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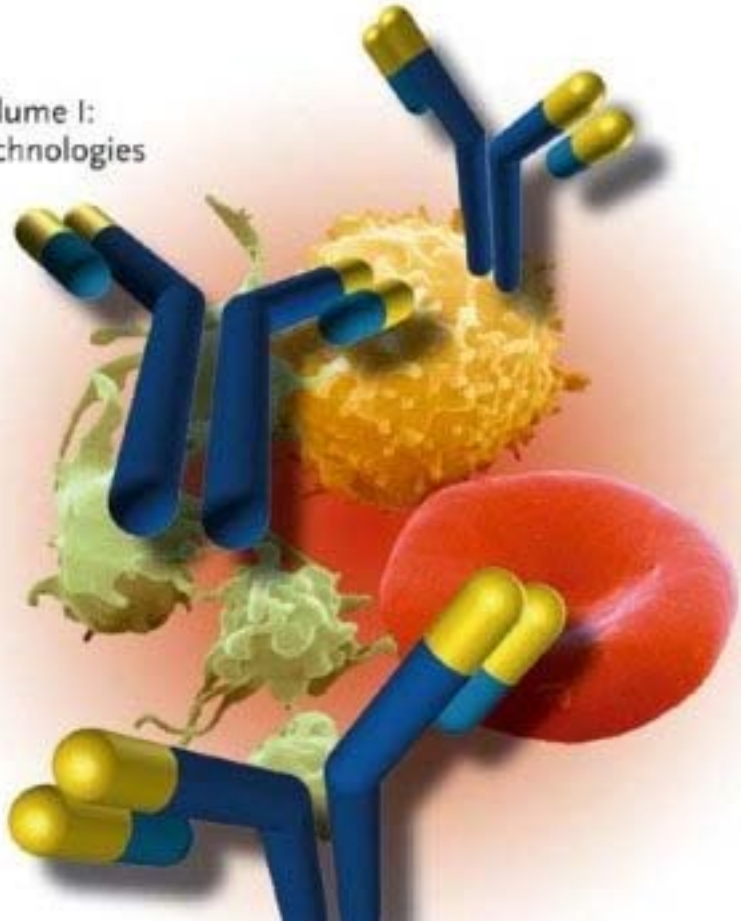
Handbook of Therapeutic Antibodies

Volume I:
Technologies



Handbook of Therapeutic Antibodies

Edited by
Stefan Dübel

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Handbook of Therapeutic Antibodies

Volume I

Edited by
Stefan Dübel

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Dedication

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

Stefan Dübel

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A Greeting by the Editor

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

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

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

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

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

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

Braunschweig, December 2006 Stefan Dübel

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Foreword

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

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

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

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

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

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

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

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Introduction

1
Therapeutic Antibodies – From Past to Future

Stefan Dübel

1.1
An Exciting Start – and a Long Trek

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

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

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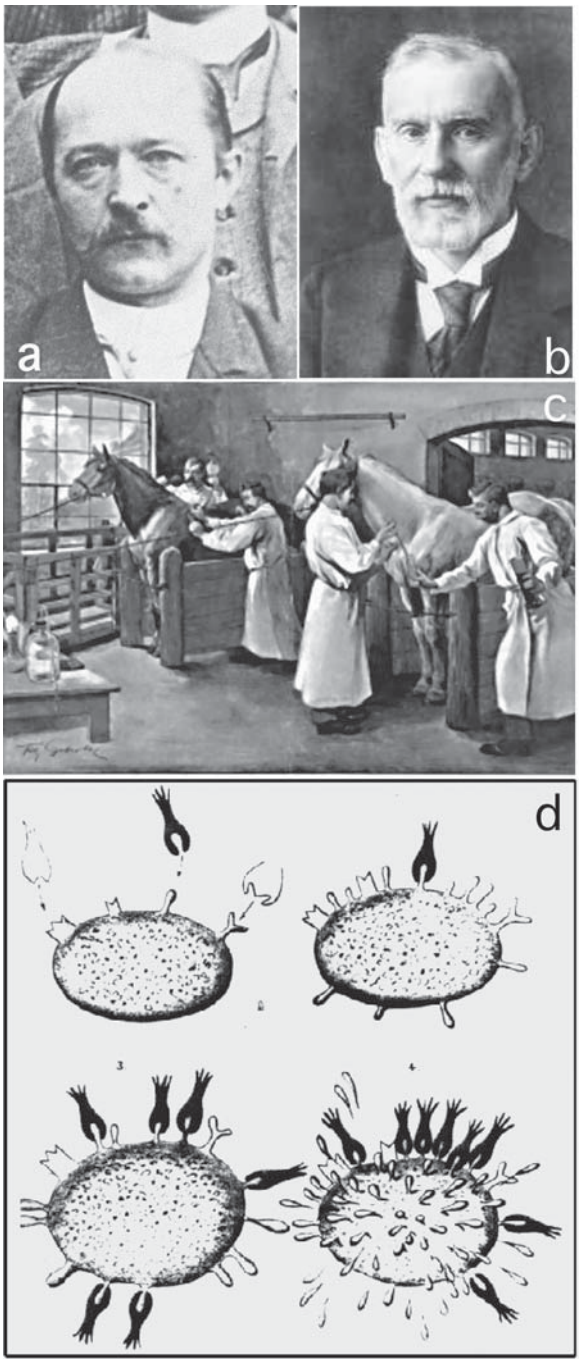

