivalent" human antibody in several steps. Adalimumab is on its way to becoming a highly successful biopharmaceutical drug for the treatment of rheumatoid arthritis and possibly also psoriasis.

Notwithstanding this spectacular success, the reliance on immunization is a major limitation in all antibody generation methods that use this step. For example, the possibility of directing the response to a particular binding epitope during immunization is very limited and usually restricted to screening hybridomas [26]. It may well be that the epitope desired for the biological effect is not particularly favored, and not one which leads to high-affinity antibodies. Furthermore, the animal repertoire is limited to those variations that the immune system introduces during somatic mutation, and these are only a

small subset of the total theoretical repertoire. Therefore, the process of somatic mutation does not provide an exhaustive screen for the highest affinity or even for the optimal biological function. In contrast, modern library-based methods (see Section 2.3.3.1) provide the opportunity to select for antibodies with defined affinities, for particular epitope specificities, and even for pre-defined cross-reactivity patterns [27–29].

2.3.3

Fully Human Antibodies

Despite the outstanding achievements made with the techniques of chimerization and humanization, a means of routinely accessing fully human antibodies has always been a goal for developers of therapeutic antibodies. Historically, the first method for making human IgGs was the immortalization of human B cells with EBV. Since this method is rather inefficient, it has not been widely used. Recently, however, a new method has been introduced that dramatically increased the efficiency of transformation [30]. This offers the opportunity to immortalize memory B cells from patients after an infection, and potentially even from cancer patients, and thus complements cloning of such antibodies from patients and recovery of antibodies by display technologies [31]. Today, the most widely used technologies for making fully human antibodies are either library-based methods or transgenic mouse approaches. The fact that over 30 antibodies based on these technologies are currently in clinical trials indicates how well established they have become.

2.3.3.1 Library-based Methods

In the late 1980s, work was commenced on constructing antibody libraries. This development took antibody generation in a new direction, for the first time obviating the need for immunization of an animal. Library methods for antibody generation brought together several new technological developments, including construction of the library itself, but equally importantly, methods of screening the resulting library. This section summarizes both library construction and screening methods.

Library construction The first antibody libraries that had not been obtained from immunized animals were based on human genetic material isolated from natural sources [32, 33]. The most convenient sources of the appropriate genetic material are DNA or mRNA from human peripheral B cells, bone marrow B cells or tonsil B cells. The resulting library reflects the make-up of the human repertoire of the particular donor(s) and will to some degree reflect amplifications from recent infection events. Often, blood from many donors is pooled for this reason [33]. This bias can also be exploited – if peripheral blood lymphocytes from a patient with an infectious disease are used, such a library is an excellent source of antibodies against the infectious agent [31]. It is also possible to use non-rearranged genomic DNA as a starting point to make a library [34]. As the antibody genes in their germline configuration do not contain the V, D or J segments (and would thus be lacking CDR3 and the last β -strand of the domain), these elements need to be added in a subsequent PCR assembly.

An alternative approach, giving the highest level of control over the process, is to synthesize the antibody genes completely [35]. In this case the frameworks can be cho-

sen to represent the optimal diversity of the antibody repertoire. In the case of HuCAL GOLD (unpublished), a fully synthetic human combinatorial library, seven frameworks for V_H and seven for V_L are used, giving 49 V_H - V_L combinations (see Fig. 2.4).

The system also comprises pre-synthesized libraries of cassettes for all six CDRs, each of which reflects the composition of the corresponding human CDRs. To maximize diversity yet maintain structural integrity, the CDRs are not sim-

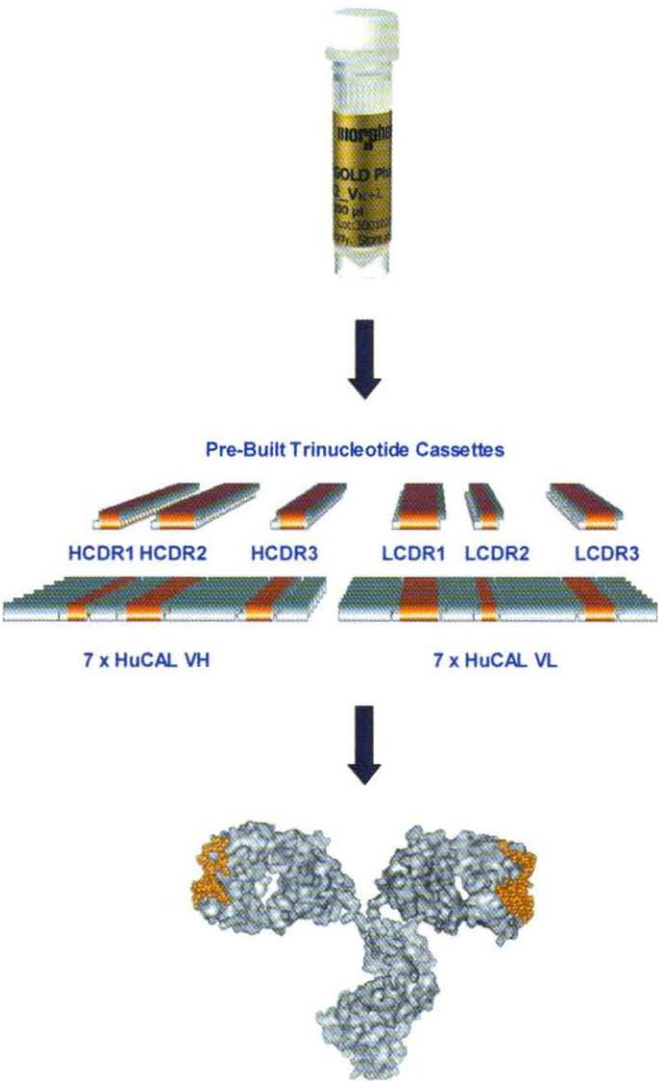


Fig. 2.4 The schematic structure of the genes underlying the HuCAL library, showing the modularity of the CDRs and the pre-assembled cassettes used during optimization.

ply randomized, but are diversified according to structural criteria, keeping a few key anchor residues constant and limiting the diversity of others to those observed in nature or compatible with the loop structure. Furthermore, the diversity of CDR1 and CDR2 is close to what is observed for a particular subtype, maximizing similarity to the germline. Conversely, the great length variation normally observed in CDR-H3 is also recaptured in HuCAL, allowing the resulting antibodies to bind to epitopes with a great variety of shapes and composition. Using this strategy, key structural residues are maintained and the variation observed in rearranged human antibody sequences, reflecting the process of affinity maturation and subsequent selection as documented in sequences of human antibodies, is well mimicked.

The HuCAL GOLD library also incorporates unique restriction sites bracketing the CDRs (Fig. 2.4), a feature made possible by the use of chemical synthesis for construction of the encoding genes. The ends of the CDR cassettes match the restriction sites bracketing their positions in the HuCAL library. A fully modular system is the result. The benefit of such a system is that antibody optimization can be rapidly and systematically carried out by replacing CDRs in turn to create new sublibraries based on one or more “hits” from a first screening. Multiple examples have shown that this is a reliable means of generating antibodies with predefined properties, while still retaining 100% “humanness” in the sequence. Such systematic optimization of antibodies builds on their inherent affinity and specificity to create substances with drug-like characteristics, including predefined cross-reactivity patterns as well as the ability to activate, deactivate and/or block certain biological processes.

The question sometimes arises as to why multiple frameworks are desirable in antibody libraries. There is a good reason why nature uses more than one framework in the antibody repertoire. As already mentioned above, antigens not only contact the CDRs, but also framework residues (Fig. 2.3). For example, large antigens make additional contact to the outer loop, or “CDR4” mentioned above, while small haptens, but also side chains of peptide antigens, often bind in a deep cavity near the pseudo-2-fold axis of the antibody [15]. These residues formally belong to the framework and thus vary between subgroups. Therefore, in order to capture the diversity of the human repertoire, a similar diversity of frameworks is necessary.

Single-framework libraries have also been made, and with a large enough diversity, high-affinity binders against many targets can be obtained [36, 37]. It must be remembered, however, that in the case of making therapeutic antibodies, usually only a very small subset of epitopes is of any utility. Therefore, obtaining high-affinity binders is a necessary, but not sufficient, condition for making a therapeutically active antibody. In general it will thus be necessary to have a technology available that can generate high-affinity binders to *any* epitope, so that cellular assays and preclinical experiments in animals can be used to identify the binders with highest potency. For example, a therapeutic antibody may need to block an interaction. In this case the antibody must bind to that part of the target engaged in the interaction, no matter whether it is protruding, recessed or flat. It is immediately obvious why a good antibody repertoire must therefore be able to bind any shape on any target molecule.

Libraries need to comprise diversity in the variable region if they are to effectively

mimic the natural immune repertoire. The molecular arrangement of the library (scFv fragment or Fab fragments being the dominant formats) is, however, mostly dictated by the selection strategy used (discussed below). For example, in phage display both scFv and Fab libraries can be used, as they can be in yeast display. In contrast, in ribosome display only single polypeptides can be used, as in scFv fragments.

Screening technologies An essential technological adjunct to the creation of an antibody library is an effective means of screening it for binders having the desired characteristics. Over recent years, several technologies have been developed that allow the selection of proteins, including antibodies, from large repertoires. Some methods also allow directed molecular evolution [38]. All of them have in common that the fact that the phenotype of the protein (in the case of an antibody, its binding specificity) must be connected to the genotype (the sequence encoding the antibody in question). A schematic overview of these concepts is shown in Fig. 2.5. On affinity “enrichment” of the antibody, its DNA sequence is thereby enriched as well, allowing the identification of the precise molecular composition that gave the desired binding phenotype.

While selections in model systems and from complex antibody libraries have been described using a number of these technologies, we restrict the discussion here to a brief description of the three methods with which the greatest experience in the field of antibodies has been obtained to date.

Historically, the first technology for the selection of polypeptides from repertoires was display using filamentous phages (Fig. 2.5a), which was first demonstrated

for peptides [39] and subsequently adapted to antibodies [40, 41]. *Escherichia coli* are transformed with the library of interest in the form of an expression plasmid that encodes the antibody (either as an scFv fragment or as a Fab fragment) in fusion with a minor coat protein (usually g3p or its C-terminal domain) of the phage. Note that each *E. coli* cell receives, in general, only one plasmid molecule and will thus encode only one molecular species of antibody. When a helper phage is added to these bacteria, new phage particles are produced that incorporate the fusion protein into their coat and thus “display” the particular antibody, while packaging the plasmid, which contains the coding information of the antibody. The phage population is then added to an immobilized target and those phages that do not bind specifically to the target are removed by washing. As a result, phage-carrying antibodies with the desired binding properties can be enriched and, in principle, recovered by an appropriate elution step. Successful proof-of-principle experiments were initially reported for scFv and Fab fragments [40, 41], and many sources of libraries have been used with this selection technology since then. While space limitations do not permit a full account of the many permutations of this fundamental concept, we want to stress that it has found broad utility in selecting antibodies against purified targets, selected epitopes, but also antigens on whole cells [42]. Most antibody selections have been carried out with this technology.

