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Development of Antibody-Based Therapeutics

Translational Considerations

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This book is dedicated to all patients who are in need of improved therapies and who inspire us to develop better drugs

Preface

Monoclonal antibodies have become a key therapeutic modality for a broad range of diseases. The therapeutic potential of monoclonal antibodies is derived from their exquisite specificity and high affinity binding to their antigen target. The therapeutic utility of monoclonal antibodies was quickly realized after the development of hybridoma technology by Kohler and Milstein in 1975.

The first generation of therapeutic antibodies was of murine origin. These antibodies were of limited therapeutic value because patients who received these agents developed an immune response to the mouse protein, referred to as human anti-mouse antibody (HAMA) response. HAMA responses negatively impacted the efficacy of antibodies of murine origin; this limitation fostered the development of new antibody technologies to reduce the immunogenicity of murine antibodies by making them more human-like. These technologies, employing recombinant DNA methods, led to the development of chimeric antibodies; chimeric antbodies maintain the murine variable region linked to human constant regions and retain approximately 35% of murine protein sequences. Additional improvements in recombinant DNA technology led to the development of humanized antibodies, which retain about 5–10% of murine protein sequences.

With further advances in antibody technology, two major platforms are now employed to generate fully human monoclonal antibodies. One platform relies on display technologies, namely phage, ribosomes, or yeast that display human antibody variable regions. The second major platform relies on transgenic mice that have been genetically engineered to produce human antibodies.

A direct consequence of the above-described technological advances has been a significant investment on the part of the biotechnology and pharmaceutical industry to develop antibodies and an exponential growth in the therapeutic market for these agents. Moreover, several therapeutic monoclonal antibodies have attained blockbuster status with sales exceeding the billion-dollar mark and beyond.

Despite the exponential growth in the therapeutic market of monoclonal antibodies, it is also important to note that there still remains a considerable unmet medical need in the three main areas of study for investigational human

monoclonal antibodies: cancer, immunological, and infectious diseases. It is expected that therapeutic monoclonal antibodies will provide valuable new treatment options for these diseases.

The main objective of this volume is to provide a comprehensive overview of the translational considerations for developing antibody-based therapeutics from discovery to the clinic. The initiating event that ultimately led to the publication of this endeavor originated from a perennial annual short course at the Protein Engineering Summit (PEGS) that we introduced in 2008 and still teach currently. From our experiences with this course, we realized that many scientists, both in the academic and biotechnology/pharmaceutical community, do not possess in-depth knowledge of all aspects of antibody drug discovery and development; we therefore concluded a more formal and thorough discussion was warranted.

The topics covered have been carefully selected. Each chapter focuses on a specific aspect of translational strategies during the development of antibody-based therapeutics. Although some topics may not appear to be directly concerned with translational considerations or are technical in nature, addressing the ancillary aspects of antibody drug discovery and development should provide the reader with a broader understanding of the strategies involved in the drug development process of these agents. We envision that someone who has little if any current knowledge about therapeutic antibodies will be able to read this book and glean substantial insights from leading scientists across a broad range of expertise.

We are indebted to our many colleagues for their contributions to this endeavor.

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Chapter 1
Translational Strategies for Development of Antibody-Based Therapeutics: An Overview

Mohammad Tabrizi, Gadi Gazit Bornstein and Scott L. Klakamp

Abstract With scientific advances, it is possible to rapidly and effectively generate highly tailored and specific antibody-based therapeutics that interact with a diverse array of soluble or cell-associated target antigens. Much like traditional small molecule drugs, a major challenge during the development of antibody-based therapeutics is maintaining an effective information flow and translation of accumulated knowledge throughout the various development phases. The design of effective translational strategies from the early stages of the development process for antibody-based therapeutics is not only necessary to lessen the development time and cost, but also to foster implementation of rational decision making processes throughout various development phases. In this book, we have attempted to provide a comprehensive discussion of various topics critical for establishing successful translational strategies for the development of antibody-based therapeutics.

"Nothing before had ever made me thoroughly realize, though I had read various scientific books, that science consists in grouping facts so that general laws or conclusions may be drawn from them"

Charles Darwin
The greatest translational scientist of all time.

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The biologics market continues to witness an impressive rate of growth, and the antibody-based therapeutic market in particular has contributed remarkably to the expansion of this segment within the pharmaceutical industry. In recent years, the pharmaceutical industry has borne witness to major challenges, including sluggish prescription trends, intensifying generic competition, and a limited late-stage pipeline. The next few years are expected to reflect a significant imbalance between new product introductions and patent losses of major blockbuster drugs. As a result of these changes, antibody-based therapeutics have entered the center stage of drug discovery for many pharmaceutical companies, and have provided a major shift from small molecules to a broader portfolio containing both protein and chemical therapeutic agents. The robust late-stage biologics pipeline within the biotech sector has drawn an increasing amount of interest from the large pharmaceutical industry. The biotech industry has increasingly become the engine for innovation and as a consequence, has been the target of recent acquisitions by global pharmaceutical companies to restock their product pipelines.

Advancements in science and the confluence of technologies has made it possible to rapidly and effectively generate highly tailored antibody-based therapeutics against a diverse array of targets, hence a greater diversity in marketed antibodies is anticipated. Whereas to date all of the monoclonal antibodies are full length "native" molecules, alternative structures and formats are seen to potentially offer advantages in certain settings and disease indications. The evolution of therapeutic antibodies has encompassed multiple engineering efforts in the hope of improving the efficacy, safety, and duration of effect for antibody-based drugs. Advances in protein engineering technologies have afforded investigators the possibility to overcome problems associated with introducing foreign antibodies into humans. These efforts have included antibody chimerization, humanization, and the more recent development of fully human antibodies, all of which have reduced anti-drug immune responses. Recent efforts have also focused on engineering antibody variable regions that encode multiple specificities into a single molecular entity. As simultaneous binding of several targets might yield better therapeutic efficacy than binding to a single target, engineering of antibody-based therapeutics to bind two or more unique targets within a single molecular entity has been undertaken, but this approach has faced new challenges. Many advances in protein engineering have also resulted in the capability to modulate an antibody's ability to interact with cells and serum components of the immune system. Hence, manipulation of antibody glycosylation and/or the amino acid sequence within the Fc domain has had a significant impact on eliciting improved effector function activity.

With the increasing number of patent expiries of innovative biologic agents and greater clarity of regulatory requirements for development of biologics, much attention has been directed to the development of next generation antibody-based therapeutics. Continued innovation in the antibody field has been fueled by improved understanding of the disease biology and advances in the technologies available for antibody generation. As the most prevalent therapeutic indications have become increasingly crowded by virtue of the growing number of commercially marketed

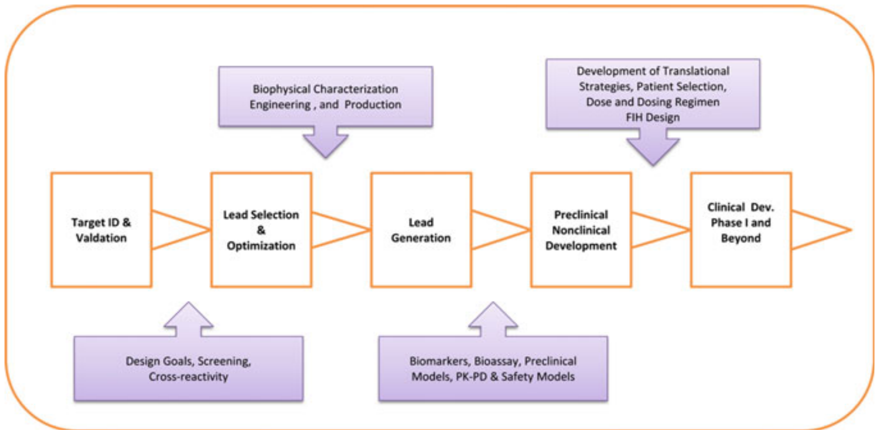

Fig. 1.1 Continuum of information flow during the design of effective translational strategies in development of antibody-based therapeutics

agents, it has become important for new antibodies to demonstrate an improved therapeutic activity over existing treatment options. Products that are differentiated in a meaningful manner against existing therapies are more likely to garner market penetration and thus continued market expansion. Therefore, the key challenge for the biotechnology industry is to continue generating products that can counteract market erosion caused by increasing pricing pressures, reimbursement issues, and biosimilar legislation.

The number of approved antibody-based therapeutics in the US underscores the clinical efficacy of this class of therapeutics in oncology and inflammatory diseases. However, a major challenge in the development of this class of biologics, much like small molecule drugs, is maintaining the effective flow of information and translation of accumulated knowledge throughout the drug development process (Fig. 1.1). The design of effective translational strategies from the early stages of the development process for antibody-based therapeutics is not only necessary to lessen the development time and cost, but also to foster implementation of rational decision-making processes throughout various development phases. Successful strategies for development of antibody-based therapeutics require integration of knowledge with respect to the target antigen properties, target pharmacology, antigen isoforms, and pharmacological redundancy in health and disease, as well as drug design criteria, such as isotype, affinity, pharmacokinetics (PK), pharmacodynamics (PD), and safety.

A deep understanding of both the biology and the pathology of disease is essential for target identification and validation. This knowledge enables the rational design of antibody therapeutic attributes including the mechanism of action, specificity, potency, isotype subclass, affinity, and half-life. Consideration and utilization of the appropriate animal models will enable optimal translation to the clinic and support of clinical trial design. Selection of a target antigen is the

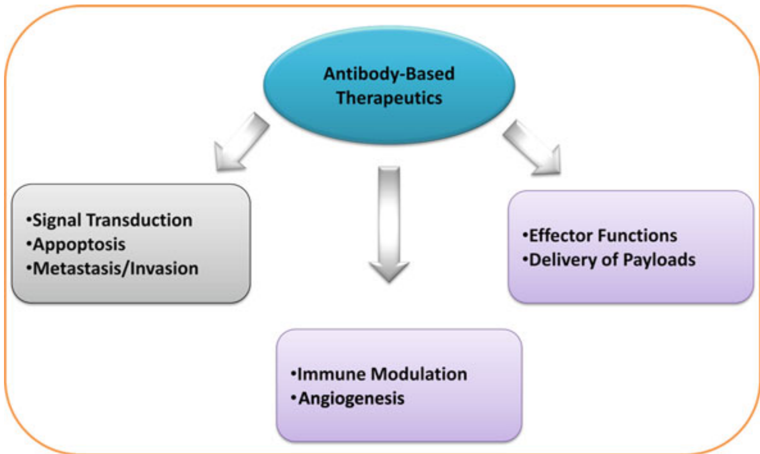

Fig. 1.2 Modes of action for antibody-based therapeutics

first step in generation of an antibody-based therapeutic. Understanding target antigen biology and its role in the pathogenesis of disease is of primary importance. Surveying appropriate tissues for validation of target expression by immunohistochemistry, or equivalent methodologies, is vital to establishing disease linkage and verifying the target antigen is not abundantly expressed in normal tissues. Also, functional validation of the target is critical. Functional redundancy of the target is an additional consideration; if the target antigen belongs to a conserved protein family, down-modulation of the target may not result in the desired phenotypic outcome.

Antibodies can mediate their biological activities via multiple mechanisms (Fig. 1.2). Growth factor receptors and/or their activating ligands are often overexpressed in a number of malignancies and can promote tumor cell growth and resistance to chemotherapeutic agents. By binding to growth factor receptors or their cognate ligands, antibodies can interfere with ligand binding and hence disrupt signaling pathways. Alternatively, antibody-based therapeutics can sterically prevent the receptor from assuming the requisite conformation for dimerization required for signal transduction. Interference of signal transduction pathways can thus mediate apoptosis and/or inhibit cellular proliferation. Antibodies also exhibit the potential to bind, and thereby inhibit the biological activity of molecular targets implicated in the invasion or metastasis of tumor cells. They can target antigens uniquely expressed on the neovasculature of tumors or growth factors that promote angiogenesis, thereby impacting the blood supply to tumors. Furthermore, antibodies can modulate the immune system by enhancing tumor antigen-specific immune responses, or alternatively, suppressing immune activity, as is the case for the treatment of autoimmune disease. Additionally, antibodies can target tumor cells by mediating effector function, namely CDC (complement-dependent cytotoxicity) and/or ADCC (antibody-dependent cell-mediated cytotoxicity). As described

earlier, a promising means for augmenting the antitumor potency of antibodies is through enhancement of effector function via engineering of IgG1 Fc variants. Antibodies can also be effective agents for the delivery of a cytotoxic payload, such as a chemotherapy drug, toxin, or radioisotope. The basic premise of an antibody drug conjugate is to confer higher tumor selectivity to a cytotoxic drug that is too toxic to be used on its own, or alternatively, to bestow improved cell-killing activity to a monoclonal antibody that is tumor-selective but inadequately cytotoxic.

Development of therapeutic monoclonal antibodies requires rigorous measurements of the kinetic and thermodynamic binding properties of antibody–antigen complexes for drug candidate optimization and the design of clinical dosing strategies. Several complex factors can influence the ideal affinity required for a therapeutic antibody, namely the nature and prevalence of the therapeutic target in the diseased tissue, as well as the desired functionality of the antibody. Hence, predicting the optimal efficacious affinity for a therapeutic antibody to its target antigen is challenging. As a result, biophysical measurements are one of the critical components necessary for developing effective translational strategies with respect to lead selection, evaluation of the relevant (appropriate) animal species for the conduct of safety and efficacy studies, and the design of effective clinical dosing strategies. Additionally, biophysical techniques prove highly effective in evaluation of cross-reactivity to orthologous antigens from species other than human. In particular, surface plasmon resonance biosenors play an especially important role in establishing various epitope classes (or bins) that allow further classification of lead antibody candidates in terms of their binding behavior.

Establishing relevant bioanalytical (BA) methodologies from early preclinical stages is critical for implementation of effective strategies necessary for successful translation of information into the later drug development phases. Robust and effective BA methodologies assist in addressing important questions regarding PK, immunogenicity (IM), and PD of drug candidates. Moreover, BA methodologies are critical for translation of exposure-response data from preclinical efficacy and nonclinical safety studies in support of the effective design of first-in-human clinical programs. In order to achieve these objectives, BA methods must be well characterized and provide a certain degree of robustness even at early stages of preclinical development. Evaluation of relevant biomarkers in appropriate animal models can greatly enhance translation of exposure-response relationships across species. When appropriate immunoassay methodologies are available, relationships between antibody PK and the ensuing effects on proof of mechanism and proof of principle biomarkers can be effectively examined. Application of biomarkers should guide the selection of safe and effective first-generation leads for advancement through various development stages. Additionally, relevant biomarkers can further provide a clear opportunity for evaluation of differentiating characteristics relevant to development of second-generation antibody-based candidates and drive lead evaluation during the preclinical phases.

Characterization of safety in relevant species is pivotal to effective translational strategies. The purpose of preclinical safety evaluation for small and large molecules is to identify potential risks to humans. These data are used to recommend a safe

starting dose and guide dose escalation schemes, as well as other risk mitigation strategies during early clinical development. The objective is to reveal potential target organs of toxicity with an assessment of dose-response, reversibility, monitorability, as well as establishing no-observed adverse effect levels, or minimally anticipated biological effect levels. It is essential therefore that these pivotal preclinical studies are conducted in a pharmacologically relevant species. Safety concerns associated with many monoclonal antibodies are often an extension of their intended pharmacological activity. This "exaggerated" pharmacological response may be the result of a more profound modulation of the target, or may occur as a consequence of antigen expression on non-target cells. Non-specific effects can also occur following dosing with monoclonal antibodies. When an appropriate species cannot be identified, as in the case when antigen target is not expressed in animals, or when there is low conservation of the epitope across species, additional approaches should be considered. This might include the use of a surrogate antibody(s) that exhibits similar characteristics to that of the intended therapeutic antibody, or the generation of transgenic animals that express the human antigen. Development and validation of transgenic animals or surrogate antibodies is likely to incur significant time and resource demands. The transgenic animal must be characterized for antigen expression and functional integrity. In the case of surrogates, an antibody is required that reflects as closely as possible the characteristics of the clinical candidate with respect to affinity, isotype, and functional activity.

Conducting PK and PD studies in appropriate animal models can greatly enhance the translation of information across species. When appropriate immunoassay methodologies are available, relationships between antibody exposure and the effect on free antigen reduction or antigen binding to the antibody can be evaluated. Evaluation of the relationships between the free antigen and antibody concentrations in vivo can provide invaluable information regarding the antibody potency, EC_{50} (antibody concentrations resulting in 50% suppression of the antigen) and the maximum system efficiency, E_{max}. The relevant experimental knowledge obtained from biophysical studies can also allow correction for affinity differences between the orthologous antigens in man and the relevant animal model. Additionally, information about antigen concentrations can be obtained experimentally by direct measurements of the target antigen in patients and be related to the concentrations in preclinical models.

In this book, we have attempted to provide a comprehensive discussion of various topics critical for establishing successful translational strategies for the development of antibody-based therapeutics. An understanding of the relationship between the "unit dose" and "unit effect" with respect to both beneficial and deleterious effects is essential for developing an effective translational strategy that will deliver a superior therapeutic candidate into clinical development. With this objective in mind, we have assembled topics that highlight a science-based approach with the underlying theme of "translatability" throughout the various drug development phases in each chapter. The ensuing chapters were prepared by

scientific experts in the field to whom we are greatly indebted for their valuable contributions to enable publication of this unique book. Undertaking this endeavor was an extremely rewarding and enjoyable scientific journey and we hope that the interested reader finds this collection informative and a valuable resource.

Chapter 2
Discovery Process for Antibody-Based Therapeutics

Heather H. Shih

Abstract Antibody-based therapeutics have entered the center stage of drug discovery as a result of a major shift in focus of many pharmaceutical companies from small molecules to a broader portfolio containing both protein and chemical therapeutic agents. The field is benefiting from both an increased understanding of the mechanistic basis of antibody-derived therapeutics and the development of sophisticated technologies to derive safe and targeted biotherapeutics. This chapter provides a general overview of the discovery process relevant for generation of antibody-based therapeutics. The discussion elaborates on target selection and validation, screening preparation, lead identification and optimization, as well as clinical candidate selection. In addition, an overview of immunogenicity, a unique challenge for protein-based therapeutics, is provided. A case study is also included to illustrate the discovery process for bapineuzumab, a humanized anti-amyloid beta (Aβ) monoclonal antibody, currently in Phase III clinical trials for the treatment of Alzheimer's disease.

Introduction

Drug discovery is a sophisticated process that integrates scientific innovation with cutting-edge technologies. Development of novel protein therapeutics or biologics has gained significant momentum in the biopharmaceutical sector in recent years. Additionally, the approval process for biosimilars and generic biological drugs is not well-defined and is currently under evaluation. Due to the complex molecular

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and functional properties associated with protein drugs, establishing pharmaceutical equivalency in terms of both safety and efficacy for biosimilars relative to their brand name counterparts is complex. Moreover, the regulatory path for approval of these agents is yet to be clearly defined. Although the biosimilar industry is growing aggressively, the major players in the biopharmaceutical sector continue to invest significant resources in discovery and development of new and novel biotherapeutics (Genazzani et al. 2007).

The shift in emphasis toward development of biotherapeutics is in part manifested by the growing preponderance of biologic agents in the portfolios of major biopharmaceutical companies. For antibody-based therapeutics, which include monoclonal antibodies, antibody-derived variants (e.g. camelid nanobody), and Fc fusion proteins, close to 40 drugs are on the market with another 30 in the late clinical phase (Reichert 2011). With the flurry of industrial activities focusing on developing novel biologics, a major effort in the biopharmaceutical industry is devoted to establishing sophisticated industrial processes for preclinical discovery and manufacturing of viable therapeutics.

The overall process for developing antibody-based therapeutics can be divided into five phases, i.e., target selection and validation, screening preparation, generation of early candidates ("hits"), selection of advanced candidates ("leads"), lead optimization, and clinical candidate selection. An overview of the drug discovery process prior to the selection of a clinical candidate is shown in Fig. 2.1. As with traditional small molecule drugs, the discovery process typically begins with selection of a validated target and a proposal for therapeutic modulation of the intended target. During the screening phase, all relevant reagents and assays are developed and tested. Screening is then carried out to generate candidate antibodies with desirable molecular and functional attributes that can be potentially translated for application in the anticipated therapeutic indication(s). At the end of this phase, a successful screen will result in the identification of one or more promising leads deemed favorable for further development. Next, the lead antibody is optimized to endow drug-like properties such as optimal target-binding affinity, manufacturability, and other biopharmaceutical properties when possible. The optimized candidate is subjected to broad and stringent in vitro and in vivo evaluation in order to determine whether it is suitable for further development. This chapter provides an overview of the preclinical drug discovery process.

Therapeutic Candidate Discovery

Target Selection

A drug discovery project may be perceived as an experimental approach for establishing that a selected biological target can be therapeutically modulated. In the case of antibody-based therapeutics, the therapeutic molecule must also be

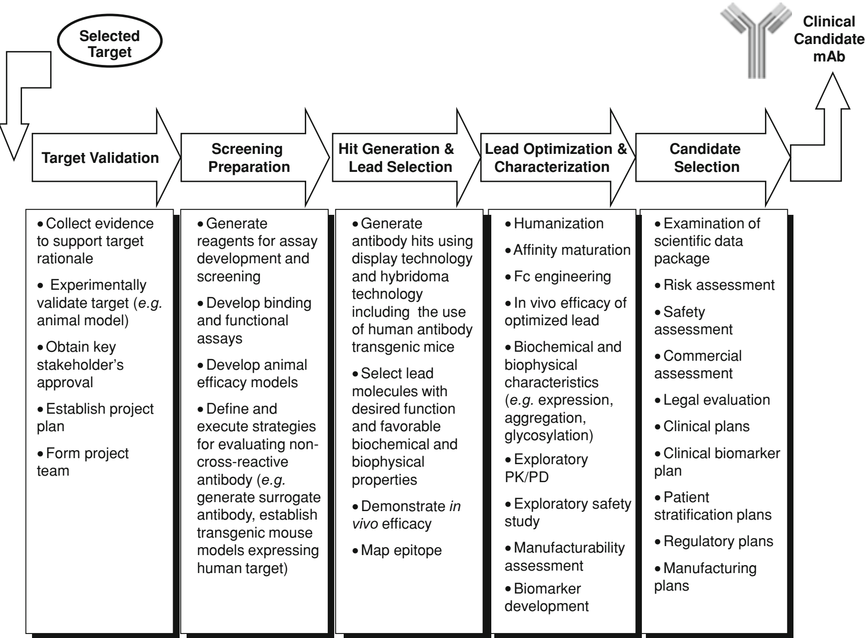


Fig. 2.1 Overview of discovery process for therapeutic monoclonal antibodies leading to the selection of a clinical candidate. The overall process can be divided into five stages: target validation, screening preparation, hit generation and lead selection, lead optimization and characterization, and candidate selection. Key activities at each of the five stages are listed in the text boxes

amenable to manufacturing in large quantities and amenable to effective delivery to human patients in order to achieve a beneficial therapeutic outcome. The selected therapeutic target is often described as "validated" to imply that there is adequate scientific evidence for its disease association and therapeutic potential. The following criteria can be used to define a validated target for an antibody-based therapeutic project: (1) the biological or pathological functions of the target are well-defined, (2) the pathological role of the target has been validated in the relevant animal models—for example, the deletion of the mouse ortholog and/or overexpression of the mouse protein have been shown to mimic the human pathology, (3) antibody-based intervention of the target has been demonstrated to achieve the desired therapeutic outcome in an animal model mimicking the human disease, (4) human genetic data have established a definitive association of the target with a specific human disease, and (5) the target resides within a molecular pathway that has been therapeutically manipulated by other means such as protein, peptide, or small molecule therapeutics. The majority of targets selected for antibody-based therapeutic projects meet some but not all of the above criteria. Therefore, additional target validation efforts are often a critical component of antibody-based therapeutics programs.

A target can be selected by various means. A "literature target" is a molecule with proven or implied disease association in human patients based on published data. Other target discovery efforts may originate from "omic" studies including transcriptional profiling and proteomics experiments that lead to the discovery of genes and proteins with aberrant expression patterns under pathological conditions. The genome-wide association studies in recent years offer yet another source for discovery of new targets (Chap. 8).

Several factors should be taken into consideration regarding the selection of a viable target for an antibody-based therapeutic project. First, the target molecule should reside in a physiological location accessible to a systemically administered therapeutic antibody. As such, the targeted moiety should be present either on the cell surface (cell surface target), in the extracellular tissue compartment (extracellular target), or in circulation (soluble target). In addition, the target should be expressed in a pathological tissue that is accessible to the therapeutic antibody delivered via systemic circulation. Brain targets are notoriously difficult for modulation by large protein therapeutics due to the presence of the blood–brain barrier that restricts the passage of large molecules from blood into the brain. Although antibody therapeutics are being developed to treat neurological diseases, such as Alzheimer's disease (AD), it is still debatable whether the major site of drug activity is in the periphery or in the central nervous system (see discussion in Sect. 2.2.7). For a soluble target, its pathological concentration should be present at a level that can be stoichiometrically bound by an administered antibody therapeutic. The peak serum concentration for an antibody therapeutic can fall within nM to μM ranges; hence, a soluble target with a serum concentration significantly exceeding this level may not be sufficiently bound by the therapeutic antibody in order to achieve the desired therapeutic outcome. Related to this caveat, many cell surface receptors are shed from the cell membrane; the shed soluble receptor is released into the circulation (sometimes referred to as "decoy receptor") and may function as a sink, thereby diverting a receptor-binding antibody from modulating the membrane form of the target (See Chap. 6).