Fig. 1.1 We have come a long way since the first methods for the generation of antibody based therapeutics were established (c), pioneered by (a) Emil von Behring and (b) Paul Ehrlich in the last decade of the nineteenth century. (d) Drawing from Paul Ehrlich on the lateral chain theory (lateral chains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

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

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

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

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

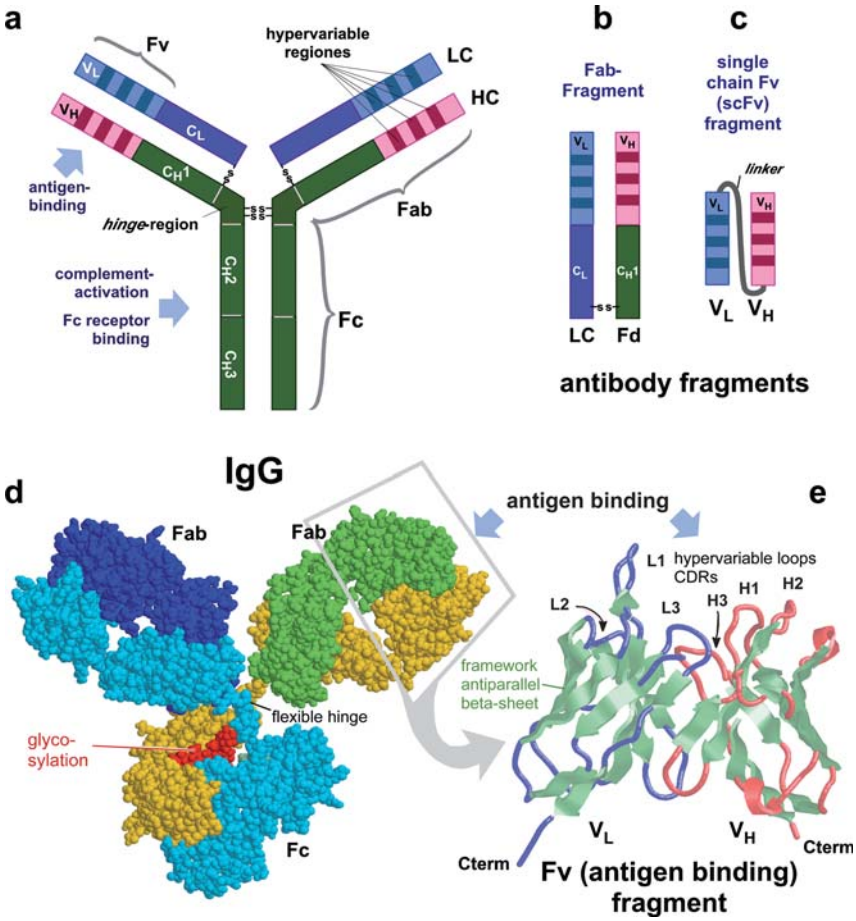

Fig. 1.2 Introduction to antibody structure. (a, d) IgG is a heterotetrameric protein assembled from two identical heavy and light chains (HC, LC), assembled by disulfide bonds. (b) Fab fragments contain the antigen-binding region, and can be generated by proteolysis or recombinant production. (c) In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