A variant on this method uses a disulfide bond to link the antibody to the coat protein of the phage, in which a pendant cysteine residue is introduced [43]. The advantage of this method is that the elution step can be replaced by mild reduction of the disulfide bond, resulting in reliable re-

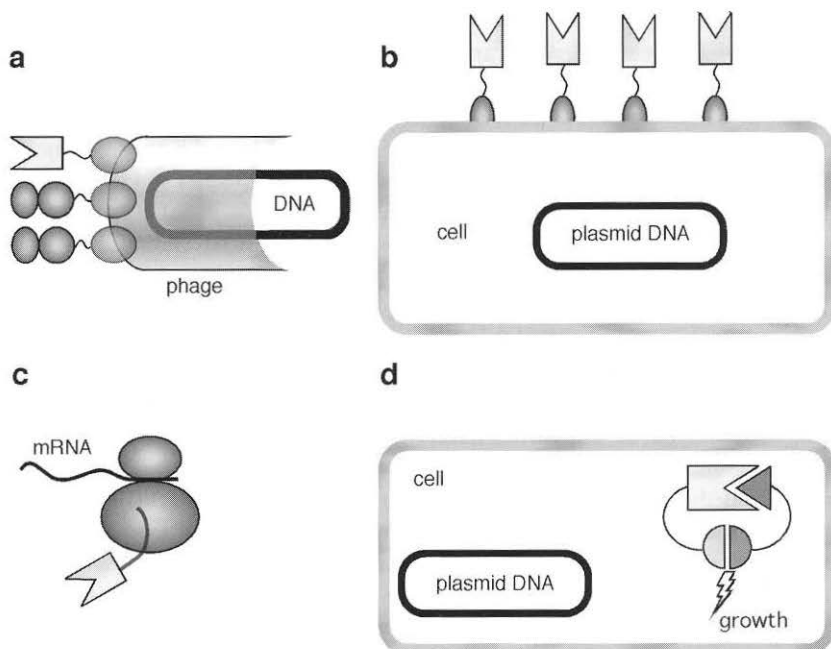


Fig. 2.5 The linking of phenotype and genotype in selection systems for antibodies. For more details on (a–c), see text. (a) In phage display, the antibody is fused to the minor coat protein g3p of filamentous phage, while the DNA is on the inside of the phage. (b) In yeast or bacterial cell surface display, the antibody is displayed on the outer surface of the cell, while the genetic information is encoded on a plasmid inside the cell. (c) In ribosome display, mRNA and protein product are linked by the ribosome, and the selection takes place in an *in vitro* translation system. Addition-

ally, antibodies can be screened and selected intracellularly (d), and selection for growth puts high demands on the folding efficiency of the antibody, while the selection for affinity is not very stringent. Intracellular screening is achieved by fusing the antigen and antibody to two protein halves which, when brought together by the antibody–antigen interaction, allow cellular growth by allowing transcription of a selectable marker (“yeast two-hybrid system”) [154] or reconstitution of a selectable enzyme activity (“protein-fragment complementation assay”) [155].

covery of enriched phage. In contrast, elution that relies on a change of pH or a competing ligand may not necessarily lead to recovery of the most interesting antibodies, i.e., those that bind to the target with the highest affinity.

A second technology that has been used more widely recently is yeast (*Saccharomyces cerevisiae*) display [44] (Fig. 2.5b). In this case, yeast cells are transformed with a plasmid library, which encodes a fusion with yeast α -agglutinin (Aga1p/Aga2p).

Again, this has been carried out both for scFv [45] and Fab [46] libraries. While transformation of yeast cells is somewhat more difficult than that of *E. coli*, methodologies do exist to achieve this. With antibodies displayed on the surface of yeast, not only a “mechanical” enrichment (e.g., with magnetic beads) is possible, but also the use of cell sorters together with a fluorescently labeled target. Therefore, thresholds for affinity can be defined and the affinity can be measured directly on

the yeast cells by titration. It has been reported that the yeast libraries can be amplified without changing their composition, i.e., particular clones do not seem to be enriched or depleted upon copying the library [45].

A third display technology that has been used to screen antibody libraries is ribosome display [47–50] (Fig. 2.5c). In contrast to the other methods, this works entirely *in vitro*, without using any cells at all. The library has to be in the scFv format, as only a single polypeptide chain can be displayed at a time. A library of PCR fragments is used, encoding a promoter and the open reading frame of the scFv fragment, fused to a “spacer”, which runs to the physical end of the fragment and does not encode a stop codon. The function of the spacer is to allow the scFv fragment to exit from the ribosomal tunnel and fold into its correct three-dimensional structure. An *in vitro* translation is thus carried out with a quantity of ribosomes stoichiometric to mRNA. The mRNA is translated to the end and remains connected to the tRNA within the ribosome, the scFv protein thereby remaining connected to the ribosome, which is also still attached to the encoding mRNA. Thereby, the antibody and its encoding mRNA remain linked. The main advantage of this method is that very large libraries can be used (typically 10^{12} different variants), as unlike in the other technologies no diversity is lost in the transformation step of *E. coli* or yeast. Furthermore, by using polymerases without proof-reading capability or even, deliberately, error-prone polymerase chain reaction or other methods to increase diversity, combined with a stringent selection for affinity, binders with picomolar affinity can be selected, thereby mimicking somatic mutation *in vitro* [49, 50].

2.3.3.2 Transgenic Mice

A scientifically elegant development was the creation of transgenic mice, in which part of the human antibody repertoire is inserted into the mouse genome (see also Part III, Chapter 4) [51–58]. This has been achieved in a variety of ways, e.g., with yeast artificial chromosomes or pieces of human chromosomes and homologous recombination (see also Part III, Chapter 2). In order to get an efficient response of human antibodies, the mouse repertoire needs to be inactivated. This has been done by targeted deletion of the J_H and J_K region (together with the constant κ region), to prevent V(D)J rearrangement of the murine antibody genes. This strategy permits well-established methods for the generation of mouse mAbs to be used to produce fully human antibodies. Historically, most therapeutic applications have used whole IgGs and the transgenic mouse approach produces them directly. In the case of the library approaches, which use antibody fragments during selection, a conversion to IgGs involves an additional (very straightforward) step. It can be expected, however, that a greater variety of formats for therapeutic antibodies will be employed in the future (see also Part V, Chapters 1 and 6), where the library technologies would have an additional advantage, since when transgenic mice are used, the antibody genes have first to be isolated by molecular cloning.

While a great scientific achievement, the disadvantages mentioned above in connection with immunization pertain here: lack of full control over the target epitope during the immunization process and inability to pre-determine cross-reactivity and affinity. In addition, and not unexpectedly, proteins that are highly conserved between man and mouse may not be immunogenic in this system.

2.4

Reaching the Target: The Importance of Specificity, Affinity and Format

An advantage of antibodies as potential therapeutics is their inherent affinity and specificity for their binding partner. These properties are a prerequisite for their therapeutic effectiveness. A third property is also crucial to the performance of a therapeutic antibody, i.e., its format. The importance of these molecular properties for the application of antibodies as biopharmaceuticals is considered here.

2.4.1

Epitope Specificity

Specificity for target is one of the main properties that distinguishes antibodies from other bioactive molecules. The first factor to consider is *where* exactly the antibody binds on the target. Some cases are easy to understand at the molecular level, such as in the case of blocking the action of a cytokine, such as TNF- α . This inflammatory cytokine is produced too abundantly in a number of diseases, such as inflammatory bowel disease (Crohn's disease and ulcerative colitis), rheumatoid arthritis and psoriasis. The therapeutic strategy thus consists of preventing the binding of this soluble, homo-trimeric molecule to its receptor. The antibody, obviously, must bind in a way that it overlaps with the binding interface to the receptor – it must bind to a “neutralizing” epitope.

In some cases, such as the one mentioned, such binders are quite straightforward to select, as the receptor contact surface is large. Indeed, TNF- α is a popular target for antibody and other protein-based therapeutic approaches, developers being encouraged by the success of etanercept (Enbrel, a soluble receptor-Fc fusion pro-

tein), infliximab (Remicade) and adalimumab (Humira) for the treatment of arthritis, psoriasis and Crohn's disease [59]. All three molecules are therapeutically active in rheumatoid arthritis. Interestingly however, the soluble receptor etanercept shows no activity in Crohn's disease, while infliximab does. A possible reason has been proposed [59]: to be effective, the transmembrane form of TNF- α must be targeted, which is present on T cells, where it may have a slightly different conformation, and this conformation is not recognized by the receptor, but by the antibody infliximab. Only the latter thus helps controlling inflammation by inducing apoptosis in T cells.

The antibody-binding site does not have to be identical to that of the receptor, it only has to overlap in such a way as to prevent simultaneous binding of the target to its receptor. In cases other than the ones described, this can be more difficult to achieve, e.g., if the binding site is small and not favored for binding. In such a case, using antibody library technologies it is often possible to “guide” the selection to the relevant epitope. This can be done in a variety of ways, such as using the “real” partner (e.g., the soluble receptor) as a competitor or using mutants of the soluble molecule to pre-bind all antibodies that are not directed to the desired epitope. The interested reader is directed to publications in which the technical approaches are discussed in more detail (see, e.g., [60]).

It is almost always necessary to test the binders so obtained in cell-based assays, in order to verify that they react with the antigen in its proper context on the cell. In some cases, on the other hand, it may not even be possible to obtain any soluble version of the protein of interest. In this case, selections can be carried out on whole cells and many of the same strategies ap-

plied. Again, the technical approaches and remaining challenges have been described [26–28], but space limitations do not permit us to discuss this in detail.

Blocking (or neutralizing) epitopes are easy to conceptualize. It should be noted, however, that in many cases the rationale why only binders to a particular epitope give a biological response is not at all clear. This is illustrated with two antibodies against HER2, a member of the epidermal growth factor receptor family overexpressed in about 25–30% of women with breast cancer and correlated with a poor prognosis (see also Part I, Chapter 5). The recently described crystal structure of 2C4 (Pertuzumab, Omnitarg) in complex with HER2 shows that this antibody inhibits the homodimerization of HER2 and also its heterodimerization with other members of the family, and thus signaling [61]. However, the antibody trastuzumab (Herceptin, 4D5) also inhibits signaling, yet without inhibiting dimerization [62, 63], and it binds close to the membrane, as shown in the crystal structure of its complex with HER2 [64]. The mechanistic reason for its inhibitory action, which is limited to tumors with high levels of homodimers of HER2, is still not entirely clear, and it is likely that internalization and/or proteolytic shedding are two of the factors influenced by trastuzumab binding, which then decrease signaling [62, 63]. Additionally, there is evidence [65] that Fc receptors on natural killer (NK) cells are recruited by the exposed Fc part of trastuzumab while bound to HER2 and this action is providing part of the therapeutic effect of this antibody. It is almost certain that the combined effect of all these factors is what gives Herceptin its efficacy.

With all the excitement surrounding this biopharmaceutical, it should not be forgotten that, for example, with Herceptin as

the sole treatment in metastatic breast cancer, only eight complete responses amongst 222 women were seen in a phase III trial [66]. Similarly, only 3–23% of all patients receiving Rituximab for relapsed or indolent refractory B cell non-Hodgkin's lymphoma showed a complete response [67]. These two examples, both concerning FDA-approved antibodies (see also Part VII, Chapter 4), underline the urgent need for further work in understanding the action – and subsequent improvement – of such antibodies, as well as the biological function of potential target antigens.

In cases where the structure and function of the target are known, presenting the “relevant” epitope in order to generate binders of the desired specificity is still the key challenge. A prime example is the difficulty in generating protective antibodies against HIV [68] (see also Part II, Chapter 7). The surface proteins of the virus, gp120 and gp41, are highly immunogenic, regardless of whether they are presented in the context of the virus particle (in infected patients) or as the soluble protein (as shed protein in infected patients). Antibodies can also be obtained readily, using immunization or display technologies. However, the great majority of antibodies that result are directed against non-protective surface epitopes. Indeed, the isolated protein does not even present the “protective” epitope, which is recessed between trimeric subunits when arranged as present on the virus. However, the virus particle normally does not elicit protective antibodies either, and thus it has been so far impossible to obtain an AIDS vaccine. Since HIV biology is well studied, a number of strategies are currently under way to overcome this challenge [69] (see also Part II, Chapter 8). Very few broadly neutralizing antibodies have been cloned from infected patients, but the mechanism of

neutralization is not clear for all of them [70].

The difficulty of generating broadly neutralizing antibodies against HIV may have attracted much attention, but it is likely that inaccessible neutralizing epitopes, and epitopes that are only formed after an initial binding event, are much more common also in other biological systems and not restricted to infective agents.

In some cases, even the precise molecular definition of the desired epitope is unclear. Accordingly, the preclinical observation will frequently be that only very few antibodies will show a biological effect, even though many others bind to the same target with high affinity. It will usually be of great benefit to attempt to understand, at the molecular and structural level, the key features distinguishing the biologically active antibodies from the others. In the case of cellular targets, this may involve differences in receptor multimerization (the antibody either inducing or preventing it), in the ensuing receptor internalization, proteolytic shedding, blocking (or enhancing) the binding of an external ligand, or making the antibody Fc part accessible – while binding to the surface target – to macrophages, neutrophils, monocytes or NK cells carrying Fc receptors. It immediately follows that the type of Fc desired (or its desired absence) is another parameter important for engineering and it requires an understanding of the mode of action of the antibody.