Another consideration is establishing whether or not antibody-mediated cross-linking of the cell surface antigen results in receptor internalization and/or the activation of downstream signaling which could be either desirable or deleterious. In such cases, it is important to develop a cellular model where the target of interest is expressed on the cell surface and the downstream signaling readout can be measured to allow examination of the biological effects from antibody-mediated endocytosis and/or cross-linking of the targeted receptor. Additionally, differential expression of the target antigen in diseased (i.e. tumors) versus normal tissues is a critical consideration for selection of a viable target as safety concerns may arise due to modulation of the target in normal tissues. For example, the VEGF system is a key mediator of normal and disease-associated angiogenesis. Anti-VEGF antibodies such as bevacizumab are a class of anti-angiogenic agents used in the treatment of cancer and macular degeneration. In theory, these antibodies would also inhibit normal angiogenesis, and the safety risks associated with bleeding have actually been observed with VEGF modulation in patients

(Wong and Joussen 2010). Although few projects with validated targets are terminated entirely based on safety concerns, evaluation of the available literature for determination of critical factors such as tissue distribution patterns and the physiological functions attributed to the target can be helpful in understanding potential safety concerns. For example, desirable inhibition of a target protein expressed in the skeletal muscle may also lead to an unintended modulation of the same protein expressed in the cardiac muscle, which could lead to deleterious toxicity effects in the heart. Experimental approaches such as siRNA-mediated gene silencing and tissue-specific gene knockout studies can be used in evaluation of potential safety consequences although these approaches may be limited (See Chap. 8).

Project Planning

"Start with the end in mind."—Stephen R. Covey. This was the take-home message given by my instructor at a company internal drug development course that has since stayed with me. For an antibody discovery project, the end goal is to advance a candidate antibody into clinical trials. In order to reach this end, often after 5–10 years of discovery activities, a project should start with a clear path forward for both the long- and short-term goals.

Once a target is selected, the therapeutic targeting strategy should be defined. For example, a common molecular mechanism for an antibody-based therapeutic is the blockade of a ligand-receptor interaction, for which there are three conceivable targeting strategies: an anti-ligand antibody, an anti-receptor antibody, and a receptor-Fc fusion protein. In theory, all three antibody-based therapeutics should achieve similar clinical outcomes. In reality however, different therapeutic entities modulating the same molecular mechanism can demonstrate unique clinical outcomes due to unique biology associated with the receptor versus the ligand as well as unique attributes possessed by the therapeutic molecule itself. Therefore, it is important to evaluate various targeting strategies and move forward either with the most strategic and/or feasible approach. Alternatively, two or more parallel approaches can be initiated and all candidate molecules can be later ranked to enable selection of the best approach. It is also worth mentioning that development of an antibody therapeutic does not exclude the effort to develop a small molecule drug modulating the same molecular target. For example, the monoclonal antibody therapeutic cetuximab and small molecule drugs gefitinib and erlotinib all target EGF receptor (Imai and Takaoka 2006). Resources allowing, an antibody-based therapeutic project can be carried out in parallel with a small molecule project for the same target if warranted. In general, due to the exquisite specificity observed with antibody-based therapeutics, a well-designed antibody is less likely to elicit adverse effects compared to a small molecule drug but is significantly more costly to produce.

The proposed targeting strategy and the underlying pharmacology should dictate the intended molecular characteristics of the therapeutic antibody under development. These considerations should include an understanding of the target epitope and its correlation with cellular signaling, binding specificity and affinity, species cross-reactivity, antigen expression profile, effector function(s) recruitment, and the anticipated clinical dose and dosing frequency. Epitopes and binding affinities are now recognized as key determinants of therapeutic mechanisms of an antibody (Chaps. 6 and 18). For example, trastuzumab and pertuzumab are two clinical mAbs that bind different epitopes on HER2. Trastuzumab is believed to inhibit ligand-independent activation of HER2 by blocking HER2 and HER3 complex formation, whereas pertuzumab targets the dimerization epitope of the HER2 receptor directly (Junttila et al. 2010). The effector functions of an antibody refer to antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Specific antibody-mediated therapeutic action such as anti-tumor activity relies heavily on the effector function of IgG to engage immune cells to kill cancer cells, whereas in other applications such as targeting cell surface receptors on immune cells, it is necessary to attenuate or eliminate the effector functions of the therapeutic antibody (Chap. 4). Several marketed antibody therapeutics such as eculizumab (anti-C5 antibody) and abtacept (CTLA4-Fc) have purposely engineered the Fc region to reduce the effector functions of these molecules to improve the safety profiles of these products.

Species cross-reactivity is a practical consideration for many antibody discovery programs, which should not be confused with antibody specificity (Chap. 10). It is a desirable feature for a candidate antibody and refers to the ability of the antibody to bind and functionally interact with the orthologous proteins from various animal species used as models for evaluation of in vivo efficacy, pharmacokinetic and pharmacodynamic (PK/PD), and safety. The animals routinely used for these purposes include but are not limited to mouse, rat, rabbit, and cynomolgus monkeys (Chap. 10). For some programs, the exquisite binding specificity of an antibody candidate paradoxically creates an issue for the program with its lack of cross-reactivity. A common practice is to intentionally screen for antibody candidates that bind and functionally interact with both the human target and its rodent ortholog, most commonly mouse (Fig. 2.2). In addition, cross-reactivity of the lead antibody to the monkey ortholog must be evaluated to facilitate the IND-enabling toxicity studies in monkeys (Chap. 10).

What if a cross-reactive antibody cannot be generated? First, a simple bioinformatic exercise can help assess the probability of obtaining cross-reactive antibodies to a selected human target. Amino acid sequences for the relevant orthologs can be easily retrieved from the public domain and aligned to determine sequence homology, which serves as a rough predictor of the likelihood for obtaining cross-reactive antibodies (in general, there is a high probability for an antibody to be cross-reactive to an ortholog when the antigens share greater than 90% sequence identity, though in some instances the identity and homology in the relevant epitope sequence will be the major determining factor, see Chap. 10). In the absence of cross-reactivity, several strategies have been considered.

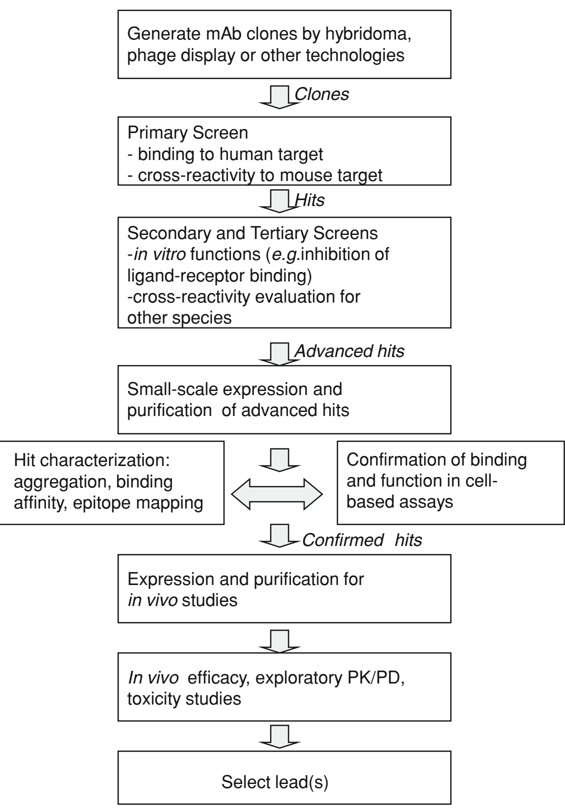


Fig. 2.2 A representative screening paradigm depicting the experimental flow of a typical screening process from the generation of antibody hits to the selection of leads

A surrogate antibody generated either prior to or in parallel to the therapeutic candidate can be used to enable preclinical proof-of-concept efficacy studies (Chap. 10). By definition, a surrogate is a functionally equivalent antibody to the therapeutic candidate while binding specifically to the target ortholog expressed in the intended animal species (Tabrizi et al. 2009). For example, anti-cytokine antibody projects often encounter low sequence homology between human and mouse cytokine orthologs. During the process of generating the lead therapeutic candidates, an anti-mouse cytokine antibody can be generated to facilitate the conduct of proof-of-concept studies in rodent efficacy models. An increasingly popular approach is generation of "human knock-in/knock-out" mice where the gene encoding the human target protein is inserted into the locus encoding the mouse target ortholog within the mouse genome. These "knock-in/knock-out" mice will only express the human target protein but not the endogenous mouse ortholog. Alternatively, transgenic mice can be produced where the human target protein is expressed in the presence of the endogenous mouse target protein. These genetically modified mice are increasingly employed for efficacy, PK, and toxicity studies for the evaluation of non-cross-reactive antibody candidates.

A screening paradigm is a frequently employed to summarize the screening strategy and process flow, thus providing a framework for the execution of an antibody-based therapeutic program. The key information captured by a screening paradigm includes the screening assays (i.e. primary, secondary, and tertiary assays), in vivo plans (i.e. efficacy, PK/PD, toxicity studies), go/no-go decision points, and estimated timelines for each process. A generic screening paradigm is shown in Fig. 2.2.

In addition to the overall goals and specific molecular features of the candidate antibody, other facets of the project plan include the intellectual property claims around the target protein and competitive landscape for the proposed therapeutic approach, the commercial value of the program, and potential safety issues related to modulating the target.

The development of a backup therapeutic candidate is an important strategic component of the project plan. If the lead molecule encounters unexpected issues in preclinical development or early clinical testing, the backup molecule can readily become the lead candidate without much loss of time. Furthermore, if the lead molecule successfully enters the market, the backup molecule can become a second-generation drug with differentiated and/or improved therapeutic features. The backup molecule can be co-developed with the lead molecule and strategically "parked" prior to clinical testing. Several instances whereby potential backup molecules may be warranted include: (1) an antibody that binds to a different epitope on the same target protein, (2) a fully human antibody while the lead is a chimeric or humanized antibody, or (3) an antibody that targets a different protein in the same biological pathway (for example, in the case of an antibody-mediated blockade of a receptor-ligand interaction, a ligand-targeting antibody can serve as a backup molecule for a lead receptor-targeting antibody).

Although this section does not describe a distinct phase of antibody drug discovery, project planning is a critical prelude to any successful execution of a drug discovery project. A project plan should be formulated at the start of an antibody discovery program to clearly define the scientific rationale, outline the long-term goals, and experimental plans. It should establish estimated timelines for various phases, interjected with milestone decision points with clearly defined go/no go criteria. Since a drug discovery program becomes increasingly costly as it advances toward the clinic, a timely termination of failing projects before they reach late-stage development has significant cost saving benefits. Lastly, every drug discovery path is never a straightforward process, but rather a dynamic one that may require flexibility as a result of unforeseeable issues and challenges. Thus, project plans organically evolve with the discovery process and must be revised and updated on a regular basis.

Once a project plan is endorsed by the key stakeholders, a project team is then assembled, which minimally consists of a team leader who is often the biology lead and an antibody engineer or technologist. The team composition varies with the stage of a project and increases in complexity with respect to the required expertise as the project progresses throughout the development process (see "Selection of the Clinical Candidate").

Screening Preparation

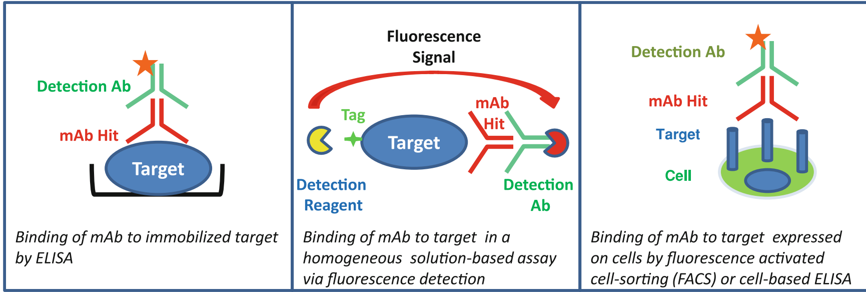
The screening preparation phase, rather than the screening phase, is often the bottleneck of the early discovery process. High quality reagents and optimized functional assays are the key steps for a successful screening phase. A common mistake is to rush into the antibody generation process before reagents and assays are fully in place. Such attempts to save time by cutting corners during the screening preparation will often result in the downstream loss of time and waste of resources. To avoid a "garbage-in and garbage-out" scenario, it is strongly recommended to have all reagents in hand and assays validated prior to initiating antibody generation (e.g. immunization of mice or phage library selections).

Reagents include materials used for the development of screening assays, antibody generation, and screening, as well as target validation and mechanistic studies. Common reagents include cDNA, expression plasmids, cell lines, purified proteins, control and reference antibodies, and target orthologs used for testing species cross-reactivity of the candidate antibody. Reagent generation is routinely outsourced to subsidize internal drug discovery activities at many pharmaceutical companies. It is critical to validate the quality of outsourced materials prior to their application during the drug discovery process. For example, proteins purified by external vendors should be evaluated in-house for the degree of purity and aggregation, presence of endotoxin, rodent virus contamination, and bioactivity.

Screening assays typically include primary, secondary, and tertiary assays. The primary screening assay typically measures the binding of an antibody to the target of interest to identify "hits." An enzyme-linked immunosorbant assay (ELISA) in 96-well or 384-well highthroughput format is commonly used as the primary assay where an antibody undergoing screening is allowed to bind to a target molecule immobilized on an ELISA plate. The bound antibody is subsequently detected with a secondary reagent. The assay is easy to set up and straightforward to operate. In addition, coating the target antigen on an ELISA plate at high density increases the avidity of antibody binding and enhances the detection of weak binders. However, the ELISA format includes many washing steps and is not easy to adapt to automation. Other commonly employed primary binding assay formats include homogenous solution-based fluorescent assays or cell-based binding assays such as fluorescence activated cell sorting. These assays, in contrast to ELISA methods, should allow presentation of the target proteins in their native conformation. A cartoon representation of these assays is shown in Fig. 2.3.

Secondary and tertiary assays are designed to measure the desired bioactivity of candidate antibodies in addition to their ability to bind the target. The sequence to apply various so-called functional assays is arbitrary, which is often based on the throughput and ease of operation. A secondary assay can be a plate-based functional assay in high throughput screening format, whereas a tertiary assay is a low-throughput cell-based assay of significant biological relevance. For example, to identify antibodies that block ligand-receptor interactions, a plate-based ligand/receptor binding ELISA can be used as a secondary assay, whereas a cell-based

Representative antibody binding assays to screen target-specific binders



Representative functional assays to detect blockade of ligand-receptor interaction

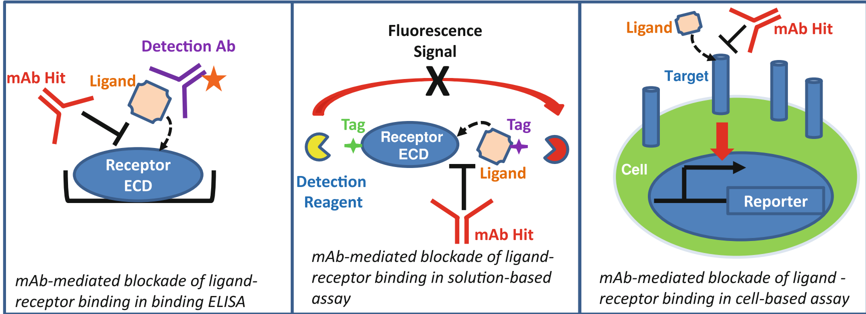


Fig. 2.3 Depiction of typical binding assays and functional assays used for screening antibodies. ECD stands for extracellular domain

ligand/receptor binding assay with a signaling readout can be included as a tertiary assay. If feasible, a functional assay using primary human cells can serve as a physiologically relevant cellular system and should be included in the screening strategy.

Screening assays must be optimized for a high signal-to-noise ratio, plate-to-plate variability, and compatibility with screening samples. Typically, an optimized assay has a greater than 3- to 5-fold signal-to-noise ratio with minimal plate-to-plate variation, and is compatible with mock samples representative of the particular screening method. For example, a cell-based functional assay used for screening hybridoma hits should be tested for compatibility with hybridoma supernatant to rule out variables such as serum effects or quenching of fluorescent signal by the coloration of hybridoma culture medium. Notably, the assay optimization criteria are not as stringent for antibody-based therapeutics as for small molecules. The exquisite binding specificity of antibody-based therapeutics often translates into high assay signals and low false positive rates in a screening assay.

A reference antibody, or a positive control antibody, is a valuable tool to help with assay validation. Furthermore, a reference antibody is often used in in vivo proof-of-concept studies either to validate the target or establish an efficacy model.

The reference antibody can be a commercially available monoclonal antibody with function similar to the intended therapeutic candidate, a polyclonal antibody functionally interacting with the target protein of interest, or an antibody reconstructed from sequences available in the public domain (e.g. a competitor's patented antibody). A negative control antibody is also critical, particularly in cell-based assays and in vivo efficacy studies to determine any biological effects associated with the effector functions of an IgG molecule independent of its target-binding function. In relation to the reference antibody, the negative control antibody should be a species and isotype-matched antibody that does not bind to any proteins expressed in the model system (e.g. an anti-green fluorescence protein antibody).

As part of the screening preparation, the development of animal models should be initiated, which in some cases may take years. In this case, the planned animal model does not recapitulate the scope of human pathology, other complementary plans such as ex vivo models and primary human cellular systems should be established. In addition, as discussed in "Project Planning", an in vivo model for testing a non-cross-reactive lead antibody should be developed in advance for a project where a low probability for generating cross-reactive antibodies is anticipated.

Hit Generation and Lead Selection

The most commonly used technologies for generating early antibody candidates ("hits") are hybridoma and phage display platforms. Many of the currently on the market therapeutic antibodies have been generated using traditional hybridoma technology developed by Kohler and Milstein (Kohler and Milstein 1975). Currently, in the antibody therapeutics field there is a strong trend toward developing fully human antibodies, either by using humanized mice that express human IgGs in place of mouse IgGs, or by using phage display technology to screen naïve and synthetic human antibody libraries. The technologies to generate human antibodies are described in detail in Chap. 3.

Different antibody generation technologies each have their unique pros and cons. Hybridoma is a classical technology that often yields high-affinity rodent antibodies with desired functional activities. In addition, humanization has become a standard practice to reduce the rodent sequence content in the candidate antibody and humanized antibodies are generally safe for use in human patients. The fact that close to 50% of currently approved antibodies are humanized suggests that hybridoma technology may remain a mainstream technology to derive antibody therapeutics. Human IgG-expressing transgenic mouse technology has contributed to six out of the seven FDA-approved antibodies and another two are pending approval, indicating that application of this technology is on the rise (Nelson et al. 2010). However, the restricted accessibility to this technology due to intellectual property rights may limit its wide application. Screening human antibody libraries

using phage display technologies enables the direct generation of human antibody without the need for humanization. Moreover, phage display platforms enable rapid identification of early hits and allow for highly controlled experimental conditions to favor the isolation of antibodies for difficult antigens, such as proteins exhibiting high homology between humans and rodents, and toxic immunogens. The initial antibody hits generated by this method may exhibit low affinity, in which case further affinity maturation may be required. Intellectual property rights also limit the use of this technology. When feasible, one may consider conducting both immunization and non-immunization approaches in parallel to generate a robust panel of candidate antibodies. As discussed, an antibody isolated via a technology platform that is different from that of the lead molecule can be considered as a backup molecule for the program.

Lead selection refers to the process by which the early hits are interrogated in a vigorous, multi-stepped screening process to select a lead molecule(s) that meets pre-established criteria for advancement into the next drug discovery stage. As shown in the screening paradigm (Fig. 2.2), screening via secondary and tertiary functional assays allows a rapid filtering of hundreds of hits down to a handful of molecules. These can then be purified at small scale (milligrams) as full IgG molecules to allow more detailed characterization, including a confirmation of binding and functional activities as well as biochemical and biophysical analyses. Common molecular analysis includes determination of expression levels from mammalian expression systems, aggregation analysis by size exclusion chromatography, SDS-PAGE, Western blot analysis, determination of target protein binding affinity by Biacore and KinExA analysis, and crude epitope mapping. Elimination of hits can be based on suboptimal target binding affinity, a lack of robust biological function, or poor biochemical and/or biophysical attributes. If none of the hits exhibits highly favorable attributes, a suboptimal hit may be subjected to molecular optimization to improve its biochemical and biophysical characteristics.

Upon completion of in vitro characterization, the selected hit antibodies are ready for expression and purification in sufficient quantity (typically a hundred milligrams to grams) for in vivo efficacy testing. If needed, a crude PK study can be conducted prior to the efficacy study to help establish the dosing regimen. For a cross-reactive antibody, exploratory PK/PD and toxicity studies can be combined along with the efficacy studies in the relevant animal models. Typically the lead molecule is selected based on demonstrated in vivo efficacy, which is often a go/no-go decision point for the program.

Lead Optimization and Characterization

The lead molecule selected from the initial screening often requires additional molecular engineering to endow drug-like properties before becoming a clinical candidate. Common lead optimization practice includes humanization of a rodent

antibody, affinity maturation, and Fc engineering. The technical details of these engineering methods are described in Chap. 4. An overview of these methods is provided here.

Humanization has become a standard and widely used technology to reduce the immunogenicity of a therapeutic antibody initially derived from rodents. The process refers to the replacement of more than 90% of rodent IgG sequence in the parental antibody molecule with human IgG sequence. In addition to humanizing rodent antibodies, ongoing efforts in the field are also devoted to the conversion of other non-human antibodies into human therapeutics, and humanization has been applied to therapeutic candidates derived from rabbit, chicken, and camelids (Steinberger et al. 2000; Tsurushita et al. 2004; Vincke et al. 2009).

Affinity maturation is often applied to antibody leads selected from a naïve human library using a display technology. These leads may have relatively low (10–100 nM) target binding affinities but can be enhanced using various affinity maturation technologies to reach a desired affinity range (normally 0.1–10 nM). In addition, in some special cases where a high affinity antibody is required, further affinity maturation is applied to antibodies that already exhibit low nM binding affinities. For example, an extremely high affinity antibody (i.e. pM range) may be needed to effectively block the binding of a cytokine to its receptor (Owyang et al. 2011). However, it is worth noting that high affinity does not always correlate with improved efficacy. A high affinity antibody binding to a rapidly internalizing target may promote the rapid clearance and elimination of the antibody from circulation, resulting in an unfavorable short in vivo half-life.

The Fc region of an IgG1 molecule is a functional molecular entity mediating: (1) ADCC via binding to Fcγ receptors (FcγR) on natural killer (NK) cells, (2) CDC via C1q binding, and (3) the increase in the in vivo half-life via binding to the neonatal Fc receptor (FcRn). Alteration of each of these activities has been explored to modulate the function of IgGs in specific applications. For example, ADCC enhancement is explored to enhance antibody-mediated tumor cell killing, which can be achieved via enhanced binding of Fc to FcγR by engineering site-directed mutations in the contact residues, or ablation of fucosylation of the Fc. In addition, site-directed mutations in the Fc/FcRn contact site have been engineered to increase the half-life of the IgG molecule (Strohl 2009). After the generation of an optimized lead, functional and molecular characterization is carried out to confirm its in vitro and in vivo activity and favorable molecular attributes as a therapeutic candidate.

Selection of the Clinical Candidate

The optimized lead molecule must undergo a series of stringent assessments that constitute the candidate selection process; at the end of this process a critical decision is made regarding whether the antibody qualifies as a clinical candidate. Selection of a clinical candidate is a milestone decision marking the stakeholder's

commitment to advance a therapeutic antibody candidate into clinical trials in human patients. The core criteria that must be met before proceeding include: (1) a clear demonstration of efficacy of the antibody candidate in cellular and/or animal models that has been deemed translatable to efficacy in human disease, (2) dose–response studies that have been completed in animals to guide the dosing regimen in early clinical development, (3) preclinical pharmacology and PK studies that have been completed to support the clinical dosing route and regimen, (4) preclinical pharmacology safety risk that has been deemed low and/or acceptable, (5) demonstration of required biochemical and biophysical properties of the candidate antibody and an optimal formulation of the clinical material, and (6) manufacturability of the candidate molecule that has been vigorously assessed and a process to prepare large quantities of clinical material has been developed (See Chap. 15). In addition to the above core criteria, the following should also be met: (1) patent claims on the candidate antibody have been filed and any intellectual property concerns have been properly addressed, (2) application of biomarkers has been incorporated into the early clinical plans (Chap. 13), (3) preliminary global market research has been conducted and competitive positioning information has been acquired, and (4) preliminary target product profile and early clinical plans have been defined.

Candidate selection also represents a transition from the early discovery phase to the clinical development phase. During this transition, a candidate or several candidates are typically assessed for optimization to facilitate process development and manufacturability. This usually involves an assessment of expression or titer based on data available from the discovery process that may include data from transient expression or pools derived from stable transfection into a CHO host cell line. In transient HEK-293 systems, titers below 50 mg/l may present challenges in supplying material to enable discovery research. While there does not appear to be a direct correlation between expression titer in a transient system and titer in the subsequent stable mammalian cell line, transient expression titers below 50 mg/l would be a potential concern; such expression levels would likely require close monitoring during development to ensure acceptable expression titers are achieved in stably transfected mammalian cell lines.

Evaluation of the propensity of an antibody candidate to aggregate and to undergo degradation in a preferred formulation or set of formulations is an important part of the early assessment process. Aggregation can occur during all phases of production and controlling the levels of aggregate in the final product can be challenging. In addition to aggregation, significant degradation pathways, such as oxidation, deamidation, isomerization, and peptide bond cleavage are also evaluated early, typically at multiple temperatures (Chaps. 4 and 15). Often, accelerated stability studies are carried out under more extreme conditions to understand the major degradation pathways for a specific candidate or set of candidates. It is important to recognize that since different degradation pathways may be accelerated at different rates, these studies need to be analyzed carefully and may not represent the distribution or even the specific composition of the various impurities under standard conditions (Daugherty and Randall 2010; Wang

et al. 2007). The early assessment of candidates is largely intended to identify those that may have significant challenges during development. If multiple candidates are being considered for development, the selection can be based on a panel of data including, but not limited to, efficacy, tolerability, and stability. Early formulation studies can help to inform the selection decision, and if a candidate shows particularly poor stability during the early assessment, it can be a significant determining factor in candidate selection.