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

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

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

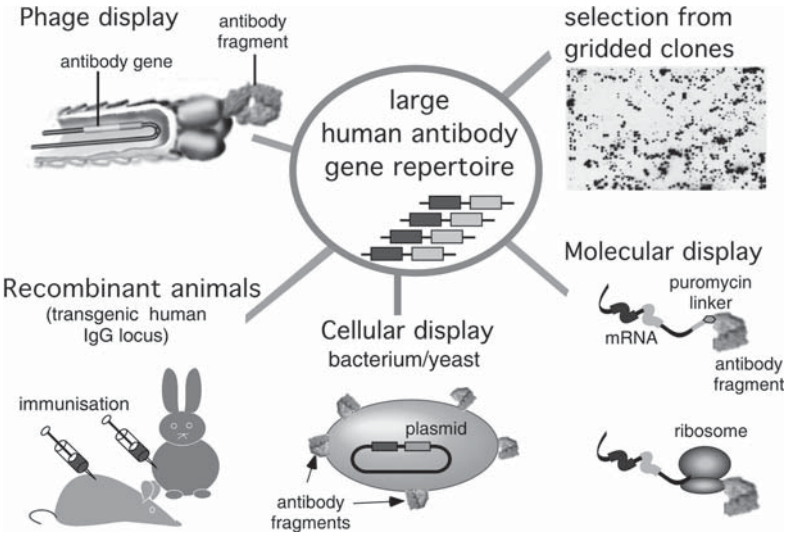

Fig. 1.3 Systems employed for the generation of human antibodies. They all include a heterologous expression of a repertoire of human immunoglobulin genes. Selection is achieved either *in vivo* by immunization (in case of the recombinant animals) or *in vitro* by binding to the antigen, allowing clonal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

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

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

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

1.2 The Gold Rush

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

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

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

1.3 Success and Disappointment

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

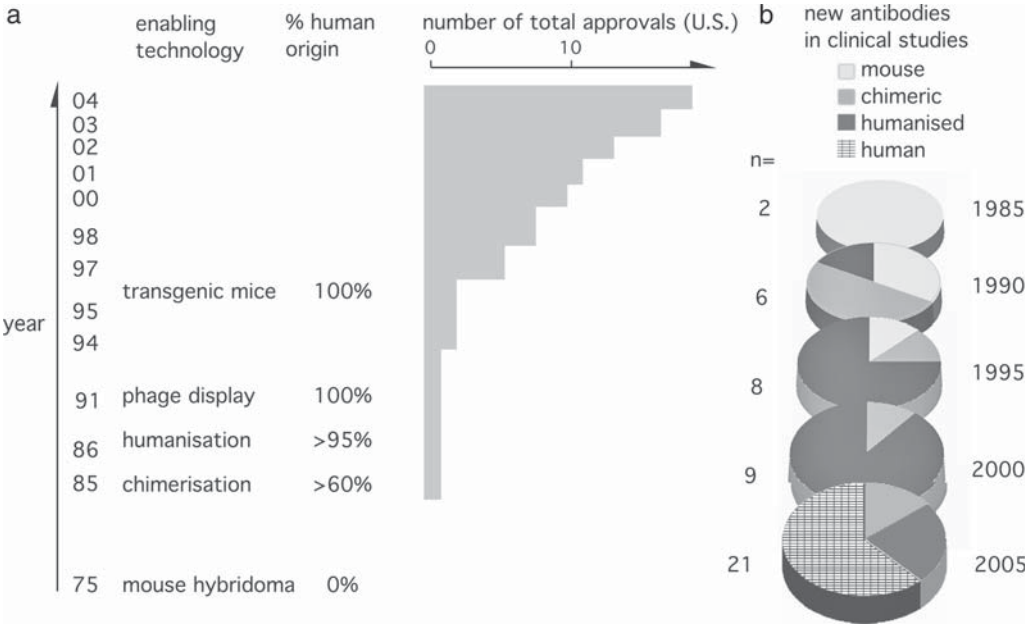

Fig. 1.4 Development history of therapeutic antibodies and the technologies for their generation. Data assembled from Reichert (2001), Reichert et al. (2005), Fabrizi (2005) and public domain sources of the companies.