A field of medicine where this lack of mechanistic understanding at the level of molecular structure is particularly notable and hampering progress is the treatment of cancer (see also Part II, Chapters 5 and 6). There are great challenges in targeting solid tumors, brought about by the enormous difficulty inherent in obtaining significant and selective enrichment of the antibody at the

tumor site. As a consequence, the majority of antibodies approved today are directed against leukemia, myeloma and lymphoma (see also Part V, Chapters 5 and 6). In these cases, two factors favor clinically successful treatment with antibodies: (1) the target cells are easily accessible in the bone marrow, lymph nodes or blood, and (2) the tumors respond well to radiation and chemotherapy. Furthermore, they can be selectively targeted via several cell-lineage-specific markers. For example, CD20 is a marker specific for B cells (a more detailed description is given in Section 2.5.2). In this case, the antigen is not restricted to the diseased cell, but the redundancy and the self-regeneration of the immune system can sustain the temporary depletion of B cells. It is in general much more difficult to identify selective markers for solid tumors [71]. The number of such tumor markers that are suited for targeted therapy is small: despite massive attempts using a variety of techniques, including screening of healthy and diseased tissues with antibody libraries, only very few new tumor-associated surface proteins have been added to the list over the years [72–74].

2.4.2 Affinity

An important factor determining therapeutic efficacy is affinity. If the goal is to block the action of a soluble target such as a cytokine, then as little as possible of the cytokine should remain in an active form. The affinity directly determines the amount of cytokine that will be free at equilibrium. In general, the affinity should thus be as high as possible for such applications. It should be noted that in many cases the soluble protein to be inhibited is a monomer (trimeric TNF- α and its homologs being more unusual in this respect),

so that the true monovalent thermodynamic affinity, i.e., the affinity of a single binding site, is the property of interest.

Another example where the importance of affinity has been clearly highlighted is the protective function of antibodies against toxic or infectious agents. For example, post-challenge protection against the anthrax toxin, a tripartite protein, correlated well with the dissociation constant of the antibody, all other properties being equal [75].

If the antibody is to be used for cellular targeting, however, more complicated relationships apply. A number of investigations in tumor targeting have uncovered some of these trends [76–78]. From these and other studies, it appears that tumor targeting in general improves with affinity, but seems to reach a plateau at affinities around 10^{-9} M. The steady-state concentration at the tumor does not get higher with higher affinity, as the dissociation rate from the tumor antigen is no longer limiting steady-state concentration once dissociation is very slow. Instead, cellular uptake and bulk flow become dominant parameters, and these are influenced by the format of the molecule. One should draw such conclusions on the importance of affinity only from comparisons of molecules which are point mutants of each other, but have otherwise exactly the same format – as different epitopes (some eliciting antigen internalization, others preventing dimerization), formats, molecular size, etc., will change targeting efficacy for different reasons (see Section 2.4.3).

Affinity is increased in the immune system by somatic mutation [79–81]. Space limitations do not allow us to discuss in detail strategies for the affinity maturation of antibodies using the various display technologies, and how the selection of high-affinity binders can be favored. This

is, however, now possible in a variety of ways and the interested reader is referred to a number of articles [82–87].

2.4.3

Format and its Impact on Pharmacokinetics

An important factor to consider is also the format of the final therapeutic molecule. The availability of bivalent immunoglobulins as well as monovalent Fab and scFv fragments makes valency an important consideration in drug design. If the protein to be blocked is soluble and contains only one copy of the epitope, as is, for example, the case in many cytokines and protein hormones, no interaction strength is gained by having IgG molecules over Fab fragments or scFv fragments. This is also true for targets on the cell surface, if they are so far apart that the two arms of an IgG cannot reach two identical epitopes. In this case again, the binding affinity and thus the blocking affinity of a monovalent antibody fragment will be identical to that of a bivalent one.

Nevertheless, the longer half-life of IgG molecules may be advantageous as it guarantees a longer duration of the blocking function. The half-lives of therapeutic antibodies have been reviewed [88] and were mentioned in Section 2.2 in the context of immunogenicity (see also Part VI, Chapters 1 and 2). The longer half-life of an IgG is caused not only by the higher molecular weight of the IgG and thus the inability to be cleared through the kidney, but mostly because of a particular mechanism selectively protecting IgG from normal serum protein catabolism [89]. Over 5 days, the human vascular endothelium engulfs all serum by endocytosis. The content is processed through a complex network of endosomes and tubules with decreasing pH. The neonatal Fc receptor

(FcRn; also termed Brambell receptor after its discoverer) is expressed in hepatocytes, endothelial cells and phagocytic cells of the reticuloendothelial system, the main location of protein catabolism. The receptor binds the Fc part of IgGs using charged histidines and thus prevents antibodies from ending up in lysosomes. Instead, FcRn with the bound IgG recycles to the same cell surface, releasing the intact IgG at the serum pH of 7.4. By this mechanism, the half-life of IgG is increased by a factor of 10 compared to the absence of this receptor in transgenic animals [89, 90]. In short, the use of whole IgG guarantees a long blocking function through its long half-life, even if it binds only monovalently to its target.

Murine IgG does not bind to human FcRn, and this explains the shorter half-life of murine antibodies in human patients, typically 12 to 48 h [91]. The half-lives of endogenous human IgG isotypes have been well studied and they do differ – 3 weeks for IgG1, IgG2 and IgG4, while IgG3 has a half-life of 1 week [88, 92].

Abciximab (ReoPro), mentioned in Section 2.3.1 as an example of a chimeric antibody, is unique among therapeutic antibodies marketed thus far in being a Fab fragment. Its half-life in plasma is only 20–30 min [93], but when interacting with platelets, this rises to 4 h. It is now clear that the antibody is dynamically redistributed between individual target molecules and platelets in less than 1 h. Thus, while the half-life of dissociation from each individual integrin molecule is rather fast, the high local concentration of integrin molecules on the platelets provides the drug with a long platelet-bound half-life, with antibody detected on platelets as long as 2 weeks after therapy. This leads to a prolonged inhibition of platelet function in response to shear stress for 72 h to 1 week [94].

In the targeting of solid tumors, the situation is again far more complicated. Large IgG molecules, because of their long life time in serum, maintain a very high steady-state concentration. From this reservoir, levels at the tumor can reach very high percentages (20–30% of the injected dose per gram), but tumor to blood ratios are very small. The problem is that diffusion of large proteins such as IgG through a solid tumor is very slow and inefficient, because the antibody is in competition with removal by bulk flow. In addition, the tumor has a high hydrostatic interstitial pressure, is heterogeneous in composition and density of antigen expression, and has reduced vasculature. Furthermore, because of the slow accumulation and the long half-life, the antibody will at no time be truly selectively enriched at the tumor (when expressed as the percentage of the injected dose per gram of tissue or blood).

This essentially constitutes a limitation on the use of toxic molecules or radioactive isotopes (see Section 2.5.7) being conjugated to the antibody in many applications: at a dose approaching the toxicity limit, the tumoricidal effect is often not yet reached [95] (see also Part V, Chapters 1 and 6). The dose-limiting organ is usually determined by the action of the free drug, as some amount of drug can be cleaved non-specifically. Since IgGs are mainly degraded in the liver, there may be concerns for liver toxicity as well for antibody–toxin conjugates. In contrast, for radionuclides, bone marrow is usually the dose-limiting organ (see below and Section 2.5.7), as some radionuclides (e.g., yttrium) can be incorporated in bone marrow [96] (see also Part V, Chapters 4 and 5).

Radioimmunotherapy provides an example of a setting in which a shorter half-life can be advantageous. Currently, two antibody–radioisotope conjugates are on the

market, both for the treatment of non-Hodgkin's lymphoma, i.e., ibritumomab tiuxetan (Zevalin) and tositumomab (Bexxar) (see also Part V, Chapter 7). Zevalin and Bexxar carry ^{90}Y and ^{131}I , respectively, but both are mouse antibodies. As detailed above, in the radioimmunotherapy setting, one of the main challenges is maximizing the dosage of radioactivity reaching the targeted tumor cells without delivering dangerous levels of non-specific radiation to organs, notably the bone marrow, the organ where hematopoietic stem cells are generated, the precursors of all blood cells. This balancing act requires that the antibody has a relatively short half-life, which is the reason why murine antibodies have been favored in this setting. The half-lives of the two marketed products illustrate this point: both ibritumomab tiuxetan [97, 98] and tositumomab [99] have half-lives of 1–3 days. To safely use rituximab in radioimmunotherapy, a chimeric antibody whose antigen (CD20) is the same as that of tositumomab would require dosimetry of individual patients [100].

In other words, the degree of humanness of an antibody, or in molecular terms, the lower affinity to the FcRn and thus the lack of selective prevention of degradation, can be used to achieve a half-life that is required for a particular therapeutic window. It is likely, however, that in the future fully human IgGs will be engineered with decreased FcRn receptor binding to obtain a particular half-life.

Smaller protein molecules (Fab fragments, scFv fragments) localize to a solid tumor much faster and also diffuse better through tumor tissue, but because of their faster excretion rate the steady-state level reached in serum is much lower. Particularly below about 25–50 kDa, clearance through the kidney becomes possible [101, 102]. It should be noted that this also

shifts safety concerns for antibodies conjugated with a toxic moiety from liver toxicity (for large proteins) to kidney toxicity (for smaller proteins), as a fraction of the recombinant molecules can be taken up by kidney parenchyma cells.

The increase in functional affinity to cell surfaces by having multiple binding sites – previously a hallmark of IgGs – has now been engineered into scFv and Fab fragments as well [103]. This “avidity effect” strongly increases the residence time on a surface-bound target molecule, provided that the antibody can reach two epitopes simultaneously. In combination with site-specific PEGylation remote from the antigen-binding site, the hydrodynamic properties and number of binding sites can now be engineered independently to achieve a compromise in the quest to optimize tumor targeting [7–9].

From these considerations it is clear that the optimal format of the antibody is dependent on the exact mode of action, its location, the effector mechanism, whether the antibody has been fused or conjugated with a toxin and what kind of toxin or toxic radioisotope is used. The use of an IgG is thus only one of many options. The advent of molecular engineering is thus pivotal to the further development of these therapeutic modalities.

2.5

Exerting an Effect at the Target

Ever since the first attempts to create antibodies for human therapy it has been recognized that, in most cases, mere binding to the target is necessary, but may not be sufficient. There may be additional prerequisites for an antibody to be a biopharmaceutical, including the blockade of a particular interaction and/or cell killing.

The largest number of therapeutic antibodies in development is in the area of cancer. This area also provides the most examples of the variety of ways in which antibodies can be used in medicine. Whereas in other diseases blockade of a particular interaction may be sufficient to have a therapeutic effect, the objective in cancer is to kill tumor cells, which usually requires some form of direct cytotoxicity. It is generally assumed [104, 105] that effective tumor killing by a naked antibody will use one or a combination of (1) blocking a growth signal, (2) delivering an inhibitory signal, (3) inducing apoptosis and (4) eliciting an immune response against the tumor. The relative importance of these factors depends on the tumor type and the targeted antigen. This section considers several such approaches in the context of anticancer drug development.

2.5.1

Blockade

Bevacizumab (Avastin) is a humanized antibody that is approved for the treatment of metastatic colorectal cancer [106] and is directed against vascular endothelial cell growth factor (VEGF), a molecule that stimulates angiogenesis. The antibody binds VEGF and thereby inhibits vascularization of tumors that overexpress the growth factor; preclinical studies show clear inhibition of tumor growth in a mouse xenograft model [107]. Bevacizumab is therefore a rare example of an anticancer antibody which exerts its effect by blocking a growth factor which is important for tumor cell proliferation, but without interacting directly with the cancer cell.

2.5.2

Naked Antibodies that Trigger Cell Killing

From a drug development perspective, an antibody that exerts its therapeutic effect without the requirement for further modification has a major advantage. Steps that require conjugation, chemical modification or new production methodology all add complexity, cost and risk to the development of a therapeutic antibody. Naked antibodies, which can be made using established, well-characterized cell lines and production/purification methods avoid these difficulties.

Several antibodies have been suggested to kill tumor cells by ADCC or CDC, or a combination of these effects. As noted above, CAMPATH is an example of an anticancer antibody that seems to work via such effects. Rituximab (Rituxan) is a chimeric antibody against CD20 that is approved for the treatment of non-Hodgkin's lymphoma [108]. The function of CD20, a well-known marker for B cell activation, is not precisely known, but it has been suggested to be involved in Ca^{2+} influx as a tetrameric molecule. Direct effects of Rituximab, including growth inhibition and apoptosis, have been shown *in vitro* and a cross-linking of lipid rafts by the antibody has been proposed, with a *trans*-activation of src kinases eventually leading to apoptosis [109]. However, it is unclear whether this contributes to the clinical benefit observed. In fact, the published data suggest that the predominant effector mechanism is ADCC, with a minor role played by complement [110]. This is the same target as that against which two radionuclide-conjugated antibodies (Zevalin and Bexxar; see Section 2.5.7) are directed, where the therapeutic mechanism is thought to be at least partially dependent on radiation damage.