Additional in vitro and ex vivo safety assessment may take place at this stage, such as screening candidate antibodies for their ability to activate immune cells. This assay has been widely adopted by the pharmaceutical industry since the TG1412 (Parexel International) Phase I trial in 2006 where a humanized "superagonistic" anti-CD28 antibody induced a systemic inflammatory response coined "cytokine storm" in six healthy volunteers. The underlying pathological mechanism was associated with TG1412 cross-linking CD28 on T cells, triggering an uncontrolled cytokine release and precipitating a life-threatening outcome (Stebbings et al. 2009).

The development of biomarkers to facilitate the selection of a targeted patient population and the measurement of defined pharmacological endpoints in clinical trials should be an effort undertaken in parallel to the lead optimization process. Biomarker development is an integral component of the drug development process and an indispensible component of clinical trials. The development of a validated biomarker often takes months to years and requires a deep understanding of the biology, pathology, and therapeutic mechanisms associated with the therapeutic program. Therefore, significant resources and a sufficient timeline must be allocated to this activity. The importance of biomarkers and their contribution in antibody therapeutic development programs are discussed in Chaps. 13 and 14.

Candidate selection is the single most important discovery milestone marking the end of the discovery activities and the beginning of the clinical testing phase of an experimental drug. This decision point is reached after a comprehensive data package is assembled on the lead molecule and evaluated by a group of experts in various disciplines including discovery sciences, manufacturing, drug safety, drug metabolism, regulatory, legal, commercial, as well as clinical. It is noteworthy that a decision to either advance or terminate a candidate molecule is rarely based on a single factor, but rather after careful and exhaustive risk-benefit calculations concerning the collective attributes of the candidate molecule.

Immunogenicity of Antibody-Based Therapeutics

The Cause of Immunogenicity

Immunogenicity remains an unresolved issue for biotherapeutics. It refers to the ability of a particular substance, in this context, a biotherapeutic agent, to elicit an immune response in patients. In the clinic, immunogenicity is quantitatively

measured in terms of levels of anti-drug antibodies (ADA) generated in the blood following administration of the biologic drug. The observed clinical ADA response is often long-lived, a result of memory B and T cell production, and characterized by high affinity, class-switched IgGs of various subclasses (Baker and Jones 2007).

Immunogenicity is believed to arise from both extrinsic and intrinsic factors associated with a biologic product. Extrinsically, both pharmaceutical production and patient biology contribute to an immunogenic response in the host. Aggregates, degradation, oxidation, and deamidation products, as well as impurities introduced into the final drug substance during its production process can significantly enhance the immunogenicity of the drug. Patient HLA genetic background, immune status, concomitant medication, and route of administration can potentially have a significant effect on the immunogenic reactions in patients. Intrinsic factors as related to the properties of a therapeutic protein, such as amino acid sequence (e.g. presence of T cell epitopes), molecular structure, therapeutic mechanism, and post-translational modifications (e.g. glycosylation), can trigger immunogenic responses in patients.

The production of anti-drug IgG molecules, characteristic of an immunogenic response, reflects an adaptive immune response associated with the activation of CD4+ helper T cells that in turn promotes B cell differentiation and isotype class switching. In theory, an administered therapeutic protein is taken up and processed by antigen-presenting cells, such as dendritic cells, and subsequently presented to CD4+ helper T cells in the form of an MHC II/antigen peptide complex in the context of the patient's HLA allotype.

Clinical Consequences of Immunogenicity

The clinical consequence of an immune response mounted in patients treated with a biotherapeutic can be benign or lead to a life-threatening condition. In the most severe cases, the ADA generated against the administered biotherapeutic can lead to neutralization of the endogenous protein(s) in patients, causing long-term undesirable toxicities (Schellekens 2005). For antibody therapeutics, acute infusion reactions are often characterized by hypersensitivity responses, ranging from mild skin reactions to severe anaphylaxis with murine and chimeric antibodies such as OK-T3 and infliximab (Maggi et al. 2011). In most cases, such responses are clinically manageable via co-administration with corticosteroids to repress inflammation, or revising the dosing regimen. The recent development of humanized and fully human therapeutic antibodies has effectively minimized this particular type of adverse event. For humanized and fully human antibodies, the observed adverse clinical responses are largely limited to altered PK properties and decreased drug efficacy due to the induction of neutralizing ADA. In infliximab-treated patients, up to 89% develop neutralizing ADA that are associated with decreased clinical efficacy (Bender et al. 2007). In some instances, an ADA

response positively correlates with the clinical efficacy of an antibody drug. For example, increased survival in non-Hodgkin lymphoma patients receiving mouse anti-lymphoma antibody Lym-1 correlates with high ADA levels; this is postulated to be due to an induction of a multilevel idiotypic cascade, generating self-antibodies that target Lym-1 on tumor cells (Azinovic et al. 2006).

The improved clinical safety of monoclonal antibody drugs is a direct result from recent advancements in antibody engineering. Immunogenic reactions resulting from the introduction of non-human antibodies (e.g. nerelimomab, a murine anti-TNF antibody, Cohen and Carlet 1996) in patients has now been largely circumvented via the humanization of rodent antibodies (Easthope and Jarvis 2001) and generation of fully human antibody therapeutics (Coenen et al. 2007). Nonetheless, even in the case of fully human antibodies, significant immunogenicity is still observed clinically, in theory partially due to the presence of natural anti-idiotypic antibodies (Gilles et al. 2000). This observation also suggests that immunogenicity may be an inherent feature associated with all antibody therapeutics. Efforts are being developed to identify T cell epitopes in the antibody therapeutic as well as to boost immune tolerance via activation of Treg cells that dampen the unwanted immunogenic response (De Groot et al. 2008). However, it remains to be determined whether these approaches will minimize the incidence of immunogenicity observed with the application of antibody therapeutics in the clinic.

Discovery Practices to Minimize Immunogenicity of a Candidate Therapeutic Antibody

Presently, the clinical immunogenic response associated with any given therapeutic antibody cannot be accurately predicted using established experimental methods. The general approach by the pharmaceutical industry is to assess the immunogenicity potential for a panel of candidate antibodies during the discovery phase and ultimately select a lead molecule with a minimally immunogenic profile as the clinical candidate. Any potential immunogenicity risk of an antibody can be reduced by minimizing the introduction of “foreignness” into the drug candidate by ensuring maximal human sequence content as well as maintaining high levels of germline sequence in the framework regions within the variable domains; employing sophisticated computer algorithms to predict in silico T cell epitopes in the variable regions of an antibody molecule, particularly in the CDRs; examining binding of synthesized peptides containing the T cell epitopes to purified MHC II proteins; conducting ex vivo T cell stimulation assays to evaluate whether peptides containing putative T cell epitopes can empirically activate T cells via binding to the MHC II complex, and modifying amino acid sequences in the parental antibody to eliminate putative T cell epitopes. However, despite an enormous effort in the biotherapeutic immunogenicity field to develop experimental methods to link

the sequence information of a therapeutic biologic to its predicted immunogenicity, the clinical correlation between this "de-epitoping" exercise and a concomitant reduction in immunogenicity response is yet to be established (Descotes 2009).

Analytical Assays for Measuring Immunogenicity

Immunogenicity of every therapeutic biologic agent, including monoclonal antibodies, must be carefully monitored in the clinic to manage potential adverse events. Since immunogenicity is measured in terms of ADA levels in patient blood, developing analytical assays to measure such responses is an essential component of the drug discovery process (See Chap. 7). The ADA measurement usually includes both a confirmatory assay that detects antibodies that bind to the drug and a neutralizing assay that detects antibodies that block the therapeutic activity of the therapeutic antibody. ELISA is a common format used for ADA screening, while other high throughput and low detection limit assays are also being adopted by the industry. In addition to developing screening assays, an immunogenicity assessment and management strategy must also be implemented prior to the initiation of clinical studies. Necessary assessment includes the risk for the given therapeutic antibody to generate an ADA response and the potential severity of the induced response. Currently, the overall practice in the pharmaceutical industry to meet regulatory requirements entails complying with the immunogenicity guideline put forth in 2008 by the Committee for Medicinal Products for Human Use (CHMP) at the European Medicines Agency (EMEA) (Jahn and Schneider 2009).

Immunogenicity and Next Generation Antibody-Based Therapeutics

Although immunogenicity alone is rarely the basis for a no-go decision during clinical candidate selection, the recent case with motavizumab suggests that it could be an issue serious enough to cost the FDA approval of a drug. Motavizumab is a follow-on therapeutic to its predecessor palivizumab, developed by MedImmune Inc., a subsidiary of AstraZeneca, for the treatment of anti-respiratory syncytical virus (RSV) in infants and small children (Wu et al. 2007). At the end of 2010, the FDA rejected the market approval application of motavizumab primarily based on safety concerns related to an induction of severe and anaphylactic allergic reactions in small children treated with this agent. For the next generation of antibody-based therapeutics, modifications to a biologic agent must be carefully evaluated to minimize the risk of eliciting immunogenicity in patients. For example, antibodies

derived from animal species other than rodents may have unique immunogenic properties. Similarly, a novel scaffold that deviates from a natural human protein (e.g. bispecific antibodies) may introduce potential T cell epitopes. Furthermore, novel targeting platforms such as antibody-drug conjugates consisting of additional moieties (i.e. linker and the toxin) may potentially present novel immunogenic epitopes to the patient's immune system.

Case Study: Discovery Process for Bapineuzumab

Alzheimer's disease is a devastating mental debilitative illness that afflicts a large and increasing percentage of the elderly population all over the globe. Currently, only a limited number of palliative treatments are available which underscores the urgent medical need for the development of therapies targeting the fundamental pathogenic mechanisms of this disease. Amongst the ongoing efforts to develop disease-modifying therapeutics, bapineuzumab (AAB-001; Johnson and Johnson/Pfizer), currently in Phase III clinical trials, is the most advanced drug under development. Here we discuss the discovery process of this antibody therapeutic candidate using information available in the public domain to illustrate many concepts described in this chapter.

Bapineuzumab is a humanized murine-derived antibody targeting β-amyloid peptides (Aβ) for the treatment of AD. The molecular target for bapineuzumab, amyloid β (Aβ), is the major protein constituent of amyloid plaques in the brain of AD patients and has long been hypothesized to play a causative role in the pathogenesis of AD (Selkoe 2001). Aβ peptides of variable lengths, particularly the 40- and 42- amino acid peptides, are proteolytic products of the amyloid precursor protein (APP) by the β- and γ-secretases. Human genetic studies have linked AD-associated gene mutations to the over-production of Aβ. Consistently, transgenic mouse models recapitulating these human genetic mutations have demonstrated that increased accumulation of Aβ in the mouse brain elicits symptoms resembling some aspects of AD pathology including the formation of brain amyloid plaques and progressive neurodegeneration. In the AD field, a prevalent theory called the "amyloid hypothesis" states that overexpressed Aβ is the initiating determinant causing AD pathogenesis and has been the driving force for the majority of drug development efforts over the past decade where the therapeutic strategy is either to remove Aβ from the brain or to prevent its production (Lichtlen and Mohajeri 2008). The amyloid hypothesis has been intensely debated for over 20 years, particularly in light of the recent failure of a late-stage clinical trial on semegasestat (Eli Lilly), a small molecule inhibitor of γ-secretase that blocks Aβ production. Despite the controversy around the amyloid hypothesis, there is irrefutable scientific evidence supporting Aβ as a validated therapeutic target.

Aβ peptides, the target of bapineuzumab, primarily reside in the brain, a physiological location considered largely inaccessible to therapeutic antibodies in circulation due

to blockade by the blood–brain barrier. In rodents, studies indicate that only 0.1% of intravenously administered anti-Aβ antibody enters the central nervous system (Pan et al. 2002). Despite this conceptual caveat, the development of a passive immunotherapy approach using a peripherally administered anti-Aβ antibody is based on the initial landmark observation that active immunization of transgenic mice overexpressing Aβ (PDAPP mice) with Aβ peptides led to a decrease in brain Aβ plaque load and a reduction in brain pathology (Schenk et al. 1999). Subsequently, a pivotal study by Bard et al. unequivocally demonstrated that peripheral administration of anti-Aβ antibodies including 3D6, the parental murine antibody for bapineuzumab in PDAPP mice, led to brain Aβ plaque clearance (Bard et al. 2000). In the above study, anti-Aβ antibodies were shown to enter the brain and directly bind Aβ amyloid plaques. Multiple therapeutic mechanisms for anti-Aβ antibodies have since been proposed (Brody and Holtzman 2008), including the "peripheral sink" hypothesis that postulates an anti-Aβ antibody can exhibit biological activity outside the brain by sequestering peripheral Aβ in an immune complex, thus altering Aβ equilibrium, resulting in a net efflux of soluble Aβ from the brain into the blood (Brody and Holtzman 2008; DeMattos et al. 2001). The development of bapineuzumab represents a unique case where the premise of an antibody drug discovery program relies upon empirical evidence (i.e. active immunization of PDAPP mice with Aβ peptide leads to plaque clearance) that defies a conventional dogma (i.e. brain diseases cannot be treated via passive immunotherapy). It highlights the potential reward of "outside-the-box" exploration of the biological system, the elusive nature of biology, as well as therapeutic action of an antibody molecule.

The parental antibody for bapineuzumab, 3D6, is a murine IgG2b antibody that was generated using traditional hybridoma technology from mice immunized with a peptide corresponding to the *N*-terminal amino acids 1–5 of Aβ conjugated to a carrier protein (Bard et al. 2003; Schenk et al. 1999). In vitro, 3D6 has been shown to bind soluble Aβ by ELISA and Aβ plaques in the brain of PDAPP mice by immunohistochemical (IHC) analysis. In addition, the antibody can actively mediate plaque clearance in an ex vivo phagocytosis assay. When tested in the PDAPP transgenic mouse model, peripheral administration of 3D6 leads to a reduction of brain amyloid burden (Bard et al. 2003, 2000). To retrofit the sequence of these experiments into a hypothetical screening paradigm, the ELISA assay measuring the binding of antibodies to Aβ can be considered the primary assay, with the IHC assay measuring antibody plaque binding as the secondary assay, and the ex vivo plaque phagocytosis assay a functional tertiary assay.

Murine antibody 3D6 selectively binds to soluble Aβ as well as brain Aβ plaques, but not APP (Bard et al. 2000). It is important to note that the epitope for a specific anti-Aβ antibody may influence its therapeutic efficacy. In vitro, antibodies targeting different epitopes on Aβ demonstrate different binding profiles for free Aβ versus plaques (Bard et al. 2003). Interestingly, a large panel of antibodies targeting distinct Aβ epitopes is under evaluation in clinical trials. Compared with bapineuzumab that binds to the N-terminus of Aβ, solanezumab (Eli Lilly, Phase III) binds to the central region of the molecule, and poneaumab (Pfizer, Phase II) binds to the C-terminus. In addition, several anti-Aβ antibodies in clinical trials are reported to target theoretically toxic Aβ oligomers (Morgan 2011).

In vivo and ex vivo evaluation of murine anti-Aβ antibodies, including 3D6, reveals a correlation between antibody effector function and plaque-removing efficacy, suggesting that antibody-mediated plaque clearance via binding to Fc receptors on brain microglial cells is a potentially important therapeutic mechanism (Bard et al. 2003). Consequently, 3D6 was humanized from its parental murine IgG2b isotype with weak effector function and isotype switched to human IgG1 to elicit potent effector function. However, in clinical trials bapineuzumab induces an inflammatory response called vasogenic edema in a subset of patients (Kerchner and Boxer 2010). It is yet to be determined whether this outcome is attributed to the effector function of bapineuzumab, and whether the elimination of its effector functions would prevent or reduce this adverse event while retaining the plaque-removing activity, as suggested in animal studies.

Humanization of 3D6 to bapineuzumab significantly reduced the murine sequence content, similar to other marketed humanized antibodies currently used for long-term therapy. The actual immunogenicity profile of bapineuzumab in human patients is yet to be reported. It is worth mentioning that ADA have been detected in Phase II clinical trials for another humanized anti-Aβ antibody, solanezumab, presently with unknown clinical implications (Siemers et al. 2010).

The clinical trial studies of bapineuzumab utilize three biomarkers: the levels of Aβ and tau in cerebrospinal fluid, brain and ventricular volume by magnetic resonance imaging, and the ^{11}C-PiB signal on positron emission tomography. These biomarkers have been developed for general AD clinical trials rather than specifically for the clinical testing of bapineuzumab (Kerchner and Boxer 2010). Chap. 14 elaborates on biomarker applications for the development of antibody-based therapeutics in brain disorders, including AD.

Development of bapineuzumab is based on the hypothesis that peripheral administration of an anti-Aβ antibody can lead to the clearance of Aβ plaques in the brain of AD patients and consequently lead to cognitive improvement. This is a highly innovative drug discovery endeavor exploring an unconventional therapeutic approach, namely treating a brain disease with passive immunotherapy. The technical process for the development of bapineuzumab is relatively straightforward and devoid of major issues. The main challenge of translating an anti-Aβ antibody into an AD therapy is the "biological black box". Significant gaps in our understanding of this disease remain—AD pathogenesis is not fully understood at the molecular and mechanistic levels, the link of plaque removal to cognitive improvement is not firmly established, a robust AD animal model is lacking, the therapeutic mechanisms of anti-Aβ antibodies are not completely elucidated, and the molecular mechanisms underlying the adverse events are not clearly understood.

The discovery of bapineuzumab helps to illustrate several facets of the enormous challenges faced by the drug discovery industry. The majority of human diseases are manifested by multifactorial and progressive pathogenic mechanisms that are difficult to modulate by a single target-based therapeutic approach. This issue is further compounded by a typical lack of complete understanding of disease biology as well as therapeutic mechanisms. Furthermore, many animal models do not faithfully recapitulate human pathology. Despite intense ongoing efforts in the

entire pharmaceutical sector to undertake novel translational approaches to overcome these challenges, it may take considerable time to reach a breakthrough that will significantly reduce the tremendous risks associated with the drug discovery process. It is worth noting that since the publication of the pivotal observation by Schenk et al. in 1999 that Aβ vaccination in the PDAPP model can clear plaques to the anticipated conclusion of the bapineuzumab Phase III trial in 2011, more than 12 years have transpired. Notably, the outcome of bapineuzumab's approval and its commercial success are both presently unclear.

Concluding Remarks

Antibody-based therapeutics has entered the center stage of drug discovery as a result of a major shift in the effort of many pharmaceutical companies. Maturation of several key recent technologies has shortened the cycle time to generate therapeutic candidate antibodies and has enhanced the safety profile of antibody therapeutics in human patients. Equally important, major efforts in the biopharmaceutical industry are devoted to establishing sophisticated industrial processes for discovery and development of viable candidates. Additional investment is directed to further shorten the development time of antibody-based therapeutics. A major focus on the clinical application of biomarkers, patient stratification to increase the efficacious signal in subpopulations, and employing PK/PD modeling to guide clinical dose selection should prove invaluable in developing biotherapeutic agents with improved clinical activity.

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Chapter 3
Technologies for the Generation of Human Antibodies

Ramesh R. Bhatt, John S. Haurum and C. Geoffrey Davis

Abstract Over the course of the last 15 years antibodies as drugs have come into their own—there are now 26 therapeutic antibodies on the market in the United States. With the passing of time, new technological developments together with competition for finite markets have continually raised the bar for the specifications of newly introduced antibodies. Our intent in this review is to provide a historical perspective on the technologies that have generated the fully human antibody drugs currently on the market as well as to impart a sense of excitement for the technologies in development that will provide the antibody drugs of the future.

Introduction

Over the course of the last 15 years antibodies as drugs have come into their own—there are now 26 therapeutic antibodies on the market in the United States. With the passing of time, new technological developments together with competition for finite markets have continually raised the bar for the specifications of newly introduced antibodies. A key concern, recognized early on, is that of

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immunogenicity. Antibodies that contain non-human sequences can induce an immune response to the antibody itself that can in turn negatively impact its pharmacokinetics or, in extreme cases, cause potentially life threatening allergic reactions.

The very first antibody to be approved for the market, muromonab-CD3, was an unmodified murine antibody (Fig. 3.1). Pursuant antibody therapeutics, with the exception of two radiolabeled antibodies, were engineered to varying degrees with the earliest being chimeric and then later entries being the products of "humanization". Antibody humanization involves the application of various algorithms to replace as much of the murine sequence as possible while seeking to retain the original binding properties. An alternative to humanization is "deimmunization", a process exercised by a few select groups that involves identifying and modifying T cell epitopes. Both approaches are typically labor intensive and time consuming.

By 2001, humanized antibodies had become established as the new standard, such that half of the ten antibodies marketed at that time were humanized. In general, these antibodies performed well in regard to the frequency of immune reactions observed in the clinic, although there were some exceptions. The following year, 2002, however, saw the approval of the first fully human antibody, adalimumab, which targets tumor necrosis factor. Adalimumab was derived from human antibody libraries through the application of phage display technology (Jespers, Roberts et al. 1994). It would be another four years before the second fully human antibody, panitumumab, an antibody targeting the epidermal growth factor receptor, would be approved in 2006.

Panitumumab was derived from transgenic mice that were the products of four different genetic manipulations: inactivation of the murine heavy chain locus, inactivation of the "murine kappa light chain locus", introduction of the majority of the human heavy chain locus, and introduction of the majority of the human kappa light chain locus (Jakobovits et al. 2007). There now exist several different versions of similarly engineered mice with more in development. The use of transgenic mice to generate new fully human antibody candidates offered a significant advance over phage display and humanization in that it reduced the labor, time, and uncertainty involved in discovering antibodies of therapeutic quality—the mice did all the heavy lifting.

During 2009 and the first half of 2010, five antibodies were approved for human use (Fig. 3.1). Four of these are fully human antibodies, and all four were derived from transgenic mouse technology. While it is likely that we will continue to see approvals for humanized antibodies for some time to come, it appears that fully human antibodies are becoming the new standard. The human antibodies currently on the market were largely derived from two strains of transgenic mice, the XenoMouse® and the HuMab mouse, that were previously available for licensing. Each of these strains has now been acquired by a pharmaceutical company, leaving only one strain, the VelociMouse still available to other companies. Other new strains are in development and are expected to be offered for use within the next couple of years. In the meantime, and afterwards, of course, display-based technologies remain a viable option, and there continue to be new technical advances

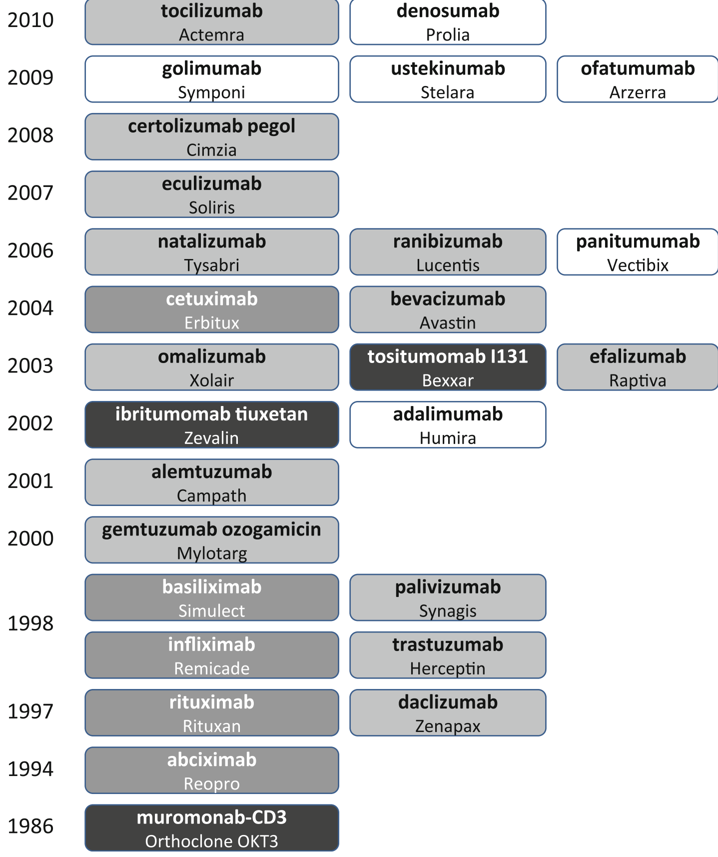

Fig. 3.1 US antibody approvals. Twenty-six therapeutic antibodies have been approved to date in the US. Approvals are shown year by year. For each product, the generic name is shown in *bold* with the brand name shown below. *Black* murine, *dark gray* chimeric, *light gray* humanized, *white* fully human

in that area as well. Some of these latter appear to offer significant advantages in shortening the time to discovery, thus potentially significantly accelerating drug development timelines.

Looking forward, we can expect to see greater diversity in marketed antibodies. Whereas to date all of the monoclonal antibodies are full length "native" molecules, alternative structures and formats are seen to potentially offer advantages in certain indications. Many of these are derived through engineering of existing monoclonals. However, some novel discovery platforms have been developed or are in progress. In this review we will restrict our attention to these novel antibody discovery platforms.