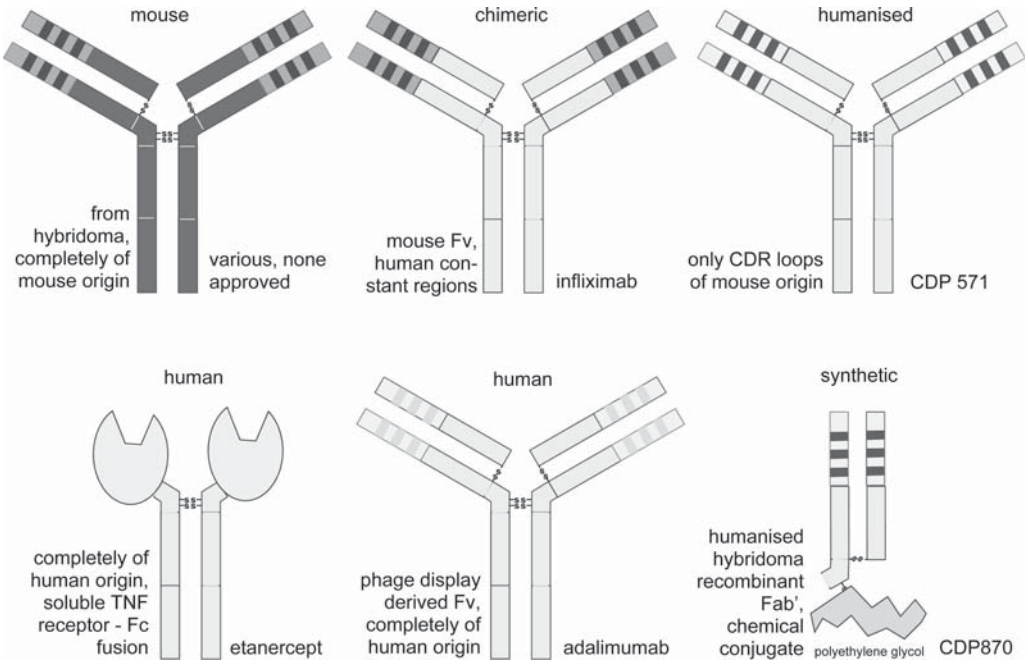

Fig. 1.5 The development of recombinant antibody variant TNF antagonists. Inspired by Rutgeerts et al. (2004).

Table 1.1 Clinical studies with therapeutic antibodies 2005.

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

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

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

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

Table 1.2

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

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

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

* Trade names are Copyright of distributing companies.

** please see addendum for nomenclature.

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

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

1.4 The Gleaming Horizon

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

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

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

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Part I
Selecting and Shaping the Antibody Molecule

2
Selection Strategies I: Monoclonal Antibodies

Gerhard Moldenhauer

2.1
Introduction

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

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

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

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

2.2 Historical Remarks

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

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

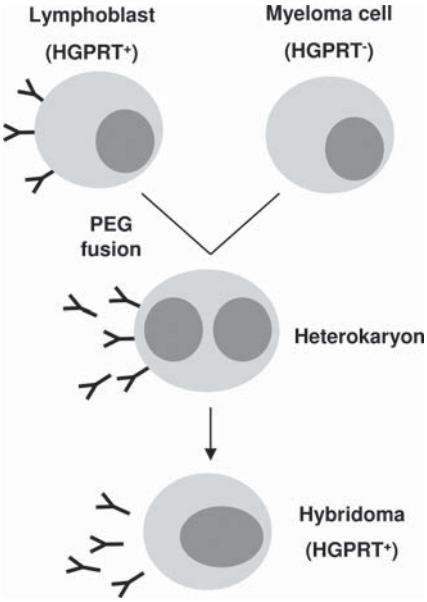

Fig. 2.1 Principle of hybridoma production by cell fusion. Both parents confer their key functions to the hybrid: production of an individual antibody (lymphoblast) and indefinite growth (myeloma cell). Introduction of a selectable marker (HGPRT) allows only hybridoma cells to proliferate.