A recent example of an anticancer antibody in development that functions via apoptosis is 1D09C3, a HuCAL-derived antibody specific for human leukocyte antigen (HLA)-DR [111, 112], which is highly expressed in B and T cell lymphomas. At the time of writing, this antibody is about to enter clinical trials for B cell lymphomas. The antibody causes cell killing selectively in activated tumor cells without the need for exogenous effector cells, via a mechanism that is caspase independent.

Safety concerns with drug- or radionuclide-conjugated antibodies are mostly due to the systemic effect of the toxic effector molecules. This problem is augmented by the high dose required, which in turn is due to the insufficient localization of the antibody, notably to solid tumors.

In contrast, naked antibodies are not systemically toxic per se. Of course, a particular antibody can be toxic by its biological effect on the chosen target – either at the intended tumor site or at another site where the target is expressed. For example, Herceptin shows incidence of cardiotoxicity, especially in combination with anthracyclines [113]. This is caused by HER2 being expressed in cardiac myoblasts, where it is involved in muscle spindle maintenance.

2.5.3

Modifying the Fc Portion to Enhance Effector Cell Recruitment

As antibodies are often able to trigger cell killing via Fc-mediated effector functions such as ADCC and CDC, much effort has been put into increasing this effect by modification of the Fc portion. A crucial experiment underlining the importance of the different receptors involved in mediating ADCC was reported recently [65].

While activator receptors, such as Fc γ RIIIA on NK cells, are responsible for arresting tumor growth by Herceptin in a transgenic mouse model, Fc γ RIIB receptors were found to be inhibitory to the killing action – mice deficient in this receptor showed much more pronounced ADCC. It is therefore tempting to modulate the relative binding to the activating and inhibitory receptor by engineering the Fc part.

Using a systematic series of point mutants in the Fc part, Presta and colleagues [114] identified residues which discriminate between binding to Fc γ RI, Fc γ RIIB and Fc γ RIIIA. A further complication is introduced by the presence of an allelic variant in human Fc γ RIIIA, which binds differentially to the mutants. Using a triple mutant IgG (S298A/E333A/K334A), improved binding to Fc γ RIIIA as well as more potent cytotoxicity in ADCC assays was observed [115]. Interestingly, in the recent crystal structure of the IgG1–Fc/Fc γ RIIIA complex, only one of these residues (Ser298) was found to make a direct contact to the receptor.

Using the crystal structure of the Fc/Fc receptor complex as a guide, other mutations in the protein sequence of the Fc part have been designed that were predicted to increase the interaction with the receptor (Dahiat et al. unpublished; Xenacor).

The Fc part is glycosylated on an asparagine residue (Asn297), and while the recent crystal structure of a complex of an Fc with the Fc γ RIII and Fc γ RIIA [116–120] shows that the oligosaccharide makes only minimal contact to the receptor, it has been known for a long time that efficient binding to the receptors requires glycosylation [121]. Structurally, the oligosaccharides attached to the conserved Asn297 of IgG are a biantennary type with a core heptasaccharide that consists of four *N*-

acetylglucosamines (GlcNAc) and three mannoses, and variable fucose addition to the core at the first GlcNAc residue [121]. From the investigation of the crystal structure of the Fc/FcR complex and a series of glycosylation truncation mutants, it became clear that the sugars act both to increase the distance (with the wild-type being optimal) and to decrease mobility of the receptor-interacting segments of C_{H2} domains [122].

One approach to improve FcγRIII interactions has been to add a bisecting GlcNAc sugar, by using engineered Chinese hamster ovary (CHO) cells expressing a glycosyl transferase [β (1,4)-N-acetylglucosaminyltransferase III] (see also Part IV, Chapters 2, 5 and 7). The resulting antibody killed neuroblastoma cells at 10- to 20-fold lower concentrations than when this bisecting GlcNAc was not present [123].

Recently, another strategy of “glyco-engineering” was reported to lead to an increased binding to FcγRIIIA and thereby to enhanced ADCC [124], i.e., the production of the antibody in host cells that do not add fucose. In a direct comparison, this strategy was more effective than adding the bisecting GlcNAc.

In an independent series of experiments using a CHO-derived cell line (Iec 13) that is unable to add fucose, this lack of fucose was shown to have no effect on binding to FcγRI (CD64) and only a marginal effect on binding to FcγRIIA and FcγRIIB (CD32), but led to a significant increase in the binding to FcγRIIIA (CD16) [125] and even led to a further increase of binding of the engineered triple mutant IgG1 S298A/E333A/K334A, which indeed translated to improved ADCC *in vitro* [126]. Interestingly, the lack of fucose correlates with an increased receptor on-rate, suggesting Fc stabilization in the active con-

formation, while the point mutations lead to a decreased off-rate, suggesting higher interaction strength [127].

In contrast, the presence and absence of fucose had no effect on binding to FcRn (which controls half-life) or C1q (which controls complement activation). The binding of the Fc part to the neonatal receptor, FcRn, can also be influenced by mutations [128, 129]. These mutations must, however, be designed to differentially affect the binding to the receptor in a pH dependent manner, such that recycling of the IgG works properly. Encouraging mouse experiments have been reported (summarized in Presta [125]), but clinical trials in patients have not yet been carried out.

The role of carbohydrate in the clearance rate of IgG remains unclear. Binding of aglycosyl IgG to the FcRn appears identical to that of the glycosylated form. Nevertheless, there is some disagreement in the literature about the influence of glycosylation on the rate of clearance. While some studies detected a difference, others did not (summarized in Shields et al. [114]).

2.5.4

Low-molecular-weight Drug Conjugates

New effector functions may be needed that exceed the efficacy of the Fc part itself, since, in a particular tumor setting, ADCC and CDC may either not be elicited well or even not at all or not be effective enough by themselves. For many years, antibodies have been conjugated to cytotoxic agents with the intention that the antibody “targets” the desired payload to the tumor, thus providing the proverbial “magic bullet”. This has been a long and winding road, with the therapeutic window frequently not opening wide enough between systemic toxicity and lack of efficacy. Nevertheless, improved molecular understanding, and

consequently better molecular design, is likely to give these approaches a renewed chance. While totally selective targeting may never be achieved, engineering of the antibody with regard to target epitope, affinity, selectivity, molecular size and thus pharmacokinetics, with the consequences of degradation of the non-localized drug-loaded antibody firmly in mind, may increase tumor selectivity above the threshold of therapeutic utility, such that more toxic conjugates can be used as drugs. IgGs may not be the preferred molecules for such approaches and the advent of recombinant technologies has greatly increased the number of possibilities regarding the molecular format.

The conjugation of antibodies to low-molecular-weight cytotoxic agents has been investigated for many years. The target in these cases should be an internalizing surface protein, as most small molecule drugs act as inhibitors of cell replication and therefore need to reach the cytoplasm or nucleus to exert their effect [130]. A case in point is gemtuzumab ozogamicin (Mylotarg), the only antibody-based drug based on this approach to reach the market. Mylotarg is a chimeric anti-CD33 antibody conjugated to the highly potent enediyne drug calicheamicin and is approved for the treatment of acute myeloid leukemia [131]. CD33 belongs to a growing family of sialic acid-binding, immunoglobulin-like lectins (siglecs). It appears to be an inhibitory receptor in myeloid lineage development [132] and is highly expressed in myeloid leukemia. The conjugation of the drug to the antibody gives a more favorable therapeutic window compared to the drug alone, despite the fact that the marketed preparation is heterogeneous, comprising a significant fraction of unconjugated antibody.

The antibody BR96, which recognizes an extended form of the Lewis Y carbohydrate

antigen present on many carcinomas [133, 134], has shown some promise as a conjugate with different small molecule drugs. Early attempts with doxorubicin (DOX), a drug of the anthracycline family, conjugated to BR96 provided excellent preclinical data. Nevertheless, phase II clinical trials of BR96-DOX for the treatment of metastatic breast cancer or gastric carcinoma showed limited efficacy, with elevated gastrointestinal toxicity, probably a consequence of the target being also expressed in healthy gastric epithelial cells [135, 136]. More recently, synergistic antitumor activity in animal models has been demonstrated for BR96-DOX in combination with the taxanes docetaxel and paclitaxel [137]. The conjugate is currently in clinical development for the treatment of non-small cell lung carcinoma in combination with docetaxel.

That factors beyond the antibody and cytotoxic agent are crucial in creating an effective biopharmaceutical is demonstrated by recent work with BR96. Conjugates comprising BR96 linked, via two different chemistries, to cytotoxic auristatin derivatives have shown promise in animal studies [138]. Clear superiority in this study was achieved by incorporating an enzyme-cleavable linker between antibody and cytotoxic agent, thereby increasing the efficiency and specificity of release of drug at the target.

In general, antibody-drug conjugates should be based on very highly potent small molecule drugs (see also Part V, Chapter 6). In addition to the cytotoxic agents mentioned above, examples of other small molecule drugs being investigated include tubulin polymerization inhibitors such as the maytansanoids, CC1065 and taxoids [130, 139]. As mentioned above, the linker chemistry, being either acid labile or enzymatically cleavable, pos-

sibly with matrix metalloproteinases as tumor-specific release mechanisms, are also factors being studied. While preclinical data are impressive, it remains to be seen whether a useful therapeutic window can be found for broad application of toxin immuno-conjugates in oncology.

2.5.5

Protein Toxin Conjugates

A conceptually similar approach is the conjugation of protein toxins to antibodies. Such toxins, typically from plants or bacteria, are enzymes that catalytically inactivate essential cellular processes such as translation. By covalently modifying a translation factor or the ribosome itself in an enzymatic process, a single enzyme molecule is sufficient to kill a cell [140–142]. The best clinically studied members of this group are *Pseudomonas* exotoxin A, a tripartite protein that enzymatically ADP-ribosylates elongation factor 2, and ricin, derived from the plant *Ricinus communis*, which modifies a critical nucleotide in eukaryotic ribosomal RNA. The natural toxins are produced with their own, unspecific uptake mechanism that allows them to infect any cell, exploiting receptor molecules ubiquitously expressed on mammalian cells. By deleting these cell-binding domains and replacing them by an internalizing antibody, typically in the single-chain format, tumor-selective killing can be achieved. The antibody thus mediates uptake of the enzyme by tumor cells.

Nevertheless, toxicity remains an issue for systemic applications of these immunotoxins, as does immunogenicity of the toxin part, which limits repeated dosing. In order to reach therapeutic levels in a solid tumor, high initial doses have to be used, but liver toxicity was observed in a trial against HER2 [143] (see also Part I,

Chapter 5). Therefore, more recent work has focused on applications in leukemia and lymphoma. Encouraging results were observed with an immunotoxin against CD22 in patients with hairy cell leukemia and chronic lymphocytic leukemia [144]. CD22 is a member of the siglec family, and serves as a receptor for sialic acid-bearing ligands expressed on erythrocytes and all leukocyte classes. CD22 appears to be primarily involved in the generation of mature B cells within the bone marrow, blood and marginal zones of lymphoid tissues [145].

A combined phase I/II trial for ricin conjugates with antibodies against the lymphocyte activation markers CD25 (the IL-2 receptor α -chain) or CD30 (a member of the TNF receptor superfamily, possibly involved among others in memory T cell development) for patients with Hodgkin's lymphoma showed some promise [146].

2.5.6

Cytokine Fusions

Since many tumors do elicit an immune response, albeit one which may be unable to eradicate the tumor, an attractive strategy would appear to be to enhance this response with immunostimulatory cytokines. In order to localize the cytokine to the tumor, fusion proteins with antibodies have been made. Constructs investigated include interleukin (IL)-2, IL-12, granulocyte macrophage colony-stimulating factor (GM-CSF) and members of the TNF superfamily [147, 148] (see also Part V, Chapters 1 and 6). In a recently reported phase I trial of a humanized mAb directed against the GD2 disialoganglioside, reversible clinical toxicities were reported together with the desired immune stimulation [149]. As is the case with bispecific antibodies (see below), the main challenge

in these approaches will be to prevent systemic engagement of the cytokine receptor by the cytokine part of the conjugate in the absence of the antibody binding to the tumor, as this is the most likely source of side-reactions. The severity of the problem will depend on the complex interplay of pharmacokinetics of the antibody reaching the tumor or the cytokine receptor.