We can also expect to see a renaissance in the development of polyclonal and oligoclonal antibody products. Polyclonal antibody preparations derived from human blood in the form of so-called intravenous immunoglobulin (IVIg) from

other species have been available for decades for the treatment of acute indications. While the potential utility of multi-specific formulations for the treatment, for example, of chronic infection or cancer where high mutation rates may limit the efficacy of a unispecific drug, immunogenicity issues and low specific activity have limited the adoption of this approach. New technologies, based both on recombinant antibody techniques as well as transgenic animals, now appear to be opening the door to the development of fully human polyclonal and oligoclonal antibody products that should offer therapeutic options distinct from those of monoclonals.

Our intent in this review is to provide an historical perspective on the technologies that have generated the fully human antibody drugs currently on the market as well as to impart a sense of excitement for the technologies in development that will provide the antibody drugs of the future. As is often the case, success is driving innovation and innovation is certain to lead to greater success. While antibody drugs have made a dramatic impact on the treatment of disease, we anticipate that the new technologies described herein for deriving fully human antibodies will increase that impact dramatically.

Transgenic Technologies for Generating Fully Human Monoclonal Antibodies

At the time of writing this chapter, the status of transgenic mice for the derivation of fully human antibodies is in flux. As noted above, the two strains of mice that originally led the field, the XenoMouse® and the HuMab Mouse, have been acquired by large pharmaceutical companies and are not likely to be available for licensing in the future. A third strain of mice, the VelociMouse appears to still be available on a limited basis, but nothing has been published on the capabilities of this mouse. Several new mouse as well as rat strains are in development by various groups but are not likely to be available for at least a year. Thus, it seems appropriate to focus this discussion on the properties and capabilities of the historical strains with the goal of providing a framework by which to evaluate future strains and to calibrate expectations.

Established Strains of Transgenic Mice

The groundwork for the commercial strains was laid by Marianne Bruggemann and colleagues. In 1989 (Bruggemann et al. 1989) she reported having generated mice carrying an unrearranged human minilocus comprised of 2 VH, 4 D, all 6 JH, and Cμ genes. These mice were able to rearrange the antibody genes to produce IgM antibodies bearing human heavy chains, albeit at low levels.

At about this time, two biotechnology companies, GenPharm International and Cell Genesys Corporation, launched more ambitious programs with the idea of generating mice that could produce completely human antibodies at sufficient frequency and quality that the mice could be useful platforms for producing therapeutic monoclonal antibodies. To this end, they used gene knockout technology to functionally inactivate both the murine heavy and kappa light chain loci. In parallel, they introduced fragments of the human heavy and light chain loci. After extensive interbreeding of the four different mouse strains, it would be possible to derive a mouse incapable of producing mouse antibodies but fully capable of producing fully human antibodies.

In 1994, both companies published on early proof-of-concept mice. Green et al. (1994) had used the technique of spheroplast fusion to introduce yeast artificial chromosomes (YACs) containing a heavy chain minilocus comprised of 4 VH, 25 D, 6 JH, Cμ, and Cδ genes together with a kappa light chain minilocus comprised of 2 Vκ, 5 Jκ, and Cκ genes. Similarly, Lonberg et al. (1994) had used pronuclear injection to introduce plasmids containing a heavy chain minilocus comprised of 4 VH, 15 D, 6 JH, Cμ, and Cγ1 genes and a kappa light chain minilocus comprised of 4 Vκ, 5 Jκ, and Cκ genes. Upon immunization of the mice finally derived from cross breeding, i.e., with functionally inactivated murine immunoglobulin loci but bearing both human heavy chain and kappa light chain miniloci, both groups saw similar results in that both the heavy and light chain loci underwent gene rearrangement with the result that fully human antigen-specific antibodies could be isolated.

These early results spurred both GenPharm and Cell Genesys to pursue the generation of more complex mice bearing a greater diversity of immunoglobulin genes and hence capable of producing a greater spectrum of antibodies. In the case of the Cell Genesys group, this was accomplished by building larger YACs such that ultimately mice were generated that bore 42 VH genes and 34 Vκ genes together with the complete complement of D and J genes (Mendez et al. 1997). Medarex (having acquired GenPharm in 1997) took the approach of crossing their mouse with a mouse bearing a complete human heavy chain locus derived by the technique of microcell-mediated chromosome transfer (Tomizuka et al. 1997; Tomizuka et al. 2000; Ishida et al. 2002). While the kappa chain locus in the product of this cross was still limited, the observation that the antibody heavy chain typically contributes more than the light chain to antigen binding suggested that these mice, too, would produce a large repertoire of antibody diversity in response to immunization with different antigens.

The productivity of these human antibody-producing mice met and surpassed early expectations. While the overall robustness of their immune systems, as judged by B cell counts and circulating IgG, was less than that of wild type mice (Green and Jakobovits 1998), it soon became clear that it was possible to derive a broad spectrum of fully human antibodies upon immunization with essentially any protein target of interest. Moreover, it was discovered, upon closer inspection, that the antibody response in these mice faithfully mimicked that of humans in nearly every aspect. Gallo et al. (2000) used reverse transcriptase PCR to sift through hundreds of recombined antibody genes from the XenoMouse® and found that

the relative frequencies of use of individual V, D, and J genes as well as the preferred sites and even the preferred substitutions for somatic hypermutation, the process that results in affinity maturation following recombination, mirrored what had been previously published from studies of human populations (Yamada et al. 1991; Brezinschek et al. 1995; Suzuki et al. 1995)

In a later generation of the XenoMouse®, the entire lambda locus was also incorporated into the mouse's genome. As was the case with all previous generations, the endogenous murine lambda locus was left intact since it had been previously determined that the frequency of hybridomas expressing antibodies containing the mouse lambda light chain remained similar to the frequency of lambda expression in wild type mice, i.e., less than 5% and, further, that these human/mouse hybrid antibodies could be easily ignored by the incorporation of an anti-human kappa ELISA assay into the early screens. Once again, a mouse endowed with human antibody genes utilized these genes in a human-like fashion—the overall frequency of utilization of human lambda light chains in mice bearing both human kappa and lambda loci was approximately 40%, essentially equivalent to that of humans (unpublished results).

From a product development perspective, there did appear to be an advantage to immunizing mice that had incorporated the human lambda locus. Besides the obvious advantage of increasing the overall diversity of antibodies meeting the initial design criteria, it was occasionally observed that for narrowly defined design criteria lambda-containing antibodies could greatly dominate the immune response.

To isolate an antibody of therapeutic quality, the emphasis is typically on numbers. Once the design criteria—performance in specific functional assays, affinity, species cross reactivity, etc.—have been defined, the objective is to derive as many monoclonal antibodies that meet those criteria as possible so that multiple candidates can then be advanced into further rounds of more refined testing to find the very best antibody for advancing into the clinic. What this means is that to take full advantage of a human antibody-producing mouse it is important to also build or access a suite of technologies for enhancing efficiencies at every level of antibody recovery and selection. This can be accomplished within the confines of hybridoma technology by employing techniques to enhance fusion efficiencies, promote hybridoma colony growth, and automate screening procedures. Alternatively, it is possible to avoid hybridomas altogether by applying display technologies for recovery of the human antibody repertoire post immunization. A potential disadvantage with this approach is that the initial heavy/light chain pairing will typically be lost, although there are techniques available for maintaining pairing through single cell PCR (Kantor et al. 1995; Yamagami et al. 1999).

Through the immunization of transgenic mice bearing extensive immunoglobulin gene repertoires coupled with robust antibody recovery and screening techniques, it has been possible to generate antibodies of therapeutic quality predictably and reliably. Typically antibodies derived from transgenic mice do not require any further affinity maturation. While it has been proposed that there

should be an "affinity ceiling" of approximately 1×10^{-10} M in mice, beyond which there can be no biological driver due to the on rate being limited by the diffusion coefficient and the off rate being limited by the rate of internalization of surface immunoglobulin (Foote and Eisen 1995), antibodies with affinities well beyond this theoretical limit have frequently been obtained, presumably as products of random mutations. Indeed, Rathanaswami et al. have reported having derived an anti-interleukin 8 antibody with subpicomolar affinity from the XenoMouse® (Rathanaswami et al. 2005).

A further consideration in designing a therapeutic antibody is the isotype required to achieve the desired effector function. For example, of the transgenic mouse-derived fully human antibodies currently on the market, ofalimumab, golimumab, and ustekinumab are IgG1 antibodies while denosumab and panitumumab are IgG2. If the choice of isotype is clear from the outset, it may be preferable to immunize a mouse bearing only that specific isotype. This facilitates early function-based screening in that no molecular manipulation of the antibodies is required. However, it should be recognized that to replace one isotype with another is a relatively straightforward exercise. With the multiple ongoing efforts to enhance Fc receptor binding (Shields et al. 2001, 2002; Lazar et al. 2006; Stavenhagen et al. 2007), complement fixation (Moore et al. 2010), and serum half-life (Hinton et al. 2004; Petkova et al. 2006; Zalevsky et al. 2010) through the introduction of specific mutations into Fc domains, it is likely that in vitro replacement of the Fc domain will become the norm.

In fact, a third transgenic mouse platform for the generation of human antibodies absolutely requires isotype replacement. The VelocImmune® platform, developed by Regeneron, is based on the concept of selectively replacing only the murine variable region genes (Vs, Ds, and Js) with their human counterparts. The rationale for this is that retention of the murine constant regions should allow for better interaction with the signaling components of the surface immunoglobulin receptor complex and may thus allow for better B cell maturation and, in turn, a more fully reconstituted B cell compartment. To date, however, there has been no published data confirming that this is the case.

One potential and often cited shortcoming of the transgenic mouse approach for generating human antibodies is the possibility that immune tolerance might prevent the mouse from reacting to human proteins that are highly homologous to mouse. While it is certainly true that making antibodies to highly conserved proteins is more difficult, there has been some success in eliciting antibodies to even 100% conserved proteins in the XenoMouse® by using a CpG-containing adjuvant (unpublished results).

Obviously, the primary driver for advancing fully human antibodies as therapeutics is to minimize the potential for immunogenicity and thus to optimize the drug half-life. How successful has the transgenic mouse approach been in this regard? Table 3.1 shows the frequency of detection of anti-drug antibody responses to all the marketed transgenic-derived fully human antibodies as well as the marketed humanized antibodies as reported in the package inserts.

Table 3.1 Frequency of immune responses to therapeutic antibodies

Generic name	Brand name	HAHA (%)
Fully human		
Panitumumab	Vectibix	<1
Ustekinumab	Stelara	1–3
Denosumab	Prolia	<1
Golimumab	Symponi	4
Ofatumumab	Arzerra	0
Humanized		
Tocilizumab	Actemra	2
Certolizumab	Cimzia	7
Eculizumab	Soliris	2
Natalizumab	Tysabri	9
Bevacizumab	Avastin	0
Omalizumab	Xolair	<1
Alemtuzumab	Campath	8
Palivizumab	Synagis	<1
Daclizumab	Zenapax	14
Trastuzumab	Herceptin	<1

Fully human antibodies derived from transgenic technologies are compared to humanized antibodies. The frequencies of human anti-human antibody (HAHA) responses are derived from the package inserts for the individual products

It is difficult to make direct comparisons from one antibody to another for all the reasons stated in every package insert, namely: "The incidence of antibody formation is highly dependent on the sensitivity and the specificity of the assay. Additionally, the observed incidence of antibody (including neutralizing antibody) positivity in an assay may be influenced by several factors including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease." However, in aggregate the data indicates that the overall incidence of a human anti-human antibody (HAHA) response to antibodies derived from transgenic mice is very low, although not obviously superior to that of humanized antibodies.

Transgenic Strains in Development

Several companies are in the process of developing new transgenic strains. There are at least three new strains of transgenic mice and one strain or transgenic rat underway. A common theme running throughout seems to attempt to preserve same-species protein interactions in the B cell receptor complex. This is based on the belief that the apparent reduction in the number of B cells and in levels of circulating immunoglobulin in the early strains may have been the result of sub-optimal signaling due to weaker interactions between heterologous proteins in the B cell receptor.

Open Monoclonal Technology, Inc. is developing a novel transgenic rat strain. This has been a particular challenge since heretofore knocking out endogenous genes had required the use of either embryonic stem cells or cell nuclear transfer, neither of which is available for the rat. However, Buelow et al. (Geurts et al. 2009) circumvented this problem by injecting into rat embryos zinc finger nucleases specifically designed to introduce double stranded breaks into an immunoglobulin gene. According to the corporate website (www.openmonoclonaltechnology.com), the human immunoglobulin transgenes will be comprised of a fully human light chain locus and a heavy chain locus that will contain human V, D, and J as well as CH1 genes but rat CH2 and CH3 genes. Thus, the rat will produce human/rat chimeric antibodies that can be readily reengineered to be fully human by replacement of the rat CH2 and CH3 segments with their human counterparts.

Ablexis (www.ablexis.com) appears to be taking a similar strategy in generating a new strain of transgenic mice in that their mice, too, will produce chimeric antibodies, although human/mouse. Ablexis has disclosed that its mice will contain both human kappa and lambda light chain loci. Kymab Ltd. (www.kymab.com) claims to be developing a mouse with a full complement of human V genes, presumably including all the human VH, Vκ, and Vλ genes. Not much else is known about their approach except that they are using cell nuclear transfer to introduce the transgenes into mouse embryos. Further, Harbour Antibodies (www.harbourantibodies.com) has announced that they are also generating a new transgenic mouse capable of making human antibodies, but no details as to its construction have been disclosed publicly to date.

Combinatorial Antibody Library Display Technologies

Ideal human monoclonal antibody discovery technologies should be: (a) derived within the context of a human immune system, (b) unbiased to immune tolerance, and (c) rapid. For obvious substantial ethical and technical reasons, it is impossible to immunize and generate human monoclonal antibodies in a manner similar to mouse-based hybridoma methods. Transgenic systems, as those described above, utilize elements of the human genetic repertoire and allow for rapid and direct identification of human monoclonal antibodies. However, transgenic systems are still influenced by immune tolerance, which can be problematic in the generation of antibodies against human targets that bear significant identity to rodent proteins and epitopes.

Through variable domain recombination, heavy and light chain pairing, and somatic hypermutation, each antibody immunoglobulin repertoire is capable of generating innumerable combinations against a nearly limitless range of targets. While these possible combinations vastly exceed the capabilities and needs of any individual, B cell maturation severely restricts the nature of these combinations through a series of developmental checkpoints. The most critical checkpoint is immune tolerance where B cells that bear heavy–light chain pairs recognizing

"self" antigens are deleted to prevent autoimmunity. However, for the purposes of therapeutic antibody discovery, heavy chain and light chain antibody combinations that recognize "self" antigens are typically the desired outcome. Combinatorial human antibody libraries arose as an effective means to isolate fully human monoclonal antibodies directly from a completely intact human immune system without the interference of immune tolerance. In addition to breaking immune tolerance, combinatorial antibody libraries utilize display systems enabling the identification of monoclonal antibodies in a manner of days rather than months.

Combinatorial Antibody Libraries Break Immune Tolerance Through Unnatural Chain Pairing

Within each individual there exists a repertoire of approximately 1×10^8 antibodies and through simple molecular biological techniques it is possible to rescue and clone the entire antibody heavy chain and light chain collections into permanent plasmid-borne collections. However, in assembling these heavy and light chain collections the combinatorial possibilities reach 1×10^{16}. As even the largest collections of combinatorial antibodies reach approximately 10^{11} it is reasonable to assume that nearly all, if not all, combinations are not original B cell clonal heavy–light chain pairings. In the case of immunized hybridomas from immunized mice, the loss of clonal heavy–light chain pairings could be disastrous; however the disruption of these original B cell clonal heavy–light chain pairings and creation of new heavy–light chain pairs provide the very fundamental ability of combinatorial antibodies to create novel binding solutions against self-proteins, effectively breaking immune tolerance.

Combinatorial Antibody Libraries Bypass Need for Immunization

Through the cloning of large combinatorial antibody libraries ($>10^{10}$) one creates permanent naïve collections that exceed a natural human repertoire by several orders and can thus be interrogated without the need for immunization to identify fully human monoclonal antibodies against a broad range of targets. The key element necessary for naïve combinatorial antibody libraries to be useful is the establishment and maintenance of combinatorial antibody clonality in a robust screenable format. In the case of traditional hybridoma-based approaches clonality is maintained by the physical isolation of individual hybridoma cells. This is possible because positive hybridoma clones can be fairly abundant following immune priming and boosting, and that physical isolation can be accomplished through arrays of physically manageable microtiter plates of growing clones.

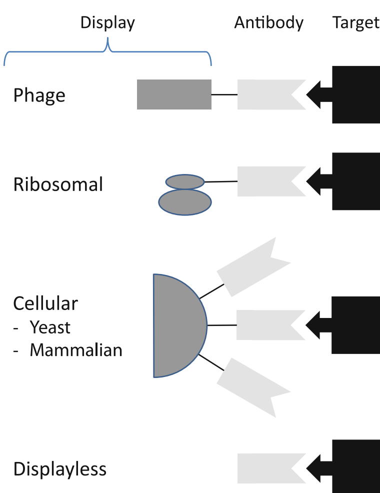

Fig. 3.2 Display systems used for antibody library selection

As positive clones against self-antigens were expected to be present at very low frequency within naïve combinatorial antibody libraries, physically arrayed multibillion member collections were obviously impractical. The key achievement to creating effective and screenable collections of antibodies was linking the binding antibody to the encoded gene and maintaining clonal segregation within the context of millions or billions of clones in a heterogeneous selective environment. As antibodies are complex heterodimeric proteins, the display of these types of proteins has followed the successes of recombinant peptide and protein library display technologies. Below we summarize the key accomplishments that led to each of the display systems utilized and highlight the continuing and future promise of these display technologies. The various systems are compared schematically in Fig. 3.2, and their principal characteristics summarized in Table 3.2.

Phage Display

Phage display encompasses several types of viral display systems but most commonly refers to those systems based upon genetic fusions to a coat protein of the ssDNA filamentous phage m13. Initially lambda phage was used to identify antibodies from binary encoded immunized antibody collections of 10^6 recombinants (Huse et al. 1989). The plaques physically isolated the expressed protein to a replicable virus that could be amplified and sequenced to identify the exact nature of both the heavy chain and light chains of each positive monoclonal antibody. Though the effort was successful and exceeded the speed and efficiency of traditional hybridomas to identify positive monoclonal antibodies, it required physical distribution of the libraries on agar plates and plaque lifts that were maximally capable of assessing repertoires of approximately 1×10^7, which was far below the natural antibody repertoire of humans and mice.

Table 3.2 Comparing display systems for antibody libraries

Format	Valency of display	Typical library size	Advantages	Weakness	Formats displayed
Phage	Monovalent and multivalent	10^{10}–10^{11}	Robust and easy to use	Introduction of diversity by cloning is slow	scFv or Fab
				Not truly monovalent	
Ribosome	Monovalent	10^{12}–10^{14}	In line mutagenesis	Not validated in de novo discovery	scFv
			Large size	scFv required	
Cell surface Yeast or mammalian	Multivalent	10^{7}–10^{10}	Expression and affinity discrimination is possible	Sorting expertise and equipment necessary for advanced selection	scFv, Fab, and IgG
			Capable of therapeutically relevant post-translational modifications	Transformation efficiencies are lower than bacterial/phage systems	
Displayless	Monovalent and bivalent	10^{4}–10^{6}	Capable of functional screens	Very small collections compared to other formats	scFv, Fab, and IgG

The key breakthrough occurred with work involved in developing a method to identify the epitope of an anti-*Eco*RI endonuclease antibody. The approach involved cloning in-frame fragments of the *Eco*RI endonuclease with the pIII coat protein of the m13 filamentous phage genome. The epitope-bearing fragment was shown to specifically interact with an antibody against *Eco*RI endonuclease, and furthermore, the resulting phage was capable of infecting *Escherichia coli* (Smith 1985). This established two key principles for the use of m13-based antibody display; first pIII, which is responsible for infection of *E. coli*, remains functional even when bearing a sizable heterologous fusion protein and secondly, the gene encoding the heterologous fusion is conveniently and stably packaged within a segregable and easily propagated virion particle (O'Connell et al. 2002).

Initial recombinant library efforts exploiting m13 phage pIII display were made with random recombinant peptide libraries and used to identify peptides against anti-peptide antibodies (Cwirla et al. 1990; Scott and Smith 1990). Peptides typically discovered from these recombinant phage peptide libraries had fairly low affinity compared to antibodies. Because pIII has a multivalent presentation of 4–6 copies per virion on the m13 phage, it is believed that the low affinity fusion leads create an apparent high affinity interaction toward target through multivalent avidity presentation of the pIII coat protein. Furthermore, the creation of additionally complex peptides and protein libraries showed that, in general, the larger the fusion, the lower the resulting titer and infectability of the resulting phage. The reason for the reduction in viral titer and infectability is thought to be attributed to destabilization of pIII to cap the virion and steric hindrance of the pIII domains involved in infective binding to *E. coli* pili. Nevertheless, each pointed toward potentially limiting features of pIII phage display. The solution to affinity ceilings, viral titers, and infectability was addressed by the development of the phagemid system for the display of hGH variants and libraries (Bass et al. 1990; Lowman et al. 1991). Essentially the phagemid system allows for the heterologous production and incorporation of recombinant pIII fusions with wild type pIII protein into a phage particle that preferentially packages the plasmid encoding the recombinant pIII fusions through the use of a pIII library plasmid and helper phage (Fig. 3.3). A beneficial aspect of the phagemid system is the ability to approximate monovalent display, thereby reducing the likelihood of recovering low affinity clones. The ability to regulate the expression and incorporation of recombinant pIII and unmodified pIII on the virion resulted in higher phage titers and high infectability. Also, having the recombinant pIII on a plasmid separate from the m13 genomic DNA allowed for more facile cloning and larger collections due to the high copy number plasmids and relatively small plasmid size compared to the m13 genome. Though antibody m13 phage libraries systems are reportedly in use for antibody discovery, phagemid libraries are by far the most common types of libraries in use with affinity panning for the screening and isolation of combinatorial monoclonal antibodies.

The engineering of the scFv antibody format reduced the binding element of an antibody to a single polypeptide, which lent itself well to the first generation of phage display antibody libraries (McCafferty et al. 1990; Marks et al. 1991;

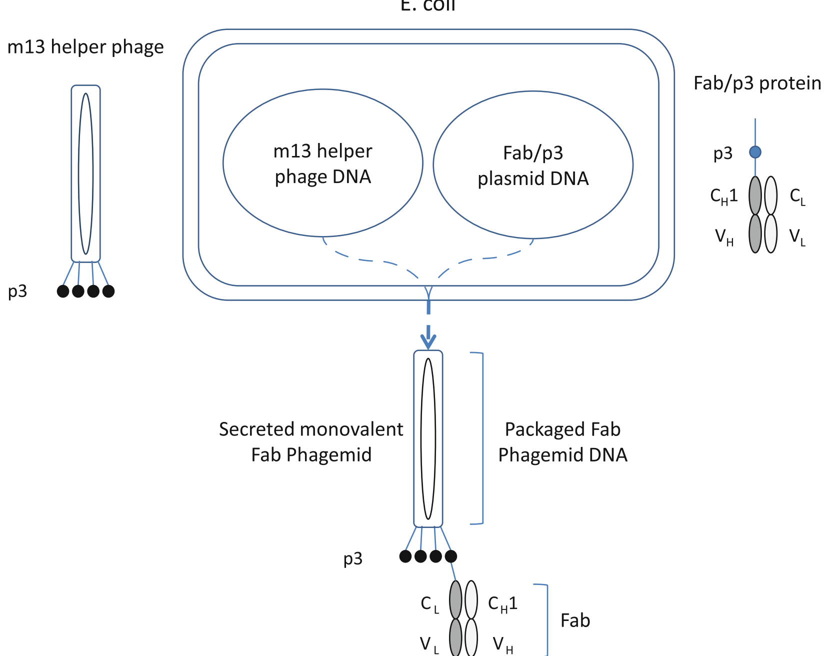

Fig. 3.3 Bicistronic phagemids expressing antibody light chain and heavy chain/p3 fusions are transformed into *E. coli*. Following infection with a helper phage, recombinant Fab phagemid particles are assembled and secreted into bacterial culture supernatants. Phagemids bearing Fabs of interest are enriched by biopanning on immobilized antigen and their specific DNA sequence is deduced

Marks et al. 1992). Continued experience with the scFv format has shown that even though the expression of the scFv antibody format on phage is convenient, the resulting soluble scFv proteins can prove difficult to work with because of a greater propensity toward aggregation than corresponding heterodimeric Fabs. Furthermore, reformatting the scFv antibodies into a heterodimeric Fab or immunoglobulin does not always maintain the binding characteristics of the parental scFv. Subsequent engineering efforts and the development of bicistronic phagemid systems have enabled the establishment of Fab-formatted antibody libraries (Barbas et al. 1991; Hoogenboom et al. 1991). In Fab display libraries it is typical to have the variable heavy and constant heavy 1 domains portion of the heavy chain fused to the pIII protein. Coexpression of soluble antibody light chain and association with the heavy chain pIII fusion protein within the *E. coli* periplasm prior to virion assembly completes the phage Fab display (Barbas et al. 1991; de Haard et al. 1999). More recently pVII/pIX fusion-based display has shown capabilities similar to that of pIII display (Gao et al. 2002; Shi et al. 2010; Tornetta et al. 2010) with two potentially beneficial attributes. The first is that by avoiding pIII as a fusion, each virion is assembled with wild type pIII, therefore

allowing infection to occur unhindered. Secondly, the pVII and pIX coat proteins are considerably smaller compared to pIII, which is toxic to *E. coli*, allowing for potentially greater recombinant protein production in *E. coli* host strains.