2.3 Antibody Structure and Function

2.3.1 Membrane-bound and Secreted Forms of Antibodies

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

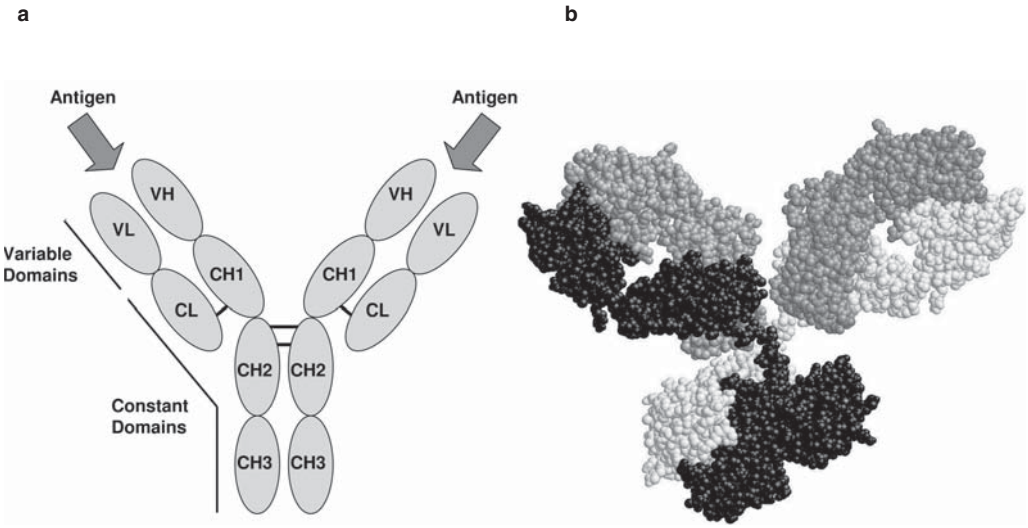

Fig. 2.2 Structure of an IgG molecule. The Y-shaped simplified representation in (a) shows the functional domains as well as the two antigen-binding sites. A more realistic space-fill model is depicted in (b) (courtesy of Dr C.W. von der Lieth, DKFZ Heidelberg).

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

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

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

2.3.2
Monoclonal Antibodies

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

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

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

2.4 Production of Monoclonal Antibodies

2.4.1 Immunization

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

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

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

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

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

2.4.2 Myeloma Cell Lines

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

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

2.4.3 Cell Fusion

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

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

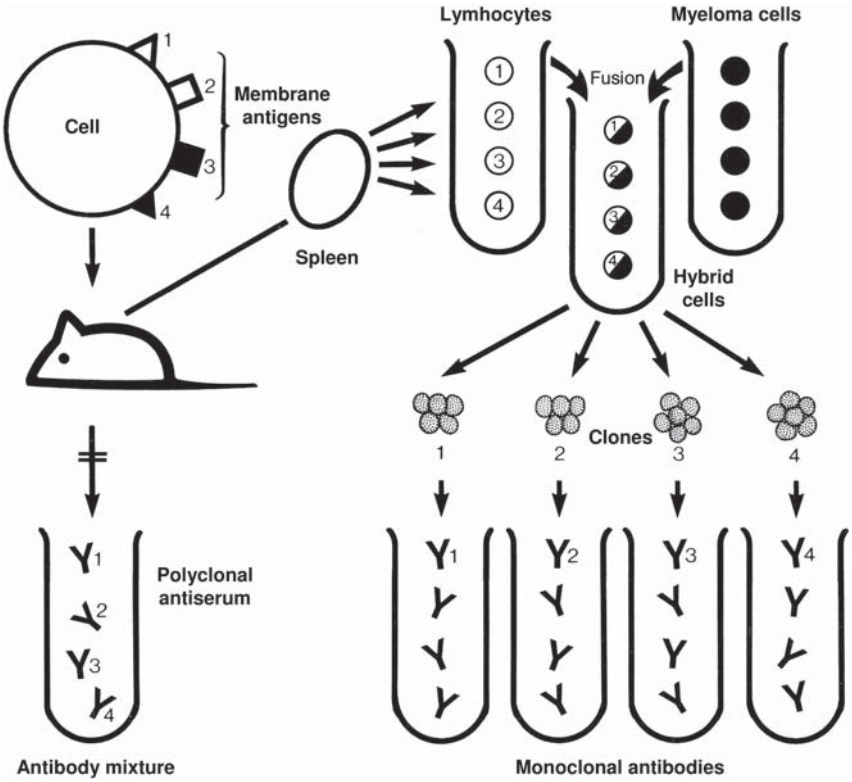

Fig. 2.3 Generation of monoclonal antibodies. Spleen cells from an immunized mouse are fused with myeloma cells to obtain hybridomas. After reduction to individual cell clones they secrete monoclonal antibodies of defined specificity.