2.5.7

Antibody-Radioisotope Conjugates

Some aspects of radioimmunotherapy have already been discussed above in the context of half-life. The conjugation of an antibody to a radioactive element that should cause radiation damage at the tumor site is an idea that has been pursued for a number of years [95] (see also Part II, Chapter 5, and Part V, Chapters 5 and 7). Again, progress with solid tumors has been modest, while encouraging results are obtained in the treatment of hematopoietic neoplasms. As noted above, the fact that [¹³¹I]tositumomab (Bexxar) and [⁹⁰Y]ibritumomab tiuxetan (Zevalin), the only FDA-approved radiolabeled therapeutic antibodies, are both of murine origin contributes significantly to a shorter half-life, which is desirable in this setting, but also creates a considerable immune response. This, however, is diminished in patients with hematopoietic disorders or prior chemotherapy. Interestingly, large quantities of the unlabeled antibody must be administered prior to or concomitantly with the radioconjugate to improve targeting. The relatively low dose that is sufficient for treating hematopoietic malignancies reduces adverse side-effects and may be the reason why a therapeutic window can be found in this case.

Improvements for solid tumors may potentially come from the use of pre-target-

ing strategies [95]. In this case the radionuclide is not directly coupled to the antibody. Instead, an antibody Fab fragment with a hapten binding function (e.g., a bi-specific antitumor×antihapten construct) is injected first and allowed to concentrate at the tumor. Once the majority has left the circulation, the radionuclide, coupled to a monomeric or dimeric hapten [95] is injected. The hapten derivative's extremely short half-life, combined with the newly generated binding sites by the noninternalizing Fab fragment on the tumor, if present on a nonshedding surface antigen, allow excellent tumor selectivity. Nevertheless, it remains to be seen whether a useful therapeutic window can be obtained, with the concern of potentially new dose-limiting mechanisms for uptake of the radionuclide.

2.5.8

Bispecific Antibodies

Attempts have also been made to use bi-specific antibodies to recruit effector cells. A number of challenges need to be overcome in this field. First, a robust method must be found by which such proteins can be produced. Initially, the co-expression of two antibodies in one cell and the separation of the one desired out of the 10 conceivable molecular forms did not seem an attractive proposition. However, a multitude of methods have been reported over the last few years [103, 150] to create bi-specific formats of the antibody with a defined molecular composition. These include (1) the co-expression of heavy chains that have been engineered to allow only the desired pairing, (2) the direct chemical linkage of two different Fab fragments, (3) a number of different bispecific recombinant antibody constructs based on scFv fragments fused to heterodimerizing pep-

tides and proteins or (4) the direct enforced pairing of the domains in so-called diabodies (see also Part IV, Chapter 16 and Part V, Chapter 1). It would be too early to favor one form over the others.

A second challenge is derived from the fact that binding with only one of the arms, e.g., the one engaging the effector cells, is a likely intermediate in the reaction: no systemic toxicity should result in such a case so as to avoid safety concerns. It is very likely that the binding to a solid tumor is much slower than binding to cells of the immune system found in the serum. The third challenge is the converse – the antibody may eventually bind to the tumor, but never reach an effector cell, because there is none there, or, for geometric reasons, its receptor cannot be reached or activated.

Factors such as these translate into practical limitations. Typically, excessively high concentrations of bispecific antibody are needed to see an effect *in vivo*. In addition, particularly in solid tumors, the effector cells are often ineffective in the absence of a local co-stimulatory signal, which usually requires the addition of an exogenous factor, a serious drawback for a viable therapeutic strategy.

A number of strategies have been developed to use bispecific antibodies to recruit different types of effector cells. Much of the earlier work sought to recruit effector cells via the IgA receptor Fc α RI or the IgG receptors Fc γ RI or Fc γ RIII. In a phase I/II trial in 16 patients, a bispecific anti-CD30 \times anti-Fc γ RIII construct led to one complete and three partial remissions plus four cases of stable disease [151]. Pretreatment with IL-2 cytokine resulted in augmented antitumor activity, possibly by an additional mechanism of activation of NK cells. In another trial, a bispecific anti-CD30 \times anti-Fc γ RI construct was tested in 10 Hodgkin's lymphoma patients [149]: one complete

and three partial remissions, plus four cases of stable disease were reported.

Quite in contrast to the situation with lymphoma, no responses were seen with solid tumors, using a bispecific anti-HER2 \times anti-Fc γ RI antibody in combination with interferon- γ or GM-CSF [152] (see also Part VIII, Chapter 3).

Attention has also focused on recruiting cytotoxic T lymphocytes (CTLs) using CD3 as the trigger. Limitations of the type mentioned above have again hampered progress in this field: to date, no clinical efficacy has been observed on systemic administration of anti-CD3 based bispecific antibodies [150].

A newer technology, which seeks to overcome some of the disadvantages of previous bispecific CTL approaches, is unique in comprising two single-chain Fv fragments linked in tandem [150]. These types of molecule have been termed "bispecific T cell engager" or "BiTE" constructs. It also has an anti-CD3 recruitment arm and a second specificity directed against a tumor marker. The molecules tested seem to have two advantages over other CTL-recruiting bispecific antibodies described previously: (1) they do not appear to require co-stimulation of T cells and (2) they appear to catalyze killing of multiple target cells by a single T cell. These advantages, if they should translate into the clinic, might offer significant dosing and cost-of-goods benefits for therapeutic antibodies of this type.

2.6

Antibodies in their Natural Habitat: Infectious Diseases

There is one field of medicine where the antibody in its classical format may indeed constitute the optimal molecular design – in the defense against infectious agents,

i.e., the normal function of an antibody. Palivizumab (Synagis) is a humanized mAb that prevents lower respiratory tract infection by respiratory syncytial virus, and it is used in pre-term infants and other young children at risk, such as with cardiopulmonary disease or immunosuppression [153].

In general, however, passive immunotherapy has not been the focus of many development projects, as small molecule antiviral and antibacterial agents would clearly be advantageous for ease of administration – provided they are available. However, the increasing spread of resistant strains of viruses and bacteria – not least caused by the indiscriminate use of antibiotics – may become one of the severe medical problems of the future. The logistics of passive immunotherapy, having available large doses of the required specificities in due time, are daunting, but perhaps not insurmountable with better production techniques, the use of well-designed cocktails of specificity and concentration on infections of great risk to global health. The multiple cases of outbreak of novel deadly diseases over the last few years, AIDS, SARS and Ebola fever, to name just a few, illustrate the need for rapid development of novel containment strategies. It is likely that recombinant antibodies will still have a role to play in this respect, as it may be faster in some cases to develop protective antibodies than a vaccine (see also Part II, Chapters 7 and 8).

2.7

Opportunities for New Therapeutic Applications Provided by Synthetic Antibodies

The advent of synthetic antibodies now allows a number of the previous key limitations in using antibodies for therapy to be

addressed. In addition to solving the problem of immunogenicity, which may be a factor preventing therapeutic utility, as explained above, the new technologies for tailor-making the antibody molecule permit new approaches to improve efficacy.

First and foremost, the relevant epitope can be more easily targeted. If its location is known at the molecular level, selections for such binders are possible, as has been delineated above, and this will often be the decisive factor in determining whether a particular antibody has any *in vivo* potency at all. Secondly, affinity can be addressed independently. Again, as described above, high affinity is almost always a benefit. In the traditional immunizations of animals, even when a large number of different mAbs is obtained, there is no guarantee that high-affinity antibodies from the panel are directed against the relevant epitope. As a rule, other places on the large surface of a protein will give many opportunities for high-affinity binding, such that it would be rather unexpected that the highest affinity antibodies happen to be directed against the epitope of choice.

It is this particular situation, where a synthetic library technology for antibody generation such as HuCAL can play out to its full advantage: affinity maturation strategies can be applied to the antibodies targeting the relevant epitope. Therefore, having *any* lead compound will usually allow the generation of a molecule, which maintains the binding at the desired location but achieves an affinity commensurate with the desired mode of action.

Because of the modularity of the HuCAL design (see above), it is possible to tailor not only one, but several antibodies that have been identified as having the desired binding specificity. Since identical restriction sites in all members of HuCAL flank the CDR cassettes, they can be exchanged

in several antibodies at once, and those with higher affinity can be selected. Thereby, it is possible to “walk” across the whole antigen-binding site and replace each CDR in turn. Since the cassettes are not random, but instead reflect the composition of the human repertoire, key structural properties of the antibody can be maintained during optimization. By separating the selection of efficacy (or binding epitope) from affinity, which can be subsequently improved for any hit, antibodies can be made to almost any specification.

The anti-HLA-DR antibody 1D09C3 mentioned above [111, 112] provides a good example of how antibody engineering can be applied to optimize antibody properties. The antibody was derived from a screen of a HuCAL library in the single-chain Fv format, which yielded a number of binders that, although of moderate affinity, efficiently killed tumor cells. The affinity of the initial lead antibodies was increased by sequential replacement of the L-CDR3 and L-CDR1, utilizing the modularity of the HuCAL gene library (see above). This process enables retention of the epitope specificity of the initial lead antibodies, which are vital for their cell-killing properties, while achieving the desired level of target affinity.

In practice, the ability to generate antibodies having particular properties from libraries is limited by the screen that is employed. Initial screens for affinity and/or specificity are used to reduce the number of potential hits from the initial library of 10^{10} to 10^3 – 10^5 , which must then be screened for a particular biological function. As delineated above, the (usually few) binders with a biological effect can then be affinity matured, such that affinity does not have to be an initial selection criterion. HuCAL thus provides a systematic means of screening large parts of sequence and

structure space for antibodies with predefined properties. Importantly, this can be done while retaining the intrinsic human composition of antibodies emerging from primary screens of the library.

2.8

Future Directions and Concluding Statements

Antibody engineering has clearly helped to solve a number of problems that have hampered early attempts in successfully using mAbs as biopharmaceuticals. Because of the lengthy development times typical of drug development, many therapeutic antibodies entering the market recently have still been made with earlier technologies and did not even fully profit from the possibilities available today.

A pivotal development of the last few years is that it is now possible to make a human antibody to practically any specification – regarding epitope, specificity, mode of binding, affinity, format and any molecule that might be linked with it. While robust manufacturing of more complicated molecules still needs to be improved, the main processes for manufacturing recombinant IgGs and fragments thereof are established.

Encouraging progress has been made in the area of neoplastic diseases of the hematopoietic system (lymphomas and leukemias). These tumors are characterized by good accessibility to the drug and the body's immune defense, high antigen density and perhaps a more homogeneous tumor cell population. In contrast, progress in the area of solid tumors has been only incremental. This is a huge need for society, and thus a great opportunity for science and medicine and the industry. It is apparent that several therapeutic antibodies are directed not only

against closely related diseases, but also against the same target (TNF- α , CD20 and CD25), while the medical need in many other areas is unmet.

The key challenge for the future is to back up today's molecular engineering capabilities with a much better molecular understanding of disease and the consequences of the application of particular molecules. A more detailed understanding of the exact molecular effect required for a particular target (blocking, dimerization, its prevention, exposure needed) will allow much better engineering of molecular properties. In particular, preclinical models must become more relevant and predictive. This is a difficult topic in complex diseases such as cancer, as not only the interaction between the therapeutic antibody and its target is of importance, but also the multitude of interactions with other cells and their surface proteins, the complex pharmacokinetics, and the detailed metabolism. For example, in many mouse models with tumors of human origin, the antigen is selectively expressed on the tumor, but not in the murine tissues. To better model the human situation, systematic approaches are dearly needed.

The use of antibodies as biopharmaceuticals to treat some of the most serious diseases affecting mankind today arises directly from impressive technological developments that have been made in this field over the last 20 years. This has been one of the most significant achievements in the field of modern biotechnology. The developments described here promise that this class of modern biopharmaceuticals will play an even more important role in the clinician's armamentarium for the foreseeable future. Molecular engineering holds the promise that the remaining problems, many of them due to incomplete molecular understanding of the most important dis-

eases, will be addressable in the future. Eventually, this will further lead to the ultimate biopharmaceutical, which, for example, "targets" the desired payload to the tumor, thus fully realizing Paul Ehrlich's vision of the proverbial "magic bullet".

Acknowledgments

We would like to thank Drs. Marlies Sproll, Uwe Zangemeister-Wittke and Michael Stumpp for critical reading of the manuscript, and Dr. Annemarie Honegger for Figs. 2.2 and 2.3.