In any respect, the clonal display of a high titer antibody phagemid was a major achievement that, combined with affinity panning, underlies the fundamental process of enrichment of pools of reactive clones. This was first accomplished by immobilizing a target of interest directly on the surface of microtiter wells and applying the high titer antibody library to the wells. After an incubation of sufficient time to allow for specific binding, non-specific binders are washed away with buffer and positive clones are typically eluted at low pH, neutralized, and used to amplify again into high titer stock. The iterative approach allows for multiple instances of positive reinforcement to enrich for the best binding solutions. In other instances, affinity panning has been performed using small, typically paramagnetic, bead surfaces derivatized with the target of interest. The main advantage in the use of beads is the greater surface area it provides that can translate into more efficient antibody selection.

Initially, human combinatorial phage display libraries derived their diversity from the immune repertoires of individuals exposed to unique infectious agents to identify antibodies against those infectious agents (Burton et al. 1991; Barbas et al. 1992; Zebedee et al. 1992). Subsequent combinatorial libraries demonstrated that collections made with sufficiently large diversity, from an individual or groups of individual donors, could be used to identify antibodies that were specific for non-pathogenic “self” targets (McCafferty et al. 1990; Marks et al. 1991). The common opinion is the productivity of such libraries is attributable to the source and diversity of heavy and light chain as well as the size of the combined collections (Ling 2003). Even though these naturally occurring combinatorial collections have proven productive against most targets, the natural occurrence of productive binding events seemingly remains random and distributive (Lloyd et al. 2009). As expected, the heavy and light chains utilized in these types of antibody libraries usually contain somatic mutations, not only in their CDRs, but also in their framework regions. However, these mutations were most likely positively reinforced in response to foreign pathogenic antigens that bore no resemblance to the desired target of the combinatorial antibody clone that now uses these chains. Simply put, not all of the framework mutations found in these first generation combinatorial antibodies were probably necessary for binding to the new target. Therefore, one way to simplify displayed antibody repertoires and create a greater number of unnatural compositions to bind self-targets was to incorporate diversity specifically into the CDRs and “hot spots” of the antibody structure within a fixed framework. This synthetic diversity approach has used random diversity (Barbas et al. 1992), biased diversity (Lee et al. 2004), and in some instances diversity reduced to use as few as four or even two amino acids per position (Fellouse et al. 2004; Fellouse et al. 2007). As a next step to expand productivity, the synthetic approach was later expanded to include not only combinatorial diversity of CDRs, but also a diversity of variable framework domains from the breadth of the immunoglobulin germline families (Knappik et al. 2000).

While the preceding examples used either naturally occurring or synthetically derived diversity, there are instances where hybrid approaches have been successful. For instance, one semi-synthetic approach has incorporated synthetically designed semi-random diversity into heavy chain CDR1 and CDR2, in combination with rescued heavy chain CDR3 diversity from natural antibody repertoires (Hoet et al. 2005). Another approach has been the use of shuffled naturally derived diversity for all three CDRs into an invariant heavy chain framework (Soderlind et al. 2000). Irrespective of the approaches for generating combinatorial diversity, phagemid and phage antibody display have dominated the field of combinatorial antibody library engineering for the past two decades, and because of its robust historical performance, phage display will likely continue to be used as the cornerstone of new formats and library approaches as they are developed.

Ribosomal Display

Through the use of combinatorial libraries and synthetic design, immune tolerance has been removed from the equation of antibody discovery, yet several practical discovery and development challenges still remain. As powerful as phage display is, it is naturally influenced by biological biases inherent in heterologous protein expression in both *E. coli* and m13 virion assembly. It is well established that numerous eukaryotic proteins are not well expressed in *E. coli* or as fusions to phage. Considering the tremendous combinatorial diversity possible with heteromeric production, assembly, and diversity of the human antibody repertoire, it is reasonable to assume that some combinations are at a devastating disadvantage to some other combinations. Taking into account the immense diversity possible from the antibody repertoire, it is reasonable to assume that at least two undesirable characteristics exist. First, it is reasonable to assume that some positive combinatorial solutions are possibly excluded from selection because their sequences or structures are incompatible with phage display. Second, the bacterial transformation step necessary to create and introduce the antibody collections fall considerably short of the combinatorial possibilities. To date, using cloning-based efforts, the largest libraries created have barely surpassed 10^{11} in diversity (Lloyd et al. 2009). Elimination of the bacterial transformation step necessary for phage production in *E. coli* and direct production of a screenable library could provide combinatorial collections that are magnitudes larger than those made by phage.

In research unrelated to antibody engineering, in vitro cell-free protein translation systems had been used as an alternative approach to produce proteins considered “toxic” to *E. coli*. To utilize cell-free in vitro protein translation systems for combinatorial libraries, a method to couple the encoded antibody gene to the antibody protein was necessary. In the case of cell-free systems, the translated mRNA needs to be maintained with the ribosome displaying the nascent antibody in a stable and screenable complex at a clonal level. The first such use

of *E. coli*-based ribosomal display involved a diverse recombinant peptide library composed of 10^{12} members (Mattheakis et al. 1994). This particular approach utilized polyproline peptides to stably complex the mRNA to the translated proteins or peptides. Additional strategies have made use of alternative approaches to form stable complexes that have included translational stalling with rare amino acid codons or, elimination of stop codons, and incorporation of covalent cross-linking agents (He and Taussig 1997). Irrespective of the approach, once a stable formation of a complex of antibody fragment and its encoding mRNA are established, the mRNAs from selected complexes are converted to cDNA, PCR amplified, transcribed, translated, and the process repeated.

Interestingly, the first report of ribosomal peptide libraries showed that high affinity clones were more prevalent than those seen in corresponding phage display libraries (Mattheakis et al. 1994). As the high affinity clones appeared to be low nanomolar, it was suggested that it was a result of a likely monovalent interaction and display. Because pIII is present in up to 5 copies per virion, most antibodies are likely present in a multivalent manner. With phage display multi-valency enables low affinity antibodies to behave and bind, through avidity, as high affinity clones. Though avidity of phage display may be useful in discovering the maximum number of possible binding solutions, it can be a hindrance to performing affinity selections to isolate clones with the highest possible affinities, such as during the active process of affinity maturation. With phagemid display, growth conditions can be established to generate a population of phage cultures that qualitatively behave similar to monovalent proteins. Still, it must be recognized that to approximate monovalent display, the growing culture very likely generates numerous phage lacking recombinant antibodies, therefore reducing the effective size of the libraries screened. Furthermore, when a naïve collection or a positive clone is expanded into a mutagenic collection, the conditions established to simulate monovalent display from a single clone does not ensure the same result from a population of recombinant phagemid. One can simply consider the conditions an approximation that is appropriate for a particular clone and that new conditions would likely be necessary for each new heavy chain and light chain combination. However, in the case of ribosomal display, it is very likely the length of the transcribed message that dictates the copy number and valency of the display. Therefore, the relevance of monovalent approximation in the case of the first peptide libraries was significant and applicable toward antibody display, and especially in the case of affinity optimization, where monovalent display enables easier affinity optimization. As such, because of the seemingly monovalent nature of the display, the most successful applications of ribosome display have been in the field of affinity maturation of antibodies (He and Taussig 1997; Hanes et al. 1998; Hanes et al. 2000; Zahnd et al. 2004).

In applying ribosomal display to affinity maturation, direct cloning and selection of mutagenic collections can be employed. However, as most in vitro display systems utilize mRNA rescue and PCR-based cDNA amplification, each step can be altered in ways to incorporate mutations to the base sequences of these collections. Most commonly, mRNA rescue can be tuned to increase the error-prone nature of the reverse

transcriptase enzyme, while another approach is to alter the PCR amplification conditions to increase mutagenic amplification (Leung et al. 1989). In yet another approach, a highly error-prone bacteriophage Q beta RNA replicase has been used in the optimization of shark antibodies (Kopsidas et al. 2006; Kopsidas et al. 2007). In any event, utilizing several variations on the approach for ribosomal display, the maturation of antibodies with picomolar affinities can be readily achieved.

Ribosomal display has been effectively employed despite the apparent monovalent display of this platform. However, because ribosomal display libraries can bypass the low efficiency bacterial transformation step required for phage display, it should offer an opportunity to surpass library sizes of phage display by several orders of magnitude. Remarkably, it is not yet apparent, from a survey of the use of antibody ribosomal display, that this advantage has been achieved or leveraged. Several plausible reasons exist for the lack of utility in antibody discovery efforts using ribosomal display. First, it is possible that ribosomal systems may be less efficient in the assembly of antibodies required for proper display. If the efficiencies were several orders less functional than phage display libraries, such a use of ribosomal display for de novo discovery would likely serve no particular advantage in assessing greater potential diversity in larger libraries. Secondly, no efficient and robust ribosomal systems have been described to translate and physically segregate the genetic material necessary for dimeric protein diversity required to assemble Fab-like molecules, such as those utilized in phage display. With phage display, the heavy and light chains are assembled in the periplasm of *E. coli* and clonality is preserved through the assembly of the completed virions. The inability to assemble, display, and clonally segregate Fabs with ribosomal display limits use to monomeric polypeptides, and for antibodies, this translates into requiring the use of scFv display. Though ribosomal display has successfully affinity matured numerous antibodies using the scFv format, for affinity maturation of antibodies, caution must be exercised to ensure that these improvements are not dependent upon the scFv format and that they carry over to the final desired IgG format.

Yeast Display

Yeast has been used to select antibodies based upon intracellular and extracellular surface display. The first antibody library-based systems involved identification of antibodies via intracellular display of antibody repertoires where yeast two-hybrid technologies were utilized to express a target as a fusion to a promoter site of a transgene and the antibodies were fused to a transactivating domain. Once a productive interaction occurs between the “antigenic bait” and a specific antibody gene, the resulting yeast clone is capable of surviving in a nutrient deprived selection media (Genetastix, company communications, www.genetastix.com).

Surface display on yeast, however, provides the opportunity to select from repertoires of antibodies on a segregatable unit that can be isolated using physical selection technologies not afforded to either phage or ribosomal display. In terms of selection, it is possible to enrich large numbers of pools with magnetic bead

separation strategies, similar to phage and ribosomes. However, because of the larger size of yeast, it is possible to physically discriminate and select clones from naïve or mutagenized collections on the basis of antibody expression levels and binding affinity by dual parameter flow cytometry (Boder and Wittrup 1997; Feldhaus et al. 2003). As such, yeast display has been successfully used in the optimization of several scFv antibodies (Boder et al. 2000; Rajpal et al. 2005).

Recently, systems have been described that are capable of producing fully bivalent IgG on the surface of yeast. Through the common use of the display technologies described above, the discovery of monoclonal antibody binders to any particular target is quite rapid and accomplished within days, considerably faster than any hybridoma-based technology. However, hybridoma technologies yield bivalent IgG that can be immediately scaled up and are appropriate for further in vitro and in vivo testing. Unlike hybridoma-derived antibodies, most display-based combinatorial libraries utilize a binding format that lacks an Fc region and is quite often monomeric. As a consequence of the antibody fragment format, most display-based monoclonals require recloning steps to recreate a full length bivalent IgG, requiring expression in orthologous mammalian systems. This reformatting and protein expression step can take a considerable amount of time and become a bottleneck to most display-based systems when compared to hybridoma-based systems. Recent examples have been presented demonstrating that yeast IgG libraries can be created, screened, and the resulting monoclonal yeast clones minimally manipulated to convert IgG expression from the cell surface tethered to a fully secreted and soluble IgG. Furthermore, the process provides an integrated acceleration of discovery to IgG suitable for further testing in a timeline of approximately one month. The obvious advantage of such a system is the ability to rapidly enable pharmacological testing of the monoclonals with greater throughput by removing the need to switch expression clone backgrounds and additional cloning to produce material suitable for pharmacological evaluation. (Adimab, public presentations, www.adimab.com).

Mammalian Display

Therapeutic antibodies are currently manufactured in mammalian host cell systems and are anticipated to be for the foreseeable future. As a consequence, there has been considerable interest in developing and optimizing mammalian display systems. Numerous reports have described the display of antibodies and antibody fragments on the surfaces of cells, but few have convincingly demonstrated de novo antibody discovery or robust optimization. Nonetheless, some very promising examples exist. In the case of discovery libraries, at least two viral transduction systems have been described. One group used a sindbis viral system to deliver surface-displayed antibody collections and successfully isolated antibodies against pathogenic protein targets (Beerli et al. 2008; 2009). In another effort, vaccinia virus was used to separately deliver light chains and surface

tethered heavy chains that were used to successfully isolate antibodies against several protein-based targets (Vaccinex, company communications, www.vaccinex.com). In either case, flow cytometry was used to isolate positive clones. Mammalian display has also been used in the area of antibody optimization. In one instance, diversity was incorporated by directed mutagenesis of a limited number of residue hotspots to create a repertoire of approximately 10^6 members that resulted in a 500-fold improvement in affinity compared to the parental clone (Zhou et al. 2010).

An interesting aspect of mammalian display is the ability to utilize molecular elements of somatic hypermutation found in B cell maturation. At least two examples are described that exploit aspects of somatic hypermutation; one successful example was expressing the protein (or antibody) of interest in a cell that is naturally capable of somatic hypermutation. More specifically, Wang et al. describe non-immunoglobulin protein optimization of a fluorescent protein in a Ramos B cell line. Iterative expression in this cell line led to red fluorescent proteins with increased photostability and far-red emissions that were better than previous structurally directed engineered variants (Wang et al. 2004). In yet another approach, overexpression of activation-induced cytidine deaminase was used to exogenously drive somatic hypermutation in cell lines not normally associated with somatic hypermutation (Martin and Scharff 2002). Recent presentations have been made regarding the utilization of recombinant activation-induced cytidine deaminase somatic hypermutation not only for the optimization of antibodies, but also for the discovery of antibodies displayed on the surface of mammalian cells. (AnaptysBio, company communications, www.anaptysbio.com).

Displayless

One interesting development in antibody display is in the area of displayless discovery strategies. These involve the screening of fully soluble antibodies or antibody fragments coupled with subsequent deconvolution of positive clones and pools. One recent successful application of this approach was shown in the identification of anti-influenza antibodies from the supernatants of stimulated and immortalized B cells. In this case the supernatants guided the selection and rescue of the positive clones (Grandea et al. 2010). In another instance, collections of antibody-secreting B cells were distributed into a semi-solid support and anti-RSV clones were physically mapped by an optics-based antigen binding ELISPOT technique (Collarini et al. 2009; Harriman et al. 2009). Finally, an alternative source of antibody diversity is from pools and clones of *E. coli* produced germline encoded Fabs. The Fabs are programmed with base level germline rearrangements to find antibody leads that can be affinity improved following the identification of the parental rearranged recombinant Fab. Thus far reports have been made regarding successful identification of low affinity hits and their optimization into high affinity variants. (Fabrus, conference presentations, www.fabrus.net)

Development of Oligoclonal or Polyclonal Antibody Products

Polyclonal antibodies have been used to treat or prevent disease in humans for more than a century. Thus, normal IVIg as well as so-called hyperimmune IVIg, collected from human blood donors with a high titer against a particular pathogen (e.g. hepatitis A virus), have a long-standing successful use against a variety of diseases, including infectious disease (tetanus, respiratory syncytial virus in premature babies, cytomegalovirus infection in transplant recipients, rabies, hepatitis A and B, measles, and varicella) and autoimmunity. Such truly polyclonal immunoglobulin products may be more effective than monoclonal antibodies in certain applications characterized by complex and highly variable disease pathogens; however, the supply of human blood-derived products that are consistent from batch to batch and with high specific activity is challenging and costly. To overcome this challenge, recombinant antibody mixtures have been developed as alternatives.

Recombinant Polyclonal Antibodies

Technologies for discovery and development of recombinant polyclonal antibodies have been developed by Symphogen. The idea behind their approach has been to generate a recombinant antibody manufacturing platform, utilizing a manufacturing cell line, which has been modified to produce a complex mixture of antibodies. One such approach has been to identify and characterize broadly the complete repertoire of antibodies against a natural antigen, e.g. the rhesus D antigen, which is the main target of rhesus D immunoglobulin used for prophylaxis of hemolytic disease of the newborn (HDN) and for treatment of idiopathic thrombocytopenic purpura (ITP). Based on a study of the range of reactivities present in anti-D, a polyclonal repertoire of antibodies was selected such that it broadly reflected the natural immune response (Andersen et al. 2007), and the resulting 25 antibodies were produced in a single batch manufacturing process known as Sympress technology (Wiberg et al. 2006). Briefly, the individual antibody expression plasmids were separately transfected into a CHO host cell line and stored before preparing a polyclonal master cell bank consisting of an equal number of input cells for each of the 25 input antibodies. Next, from the said master cell bank, a polyclonal working cell bank (pWCB) was derived before initiation of manufacturing runs, similar to the manufacturing of individual monoclonal antibodies.

A key aspect of the technology, on the one hand, is the need for highly reproducible procedures and processes to keep clonal growth variation limited between batches, and on the other, the development of analytical tools allowing the assessment and verification of process controls and batch to batch variability. Such assays include characterization of the growth and productivity of the

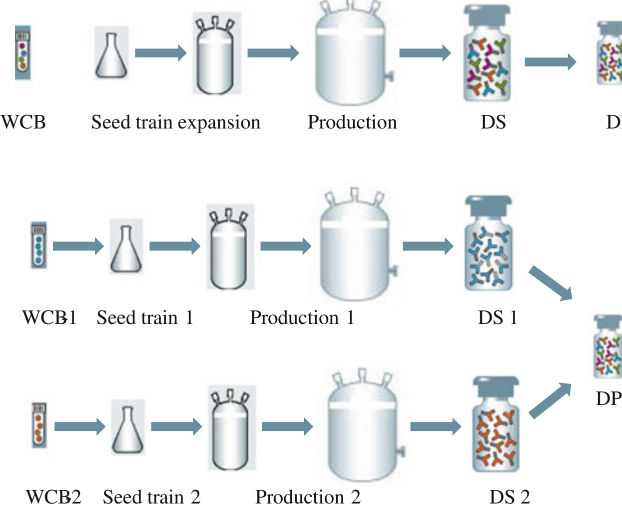

Fig. 3.4 Comparison of production of a polyclonal product from a single cell bank to production of an oligoclonal product from two separate cell banks. **a** A polyclonal working cell bank (pWCB) consisting of a mixture of monoclonal cell lines provides the starting material for generation of a drug substance (DS) and finally a vialed drug product (DP) containing a complex mixture of antibodies in a single linear process. **b** Two separate monoclonal WCBs provide the starting material for two separate production strains, yielding two separate DS that are combined to form the final DP comprised of only two different antibodies

individual antibody constituent clones in order to ensure inclusion of clones with acceptable mean characteristics. In addition, mass spectrometry and ion exchange chromatography-based analytics are incorporated for in process control as well as for comparison of batches with product specifications. The technology has reportedly provided consistent manufacturing batches and has been used to produce products which, as of 2010, are being tested in clinical trials against ITP and HDN. In Fig. 3.4, the process is compared schematically to the typical process for producing oligoclonals.

Oligoclonal Antibody Combinations

A different approach for single batch manufacturing of antibody mixtures has been proposed by Merus, where the manufacturing cell line is transfected with several antibody expression plasmids. In order to avoid the generation of heterogeneity in the form of the various permutations of the non-cognate (scrambled) heavy and light chain pairs, the antibodies are selected using display technology from a library with a shared antibody light chain, thus eliminating the contribution of diversity from the light chain (de Kruif et al. 2010). Merus is currently developing

transgenic mice as a source for antibodies sharing a common light chain (www.merus.nl). A benefit of the Merus technology is the use of a single clonal manufacturing cell bank, which eliminates the risk of drift in the relative ratio of constituent cellular clones during culture. However, the technology may be limited to the expression of oligoclonal mixtures of relatively low complexity, on the order of three to five antibodies.

Several research groups and companies are pursuing the development of antibody mixtures consisting of separately manufactured monoclonal antibody drug substances (DS), followed by the formulation of antibody mixtures into a single drug product (DP) usually in a ratio of one to one (Fig. 3.4). This approach seems straightforward from a developmental perspective and allows for the controlled manufacturing of antibody mixtures, due to the fact that the production can be based on cell lines exhibiting specific productivity profiles equal to mAb manufacturing. This means that at the end of the day, Cost of Goods Sold (COGS) are going to be more or less identical to mAb manufacturing. Naturally, such a development proposal is more costly and challenging during the development phase—as a case in point, two constituent DS batches for production of phase I and II DP will incur twice the manufacturing cost and may for this reason pose a challenge both from a financial and timeline perspective, especially for smaller biotech companies. Additionally, the development challenges include additional analytical methods development for measurement of PK/PD and immunogenicity of the mixture and its constituent molecules after in vivo administration.

From a regulatory perspective, antibody combinations are making headway. For example, a mixture of two rabies virus-specific antibodies is in multiple phase II clinical trials (www.crucell.com) and another mixture of two EGFR-specific antibodies has entered phase I clinical trials as of 2010 (www.symphogen.com). In both instances, regulatory authorities have not required separate clinical testing in humans of the individual constituent antibody components of the final product, only individual preclinical toxicology studies.

Human Polyclonal Antibodies Derived from Immunized Animals

A separate approach to generate specific, immune human polyclonal antibody products has been to create larger animals that are transgenic for the human antibody genes and use these for immunizations and production of human hyperimmune immunoglobulin products. Animals employed in this approach include rabbits (www.roche.com) and cows (Kuroiwa et al. 2002), and the technology remains in development. The promise of this technology is cheaper large-scale manufacturing of genuinely polyclonal hyperimmune human immunoglobulin, in principle against any target of choice. However, risks related to carry-over of animal protein impurities and challenges related to specific activity (titer) remain to be addressed. Also, since the product is dependent on harvest

of blood from immunized herd animals, another challenge is the maintenance of consistent product quality over time.

Alternative Fully Human Formats

The technologies described thus far fundamentally yield conventional fully human antibodies, i.e. antibodies comprised of two copies of a human heavy chain and two copies of a human light chain (H2L2). In this section, additional technologies are described that yield products still derived from human antibody genes but with compositions distinct from the standard H2L2 format.

Heavy Chain Antibodies

Domain antibodies are heavy chain variable (VHH) domain binding units that do not require, and lack, a light chain partner. The first report of a domain antibody came from the study of a murine anti-lysozyme antibody (D1.3) that bound hen egg lysozyme with an affinity of 3 nM. In the study it was reported that the heavy chain alone exhibited specific binding abilities toward hen egg lysozyme equivalent to 19 nM (Ward et al. 1989). The group reported the domain antibodies were expressed in lower yields than the corresponding Fv and were also "relatively sticky," presumably due to the exposed hydrophobic surfaces that were normally occluded by either a $V\kappa$ or $V\lambda$ domain. The authors coined the term "single domain antibodies" or "dAbs" to describe these structures. Though dAbs have not been found to exist naturally in the human repertoire, analysis of camelids showed the presence of binding antibodies that were truly devoid of light chains and constituted at least 75% of the protein A recoverable material present in their serum (Hamers-Casterman et al. 1993). Aside from the lack of light chains, analysis of the camel VH regions showed three prominent differences between these heavy chain only antibodies and standard antibodies (Muyldermans et al. 1994). First, the camel antibodies lacked classic CH1 domains and the variable domains were instead connected via either a short or long linker to a CH2 domain. Second, the camel antibodies contained numerous amino acid substitutions of conserved hydrophobic residues to hydrophilic residues, seemingly to stabilize the molecules to hydrophilic environments. Finally, the CDR3 loops were comparatively long, most likely to increase the overall interactive surface area and compensate for the lack of light chain contacts.

The total output of work with camelids has been used to increase the legitimacy and utility of human single domain molecules. However, considerable protein engineering has been necessary for the stabilization of these domain formatted molecules. In order to stabilize these domains two notable approaches have been taken. The first has been to utilize the knowledge of camel antibodies

to incorporate unique camel elements into the human frameworks to act as stabilizers ("camelization") (Riechmann and Muyldermans 1999). The second approach has been to generate human dAb phage display libraries and screen for aggregation resistant dAbs (Jespers et al. 2004). In this screen a single domain antibody framework was fully diversified within the three CDRs and the library was subjected to repeated rounds of heating and cooling with intervening periods of interaction with protein A or BSA. The resulting clones had excellent thermostability, as well as high yield and recovery from *E. coli*. DAb libraries are now being increasingly used to identify new diagnostics and therapeutics (Chen et al. 2008, 2010). They may also provide building blocks for more facile construction of multivalent and multi-specific constructs (Els Conrath et al. 2001) as compared to conventional antibodies. The most advanced dAb, derived from phage display, targets tumor necrosis factor and entered clinical trials in 2007 (www.Arana.com).

Spurred by the success with transgenic mice in generating normal human antibodies, a similar approach has now been launched for creating transgenic mice to produce human dAbs. In 2006 Janssens et al. (Janssens et al. 2006) reported on transgenic mice that upon immunization were capable of producing high affinity antibodies that were composed solely of heavy chains; these animals lacked the ability to make murine antibodies, but rather contained a locus with two camelid VHH domains and also had the full complement of human D and J genes and a human gamma constant region gene in which the coding sequence for Cγ1 had been deleted. Harbour antibodies has now taken this observation to the next step by replacing the camelid VHH domains with four human VH domains. Upon antigen challenge, these mice have produced fully human heavy chain antibodies with subnanomolar affinities. Importantly, heavy chain antibodies selected in vivo solve the solubility problem independently without having to resort to any camel-derived stabilizing sequences (Harbour Antibodies corporate presentations, www.harbourantibodies.com).

Surrobodies

Another promising fully human format under development is the Surrobody^{TM}. Surrobodies^{TM} are target-specific binding proteins based upon pre-B cell receptors. Pre-B cells are formed immediately following heavy chain V(D)J recombination. The hallmark of pre-B cells is the pre-B cell receptor, which is composed of a transmembrane anchored heavy chain associated with a surrogate light chain. The surrogate light chain is composed of two proteins, VpreB and λ5. It is postulated that the surrogate light chain performs two essential functions; first, it provides a quality control step to ensure the recombined heavy chain is properly secreted and assembled, and, second, it serves as the first opportunity to survey for immune tolerance. If the pre-B cells fail either step the clone is eliminated. However, if no such problems occur, then light chain V–J joining occurs and the remainder of B cell development proceeds (Melchers 1999; Vettermann et al. 2006).