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

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

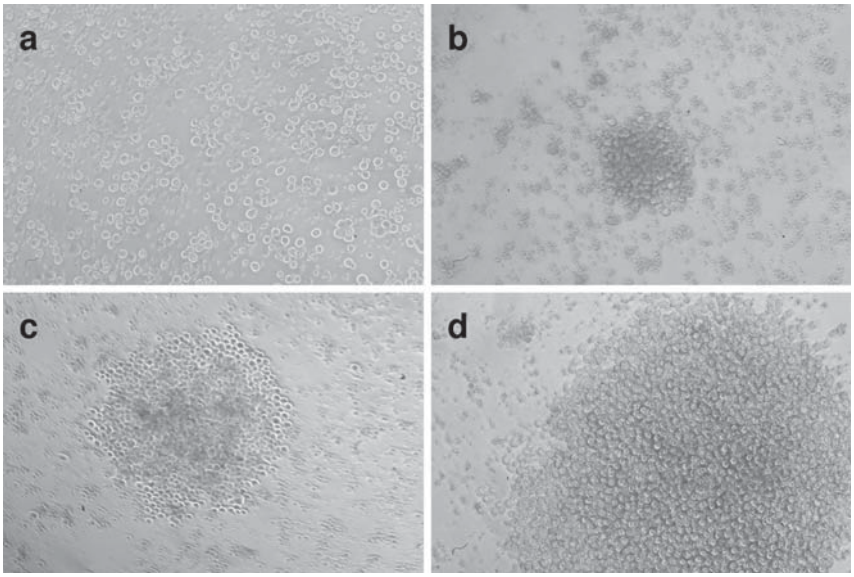

Fig. 2.4 Time course of hybridoma development visualized by phase-contrast light microscopy. Fusion mixture is plated on day 0 (a) and early hybridomas became visible after 4–6 days (b). At day 6–10 hybridomas grow vigorously (c) and are ready for screening at day 10–14 (d).

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

2.4.4 Drug Selection of Hybridomas

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

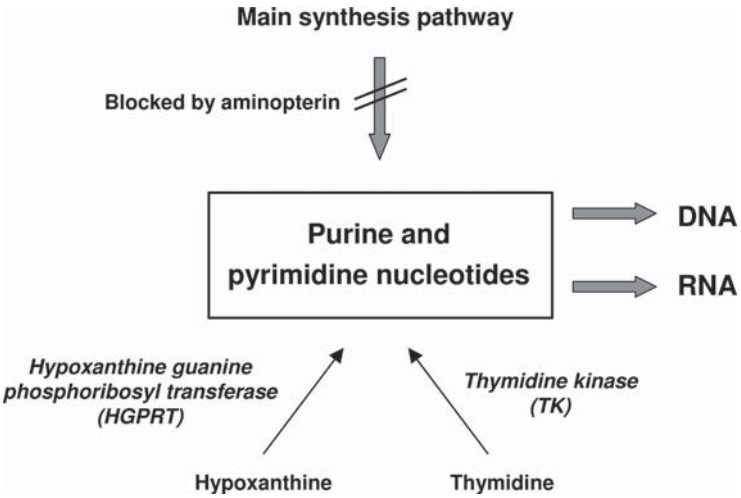

Fig. 2.5 Chemical selection of hybridomas. While the main pathway for synthesis of purine and pyrimidine nucleotides is blocked by the folic acid antagonist aminopterin, cells are forced to use the salvage pathway that requires the enzymes hypoxanthine guanine phosphoribosyl transferase (HGPRT) and thymidine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