References

- 1 Köhler, G., Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **1975**, 256, 495–497.
- 2 Schirle, M., Weinschenk, T., Stevanovic, S. Combining computer algorithms with experimental approaches permits the rapid and accurate identification of T cell epitopes from defined antigens. *J. Immunol. Methods* **2001**, 257, 1–16.
- 3 Flower, D.R. Towards *in silico* prediction of immunogenic epitopes. *Trends Immunol.* **2003**, 24, 667–674.
- 4 Flower, D.R. Databases and data mining for computational vaccinology. *Curr. Opin. Drug Discov. Dev.* **2003**, 6, 396–400.
- 5 Koren, E., Zuckerman, L.A., Mire-Sluis, A.R. Immune responses to therapeutic proteins in humans – clinical significance, assessment and prediction. *Curr. Pharm. Biotechnol.* **2002**, 3, 349–360.
- 6 Sturniolo, T., Bono, E., Ding, J., Radrizzani, L., Tuereci, O., Sahin, U., Braxenthaler, M., Gallazzi, F., Protti, M.P., Sinigaglia, F., Hammer, J. Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nat. Biotechnol.* **1999**, 17, 555–561.
- 7 Chapman, A.P., Antoniw, P., Spitali, M., West, S., Stephens, S., King, D.J. Therapeutic antibody fragments with prolonged *in vivo* half-lives. *Nat. Biotechnol.* **1999**, 17, 780–783.

- 8 Greenwald, R.B., Choe, Y.H., McGuire, J., Conover, C.D. Effective drug delivery by PEGylated drug conjugates. *Adv. Drug Deliv. Rev.* **2003**, *55*, 217–250.
- 9 Yang, K., Basu, A., Wang, M., Chintala, R., Hsieh, M.C., Liu, S., Hua, J., Zhang, Z., Zhou, J., Li, M., Phyu, H., Petti, G., Mendez, M., Janjua, H., Peng, P., Longley, C., Borowski, V., Mehlig, M., Filpula, D. Tailoring structure–function and pharmacokinetic properties of single-chain Fv proteins by site-specific PEGylation. *Protein Eng.* **2003**, *16*, 761–770.
- 10 Clark, M. Antibody humanization: a case of the “Emperor’s new clothes”? *Immunol. Today* **2000**, *21*, 397–402.
- 11 Reichert, J., Pavlou, A., Monoclonal antibodies market. *Nat. Rev. Drug Discov.* **2004**, *3*, 383–384.
- 12 Wagner, C.L., Schantz, A., Barnathan, E., Olson, A., Mascelli, M.A., Ford, J., Damaraju, L., Schaible, T., Maini, R.N., Tcheng, J.E. Consequences of immunogenicity to the therapeutic monoclonal antibodies ReoPro and Remicade. *Dev. Biol.* **2003**, *112*, 37–53.
- 13 Nurden, A.T., Nurden, P. GPIIb/IIIa antagonists and other anti-integrins. *Semin. Vascular Med.* **2003**, *3*, 123–130.
- 14 Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S., Winter, G. Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature* **1986**, *321*, 522–525.
- 15 Ewert, S., Honegger, A., Plückthun, A. Stability improvement of antibodies for extracellular and intracellular applications: CDR grafting to stable frameworks and structure-based framework engineering. *Methods* **2004**, *34*, 184–199.
- 16 Padlan, E.A. A possible procedure for reducing the immunogenicity of antibody variable domains while preserving their ligand-binding properties. *Mol. Immunol.* **1991**, *28*, 489–498.
- 17 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. Humanization of murine monoclonal antibodies through variable domain resurfacing. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 969–973.
- 18 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. A comparison of two murine monoclonal antibodies humanized by CDR-grafting and variable domain resurfacing. *Protein Eng.* **1996**, *9*, 895–904.
- 19 Helft, P.R., Schilsky, R.L., Hoke, F.J., Williams, D., Kindler, H.L., Sprague, E., DeWitte, M., Martino, H.K., Erickson, J., Pandite, L., Russo, M., Lambert, J.M., Howard, M., Ratain, M.J. A phase I study of cantuzumab mertansine administered as a single intravenous infusion once weekly in patients with advanced solid tumors. *Clin. Cancer Res.* **2004**, *10*, 4363–4368.
- 20 Tolcher, A.W., Ochoa, L., Hammond, L.A., Patnaik, A., Edwards, T., Takimoto, C., Smith, L., de Bono, J., Schwartz, G., Mays, T., Jonak, Z.L., Johnson, R., DeWitte, M., Martino, H., Audette, C., Maes, K., Chari, R. V., Lambert, J.M., Rowinsky, E.K. Cantuzumab mertansine, a maytansinoid immunoconjugate directed to the CanAg antigen: a phase I, pharmacokinetic, and biologic correlative study. *J. Clin. Oncol.* **2003**, *21*, 211–222.
- 21 Hale, G., Dyer, M.J., Clark, M.R., Phillips, J.M., Marcus, R., Riechmann, L., Winter, G., Waldmann, H. Remission induction in non-Hodgkin lymphoma with reshaped human monoclonal antibody CAMPATH-1H. *Lancet* **1988**, *2*, 1394–1399.
- 22 Rowan, W., Tite, J., Topley, P., Brett, S.J. 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* **1998**, *95*, 427–436.
- 23 Dyer, M.J. The role of CAMPATH-1 antibodies in the treatment of lymphoid malignancies. *Semin. Oncol.* **1999**, *26*, 52–57.
- 24 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., Osterborg, A., Hillmen, P. Blood concentrations of alemtuzumab and antiglobulin responses in patients with chronic lymphocytic leukemia following intravenous or subcutaneous routes of administration. *Blood* **2004**, *104*, 948–955.
- 25 Jespers, L.S., Roberts, A., Mahler, S.M., Winter, G., Hoogenboom, H.R. Guiding the selection of human antibodies from phage display repertoires to a single epitope of an antigen. *Biotechnology* **1994**, *12*, 899–903.
- 26 Jia, X.C., Raya, R., Zhang, L., Foord, O., Walker, W.L., Gallo, M.L., Haak-Frendscho, M., Green, L.L., Davis, C.G. A novel method

- of multiplexed competitive antibody binning for the characterization of monoclonal antibodies. *J. Immunol. Methods* **2004**, *288*, 91–98.
- 27 Hoogenboom, H. R., Winter, G. By-passing immunisation. Human antibodies from synthetic repertoires of germline V_H gene segments rearranged *in vitro*. *J. Mol. Biol.* **1992**, *227*, 381–388.
 - 28 Hoogenboom, H. R., Lutgerink, J. T., Pelsers, M. M., Rousch, M. J., Coote, J., Van Neer, N., De Bruine, A., Van Nieuwenhoven, F. A., Glatz, J. F., Arends, J. W. Selection-dominant and nonaccessible epitopes on cell-surface receptors revealed by cell-panning with a large phage antibody library. *Eur. J. Biochem.* **1999**, *260*, 774–784.
 - 29 Mutuberria, R., Hoogenboom, H. R., van der Linden, E., de Bruine, A. P., Roovers, R. C. Model systems to study the parameters determining the success of phage antibody selections on complex antigens. *J. Immunol. Methods* **1999**, *231*, 65–81.
 - 30 Traggiai, E., Becker, S., Subbarao, K., Kolesnikova, L., Uematsu, Y., Gismondo, M. R., Murphy, B. R., Rappuoli, R., Lanzavecchia, A. An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat. Med.* **2004**, *10*, 871–875.
 - 31 Burton, D. R., Barbas, C. F., 3rd, Persson, M. A., Koenig, S., Chanock, R. M., Lerner, R. A. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc. Natl Acad. Sci. USA* **1991**, *88*, 10134–10137.
 - 32 Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D., Winter, G. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* **1991**, *222*, 581–597.
 - 33 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. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat. Biotechnol.* **1996**, *14*, 309–314.
 - 34 Hoogenboom, H. R. Overview of antibody phage-display technology and its applications. *Methods Mol. Biol.* **2002**, *178*, 1–37.
 - 35 Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wölle, J., Plückthun, A., Virnekäs, B. Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *J. Mol. Biol.* **2000**, *296*, 57–86.
 - 36 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. Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries. *Nat. Biotechnol.* **2000**, *18*, 852–856.
 - 37 Pini, A., Viti, F., Santucci, A., Carnemolla, B., Zardi, L., Neri, P., Neri, D. Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. *J. Biol. Chem.* **1998**, *263*, 21769–21776.
 - 38 Golemis, E. (ed.). *Protein-Protein Interactions: A Molecular Cloning Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, **2002**.
 - 39 Smith, G. P. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **1985**, *228*, 1315–1317.
 - 40 McCafferty, J., Griffiths, A. D., Winter, G., Chiswell, D. J. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **1990**, *348*, 552–554.
 - 41 Bass, S., Greene, R., Wells, J. A. Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins* **1990**, *8*, 309–314.
 - 42 Barbas, C. F., 3rd, Burton, D. R., Scott, J. K., Silvermann, G. J. *Phage Display: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, **2001**.
 - 43 Ostendorp, R., Frisch, C., Urban, M. Generation, engineering and production of human antibodies using HuCAL. In *Antibodies, Vol. 2: Novel Technologies and Therapeutic Use*, Subramanian, G. (ed.). Kluwer/Plenum, New York, **2004**.
 - 44 Boder, E. T., Wittrup, K. D. Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **1997**, *15*, 553–557.
 - 45 Feldhaus, M. J., Siegel, R. W., Opreko, L. K., Coleman, J. R., Feldhaus, J. M., Yeung, Y. A., Cochran, J. R., Heinzelman, P., Colby, D., Swers, J., Graff, C., Wiley, H. S., Wittrup, K. D.

- Flow-cytometric isolation of human antibodies from a nonimmune *Saccharomyces cerevisiae* surface display library. *Nat. Biotechnol.* **2003**, *21*, 163–170.
- 46 Weaver-Feldhaus, J.M., Lou, J., Coleman, J.R., Siegel, R.W., Marks, J.D., Feldhaus, M.J. Yeast mating for combinatorial Fab library generation and surface display. *FEBS Lett.* **2004**, *564*, 24–34.
 - 47 Hanes, J., Plückthun, A. *In vitro* selection and evolution of functional proteins by using ribosome display. *Proc. Natl Acad. Sci. USA* **1997**, *94*, 4937–4942.
 - 48 Hanes, J., Jermutus, L., Weber-Bornhauser, S., Bosshard, H.R., Plückthun, A. Ribosome display efficiently selects and evolves high-affinity antibodies *in vitro* from immune libraries. *Proc. Natl Acad. Sci. USA* **1998**, *95*, 14130–14135.
 - 49 Jermutus, L., Honegger, A., Schwesinger, F., Hanes, J., Plückthun, A. Tailoring *in vitro* evolution for protein affinity or stability. *Proc. Natl Acad. Sci. USA* **2001**, *98*, 75–80.
 - 50 Hanes, J., Schaffitzel, C., Knappik, A., Plückthun, A. Picomolar affinity antibodies from a fully synthetic naive library selected and evolved by ribosome display. *Nat. Biotechnol.* **2000**, *18*, 1287–1292.
 - 51 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. High-avidity human IgG kappa monoclonal antibodies from a novel strain of minilocus transgenic mice. *Nat. Biotechnol.* **1996**, *14*, 845–851.
 - 52 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. Functional transplant of megabase human immunoglobulin loci recapitulates human antibody response in mice. *Nat. Genet.* **1997**, *15*, 146–156.
 - 53 Tomizuka, K., Yoshida, H., Uejima, H., Kugoh, H., Sato, K., Ohguma, A., Hayasaka, M., Hanaoka, K., Oshimura, M., Ishida, I. Functional expression and germline transmission of a human chromosome fragment in chimeraic mice. *Nat. Genet.* **1997**, *16*, 133–143.
 - 54 Green, L.L., Jakobovits, A. Regulation of B cell development by variable gene complexity in mice reconstituted with human immunoglobulin yeast artificial chromosomes. *J. Exp. Med.* **1998**, *188*, 483–495.
 - 55 Nicholson, I.C., Zou, X., Popov, A.V., Cook, G.P., Corps, E.M., Humphries, S., Ayling, C., Goyenechea, B., Xian, J., Taussig, M.J., Neuberger, M.S., Brüggemann, M. Antibody repertoires of four- and five-feature translocus mice carrying human immunoglobulin heavy chain and kappa and lambda light chain yeast artificial chromosomes. *J. Immunol.* **1999**, *163*, 6898–6906.
 - 56 Gallo, M.L., Ivanov, V.E., Jakobovits, A., Davis, C.G. The human immunoglobulin loci introduced into mice: V, (D) and J gene segment usage similar to that of adult humans. *Eur. J. Immunol.* **2000**, *30*, 534–540.
 - 57 Magadan, S., Valladares, M., Suarez, E., Sanjuan, I., Molina, A., Ayling, C., Davies, S.L., Zou, X., Williams, G.T., Neuberger, M.S., Brüggemann, M., Gambon, F., Diaz-Espada, F., Gonzalez-Fernandez, A. Production of antigen-specific human monoclonal antibodies: comparison of mice carrying IgH/kappa or IgH/kappa/lambda transloci. *Biotechniques* **2002**, *33*, 680–684.
 - 58 Ishida, I., Tomizuka, K., Yoshida, H., Tahara, T., Takahashi, N., Ohguma, A., Tanaka, S., Umehashi, M., Maeda, H., Nozaki, C., Halk, E., Lonberg, N. Production of human monoclonal and polyclonal antibodies in TransChromo animals. *Cloning Stem Cells* **2002**, *4*, 91–102.
 - 59 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. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. *Gastroenterology* **2003**, *124*, 1774–1785.
 - 60 Parsons, H.L., Earnshaw, J.C., Wilton, J., Johnson, K.S., Schueler, P.A., Mahoney, W., McCafferty, J. Directing phage selections towards specific epitopes. *Protein Eng.* **1996**, *9*, 1043–1049.
 - 61 Franklin, M.C., Carey, K.D., Vajdos, F.F., Leahy, D.J., de Vos, A.M., Sliwkowski, M.X. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell* **2004**, *5*, 317–328.