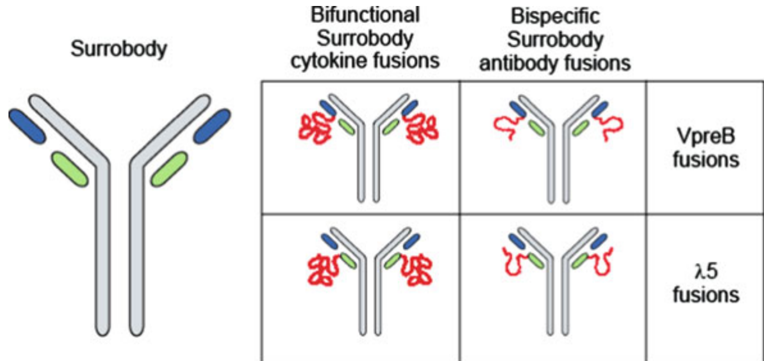

Fig. 3.5 General scheme for SLC genetic fusions for cytokines and antibodies. **a** Color-coded heteromeric Surrobody structure: *light gray chains* represent heavy chains, *blue* represents the SLC VpreB subunits, and *green* represents the SLC λ5 subunits. **b** Recombinant cytokine fusions are represented by *red chains* (*left*) and recombinant scFv antibody fusions are represented by *red chains* (*middle*). Subunit targets for fusions are indicated in right

Recent work has shown that recombinant surrogate light chains partnered with heavy chains, or Surrobodies™, are capable of binding protein targets with high affinity and specificity (Xu et al. 2008, 2010). What differentiates the Surrobody™ from other formats is the nature of the surrogate light chain that affords opportunities for additional protein engineering, not found with standard antibodies. Because the surrogate light chain bears two additional termini, it is instructive toward novel sites to utilize for additional binding energy, specificity, and function. In a structural examination in which model antibody heavy chains were complemented with a surrogate light chain, the resulting molecule did not show any binding to target and the investigators postulated the non-immunoglobulin tails of the VpreB protein occluded the heavy chain from binding target (Bankovich et al. 2007). However, the work with the phage display repertoires showed this to not be the case (Xu et al. 2008). Not only can Surrobodies™ bind target with high affinity and specificity, but they are also amenable to bi-functional and bi-specific engineering (Fig. 3.5). In subsequent work Surrobody™ bi-functionality was demonstrated with Surrobodies™ bearing IL-2, recombinantly fused to either the VpreB or λ5 tail, which were capable of simultaneously binding a cognate target and an IL-2 receptor. Bi-specificity was also established with H5N1-specific Surrobodies™ bearing H3N2-specific scFv fused to either the VpreB or λ5 tail that were capable of binding the hemagglutinins from either H5N1 or H3N2 (Xu et al. 2010). In either case, the ease of engineering this accommodating binding format suggests the Surrobody™ is a novel and facile platform for the generation of bi-functional and/or bi-specific fully human biologic agents.

Concluding Remarks

We have attempted here to provide an overview of technologies for the generation of fully human antibody therapeutic products. In doing so, we have used an arguably narrow definition of “antibody” in that we have restricted our review to technologies that yield products consisting of full length heavy and/or light chains that are derived directly from naturally occurring human genes. Of course, there are many other technologies that can and will yield useful antibody products, as has been clearly demonstrated by the clinical success of multiple humanized antibodies. In addition, there are a number of antibody-like scaffold technologies in development that may ultimately provide products with certain distinct advantages over conventional antibody products. The products of these technologies, taken together, promise to dominate the realm of therapeutic biologics for some time to come.

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Chapter 4
Application of Antibody Engineering in the Development of Next Generation Antibody-Based Therapeutics

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Abstract The evolution of therapeutic antibodies has encompassed multiple engineering efforts in the hope of improving the efficacy, safety, and duration of effects of antibody-based drugs. Advances in protein engineering technologies afforded investigators the ability to overcome problems associated with introducing foreign antibodies into humans. These efforts included antibody chimerization, humanization, and the more recent development of human antibodies, all of which reduced anti-drug immune responses. Additional efforts have engineered antibody variable regions that encode multiple specificities into a single molecular entity. Apart from optimizing antigen-binding capabilities and reducing immunogenicity, many advances have been made that modulate an antibody's ability to interact with cells and serum components of the immune system. Manipulation of antibody glycosylation or the amino acid sequence has had a significant impact on recruitment of the Fc-dependent effector functions. This chapter presents an overview of *V* region and Fc modifications and focuses on advances in engineering to tailor an antibody's function relative to the intended therapeutic need.

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Introduction

Antibodies are complex glycoproteins and key components of the adaptive immune response. There are five classes of human antibodies: IgA, IgD, IgE, IgG, and IgM. The basic structure of monomeric IgA, IgD, and IgG antibodies is comprised of two antigen-binding fragments (Fabs) coupled to a single crystallizable (Fc) fragment by a core hinge (Fig. 4.1a). In contrast, the monomeric forms of IgE and IgM antibodies lack a core hinge. IgG is the most prevalent class of antibody in serum and non-mucosal tissues and is the most common class of molecular format used in biological therapeutics. IgGs are comprised of two identical heavy chains (HCs) and two identical light chains (LCs) covalently associated with each other by disulfide bonds. Human LCs are divided into two classes, κ and λ. Each LC has a single variable domain (V_L) and a single constant domain (C_L). The HC consists of a single variable domain (V_H) and three constant domains (C_H1, C_H2, and C_H3), where the C_H3 domain is located at the C-terminus. Diverse germline IgVL and IgVJ gene repertoires encode the V_L domain, whereas V_H domains are encoded by repertoires of IgVH, IgDH, and IgJH genes (Matsuda et al. 1998; Schable et al. 1994; Tomlinson et al. 1992; Tonegawa 1983). The germline gene diversity and further variations contributed by somatic mechanisms, such as somatic hypermutation (Neuberger 2008) and gene conversion (Mage 2006), are concentrated in the complementarity-determining regions (CDRs). There are three such regions in V_L: CDR-L1, CDR-L2, and CDR-L3 and three in V_H: CDR-H1, CDR-H2, and CDR-H3. These regions are alternated with conserved regions called framework regions (FRs), four in V_L: FR-L1, FR-L2, FR-L3, and FR-L4 and four in V_H: FR-H1, FR-H2, FR-H3, and FR-H4. The six CDRs are brought together by folding and non-covalent association of the V domains to form the antigen-binding site (Fig. 4.1b), which is responsible for the fundamental properties of specificity and affinity of the antibody against the antigen.

The Fc domain links the antibody to immune effector pathways. The hinge region of IgG, as well as discrete locations further along the C_H2 domain, contain critical residues that facilitate interactions with the Fc gamma family of receptors (FcγRs) and complement. Engagement of FcγRs on immune effector cells triggers cellular responses, such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP), whereas complement fixation leads to activation and formation of the membrane attack complex, which results in cellular lysis. Additionally, IgG antibodies contain an *N*-glycosylation site at asparagine 297 in the C_H2 domain. Modification of this *N*-linked glycan can have profound effects on Fc-mediated effector functions. Amino acid residues located near the C_H2–C_H3 junction engage the MHC class I-related receptor, known as the neonatal Fc receptor (FcRn). Fc interactions with FcRn are responsible for transporting IgGs across the fetal/maternal barrier, protect IgGs from catabolism, and thereby contribute to the long circulating half-life of IgGs compared to other serum proteins of comparable size.

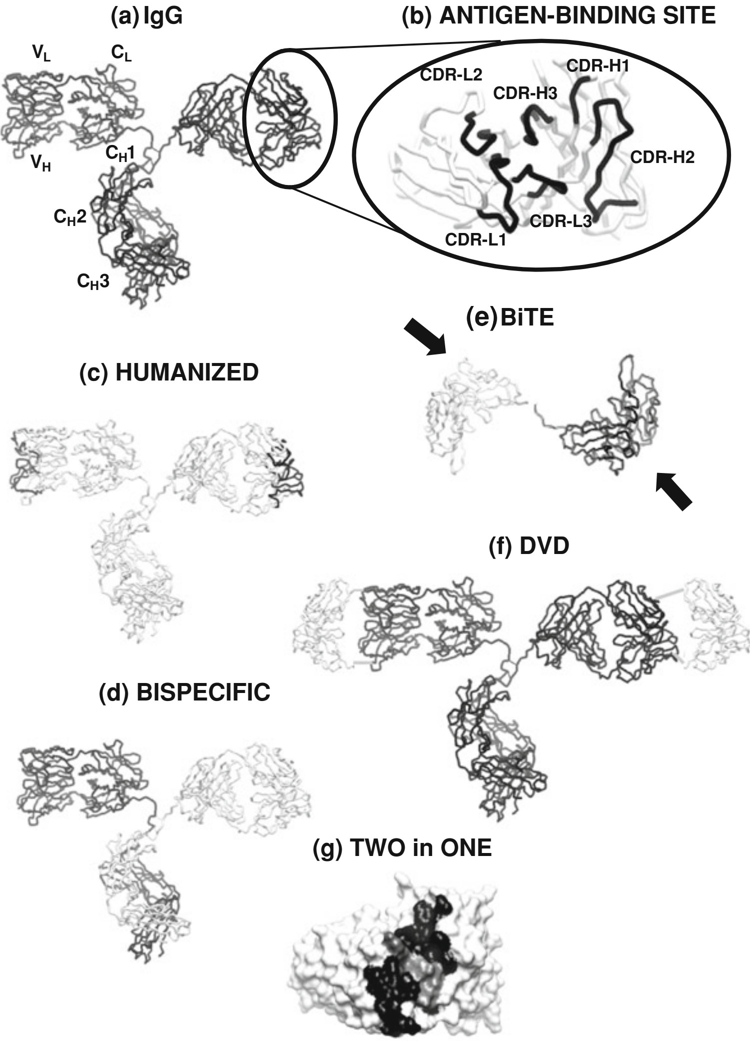

Fig. 4.1 Structure of an IgG, antigen-binding site, a humanized antibody and 3D models of antibody formats inspired on the antibody molecule used to generate bi- and multispecific molecules. **a** Structure of an IgG molecule comprising several domains. The coordinates used to generate the figure correspond to the structure with pdb code: 1IGT. **b** Fv fragment viewed from the antigen perspective. The antigen-binding site is colored in black, following Kabat's definition of CDRs. **c** Humanized antibody. The mouse CDRs are colored in *black*. **d** Bispecific format combining the LC and HC from one antibody (*black*) with LC and HC from another antibody (*gray*) to generate a hybrid IgG molecule with each Fab arm recognizing a different target. **e** Tandem of scFvs that bind two different targets, in the case of BiTE, one of the targets is CD3. *Arrows* indicate the position of the antigen-binding sites. The coordinates used to generate the figure correspond to the structure with pdb code: 2KH2. **f** DVD format, which combine Fv fragments with an IgG molecule. **g** Connolly surface of the same Fv shown in **b** now illustrating the two-in-one format. Residues determining binding to the targets are in *black* with an overlapping region in *dark gray*

The first monoclonal antibody approved by the Food and Drug Administration (FDA) for use in humans was an immunosuppressive murine anti-CD3 epsilon subunit monoclonal antibody named Orthoclone OKT3® (muromonab-CD3), indicated for the treatment or prevention of organ transplant rejection in patients receiving a donor heart, kidney, or liver (Chatenoud and Bluestone 2007). It was recognized early on that individuals receiving OKT3 developed immune responses against the murine variable and constant regions that could neutralize the immunosuppressive properties of the therapeutic (Legendre et al. 1992; Norman et al. 1988; Woodle et al. 1991). Additionally, many patients exhibited a first dose reaction within hours of OKT3 administration, characterized by increased levels of cytokines (Chatenoud et al. 1990), presumably due to interactions between the murine Fc region and human FcγRs (Alegre et al. 1994; Xu et al. 2000). Ultimately, OKT3 Fc:FcγR interactions led to cytokine release and adverse events from the resultant cytokine storm upon initial dosing, and the induction of human anti-murine antibodies resulted in rapid clearance of the injected therapeutic antibody on subsequent treatments. Some of the early antibody engineering efforts were directed toward reducing immune responses by replacing murine constant regions with human constant regions, termed chimerization (Morrison et al. 1984). These initial engineering efforts were followed by humanization (Jones et al. 1986), in vitro selection of human antibodies via phage display technologies (McCafferty et al. 1990), and engineering transgenic mice that encode human variable and constant regions to obtain human antibodies (Lonberg et al. 1994). These advances in the antibody engineering field have significantly reduced the occurrence of anti-therapeutic antibody immune responses (Nelson et al. 2010).

In addition to engineering the V regions within the Fab arms to reduce anti-drug immune responses, ongoing engineering efforts have been made to alter the Fc domain to either reduce or enhance Fc-mediated immune effector functions depending on the specific clinical situation (Labrijn et al. 2008; Presta 2008; Strohl 2009a). In situations where Fc interactions with immune cells or complement could lead to adverse events, the Fc domain can be rendered silent (Labrijn et al. 2008). In contrast, increasing Fc effector functions may improve the efficacy of some therapeutic antibodies, particularly those directed against cancer cells. Clinical studies using antibody therapeutics against cancer have shown that patients who express higher affinity polymorphisms of FcγRIIa (H131) and FcγRIIIa (V158) have longer progression-free survival than those patients expressing the lower affinity polymorphisms (R131 on FcγRIIa and F158 on FcγRIIIa) (Bibeau et al. 2009; Cartron et al. 2002; Musolino et al. 2008), implicating Fc/FcγR interactions as a contributing mechanism for tumor suppression. Extensive work has been conducted to engineer monoclonal antibodies to alter their binding capabilities to lower affinity FcγRs through amino acid modifications and glycoengineering (Presta 2008; Strohl 2009a). Fc engineering has also been performed to improve complement-dependent cytotoxicity (CDC). Additionally, investigators have been able to increase or decrease the circulating half-life of therapeutic antibodies by altering interactions with FcRn. Because of the breadth of engineering efforts, this chapter provides an overview of engineering methods

used to improve efficacy and potency of antibodies and describes select cases where antibody engineering advances led to the next generation of antibody therapeutics. We first describe strategies to select specific V regions, followed by engineering methods designed to optimize human content, and improve affinity and enhance stability optimization, as well as technologies to generate multi-specific molecules. Finally, we describe Fc engineering efforts to alter effector functions and the half-life of antibody-based therapeutics.

Strategies to Select Specific V Regions, Improve Affinity, Stability, and Generate Multispecific Antibodies

Generation of Specific V *Regions*

The traditional method to generate monoclonal antibodies as first described by Kohler and Milstein (1975) consists of immunizing a mouse, typically BALB/c, with a target, isolating B cells from the spleen and then fusing the B cells with myeloma cells. This process results in a hybridoma, which is a cell containing the genes to produce a target-specific antibody and the potential for indefinite propagation in culture. Thus, hybridoma technology ultimately permits the isolation and purification of target-specific antibodies in significant quantities by providing a source for potentially unlimited quantities. This technology has recently been expanded to generate rabbit hybridomas (Huang et al. 2007). Since rabbit antibody diversification mechanisms are different from those of mice (Mage et al. 2006), rabbit hybridoma technology has the potential to generate antibodies against targets and epitopes not accessible to the traditional murine hybridoma platform. However, mouse and rabbit antibodies are highly immunogenic in humans because they are foreign proteins. Humanization has reduced immunogenicity while preserving specificity and potency.

In an effort to isolate human antibodies and thus bypass the need for humanization, phage display technology developed by George Smith in 1985 to display peptides on the phage surface was adapted at the beginning of the 1990s (McCafferty et al. 1990) to display antibody fragments (see Chap. 3). During the 1990s and the present decade, several academic laboratories and biotechnology companies have designed and implemented human antibody phagedisplay libraries for antibody discovery (Bradbury 2010; Hoogenboom 2005). Such libraries have enabled the isolation of high affinity and specific antibodies against a wide range of molecules. Since phage display bypasses immunization, it is especially useful for obtaining antibodies against conserved targets across species and those that may be toxic where in vivo methods are ineffective and/or impractical. In addition, since phage display technologies allow access to the repertoire of genes intended for expression and display on the phage surface, the number of genes and variants can be designed or chosen to bias the repertoire toward genes with predefined

characteristics. For instance, libraries have been designed to generate molecules with a propensity to recognize certain types of generic specificities, such as proteins (Almagro et al. 2006), peptides (Cobaugh et al. 2008), and haptens (Persson et al. 2006). Furthermore, since the selection process occurs in vitro, it enables selection against different antigen concentrations, ionic strength, pH, selection matrix, and temperature, just to mention a few variables. Thus, selection conditions can be manipulated to pan the library against specificities difficult to obtain in vivo and/or isolating molecules with enhanced biophysical profiles (Jespers et al. 2004).

Another platform for discovery of specific human *V* regions that bypasses humanization was developed in the mid-1990s. In 1994, two groups (Green et al. 1994; Lonberg et al. 1994) showed that immunization of transgenic mice that contained portions of the human heavy and light chain loci generated antigen-specific human antibody responses. The genetic modifications supported the development of functional B cells that produced antibodies in response to antigen challenge, and some of the resultant antibodies underwent affinity maturation and class switch recombination (Harding and Lonberg 1995a, b). A variety of additional mouse strains, created using larger human immunoglobulin transgenes (Harding and Lonberg 1995a, b; Lonberg 2005), have followed these initial works (Pappas et al. 2009).

Finally, a recent development (Reddy et al. 2010) to isolate specific antibodies consists of high-throughput (HTP) sequencing and bioinformatics. The rationale is that the *V* gene repertoire becomes highly biased after immunization, with the most abundant genes represented at frequencies between ~1 and >10% of the total repertoire. Therefore, by sequencing enough *V* genes after immunization and ranking their usage frequency, the most frequent genes are identified, synthesized, and expressed as recombinant antibodies in bacteria or mammalian cells. Antibodies generated in this manner from six mice, each immunized with one of three antigens, were found to be antigen-specific and with affinities in the nM range (Reddy et al. 2010).

Humanization

Humanization of antibodies is designed to increase the human content of the *V* region obtained from non-human sources such as antibodies secreted by mouse or rabbit hybridomas. The first humanization method successfully applied to engineer an antibody with therapeutic value, alemtuzumab, was CDR grafting, which was developed in the 1980s (Jones et al. 1986) and is represented in Fig. 4.1c. As of July 2010, the FDA has approved 28 antibodies for therapeutic applications in humans and 13 are humanized molecules (Reichert 2010b). The success of humanization, combined with numerous patents protecting the original CDR grafting method (US Patent 5,225,539 to Winter and Jones) and its variations (US Patent 5,693,761 to Queen et al. and 5,821,337 to Carter et al.)

fueled the diversification of humanization methods in the last two decades. Some of the methods developed in the 1990s and 2000s, often called rational methods (Almagro and Strohl 2009), include resurfacing (Padlan 1991), deimmunization (De Groot et al. 2006), specificity-determining residues grafting (Tamura et al. 2000), superhumanization (Tan et al. 2002), human string content optimization (Lazar et al. 2007), and germline humanization (Pelat et al. 2008). These methods have in common the design of fewer humanized variants to be tested for binding or any other property of interest based on sequence and structural considerations. If the designed variants prove to be unsatisfactory, a new design cycle and binding assessment is initiated.

Other humanization methods, sometimes called empirical methods (Almagro and Fransson 2008), rely on selection rather than on the design cycle. These methods emerged with the invention of phage display and high-throughput screening (HTS) techniques during the 1990s. Phage display and HTS offered efficient tools to explore combinatorial libraries of billions of antibody variants and select those of interest with relative ease. A typical example of an empirical method is guided selection (Osbourn et al. 2005). This method enabled the discovery of the first phage display antibody approved by the FDA called Adalimumab (Reichert 2010b) for treatment of rheumatoid arthritis and Crohn's disease. In contrast to the rational methods to humanize antibodies, guided selection makes no assumptions on the impact of mutations on the antibody structure and binding. The method produces a human version of a non-human antibody by transitioning from non-human to human sequences via chimerical molecules with similar characteristics to those of the parental antibody. Another example of an empirical method is FR shuffling (Dall'Acqua et al. 2005), which is based on the generation of a library of humanized antibody variants by combining non-human CDRs with a repertoire of mixed human germline FRs, followed by screening for binding to antigen. Strategies combining both empirical and rational methods have also been recently published (Fransson et al. 2010).

Affinity Optimization

Affinity losses are a frequent side effect of humanization (Hwang et al. 2005). In addition, most of the antibodies isolated from phage display libraries have affinities in the low nM or high pM range (Hoet et al. 2005; Rothe et al. 2008; Shi et al. 2010). Since higher affinity antibodies can be more efficacious in some indications and/or can be administered in lower or less frequent doses, increasing the affinity to the low pM or even fM range may be desirable (see Chap. 7). Affinity optimization is typically accomplished by creating a library of antibody variants using the parent molecule as the *V* region template and display technologies, e.g., phage (Barbas et al. 1994), ribosome (Hanes et al. 1998), or yeast display (Midelfort et al. 2004) to select the variants of interest from the library. Diversity can either be introduced randomly across the parental *V* gene

(Hawkins et al. 1992, 1993) or targeted to specific regions of the *V* gene (Lowman et al. 1991). The inherent properties of ribosome display, i.e., PCR amplification between selection rounds, make it well suited for random approaches. Focused diversity is typically applied to phage and yeast display, and has the advantage over random mutagenesis in that one has more control over the consequences of changes introduced into the *V* gene. This is critical when optimizing a therapeutic antibody, since indiscriminate introduction of mutations could generate immunogenic spots and destabilizing mutations. An additional advantage of focused diversity is that variation can be concentrated in a given region of the protein, and thus the sequence space of a set of predetermined positions can be exhaustively explored using saturation mutagenesis.

Most affinity maturation strategies for antibody affinity optimization have concentrated on the antigen-binding site. Since CDRs are on average nine residues long, the sequence space generated by saturation mutagenesis cannot be exhaustively explored if more than one CDR is targeted for diversification. Therefore, libraries of individual CDRs are cloned and selected in parallel against the target (Yang et al. 1995). The best variants of this first round of selection are then combined and screened for improved binding. An alternative to this parallel approach is to conduct sequential selections by choosing the best variant in one CDR library and use it as the starting point to optimize the next CDR. The latter strategy has consistently yielded variants of improved affinity (Yang et al. 1995).

More focused strategies in which only the sequence space of the CDR-3 regions is explored have been published (Schier et al. 1996). The rationale is that CDR-3 regions are located at the center of the antigen-binding site and thus play a major role in determining the specificity and affinity of antibodies. Focusing on CDR-3s, Schier et al. (1996) were able to enhance the affinity of an anti-ErbB2 antibody by more than three orders of magnitude, down to 13 pM. The authors optimized the CDR-L3 first, followed by CDR-H3 and combined mutants with improved affinity from parallel selections. Alternatively, only a few residues within a given CDR can be diversified, which enable exploration of more than one CDR at a time. Investigators have targeted for variation residues predicted to be in contact with different types of antigens, called specificity determining residues usage (SDRU) (Fransson et al. 2010). This strategy has the potential to explore synergistic combinations of residues coming from different CDRs.

Minimizing Aggregation

Proteins have an inherent tendency to aggregate, especially at the high concentrations for which they are often formulated for therapeutic use. Hydrophobic interactions appear to be a major contributor to aggregation. For instance, a case study of engineering monoclonal antibody solubility was recently reported (Pepinsky et al. 2010). In the crystal structures of the Fab fragments of IgG1 and IgG2 subtypes, a large hydrophobic patch in the CDRs was found to involve

CDR-CDR and CDR-FR interactions. The solubility of some of these mutants was significantly improved by mutating the hydrophobic residues, adding glycans to C_H1, or switching the isotype to remove the hydrophobic interactions.

Aggregation can also be due to more specific protein–protein interactions (Wu et al. 2010). The IgG of a phage derived antibody, CNTO607, proved to be poorly soluble ($\sim$13 mg/ml), whereas the Fab fragment was soluble to more than 100 mg/ml in phosphate-buffered saline (PBS). The crystal structure of the Fab fragment revealed a tetrameric arrangement of the Fab in which the CDR-H3 loops were nestled against the elbow regions of the other Fabs of the tetramer (Teplyakov et al. 2009). It was postulated that the two Fab moieties can engage in similar IgG–IgG interactions independently, leading to the formation of large clusters and eventually insoluble aggregates due to the bivalency of the IgG. By strategically placing a glycan in the CDR-H2 to interfere with this presumed mechanism, the solubility of the resultant IgG was improved well over 100 mg/ml in PBS (Wu et al. 2010). This result suggests that antibody aggregation is perhaps a more complex issue than generally understood. Therefore, careful studies including analysis of the structures to dissect the mechanism of aggregation are an important component of engineering antibodies with increased solubility for therapeutic settings.

Enhancing Physical Stability

Physical stability can be affected by a number of factors, such as temperature, pH, pressure, denaturing agents (e.g., guanidine hydrochloride, surfactants), and mechanical disruption (e.g., shaking and shearing). Thermodynamic stability plays a critical role in the process of aggregation, as proteins with low stability tend to unfold and nucleate aggregation more easily than proteins with a higher thermal stability. The thermal transition midpoint (Tm), which can often be determined by differential scanning calorimetry (DSC), is an indicator of thermal stability. Overall, the Tm is inversely correlated with the protein's susceptibility to unfold and denature in solution and to degradation processes that depend on the tendency of the protein to unfold (Remmele RaG 2000).