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

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

2.4.5
Screening Hybridoma Cultures for Specific Antibody

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

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

2.4.5.1 **Enzyme-linked Immunosorbent Assay (ELISA)**

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

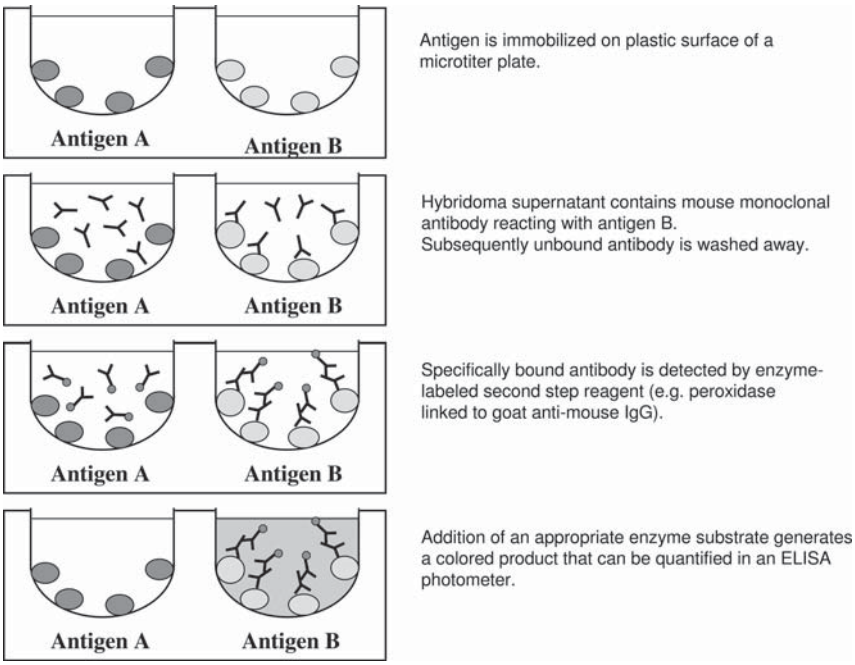

Fig. 2.6 Solid-phase ELISA for antibody screening.

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

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

2.4.5.2 Flow Cytometry

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

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

2.4.5.3 Immunohistology and Immunocytology

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

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

2.4.5.4 **Cytotoxicity Assays**

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

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

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

2.4.5.5 **Screening for Function**

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

2.4.6
Cloning

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

2.4.7
Expansion and Freezing of Hybridoma Clones

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

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

2.5 Purification and Modification of Monoclonal Antibodies

2.5.1 Mass Culture and Purification of Monoclonal Antibody

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

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

2.5.2 Fragmentation of Monoclonal IgG Antibodies

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

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

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

2.5.3
Labeling of Monoclonal Antibodies

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

2.6
Monoclonal Antibodies for Tumor Therapy

2.6.1
Leukocyte Differentiation Antigens

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

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

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

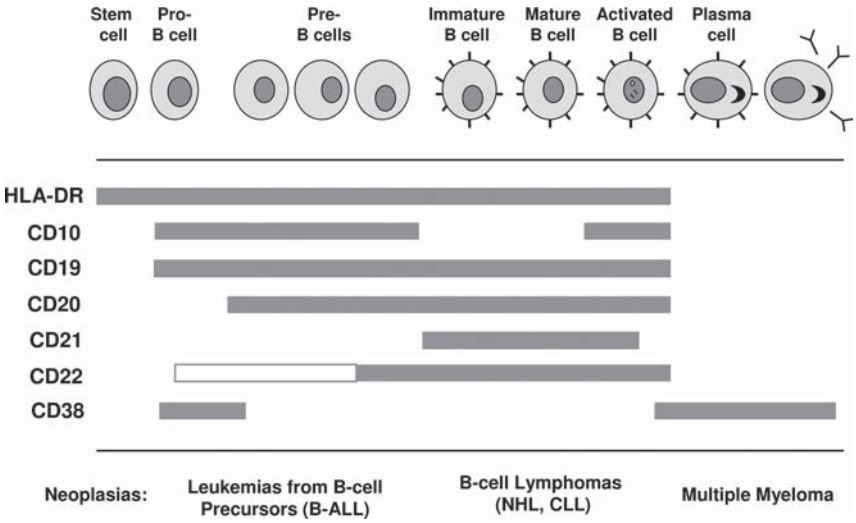

Fig. 2.7 Differentiation antigens of human B lymphocyte. Cluster of differentiation antigens (CD antigens) are expressed on certain maturation stages during B-cell ontogeny (upper panel). Monoclonal antibodies to CD antigens are also reactive with tumor-derived samples of corresponding counterparts (bottom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

Table 2.1 Target antigens on lymphocytes for monoclonal antibody therapy.

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

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

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

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

2.6.2
Epithelial Differentiation Antigens

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

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

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

2.6.3
Mechanisms of Action of Monoclonal Antibodies

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

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

Table 2.2 Therapeutic effects of monoclonal antibodies.

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

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

2.6.4
Human Monoclonal Antibodies

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

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

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

2.7 Outlook

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

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