- 62 Agus, D.B., Akita, R.W., Fox, W.D., Lewis, G.D., Higgins, B., Pisacane, P.I., Lofgren, J.A., Tindell, C., Evans, D.P., Maiese, K., Scher, H.I., Sliwkowski, M.X. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell* **2002**, 2, 127–137.
- 63 Badache, A., Hynes, N.E. A new therapeutic antibody masks ErbB2 to its partners. *Cancer Cell* **2004**, 5, 299–301.
- 64 Cho, H.S., Mason, K., Ramyar, K.X., Stanley, A.M., Gabelli, S.B., Denney, D.W., Jr., Leahy, D.J. Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature* **2003**, 421, 756–760.
- 65 Clynes, R.A., Towers, T.L., Presta, L.G., Ravetch, J.V. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat. Med.* **2000**, 6, 443–446.
- 66 Cobleigh, M.A., Vogel, C.L., Tripathy, D., Robert, N.J., Scholl, S., Fehrenbacher, L., Wolter, J.M., Paton, V., Shak, S., Lieberman, G., Slamon, D.J. 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.* **1999**, 17, 2639–2648.
- 67 Plosker, G.L., Figgitt, D.P. Rituximab: a review of its use in non-Hodgkin's lymphoma and chronic lymphocytic leukaemia. *Drugs* **2003**, 63, 803–843.
- 68 Burton, D.R. Antibodies, viruses and vaccines. *Nat. Rev. Immunol.* **2002**, 2, 706–713.
- 69 Pantophlet, R., Burton, D.R. Immunofocusing: antigen engineering to promote the induction of HIV-neutralizing antibodies. *Trends Mol. Med.* **2003**, 9, 468–473.
- 70 Burton, D.R., Desrosiers, R.C., Doms, R.W., Koff, W.C., Kwong, P.D., Moore, J.P., Nabel, G.J., Sodroski, J., Wilson, I.A., Wyatt, R.T. HIV vaccine design and the neutralizing antibody problem. *Nat. Immunol.* **2004**, 5, 233–236.
- 71 Urban, J.L., Schreiber, H. Tumor antigens. *Annu. Rev. Immunol.* **1992**, 10, 617–644.
- 72 Agnantis, N.J., Goussia, A.C., Stefanou, D. Tumor markers. An update approach for their prognostic significance. Part I. *In Vivo* **2003**, 17, 609–618.
- 73 Guillemard, V., Saragovi, H.U. Novel approaches for targeted cancer therapy. *Curr. Cancer Drug Targets* **2004**, 4, 313–326.
- 74 Roselli, M., Guadagni, F., Buonomo, O., Belardi, A., Ferroni, P., Diodati, A., Anselmi, D., Cipriani, C., Casciani, C.U., Greiner, J., Schlom, J. Tumor markers as targets for selective diagnostic and therapeutic procedures. *Anticancer Res.* **1996**, 16, 2187–2192.
- 75 Maynard, J.A., Maassen, C.B., Leppla, S.H., Brasky, K., Patterson, J.L., Iverson, B.L., Georgiou, G. Protection against anthrax toxin by recombinant antibody fragments correlates with antigen affinity. *Nat. Biotechnol.* **2002**, 20, 597–601.
- 76 Adams, G.P., Schier, R., McCall, A.M., Simmons, H.H., Horak, E.M., Alpaugh, R.K., Marks, J.D., Weiner, L.M. High affinity restricts the localization and tumor penetration of single-chain Fv antibody molecules. *Cancer Res.* **2001**, 61, 4750–4755.
- 77 Adams, G.P., Schier, R. Generating improved single-chain Fv molecules for tumor targeting. *J. Immunol. Methods* **1999**, 231, 249–260.
- 78 Verel, I., Heider, K.-H., Siegmund, M., Ostermann, E., Patzelt, E., Sproll, M., Snow, G.B., Adolf, G.R., van Dongen, G.A.M.S. Tumor targeting properties of monoclonal antibodies with different affinity for target antigen CD44V6 in nude mice bearing head-and-neck cancer xenografts. *Int. J. Cancer* **2002**, 99, 396–402.
- 79 Neuberger, M.S., Harris, R.S., Di Noia, J., Petersen-Mahrt, S.K. Immunity through DNA deamination. *Trends Biochem. Sci.* **2003**, 28, 305–312.
- 80 Besmer, E., Gourzi, P., Papavasiliou, F.N. The regulation of somatic hypermutation. *Curr. Opin. Immunol.* **2004**, 16, 241–245.
- 81 Sale, J.E., Bemark, M., Williams, G.T., Jolly, C.J., Ehrenstein, M.R., Rada, C., Milstein, C., Neuberger, M.S. *In vivo* and *in vitro* studies of immunoglobulin gene somatic hypermutation. *Philos. Trans. R. Soc. London B Biol. Sci.* **2001**, 356, 21–28.
- 82 Gram, H., Marconi, L.A., Barbas, C.F., 3rd, Collet, T.A., Lerner, R.A., Kang, A.S. *In vitro* selection and affinity maturation of antibodies from a naive combinatorial immunoglobulin library. *Proc. Natl Acad. Sci. USA* **1992**, 89, 3576–3580.
- 83 Hawkins, R.E., Russell, S.J., Winter, G. Selection of phage antibodies by binding affinity. Mimicking affinity maturation. *J. Mol. Biol.* **1992**, 226, 889–896.

- 84 Schier, R., Bye, J., Apell, G., McCall, A., Adams, G. P., Malmqvist, M., Weiner, L. M., Marks, J. D. Isolation of high-affinity monomeric human anti-c-erbB-2 single chain Fv using affinity-driven selection. *J. Mol. Biol.* **1996**, 255, 28–43.
- 85 Chen, Y., Wiesmann, C., Fuh, G., Li, B., Christinger, H. W., McKay, P., de Vos, A. M., Lowman, H. B. Selection and analysis of an optimized anti-VEGF antibody: crystal structure of an affinity-matured Fab in complex with antigen. *J. Mol. Biol.* **1999**, 293, 865–881.
- 86 Plückthun, A., Schaffitzel, C., Hanes, J., Jeremius, L. *In vitro* selection and evolution of proteins. *Adv. Protein Chem.* **2000**, 55, 367–403.
- 87 Zahnd, C., Spinelli, S., Luginbühl, B., Amstutz, P., Cambillau, C., Plückthun, A. Directed *in vitro* evolution and crystallographic analysis of a peptide-binding single chain antibody fragment (scFv) with low picomolar affinity. *J. Biol. Chem.* **2004**, 279, 18870–18877.
- 88 Roskos, L. K., Davis, C. G., Schwab, G. M. The clinical pharmacology of therapeutic monoclonal antibodies. *Drug Dev. Res.* **2004**, 61, 108–120.
- 89 Junghans, R. P. Finally! The Brambell receptor (FcRB). Mediator of transmission of immunity and protection from catabolism for IgG. *Immunol. Res.* **1997**, 16, 29–57.
- 90 Telleman, P., Junghans, R. P. The role of the Brambell receptor (FcRB) in liver: protection of endocytosed immunoglobulin G (IgG) from catabolism in hepatocytes rather than transport of IgG to bile. *Immunology* **2000**, 100, 245–251.
- 91 Trang, J. M. Pharmacokinetics and metabolism of therapeutic antibodies. In *Protein Pharmacokinetics and Metabolism*, Ferraiolo, B. L., Mohler, M. A., Gloff, C. A. (eds). Plenum, New York, **1992**.
- 92 Waldmann, T. A., Strober, W. Metabolism of immunoglobulins. *Prog. Allergy* **1969**, 13, 1–110.
- 93 Schror, K., Weber, A. A. Comparative pharmacology of GP IIb/IIIa antagonists. *J. Thromb. Thrombolysis* **2003**, 15, 71–80.
- 94 Tam, S. H., Sassoli, P. M., Jordan, R. E., Nakada, M. T. Abciximab (ReoPro, chimeric 7E3 Fab) demonstrates equivalent affinity and functional blockade of glycoprotein IIb/IIIa and $\alpha_v\beta_3$ integrins. *Circulation* **1998**, 98, 1085–1091.
- 95 Goldenberg, D. M. Advancing role of radiolabeled antibodies in the therapy of cancer. *Cancer Immunol. Immunother.* **2003**, 52, 281–296.
- 96 Wilder, R. B., DeNardo, G. L., DeNardo, S. J. Radioimmunotherapy: recent results and future directions. *J. Clin. Oncol.* **1996**, 14, 1383–1400.
- 97 Bischof Delaloye, A. The role of nuclear medicine in the treatment of non-Hodgkin's lymphoma (NHL). *Leuk. Lymphoma* **2003**, 44 (Suppl. 4), S29–S36.
- 98 Wiseman, G. A., Kornmehl, E., Leigh, B., Erwin, W. D., Podoloff, D. A., Spies, S., Sparks, R. B., Stabin, M. G., Witzig, T., White, C. A. Radiation dosimetry results and safety correlations from ^{90}Y -ibritumomab tiuxetan radioimmunotherapy for relapsed or refractory non-Hodgkin's lymphoma: combined data from 4 clinical trials. *J. Nucl. Med.* **2003**, 44, 465–474.
- 99 Kaminski, M. S., Estes, J., Zasadny, K. R., Francis, I. R., Ross, C. W., Tuck, M., Regan, D., Fisher, S., Gutierrez, J., Kroll, S., Stagg, R., Tidmarsh, G., and Wahl, R. L. Radioimmunotherapy with iodine ^{131}I tositumomab for relapsed or refractory B-cell non-Hodgkin lymphoma: updated results and long-term follow-up of the University of Michigan experience. *Blood* **2000**, 96, 1259–1266.
- 100 Scheidhauer, K., Wolf, I., Baumgartl, H. J., Von Schilling, C., Schmidt, B., Reidel, G., Peschel, C., Schwaiger, M. Biodistribution and kinetics of ^{131}I -labelled anti-CD20 MAB IDEC-C2B8 (rituximab) in relapsed non-Hodgkin's lymphoma. *Eur. J. Nucl. Med. Mol. Imaging* **2002**, 29, 1276–1282.
- 101 Maack, T. Renal handling of proteins and polypeptides. In *Handbook of Physiology, Section 8: Renal Physiology*, Windhager, E. E. (ed.). APS, Washington, DC, **1992**.
- 102 Renkin, E. M., Gilmore, J. P. Glomerular filtration. In *Handbook of Physiology: Renal Physiology*, Orloff, J., Berliner, R. W. (eds.). APS, Washington, DC, **1973**.
- 103 Plückthun, A., Pack, P. New protein engineering approaches to multivalent and bi-specific antibody fragments. *Immunotechnol.* **1997**, 3, 83–105.
- 104 Carter, P. Improving the efficacy of antibody-based cancer therapies. *Nat. Rev. Cancer* **2001**, 1, 118–129.