The different domains of an IgG, including the Fv, C_H1/C_L, C_H2, and C_H3, are relatively independent structurally and often exhibit their own specific thermal transitions. DSC investigations with recombinant glycosylated and deglycosylated versions of IgG1, IgG2, IgG4, and IgG1-Fc have enabled the identification of three typical IgG transitions (Garber and Demarest 2007). C_H2 exhibits the lowest Tm with values between 64 and 70 °C in IgG4 and IgG1, respectively. The Fab has a wide range of Tm values, typically varying from 57 to 82 °C. C_H3 is the most stable domain with a Tm in the vicinity of 80 °C. Deglycosylation generally leads to decreased stability for the C_H2 domain but has little effect on the stability of the Fab or the C_H3 domain. Thus, IgG1 molecules have the most stable Fc when compared to IgG4 based on the Tm of their C_H2 and C_H3 domains. Antibodies

with low Fab stabilities have been found to aggregate and express poorly. Fab instability has been often associated with high levels of uncommonly observed amino acids or CDR loop lengths, particularly at V_H (Garber and Demarest 2007).

One method to enhance physical stability is isotype switching, i.e., combining the Fv with the constant domains of isotypes different from the parent antibody (Pepinsky et al. 2010). The resulting antibody has an Fv fragment identical to the parental antibody but the overall stability is shifted due to changes in the intrinsic stabilities of the constant domains. If a higher stability is needed, further engineering efforts are focused upon stability improvements of the *V* domains (Ewert et al. 2003, 2004; Worn and Pluckthun 1998). General strategies to stabilize *V* domains includes increasing the intrinsic stabilities of the V_H and V_L domains by grafting CDRs onto more stable FRs or designing mutations into FRs and CDRs that stabilize the *V* domains and/or the V_H/V_L interface (Spada et al. 1998; Worn and Pluckthun 2001).

However, introducing unusual mutations into the *V* regions could increase the risks of immunogenicity. With this caveat in mind, a "germline design" approach to stabilize the Fv has recently been proposed (Luo et al. 2010). The method is based on the hypothesis that germline sequences have evolved to encode highly stable antibodies, which can accommodate destabilizing mutations during the somatic hypermutation process (Wiens et al. 1998). Thus, by combining structural analysis of the Fv fragment and sequence comparisons to the closest matching germline IgV and IgJ genes, mutations that restore the germline residues in the non-binding regions of an antibody could be designed. Substituting germline mutations back into the lead candidate antibody has resulted in significant thermal stabilization of the Fab, with some mutants exhibiting an increased Tm of more than 12 °C (Luo et al. 2010).

Avoiding Chemical Instability

Chemical instability can lead to covalent modification of the antibody through bond formation or cleavage and thus generation of heterogeneities (Wang et al. 2007). Chemical instability is an outcome of reactions, such as deamidation, oxidation, disulfide bond breakage and formation, hydrolysis, and isomerization. Chemical modification may lead to a loss of biological activity depending on the location of the modified residues. For instance, binding could be decreased if the affected amino acids are required for antigen-binding. A more indirect mechanism is also conceivable, in which alterations in the flexibility of the *V* domains impact binding.

Deamidation is a chemical reaction in which the amide functional group from an aspargine (Asn) or glutamine (Gln) is removed (Robinson 2002). Deamidation is one of the most common degradation pathways in protein pharmaceuticals that can have a significant impact on protein bioactivity, half-life, conformation, aggregation, and immunogenicity. The relative position of Asn and/or Gln in

proteins as well as the neighboring amino acids at a deamidation site may affect the rate of deamidation. Asn and Gln deamidation are also strongly influenced by buffer anions, especially phosphate. Asn is more prone to deamidation than Gln. Sequence-dependent rates of deamidation of Asn in Tris-HCl, three-dimensional protein structures, and qualitative reports of deamidation in proteins under a wide variety of solvent conditions have been combined to produce a reliable calculation method for estimating the deamidation indexes and deamidation coefficients of Asn in all proteins for which the three-dimensional structure is known. These coefficients and indexes have been found to depend about 60% on primary structure and 40% on three-dimensional structure (Robinson 2002). The rates of deamidation of Gln have been reported and may eventually allow quantitative understanding of Gln deamidation on a similar basis to that now available for Asn (Robinson et al. 2004).

Amino acids that can undergo oxidation include methionine (Met), cysteine (Cys), histidine (His), tryptophan (Trp), and tyrosine (Tyr). Although oxidation is not as common as deamidation, it can occur during storage conditions. Most oxidation reactions commonly encountered in therapeutic proteins under normal storage conditions involve Met or Cys residues. Met residues can be oxidized, even by atmospheric oxygen to Met sulfoxide and to Met sulfone under extreme oxidative conditions (Ji et al. 2009). Met residues can undergo auto-oxidation, chemical oxidation, and photo-oxidation. Cys residue is also easily oxidized to yield Cys disulfide. During long-term storage, free sulfhydryl groups may be oxidized to intrachain or interchain disulfide linkages, which may lead to protein aggregation. Trp is also well-known to be susceptible to oxidation by reactive oxygen species and photo-oxidation leading to the formation of *N*-formylkynurenine and 3-hydroxykynurenine (Ji et al. 2009). A number of factors may affect the rate of photo-oxidation of Trp, including the primary sequence of the protein, pH of the solvent, and accessibility of the residue to oxygen and solvent molecules.

Disulfide bond formation/exchange is one of the most common cross-linking pathways, leading to chemical aggregation. Free Cys residues in proteins can be easily oxidized to form disulfide bond linkages or cause thiol-disulfide exchanges, leading to protein aggregation. The rate of disulfide bond cleavage and formation is dependent on the conformation of the protein and pH of the solvent. In addition to the typical disulfide bridge of the *V* domains of antibodies, only a few unpaired Cys residues are encoded in human IgVH and IgVκ germline genes. Specifically, the only member of the IgVH-7 gene family (Tomlinson et al. 1992) and two genes from the IgVκ-1 family, 1–8 (L9) and 1D-8 (L24) (Tomlinson et al. 1995), have unpaired Cys residues in FR-3. In human antibodies, Cys residues are also germline-encoded in DH genes (Zemlin et al. 2003). In the majority of the DH2 sequences both Cys residues are preserved, allowing the formation of an intrachain disulfide bond (Zemlin et al. 2003). Since free Cys residues can lead to cross-linking, human genes encoding unpaired Cys should be avoided during the humanization process. By the same token, antibodies with CDR-H3 loops having disulfide bridges tend to be unselected as lead molecules when developing antibody-based therapeutics.

Bi- and Multi-Specificity

Simultaneous binding of several targets might yield better therapeutic efficacy than binding to a single target. In fact, engineering antibodies to bind two or more unique targets within a single molecular entity has been a long sought but challenging goal. Several molecular formats inspired by the IgG molecule have been explored during more than two decades of intense research in this field, including: (a) combining two antibodies to generate a hybrid IgG molecule with each Fab arm recognizing a different target (Fig. 4.1d), (b) fusing two antibody fragments that specifically bind different targets via a peptide or a protein linker (Fig. 4.1e), (c) attaching Fv fragments or V domains at the amino- or carboxy-terminal or both ends of a known antibody (Fig. 4.1f); and (d) engineering antigen-binding sites to bind more than one target (Fig. 4.1d).

The first bi-specific antibody obtained by combining two antibodies (approach (a)) was approved by the European Medicines Agency for the treatment of malignant ascites in 2009 (Seimetz et al. 2010). This bispecific antibody called catumaxomab (Removab®; Fresenius Biotech/TRIoN Pharma), binds to both epithelial cell adhesion molecule on tumor cells and CD3 on effector immune cells. In addition, the Fc portion can interact with FcγRs on immune cells. Thus, drugs based on this platform are called trifunctional antibodies.

An example of approach (b) is BiTE (bispecific T cell engager) technology (Wolf et al. 2005). This molecular format consists of linking scFvs with different specificities via a peptide. In this category, blinatumomab, specific for tumor-associated CD19 and T cell-expressed CD3 (Cheadle 2006) is currently in Phase II clinical trials for therapy of minimal residual disease of B cell-precursor acute lymphoblastic leukemia. Another example of this molecular format is the bispecific ErbB3/B2 created by linking the scFvs of A5 anti-ErbB3 antibody and ML3.9, an anti-ErbB2 antibody (Robinson et al. 2008). An alternative to the peptide linker to increase half-life has been developed by Merrimack Pharmaceuticals, Inc., in which the same scFvs (A5 and ML3.9) are linked by a proprietary mutated human serum albumin (http://www.merrimackpharma.com/pipeline/). This resultant molecule is in a Phase I/II clinical trial for tumors that overexpress ErbB2/3.

A range of molecular formats has been exploited in the third category (c) of bispecific formats (Caravella and Lugovskoy 2010), e.g., linking *V* domains or Fvs to a known antibody. One of the formats is the dual variable domain IgG (DVD) technology (Wu et al. 2007). This new type of antibody is the fusion of an Fv fragment to the N-terminal of an IgG molecule. Proof of concept DVDs with combined specificities for IL-12 and IL-18 or IL-1α and IL-1β have been generated and tested. DVDs can be produced as a homogeneous single, functional species displaying properties similar to conventional IgGs, thus troubleshooting drawbacks of initial technologies based on combining two antibodies [approach (i)]. ScFvs have also been attached to the N-terminus or C-terminus of the HC of an IgG via a $(G_4S)_n$ linker. Using this strategy, bispecifics that bind both TRAIL-R2 and LTβR have been reported (Michaelson et al. 2009). Variations on this theme by attaching

scFvs to the N-terminus of the LCs and either the N-terminus or C-terminus of the HCs to achieve multispecificity have also been generated and are currently being studied (Dimasi et al. 2009).

In the last category (d) to create multispecific drugs, a recent approach challenged the concept of one antigen-binding site, one specificity (Bostrom et al. 2009). This strategy is referred to as two-in-one antibodies. One example consisted of engineering the antigen-binding site of Herceptin® (trastuzumab), an FDA approved humanized antibody (Reichert 2010b) which binds to HER2, to also bind vascular endothelial growth factor (VEGF). The X-ray crystallographic structure of the two-in-one antibody in complex with HER2 and VEGF showed extensive overlap between the antibody surface areas contacting the two targets. Interestingly, alanine scanning of the binding site revealed that for the most part, distinct amino acids were involved in the specific recognition of HER2 and VEGF (Fig. 4.1d), thus emphasizing once again the difference between structural and functional paratope. Therapeutics based on the two-in-one concept could provide new avenues for antibody-based therapy consisting of reengineering existing antibodies to accommodate new specificities.

Altering Interactions with FcRn to Impact Half-Life

One way in which antibody engineering can be employed to improve the efficacy of therapeutic antibodies is to increase the therapeutic antibody's circulating half-life. This approach can provide several beneficial properties to antibody therapeutics, including decreased and/or extended dosing schedules, minimization of toxicity associated with high doses, increased bioavailability, and lower cost of goods (Roopenian and Akilesh 2007). IgG antibodies have a long circulating half-life estimated at 7–21 days (Morell et al. 1970; Spiegelberg and Weigle 1965). Antibody half-life is regulated in large part by Fc-dependent interactions with the neonatal Fc receptor (Roopenian and Akilesh 2007). FcRn is a heterodimer consisting of a transmembrane α-chain and the β_2-microglobulin light chain (β2 m). Wild-type IgG antibodies bind to FcRn at slightly acidic pH (6.0–6.5) and are released at pH 7.4, the pH of blood. At steady-state levels, FcRn is primarily expressed within endosomes. When pinocytosed, IgGs enter acidified endosomes where protonation of conserved histidine residues on the Fc domain facilitates interactions with FcRn (Roopenian and Akilesh 2007). IgGs that do not bind to FcRn are thought to be directed towards lysosomes, where they can be degraded, whereas IgGs bound to FcRn are recycled and released back into circulation upon pH neutralization. β2 m knockout mice have reduced circulating IgGs, further supporting a role for FcRn in maintaining the long half-life of IgGs (Ghetie et al. 1996). Several groups have introduced amino acid mutations in the Fc domain to alter circulating half-life (Table 4.1).

MedImmune developed a series of Fc variants by phage display and tested their ability to bind to FcRn (Dall'Acqua et al. 2002). The best characterized variant

Table 4.1 Examples of engineering efforts to alter antibody half-life

Intended function	Mutation(s)	Test species	Reference
Increased half-life	IgG1:M252Y/S254T/T256E	Cynomolgus	(Dall'Acqua et al. 2006)
Increased half-life	IgG1:T250Q/M428L	Rhesus	(Hinton et al. 2006)[a]
Increased half-life	IgG1:N434A	hFcRn transgenic mice	(Petkova et al. 2006)
Increased half-life	IgG1:N434A	Cynomolgus	(Yeung et al. 2009)
Increased half-life	IgG1:M428L/N434S	Cynomolgus	(Zalevsky et al. 2010)
Increased half-life	IgG1:T307A/E380A/N434A	hFcRn transgenic mice	(Petkova et al. 2006)
Decreased half-life	IgG1:I253A	hFcRn transgenic mice	(Petkova et al. 2006)
Decreased half-life	IgG1:P257I/N434H or D376V/N434H	Cynomolgus	(Datta-Mannan et al. 2007a)
Decreased endogenous IgG	IgG1:M252Y/S254T/T256E/H433K/N434F	Mice	(Vaccaro et al. 2005)

This table is adapted from those previously published by Strohl (2009a) and Presta (2008)
[a] The T250Q/M428L mutation did not improve in vivo half-life in a PK study performed in cynomolgus monkeys (Datta-Mannan et al. 2007b), whereas it did have a 2.3-fold slower clearance in normal mice. This suggests that the T250Q/M428L mutation may be both species-specific and antibody-specific in terms of its ability to increase half-life

was termed "YTE," consisting of three mutations in the C_H2 domain of M252Y, S254T, and T256E. The YTE variant had increased binding affinity to human FcRn at pH 6.0 but not at pH 7.4. In contrast, the YTE variant had increased binding to murine FcRn at both pH 6.0 and 7.4. Another attribute of the YTE mutations in the C_H2 region was a reduction in ADCC function. When the pharmacokinetics (PK) of YTE was studied in normal mice, the authors found that the YTE variant had a decreased half-life compared to a wild-type control. They concluded that engineering higher affinity at pH 7.4 can reduce in vivo circulating half-life. In a follow-on study, the YTE variant displayed improved binding to cynomolgus FcRn at pH 6.0, but was released at pH 7.4 (Dall'Acqua et al. 2006). A confirmatory PK study in cynomolgus monkeys demonstrated that the YTE variant had a fourfold increase in half-life compared to a wild-type control. The MedImmune YTE variant has been incorporated into their anti-RSV therapeutic, MEDI-557 (Strohl 2009a). At the time this chapter was written, MEDI-557 was undergoing clinical trials in humans. The outcome of the MEDI-557 clinical trials could prove to be an important test case for amino acid modifications used to increase antibody half-life.

In some cases, increased binding at pH 6.0 alone is not predictive of an in vivo increase in half-life. Protein Design Labs used a molecular modeling approach combined with mutagenesis to identify variants with increased binding to FcRn at pH 6.0, one of which was the variant T250Q/M428L (Hinton et al. 2004).

Introduction of this variant into human IgG1 directed against hepatitis B virus resulted in a 2.5-fold increase in serum half-life compared to a wild-type control in rhesus monkeys (Hinton et al. 2006). The inclusion of this mutation did not affect the antibody's ability to mediate ADCC or CDC, nor did it impair the antibody's ability to bind to antigen. In contrast, the same T250Q/M428L variant on an anti-TNFα IgG1 antibody had a 40-fold increase in binding to cynomolgus FcRn, but this difference did not translate into an increase in serum half-life in a cynomolgus monkey PK study (Datta-Mannan et al. 2007b). In the latter report, Lilly Research Laboratories (Datta-Mannan et al. 2007b) demonstrated that the anti-TNFα T250Q/M428L variant had an approximately 500-fold increase in affinity for murine FcRn and a 2.3-fold slower clearance than a wild-type control in a mouse PK study. Taken together, these investigations demonstrated the importance of pH-dependent binding of FcRn as well as species selection for in vivo PK studies.

Xencor recently published a double mutation intended for increased FcRn binding and half-life extension. Their double mutation of M428L/N434S had an 11-fold increase in affinity for human FcRn at pH 6.0 compared to wild-type IgG1, and a 3.2-fold increased in vivo half-life demonstrated in a PK study in cynomolgus monkeys (Zalevsky et al. 2010). This study also confirmed the variant's ability to improve therapeutic efficacy in two cancer models. The M428L/N434S variant was incorporated into either an anti-VEGF antibody or an anti-EGFR antibody and tested independently in xenograft mouse models, where the mice were both immunodeficient and expressed human FcRn. The results from both models indicated that the M428L/N434S variant extended the serum half-life of the antibodies and decreased the tumor burden compared to IgG1 wild-type controls. These studies demonstrated for the first time that amino acid alterations intended to improve half-life also improved therapeutic efficacy in preclinical animal models.

As opposed to increasing half-life, there are some circumstances where decreased half-life would be desired, such as to reduce the possibility of adverse events associated with high ADCC or CDC antibodies, or with antibodies conjugated to toxins (Presta 2008). Additionally, antibodies intended for use as a diagnostic—such as antibodies coupled to a fluorochrome for imaging purposes—rather than a therapeutic, may not require a long circulating half-life (Presta 2008). The single amino acid mutation of I253A abrogated binding to murine FcRn at both pH 6.0 and 7.0; a significant decrease in the in vivo half-life was also observed, such that the variant could not be detected in serum 3 days after injection (Petkova et al. 2006). The use of amino acid modifications within the Fc domain of an IgG to decrease binding to FcRn could afford investigators the possibility of fine-tuning an antibody's half-life, as opposed to using Fab and $F(ab')_2$ fragments which have very short serum half-lives—as little as a few hours—due to the lack of an Fc domain.

Engineering efforts to manipulate FcRn binding or the function of the FcRn receptor can also increase the degradation of endogenous antibodies. In several autoimmune disorders, autoantibodies can contribute to pathology, such as those associated with autoimmune hemolytic anemia, idiopathic thrombocytopenic

purpora, systemic lupus erythematosus, or myasthenia gravis. Additionally, it has been suggested that FcRn contributes to the persistence of autoantibodies (Akilesh et al. 2004). One proposed way to ameliorate autoantibody-mediated pathology in autoimmune disorders is to decrease the persistence of autoantibodies by using Abdegs (antibodies that enhance IgG degradation) (Vaccaro et al. 2005). An Abdeg engineered to bind to FcRn with higher affinity at both pH 6.0 and 7.4 could potentially saturate endogenous FcRn to inhibit its salvaging function, resulting in decreased host antibody concentrations due to increased degradation and decreased half-life. Ward et al. (Vaccaro et al. 2005) devised an IgG1 variant with the mutations M252Y, S254T, T256E, H433K, and N434F (MST-HN) for its ability to interact with FcRn, and its effect on endogenous IgGs. They showed that the MST-HN variant had increased binding to both human and mouse FcRn at pH 6.0 and 7.4. Furthermore, mice injected with the variant had an overall decrease in serum IgG concentrations compared to mice injected with an IgG1 wild-type control. It remains to be seen whether or not this novel approach can ameliorate autoantibody-mediated disease.

Silencing Fc-Dependent Effector Functions

There are some cases where Fc-dependent effector functions could prove to be detrimental, such as when antibodies target immune cell-surface receptors, and the intended function is receptor blocking. Cross-linking cell-bound antibodies by FcγR receptors can unintentionally lead to immune cell activation and cytokine storm, as was the case with OKT3 (Alegre et al. 1994; Xu et al. 2000). Additionally, the presence of an Fc domain capable of interacting with FcγRs or complement could lead to cell-killing instead of merely blocking a cell-surface receptor. Therefore, considerable efforts have been made to engineer antibody Fc domains that are devoid of effector functions. Some early attempts to silence the Fc domain utilized the knowledge that the lower hinge of IgGs is critical for both FcγR-binding and activation of the complement cascade, particularly the IgG1 sequence of E233/L234/L235/G236/G237/P238 (Brezski and Jordan 2010). Several investigators mutated the lower hinge of OKT3, including the human IgG4 variants L235E (Alegre et al. 1992) and F234A/L235A (Alegre et al. 1994), and the human IgG1 variant L234A/L235A (Xu et al. 2000), all resulting in decreased FcγR interactions.

Other investigators have used isotype selection to take advantage of classically non-activating Fc domains (Jefferis 2007). The human isotypes of IgG1 and IgG3 are typically attributed with the highest cell-killing functions due to their ability to effectively initiate NK cell-mediated ADCC and complement-mediated cell lysis. In contrast, IgG2 and IgG4 fail to activate NK cell-mediated ADCC and fix complement, and therefore were considered silent. However, increasing evidence suggests that IgG2 and IgG4 can both have Fc effector functions independent of NK cell, ADCC, and CDC, and each isotype has unique biophysical properties.

Although IgG2 has reduced binding to FcγRI and FcγRIIIa, IgG2 can interact with the higher affinity polymorphism of FcγRIIa, H131 (Bruhns et al. 2009). Schneider-Merck et al. (2010) showed that IgG2 can effectively engage FcγRIIa-expressing cells of the myeloid lineage, resulting in myeloid-mediated target cell-killing. They also corroborated that IgG2 does not trigger appreciable NK cell-mediated ADCC, because the only activating FcγR expressed on human NK cells is FcγRIIIa. Human IgG2 also has several structural isoforms as a result of differential disulfide linkages in the hinge region, which can affect its potency (Dillon et al. 2008; Wypych et al. 2008). Additionally, IgG2 can form covalent dimers with other IgG2s, increasing avidity-based interactions and potentially augmenting cell-surface receptor cross-linking capabilities (Yoo et al. 2003). IgG4 has reduced binding to FcγRIIa and FcγRIIIa, but only has a tenfold decrease in binding to FcγRI compared to IgG1 (Woof et al. 1986). Cells of the myeloid lineage, especially IFNγ activated macrophages that express activating FcγRs, including FcγRI, can facilitate cell-killing via IgG4 antibodies (Steplewski et al. 1988). Indeed, IgG4 Fc-dependent depletion of target cells has been demonstrated in humans (Isaacs et al. 1996). IgG4 also has the unique property of Fab arm exchange both in vitro and in vivo (van der Neut Kolfschoten et al. 2007), which results in functional monovalency (Schuurman et al. 1999). Two factors contribute to Fab arm exchange; one is the “CPSC” core hinge that allows intraHC disulfide bonds that replaces the interHC disulfide bonds. The second is weaker non-covalent interactions in the C_H3 region compared to other isotypes (van der Neut Kolfschoten et al. 2007). Mutation of the core hinge and/or C_H3 determinants can increase the stability of the molecule (Angal et al. 1993). Because of these properties, investigators have adopted additional strategies other than isotype selection alone to silence Fc effector functions.

Complete removal of the Fc glycan by mutation of the N-linked glycosylation site, such as N297A or N297Q, has been associated with decreased binding to FcγRs (Bolt et al. 1993; Walker et al. 1989). The Fc-linked glycan makes contacts with amino acid residues within the C_H2 domain, which is thought to stabilize an open conformation, influencing FcγR interactions (Jefferis and Lund 2002). Crystal structure analysis of an aglycosylated murine Fc compared to a wild-type glycosylated murine Fc indicated that the aglycosylated C_H2 takes on a “closed” conformation, perhaps limiting FcγR interactions (Feige et al. 2009). At least one aglycosylated antibody, GSK/Tolerx’s anti-CD3 otelixizumab, is currently in late stage clinical trials (Keymeulen et al. 2005).

Although the Fc domain of the human IgG class of antibodies share considerable homology, as previously discussed, each isotype has different properties with regards to engaging FcγRs and complement (Jefferis 2007; Strohl 2009a). The approved therapeutic eculizumab from Alexion Pharmaceuticals, Inc. combined the C_H1 and hinge region of IgG2 with the C_H2 and C_H3 regions of IgG4, resulting in reduced binding to FcγRs and complement activation (Rother et al. 2007). This approach has been refined by mutating single or multiple amino acids within the hinge or Fc region of an activating isotype to corresponding amino acids from non-activating isotypes. Williamson et al. (Ghevaert et al. 2008) mutated the Fc domain

Table 4.2 Examples of modifications for decreased effector functions

Intended Function	Mutation(s)	Reference
Decreased ADCC, ADCP	IgG1:N297A	(Bolt et al. 1993)
Decreased ADCC, ADCP, cytokine storm	IgG4:F234A/L235A	(Alegre et al. 1994)
Decreased ADCC, ADCP, cytokine storm	IgG1:L234A/L235A	(Xu et al. 2000)
Decreased ADCC, ADCP, cytokine storm	IgG2:V234A/G237A	(Cole et al. 1999)
Decreased ADCC, ADCP, CDC	IgG2/4:IgG2 sequence 118-260[a]; IgG4 sequence 261-447[a]	(Rother et al. 2007)
Decreased ADCC, ADCP, CDC	IgG1:K214T/E233P/L234 V/L235A/ G236-deleted/A327G/P331A/ D356E/L358 M	(Ghevaert et al. 2008)
Decreased ADCC, ADCP, CDC	IgG2:H268Q/V309L/A330S/P331S	(An et al. 2009)
Decreased ADCC, ADCP	IgG1:C226S/C229S/E233P/L234V/ L235A	(McEarchern et al. 2007)
Enhanced to the inhibitory FcγRIIb	IgG1:S267E/L328F	(Chu et al. 2008)

The above table is adapted from one previously published by Strohl (2009a)
[a] These numbers refer to the EU system (Edelman et al. 1969)

of IgG1 to render it silent in terms of cell-killing by incorporating non-activating amino acids from IgG2 and IgG4 (see Table 4.2). Importantly, this study and others have shown that it is possible to introduce silencing mutations into the C_H2 region of an antibody without affecting the antibody's ability to bind to FcRn (An et al. 2009). Therefore, it is possible to engineer antibodies devoid of cell-killing functions that can still maintain a long circulating half-life. Results from clinical trials implementing Fc-silencing technologies should provide information on whether or not amino acid mutations result in immune responses, and if these antibodies are indeed silent in humans.