- 105 Glennie, M. J., Johnson, P. W. Clinical trials of antibody therapy. *Immunol. Today* **2000**, 21, 403–410.
- 106 Zondor, S. D., Medina, P. J. Bevacizumab: an angiogenesis inhibitor with efficacy in colorectal and other malignancies. *New Drug Dev.* **2004**, 38, 1258–1264.
- 107 Kim, K. J., Li, B. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* **1993**, 362, 841–844.
- 108 Cartron, G., Watier, H., Golay, J., Solal-Celigny, P. From the bench to the bedside: ways to improve rituximab efficacy. *Blood* **2004**, 104, 2635–2642.
- 109 Deans, J. P., Li, H., Polyak, M. J. CD20-mediated apoptosis: signalling through lipid rafts. *Immunology* **2002**, 107, 176–182.
- 110 Maloney, D. G., Smith, B., Rose, A. Rituximab: mechanism of action and resistance. *Semin. Oncol.* **2002**, 29, 2–9.
- 111 Nagy, Z. A., Mooney, N. A. A novel, alternative pathway of apoptosis triggered through class II major histocompatibility complex molecules. *J. Mol. Med.* **2003**, 81, 757–765.
- 112 Nagy, Z. A., Hubner, B., Löhning, C., Rauchenberger, R., Reiffert, S., Thomassen-Wolf, E., Zahn, S., Leyer, S., Schier, E. M., Zahradnik, A., Brunner, C., Lobenstein, K., Rattel, B., Stanglmaier, M., Hallek, M., Wing, M., Anderson, S., Dunn, M., Kretschmar, T., Tesar, M. Fully human, HLA-DR-specific monoclonal antibodies efficiently induce programmed death of malignant lymphoid cells. *Nat. Med.* **2002**, 8, 801–807.
- 113 Cook-Bruns, N. Retrospective analysis of the safety of Herceptin immunotherapy in metastatic breast cancer. *Oncology* **2001**, 61 (Suppl. 2), 58–66.
- 114 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. 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.* **2001**, 276, 6591–6604.
- 115 Presta, L. G. Engineering antibodies for therapy. *Curr. Pharm. Biotechnol.* **2002**, 3, 237–256.
- 116 Maxwell, K. F., Powell, M. S., Hulett, M. D., Barton, P. A., McKenzie, I. F., Garrett, T. P., Hogarth, P. M. Crystal structure of the human leukocyte Fc receptor, FcγRIIa. *Nat. Struct. Biol.* **1999**, 6, 437–442.
- 117 Radaev, S., Motyka, S., Fridman, W. H., Sautes-Fridman, C., Sun, P. D. The structure of a human type III Fcγ receptor in complex with Fc. *J. Biol. Chem.* **2001**, 276, 16469–16477.
- 118 Sonderrmann, P., Huber, R., Oosthuizen, V., Jacob, U. The 3.2-Å crystal structure of the human IgG1 Fc fragment–FcγRIII complex. *Nature* **2000**, 406, 267–273.
- 119 Sonderrmann, P., Kaiser, J., Jacob, U. Molecular basis for immune complex recognition: a comparison of Fc-receptor structures. *J. Mol. Biol.* **2001**, 309, 737–749.
- 120 Zhang, Y., Boesen, C. C., Radaev, S., Brooks, A. G., Fridman, W. H., Sautes-Fridman, C., Sun, P. D. Crystal structure of the extracellular domain of a human FcγRIII. *Immunity* **2000**, 13, 387–395.
- 121 Jefferis, R., Lund, J., Pound, J. D. IgG-Fc-mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation. *Immunol. Rev.* **1998**, 163, 59–76.
- 122 Krapp, S., Mimura, Y., Jefferis, R., Huber, R., Sonderrmann, P. Structural analysis of human IgG-Fc glycoforms reveals a correlation between glycosylation and structural integrity. *J. Mol. Biol.* **2003**, 325, 979–989.
- 123 Umana, P., Jean-Mairet, J., Moudry, R., Amstutz, H., Bailey, J. E. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat. Biotechnol.* **1999**, 17, 176–180.
- 124 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. 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.* **2003**, 278, 3466–3473.
- 125 Presta, L. G. Antibody engineering for therapeutics. *Curr. Opin. Struct. Biol.* **2003**, 13, 519–525.
- 126 Shields, R. L., Lai, J., Keck, R., O'Connell, L. Y., Hong, K., Meng, Y. G., Weikert, S. H., Presta, L. G. Lack of fucose on human IgG1 N-linked oligosaccharide improves binding to human FcγRIII and antibody-dependent

- cellular toxicity. *J. Biol. Chem.* **2002**, 277, 26733–26740.
- 127 Okazaki, A., Shoji-Hosaka, E., Nakamura, K., Wakitani, M., Uchida, K., Kakita, S., Tsutomoto, K., Kumagai, I., Shitara, K. Fucose depletion from human IgG1 oligosaccharide enhances binding enthalpy and association rate between IgG1 and FcγRIIIa. *J. Mol. Biol.* **2004**, 336, 1239–1249.
 - 128 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. Increasing the affinity of a human IgG1, for the neonatal Fc receptor: biological consequences. *J. Immunol.* **2002**, 169, 5171–5180.
 - 129 Hinton, P. R., Johlfs, M. G., Xiong, J. M., Hainestad, K., Ong, K. C., Bullock, C., Keller, S., Tang, M. T., Tso, J. Y., Vasquez, M., Tsurushita, N. Engineered human IgG antibodies with longer serum half-lives in primates. *J. Biol. Chem.* **2004**, 279, 6213–6216.
 - 130 Trail, P. A., King, H. D., Dubowchik, G. M. Monoclonal antibody drug immunoconjugates for targeted treatment of cancer. *Cancer Immunol. Immunother.* **2003**, 52, 328–337.
 - 131 Voutsadakis, I. A. Gemtuzumab ozogamicin (CMA-676, Mylotarg) for the treatment of CD33⁺ acute myeloid leukemia. *Anticancer Drugs* **2002**, 13, 685–692.
 - 132 Paul, S. P., Taylor, L. S., Stansbury, E. K., McVicar, D. W. Myeloid specific human CD33 is an inhibitory receptor with differential ITIM function in recruiting the phosphatases SHP-1 and SHP-2. *Blood* **2000**, 96, 483–490.
 - 133 Hellström, I., Garrigues, H. J., Garrigues, U., Hellström, K. E. Highly tumor-reactive, internalizing, mouse monoclonal antibodies to Le_y-related cell surface antigens. *Cancer Res.* **1990**, 50, 2183–2190.
 - 134 Saleh, M. N., Sugarman, S., Murray, J., Ostroff, J. B., Healey, D., Jones, D., Daniel, C. R., LeBherz, D., Brewer, H., Onetto, N., LoBuglio, A. F. Phase I trial of the anti-Lewis Y drug immunoconjugate BR96–doxorubicin in patients with lewis Y-expressing epithelial tumors. *J. Clin. Oncol.* **2000**, 18, 2282–2292.
 - 135 Ajani, J. A., Kelsen, D. P., Haller, D., Hargraves, K., Healey, D. A multi-institutional phase II study of BMS-182248-01 (BR96–doxorubicin conjugate) administered every 21 days in patients with advanced gastric adenocarcinoma. *Cancer J.* **2000**, 6, 78–81.
 - 136 Tolcher, A. W., Sugarman, S., Gelmon, K. A., Cohen, R., Saleh, M., Isaacs, C., Young, L., Healey, D., Onetto, N., Slichenmyer, W. Randomized phase II study of BR96–doxorubicin conjugate in patients with metastatic breast cancer. *J. Clin. Oncol.* **1999**, 17, 478–484.
 - 137 Wahl, A. F., Donaldson, K. L., Mixan, B. J., Trail P. A., Siegall, C. B. Selective tumor sensitization to taxanes with the mAb–drug conjugate cBR96–doxorubicin. *Int. J. Cancer* **2001**, 93, 590–600.
 - 138 Doronina, S. O., Toki, B. E., Torgov, M. Y., Mendelsohn, B. A., Cervený, C. G., Chace, D. F., DeBlanc, R. L., Gearing, R. P., Bovee, T. D., Siegall, C. B., Francisco, J. A., Wahl, A. F., Meyer, D. L., Senter, P. D. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat. Biotechnol.* **2003**, 21, 778–784.
 - 139 Prinz, H. Recent advances in the field of tubulin polymerization inhibitors. *Expert Rev. Anticancer Ther.* **2002**, 2, 695–708.
 - 140 Falnes, P. O., Sandvig, K. Penetration of protein toxins into cells. *Curr. Opin. Cell Biol.* **2000**, 12, 407–413.
 - 141 Perentesis, J. P., Miller, S. P., Bodley, J. W. Protein toxin inhibitors of protein synthesis. *Biofactors* **1992**, 3, 173–184.
 - 142 Stirpe, F. Ribosome-inactivating proteins. *Toxicol.* **2004**, 44, 371–383.
 - 143 Pai-Scherf, L. H., Villa, J., Pearson, D., Watson, T., Liu, E., Willingham, M. C., Pastan, I. Hepatotoxicity in cancer patients receiving erb-38, a recombinant immunotoxin that targets the erbB2 receptor. *Clin. Cancer Res.* **1999**, 5, 2311–2315.
 - 144 Pastan, I. Immunotoxins containing Pseudomonas exotoxin A: a short history. *Cancer Immunol. Immunother.* **2003**, 52, 338–341.
 - 145 Poe, J. C., Fujimoto, Y., Hasegawa, M., Haas, K. M., Miller, A. S., Sanford, I. G., Bock, C. B., Fujimoto, M., Tedder, T. F. CD22 regulates B lymphocyte function *in vivo* through both ligand-dependent and ligand-independent mechanisms. *Nat. Immunol.* **2004**, 5, 1078–1087.
 - 146 Schnell, R., Borchmann, P., Staak, J. O., Schindler, J., Ghetie, V., Vitetta, E. S., Engert, A. Clinical evaluation of ricin A-chain immunotoxins in patients with Hodgkin's lymphoma. *Ann. Oncol.* **2003**, 14, 729–736.
 - 147 Helguera, G., Morrison, S. L., Penichet, M. L. Antibody–cytokine fusion proteins: harness-

- ing the combined power of cytokines and antibodies for cancer therapy. *Clin. Immunol.* **2002**, *105*, 233–246.
- 148 Gillies, S.D., Lan, Y., Brunkhorst, B., Wong, W.K., Li, Y., Lo, K.M. Bi-functional cytokine fusion proteins for gene therapy and antibody-targeted treatment of cancer. *Cancer Immunol. Immunother.* **2002**, *51*, 449–460.
 - 149 Borchmann, P., Schnell, R., Fuss, I., Manzke, O., Davis, T., Lewis, L.D., Behnke, D., Wickenhauser, C., Schiller, P., Diehl, V., Engert, A. Phase 1 trial of the novel bispecific molecule H22×Ki-4 in patients with refractory Hodgkin lymphoma. *Blood* **2002**, *100*, 3101–3107.
 - 150 Kufer, P., Lutterbuse, R., Baeuerle, P.A. A revival of bispecific antibodies. *Trends Biotechnol.* **2004**, *22*, 238–244.
 - 151 Hartmann, F., Renner, C., Jung, W., da Costa, L., Tembrink, S., Held, G., Sek, A., König, J., Bauer, S., Kloft, M., Pfreundschuh, M. Anti-CD16/CD30 bispecific antibody treatment for Hodgkin's disease: role of infusion schedule and costimulation with cytokines. *Clin. Cancer Res.* **2001**, *7*, 1873–1881.
 - 152 James, N.D., Atherton, P.J., Jones, J., Howie, A.J., Tchekmedyan, S., Curnow, R.T. A phase II study of the bispecific antibody MDX-H210 (anti-HER2×CD64) with GM-CSF in HER2⁺ advanced prostate cancer. *Br. J. Cancer* **2001**, *85*, 152–156.
 - 153 Pollack, P., Groothuis, J.R. Development and use of palivizumab (Synagis): a passive immunoprophylactic agent for RSV. *J. Infection Chemother.* **2002**, *8*, 201–206.
 - 154 Visintin, M., Tse, E., Axelsson, H., Rabbitts, T.H., Cattaneo, A. Selection of antibodies for intracellular function using a two-hybrid *in vivo* system. *Proc. Natl Acad. Sci. USA* **1999**, *96*, 11723–11728.
 - 155 Mössner, E., Koch, H., Plückthun, A. Fast selection of antibodies without antigen purification: adaption of the protein fragment complementation assay to select antigen–antibody pairs. *J. Mol. Biol.* **2001**, *308*, 115–122.