Engineering for Effector Cell Recruitment

Glyco-Engineering for Effector Cell Recruitment

The composition of the N-linked glycan attached at N297 can impact Fc-mediated effector function of antibodies. A high degree of carbohydrate heterogeneity exists for both human serum antibodies and cell line produced antibodies. The core structure of the carbohydrate contains *N*-acetylglucosamine (GlcNac) and mannose, with additional terminal sugar modifications, such as a core fucose, galactose, bisecting GlcNAc, and sialic acid (Raju 2008). Umana et al. (1999) showed that alteration of antibody glycosylation patterns using an engineered cell line that

produced bisected, non-fucosylated oligosaccharides improved ADCC function. Genentech/Roche (Shields et al. 2002) later used an engineered Chinese hamster ovary (CHO) cell line to produce completely non-fucosylated antibodies that otherwise had oligosaccharides comparable to antibodies produced in normal CHO cells and the oligosaccharides found in human serum antibodies. These non-fucosylated antibodies had up to 50-fold increased binding to FcγRIIIa that translated into improved in vitro ADCC capacity compared to wild type IgG1 controls. There are several proposed ways in which low or non-fucosylated IgGs enhance binding to FcγRIIIa and increased ADCC capacity. On the molecular level, Glycart Biotechnology AG (Ferrara et al. 2006) has proposed that regions of the Fc domain only exposed when an antibody is non-fucosylated can have productive interactions with a carbohydrate attached at N162 on FcγRIIIa and FcγRIIIb, resulting in higher affinity binding. In terms of increasing ADCC activity ex vivo, Kyowa Hakko (Iida et al. 2006) demonstrated that non-fucosylated antibodies can overcome the inhibitory effect of competing serum IgGs for FcγRIIIa, resulting in increased ADCC. An additional proposed benefit of non-fucosylated antibodies is that they have been shown to increase NK-mediated ADCC with cells expressing the lower affinity polymorphism of FcγRIIIa, F158 (Niwa et al. 2004).

Several in vivo studies were performed to test the efficacy of low or non-fucosylated antibodies in preclinical animal models. Scallon et al. (2007) demonstrated that antibodies with lower levels of fucose have increased ability to functionally interact with FcγRs in vivo in normal BALB/c mice, indicating that complete non-fucosylation may not be needed to achieve enhanced efficacy. GA-101 is a non-fucosylated anti-CD20 antibody being developed by Genentech/Roche using technology from Glycart Biotechnology AG (Robak 2009). Mossner et al. (2010) have demonstrated that GA-101 had increased in vitro cell-killing capacity compared to the anti-CD20 therapeutic rituximab by both direct cell death induction and Fc-dependent B cell depletion. Additionally, GA-101 had increased in vivo efficacy in murine xenograft models compared to rituximab. GA-101 and rituximab had similar abilities to deplete peripheral blood B cells in a cynomolgus monkey study; however, GA-101 depleted more splenic and lymph node B cells compared to rituximab. A non-fucosylated variant of an anti-HER2 IgG1 was recently tested for in vivo efficacy in transgenic mice (Junttila et al. 2010). The mice lacked murine FcγRI and murine FcγRIII but expressed human FcγRIIIa, which was shown to be present on murine NK cells and macrophages. In an orthotopic xenograft model, the authors demonstrated that non-fucosylated anti-HER2 antibodies had an approximately twofold increase in tumor suppression compared to normally fucosylated IgG1 wild type controls. These studies and others have demonstrated that low or non-fucosylated antibodies can increase the in vivo efficacy of antibody therapeutics where interactions with FcγRs are a contributing mechanism of action.

Of the non-fucosylated antibodies that showed promise in preclinical animal models, several are being evaluated in clinical settings. The previously mentioned GA-101 is undergoing phase I and phase I/IIa clinical trials that should provide

information on the safety and anti-tumor effects of GA-101 (Robak 2009). A recent clinical assessment of Amgen/Kyowa Hakko's KW-0761, a non-fucosylated anti-CCR4 antibody, demonstrated that 5 out of 16 patients achieved objective responses (Yamamoto et al. 2010). Additional examples of non-fucosylated antibodies in the clinic include MedImmune's anti-IL5R benralizumab (MEDI-563) using BioWa's Potelligent technology and Medarex's anti-CD30 (MDX-1401), also using BioWa's Potelligent technology (Strohl 2011). The results of these and other clinical trials involving low or non-fucosylated antibodies should help determine if glyco-engineering, specifically efforts that alter the fucose content of antibodies, can translate into improved performance of third generation antibody therapeutics.

Amino Acid Modifications for Enhanced Effector Cell Recruitment

Augmenting Fc-dependent effector functions through amino acid mutations has been studied using numerous techniques including random mutations, high resolution mapping through alanine-scanning, and computational structure-based analyses (Kubota et al. 2009). Examples of Fc mutations that increase Fc-dependent cell-killing functions are shown in Table 4.3. Genentech/Roche (Shields et al. 2001) studied which amino acids influence Fc:FcγR interactions by performing an alanine-scan of solvent exposed amino acids in the Fc region of IgG1. The authors scored how each individual alanine mutation affected binding to FcγRs and FcRn. The study identified multiple hot-spots of FcγR:Fc interactions and engineered a panel of variants based on the data obtained. Of note was the triple mutation, S298A/E333A/K334A that displayed increased binding to FcγRIIIa and increased ADCC capacity. Another potential benefit of utilizing amino acid mutations in the Fc region is the ability to fine-tune the effector functions of a given antibody. For example, injection site reactions have been linked to complement activation (van der Kolk et al. 2001); therefore, it would be of interest to increase FcγR interactions while ablating CDC activity. Xencor demonstrated the ability to modify differential effector functions by using a computational analysis and high throughput screening to test a series of Fc variants (Lazar et al. 2006). The S239D/I332E mutation increased binding to FcγRIIa and FcγRIIIa without affecting the variant's ability to mediate CDC. Addition of the mutation A330L to S239D/I332E diminished CDC capacity without affecting interactions with FcγRIIa and FcγRIIIa. Xencor later identified a series of variants that augmented FcγRIIa binding to take advantage of FcγRIIa-expressing myeloidlineage cells (Richards et al. 2008). Several variants, including G236A/S239D/I332E, had increased macrophage-mediated ADCP capacity. Taken together, these studies and others have not only added insights into the basic biology of FcγR:Fc interactions, but also defined numerous variants that could prove useful for engineering the next generation of fit-for-purpose antibodies (Strohl 2009a).

Table 4.3 Examples of Fc modifications for increased effector functions

Intended function	Mutation(s)	Reference
Increased ADCC, ADCP	IgG1:S298A/E333A/K334A	(Shields et al. 2001)
Increased ADCC, ADCP	IgG1:S239D/I332E	(Lazar et al. 2006)
Increased ADCC, ADCP; reduced CDC	IgG1:S239D/A330L/I332E	(Lazar et al. 2006)
Increased ADCC	IgG1:F243L/R292P/Y300L; IgG1:F243L/R292P/Y300L/P396L; IgG1:F243L/R292P/Y300L/V305I/P396L	(Stavenhagen et al. 2007)
Increased ADCP	IgG1:G236A; IgG1:G236A/S239D/I332E	(Richards et al. 2008)
Increased C1q binding and CDC, normal ADCC	IgG1:K326A/E333A	(Idusogie et al. 2001)
Increased C1q binding and CDC, reduced ADCC, increased C1q binding and CDC compared to IgG2wt	IgG1:K326W/E333S; IgG2:E333S	(Idusogie et al. 2001)
Increased C1q binding and CDC	IgG1:H268F/S324T; IgG1:S267E/H268F; IgG1:S267E/S324T; IgG1:S267E/H268F/S324T	(Moore et al. 2010)
Aglycosylated mutants with increased binding to FcγRIIa(R131) over IgG1 wildtype	IgG1:S298G/T299A	(Sazinsky et al. 2008)
Aglycosylated mutants with low nM binding to FcγRI	IgG1:E382V/M428I	(Jung et al. 2010)

This table is adapted from those previously published by Strohl (2009a) and Presta (2008)

As previously discussed, the Fc-glycan can profoundly influence FcγR-mediated functions. Therefore, many investigators produced antibody Fc variants in mammalian cell lines due to the importance of glycosylation in FcγR interactions. Non-mammalian display technologies were often avoided because they would not incorporate mammalian glycosylation patterns. Two recent studies have avoided that problem by engineering variants that lacked the glycan at N297 within the Fc domain, but contained compensating mutations in the C_H2 and/or C_H3 region using yeast and bacteria display technologies, respectively. Wittrup et al. (Sazinsky et al. 2008) designed mutations around the canonical Asn-X-Ser/Thr N-linked glycosylation motif and identified several mutants that were capable of binding to FcγRIIa compared to an aglycosylated control antibody, in particular the S298G/T299A variant. Using a human FcγRIIa transgenic mouse model, the authors demonstrated a significant in vivo reduction in antibody targeted platelets with the S298G/T299A variant compared to an aglycosylated N297A variant. Georgiou et al. (Jung et al. 2010) developed a bacterial display system to screen for aglycosylated Fc variants that displayed binding to the high affinity FcγRI receptor. They identified two mutations in the C_H3 region, E382V/M428I, that

displayed low nM binding to FcγRI, but had significantly reduced binding to the other FcγRs compared to a glycosylated control. The E382V/M428I variant showed potent anti-tumor activity in an in vitro ADCC assay using purified dendritc cells. These two studies highlight that high throughput; non-mammalian screening technologies could potentially provide a source for Fc variants with unique properties.

Concluding Remarks

As of July 2010, 28 therapeutic antibodies (or antibody fragments) have been approved for use in human therapy. Of these, 3 are murine, 5 are chimeric, 13 are humanized, and 7 are human (Reichert 2010b). The trend toward antibodies with higher human content and fully human antibodies is evident. Additionally, there are two Fc modified therapeutic antibodies on the market, eculizumab, which is approved in the US, and catumaxomab, which is approved in Europe (Strohl 2009a). The latter is also a trifunctional molecule. Thus, modifications of human antibodies to enhance functions as well as strategies to design molecules that engage more than one target have been guiding next generation antibodies with new therapeutic capabilities. Many more engineered monoclonal antibodies are undergoing clinical trials (Reichert 2010a). It was recently estimated that the probability of success (POS) for antibodies transitioning between Phase I and Phase II was 62%, whereas the POS for antibodies transitioning between Phase III to the market was 75% (Strohl 2009b). Although these approval rates are relatively high, efforts to differentiate antibody therapeutics from their earliest embodiments are paving the way for diversification of methods to engineer more potent, robust, and efficacious antibodies. Advancement of the next generation of engineered antibodies through clinical trials should provide insightful information as to how to fine-tune an antibody's function to improve therapeutic efficacy.

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Chapter 5
Biophysical Considerations for Development of Antibody-Based Therapeutics

Andrew W. Drake and Giuseppe A. Papalia

Abstract Development of therapeutic monoclonal antibodies (mAbs) requires rigorous measurements of the kinetic and thermodynamic binding properties of antibody–antigen complexes for drug candidate optimization and the design of clinical dosing strategies. For measuring the dissociation equilibrium constants of mAbs binding reversibly to antigens, two premier technologies are commonly used: Biacore surface plasmon resonance (SPR) and the solution-based kinetic exclusion assay (KinExA). This chapter details the correct experimental design, the proper use of the instrumentation, optimal data processing, instrument limitations and potential sources of artifacts, as well as a rigorous comparison between SPR and KinExA approaches. Biacore applications for high-throughput kinetic screening and epitope binning are briefly presented. Additionally, the use of cell-based affinity assays using fluorescence activated cell sorting and KinExA is discussed for instances where purified antigens outside a cell membrane lose their native structure and/or functionality.

Introduction

Development of therapeutic monoclonal antibodies (mAbs) requires rigorous measurements of the kinetic and thermodynamic binding properties of antibody–antigen complexes for antibody drug candidate efficacy optimization and the design of clinical dosing strategies via pharmacokinetic/pharmacodynamic modeling.

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Quantitatively describing the binding characteristics of antibody–antigen complexes is possible by considering the interaction to be a simple bimolecular reversible interaction. Subsequently, chemical binding principles can be applied to describe the association rate constant (k_a), the dissociation rate constant (k_d), and the equilibrium dissociation constant (K_D), also known as the "affinity" of the interaction. The association rate constant is a measure of how fast two reactants A and B form AB complex:

$$A + B \xrightarrow{k_a} AB \tag{5.1}$$

where k_a, often referred to as the "on-rate", is defined as the number of AB complexes formed per second/unit volume in a one molar solution of A and B and has units of inverse molarity-seconds (M^{-1} s^{-1}). The forward reaction rate (r_a) is directly proportional to k_a and to the concentrations of A and B ([A] and [B], respectively) and is described by the differential rate law:

$$r_a = k_a[A]\,[B] \tag{5.2}$$

The dissociation rate constant describes the dissociation of AB formed in Eq. 5.1 above:

$$AB \xrightarrow{k_d} A + B \tag{5.3}$$

where k_d is defined as the fraction of complexes that decays per second and has units of inverse seconds (s^{-1}). The dissociation rate constant is a measure of the inertness of the AB complex, and is often referred to as the "off-rate". The rate of the dissociation of AB (r_d) is directly proportional to k_d and to the concentration of formed AB ([AB]) and is described by the differential rate law:

$$r_d = k_d[AB] \tag{5.4}$$

Note the contrast between the reaction rates (r_a, r_d) and the rate constants (k_a, k_d): reaction rates change as [A], [B], and [AB] change over the course of a bimolecular interaction while k_a and k_d are characteristic *constants* which quantitatively describe the more important and useful intrinsic binding properties of, in this example, the interaction between binding partners A and B. At equilibrium, the rate of complex formation (r_a) in Eq. 5.2 equals the rate of complex dissociation (r_d) in Eq. 5.4. Hence at equilibrium:

$$k_a[A][B] = k_d[AB] \tag{5.5}$$

Rearranging Eq. 5.5 derives an expression for the equilibrium dissociation constant:

$$\frac{[A][B]}{[AB]} = \frac{k_d}{k_a} = K_D \tag{5.6}$$

where K_D is expressed in units of molarity from the ratio k_d/k_a and describes the overall strength of the reversible association between A and B. The lower the

magnitude of K_D, driven by either a high k_a, indicating a fast formation of AB, or a low k_d, indicating a slow dissociation of AB, or driven by a combination of both a high k_a and a low k_d, the more stable or "tighter" the interaction between A and B, referred to as a "high affinity" interaction.

Several complex factors can influence the ideal K_D for a therapeutic mAb, namely the nature and prevalence of the therapeutic target in the diseased tissue, as well as the desired functionality of the mAb. Hence, predicting the optimal efficacious affinity for a therapeutic mAb to its target antigen is difficult (discussed in Chap. 6). In general, however, affinities ranging from 1 nM to less than 10 pM should not be unreasonable design goals. Measuring K_D, k_a, and k_d for such high affinity mAbs is challenging to the biophysical chemist for three reasons: (1) the time to reach equilibrium for the mAb-antigen complex can be on the order of days, (2) the k_d can be so low that long periods of data acquisition may be needed for an accurate off-rate measurement, and (3) whenever the k_d isn't unusually slow ($>5 \times 10^{-4}$ s^{-1}), the k_a can be extremely fast ($>1 \times 10^{7}$ M^{-1} s^{-1}). However, these kinetic rate constants and affinities can be reliably estimated when the correct instrumentation is utilized properly, when the experiments are designed correctly, and data are processed optimally. Fluorescence polarization anisotropy can be used to measure the binding affinity between two molecules by monitoring the increase of polarized fluorescence emission as polarized light excites a solution sample containing a fluorescent-tagged molecule binding to a larger molecule. The larger complex formed "tumbles" in solution more slowly thus emitting more polarized light as compared to the smaller uncomplexed fluorescent-tagged partner which "tumbles" in solution more rapidly (Nasir and Jolley 1999; Jameson and Mocz 2005). However, with larger antigens (>60 kDa) there often is not enough of a molecular size discrepancy between unbound antigen and the antibody–antigen complex to affect a detectible difference in rotational diffusion (Nasir and Jolley 1999). Fluorescence polarization anisotropy also requires that one of the binding partners be modified with a fluorophore. A common label-free method used to measure the binding affinity between macromolecules is isothermal titration calorimetry (ITC) (Doyle 1997). In ITC, the amount of heat absorbed or released is monitored as a sample of a macromolecule associates with another macromolecule in an adiabatic chamber, depending on whether the association is endothermic or exothermic in nature (Pierce et al. 1999). Measuring the magnitude of heat change as a function of the sample concentration injected into the adiabatic chamber allows for a direct calculation of the equilibrium dissociation constant. ITC is limited, however, in that relatively high (micromolar) protein concentrations are needed to detect the heat of binding within the sensitivity of the calorimeter. The sensitivity of ITC heat detection is often limited when lower protein concentrations are required for measuring subnanomolar protein–protein affinities (Doyle 1997).

This chapter describes in detail surface- , solution- , and cell-based biophysical techniques ideally suited for measuring the binding constants of therapeutic monoclonal antibody–antigen complexes. Each of the three experimental formats is critically evaluated and comparisons between the technologies are supported

with examples of previous studies from the literature. Theoretical binding concepts that must be considered in properly designing an experiment and evaluating data using these biophysical methodologies are also discussed.

Biacore Technology

The premier technology for measuring the binding affinities of antibody–antigen complexes is surface plasmon resonance (SPR)-based Biacore instrumentation (Karlsson and Fält 1997). With Biacore, one of the binding partners is captured or covalently immobilized (the "ligand") to a modified gold surface while the other reactant (the "analyte") is flowed across the surface. The most common commercially available Biacore gold surface modification is a dextran matrix containing carboxyl groups for covalent protein coupling. As the flowed reactant binds to the surface, plasmons, or quantum mechanical electron density waves on the gold surface generated by an evanescent wave of multi-angled totally internally reflected light are influenced by the bound protein mass. A resonance effect between surface plasmons and the evanescent wave shadows the intensity of the totally internally reflected light at an angle dependant on the amount of protein mass bound to the surface. As protein mass binds to, or dissociates from the surface over time, the shadowed reflected angle shifts in proportion to the mass change at the surface. The magnitude of these detected angle shifts is then converted into arbitrary resonance units (RU). In simpler terms, as the flowed protein binds to the biosensor surface, the detected RU signal increases; as the protein dissociates from the surface, the detected RU signal decreases, thus providing real time binding of the reactants. The signal trace as a function of time recorded by the Biacore instrument, called a "sensorgram", contains binding association and dissociation phase information, allowing for a direct measurement of k_a and k_d for most high-affinity interactions. The K_D can then be calculated from the quotient k_d/k_a (Eq. 5.6). Standard Biacore instruments feature the ability to inject analyte over four independent flow cells individually or simultaneously. Figure 5.1 illustrates the kinetic binding information contained in a schematic of a Biacore sensorgram.

Because one binding partner is immobilized to a biosensor surface with Biacore, the technology is often criticized for having an experimental geometry which makes it prone to generating artifactual data. The inception of the surface dextran was to provide a solution-like environment where immobilized protein can maintain some degree of rotational freedom (Karlsson et al. 1994), but there is concern in the literature that affinities measured using Biacore biosensors will differ from pure "solution-phase" affinity measurements where neither of the binding partners is tethered to a surface. These concerns might have some merit if not for the fact that most published Biacore data were the result of incorrect experimental design and less than optimal data processing techniques. In addition, a majority of published articles that contain Biacore data provide insufficient information with regard to their Biacore experimental protocol and often forego

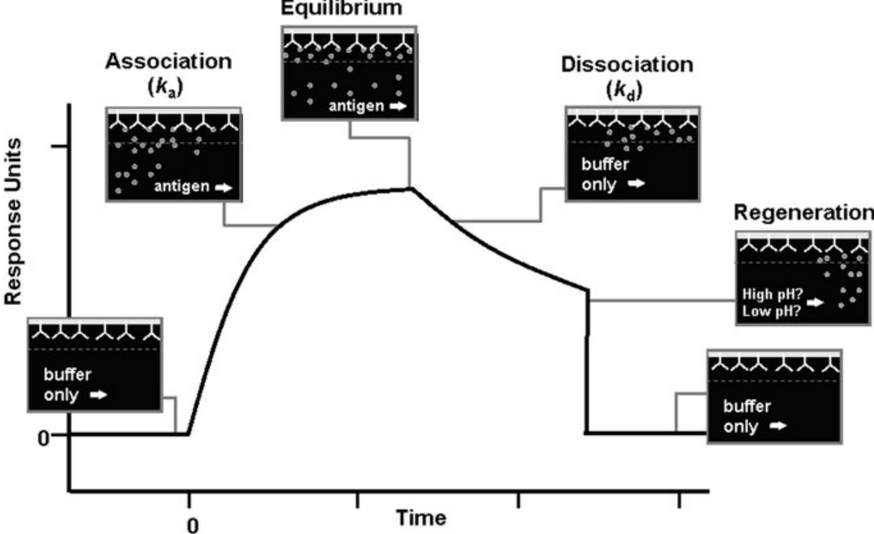

Fig. 5.1 A model Biacore sensorgram in which antigen (the "analyte") is injected over surface-immobilized antibody (the "ligand"). As analyte binds reversibly to the immobilized ligand, association phase data in the sensorgram contain association rate constant (k_a) information and even some dissociation constant information (k_d). The sensorgram response decreases as analyte dissociates from the biosensor surface, providing solely dissociation rate constant (k_d) information. A simple bimolecular kinetic model estimates k_a and k_d and the quotient k_d/k_a equals the affinity (K_D). Biacore surfaces are normally regenerated with short pulses of acidic or basic reagents so that multiple sensorgram replicates of various analyte concentrations can be acquired. (Tabrizi et al. 2009, reproduced with permission.)

displaying sensorgram data, instead providing only a table of numerical results. The reader is then prevented from reproducing the experiment and is unable to fully critique whether or not the Biacore data were either generated correctly or were of sufficient quality to provide reliable results (Rich and Myszka 2010). Myszka (1999b) was the first to provide an extensive annual review of the biosensor literature reporting the use of Biacore and other biosensor technologies. Rich and Myszka (2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2010) have since continued the arduous task of annually reviewing and critiquing the biosensor literature. In their most recent review, Rich and Myszka (2010) noted that out of over 1,400 papers that published biosensor data, only 5% appeared to have shown a proper use of the technology and a proper communication of their use of the technology. Again, the technology itself is often blamed for providing erroneous kinetic data when the real culprit is often from the erroneous use of the technology or from the condition of the reagent samples being studied (Rich and Myszka 2010). Most potential sources for generating artifactual data in a Biacore biosensor instrument can be avoided with the proper experimental design and processing of the data while using the highest quality protein reagents.

Biacore Experimental Design

When measuring the binding kinetics of an antibody–antigen interaction using a Biacore instrument, the bivalency of the antibody dictates that it should be immobilized to the surface while the antigen should be injected over the biosensor surface. This orientation (Fig. 5.2a) not only ensures that the more characteristic site-binding equilibrium dissociation constant is being measured, but also provides an experimental design which would generate kinetic data that could most likely be described by a simple 1:1 kinetic binding model wherein k_a and k_d can be reliably estimated. The incorrect reverse orientation shown in Fig. 5.2b where antibody flows over immobilized antigen promotes crosslinking of the bivalent antibody to the immobilized antigen (avidity). This is problematic for two reasons: (1) avidly binding mAb can yield artificially low measurements for k_d leading to an artificially tight K_D, and (2) the risk of generating complex kinetic sensorgram data is greatly increased. The latter point is especially crucial since interpretation of complex kinetics can often be driven by assumptions made without additional supporting data that should normally require an inordinate and painstaking amount of time and resources to select a sensible binding mechanism to describe the complex sensorgrams. In fact, several different complex models could successfully fit the same complex Biacore data set while estimating different rate constants. In theory, this complexity could be avoided if a low enough surface density of antigen is immobilized as shown in Fig. 5.2c. Experience has shown, however, that even when antigen is immobilized at a low surface capacity it is still difficult to rid sensorgrams of kinetic complexity when mAb is flowed across a surface with immobilized antigen.

The experimental design when using Biacore to measure antibody–antigen binding kinetics becomes more challenging when the antigen is itself multivalent or if a monovalent antigen tends to form soluble multimers in solution. Here a higher probability of multiple antigen cross-linking exists, thus increasing the probability of observing complex kinetic sensorgrams. Figure 5.2c depicts the most reasonable experimental approach when both reactants are multivalent: immobilize the minimal amount of either multivalent binding partner that appears to maintain a detectible and usable RU response when the other multivalent protein is injected over the surface. This design may decrease the occurrence of multistep binding at the surface but it does not necessarily guarantee that the resulting binding sensorgrams can be described by a simple 1:1 binding model. Even in cases where, for example, kinetic sensorgrams of multivalent antigen binding to immobilized antibody do fit well to a simple 1:1 interaction model, it is still ambiguous whether the K_D measured represents the intrinsic site (microscopic) binding constant or a stoichiometric (macroscopic) binding constant representing two sites from the multivalent antigen interacting with the two mAb binding sites. In other words, it is possible, although unlikely, that the interaction between multiple binding sites of a multimeric antigen and the two binding sites of

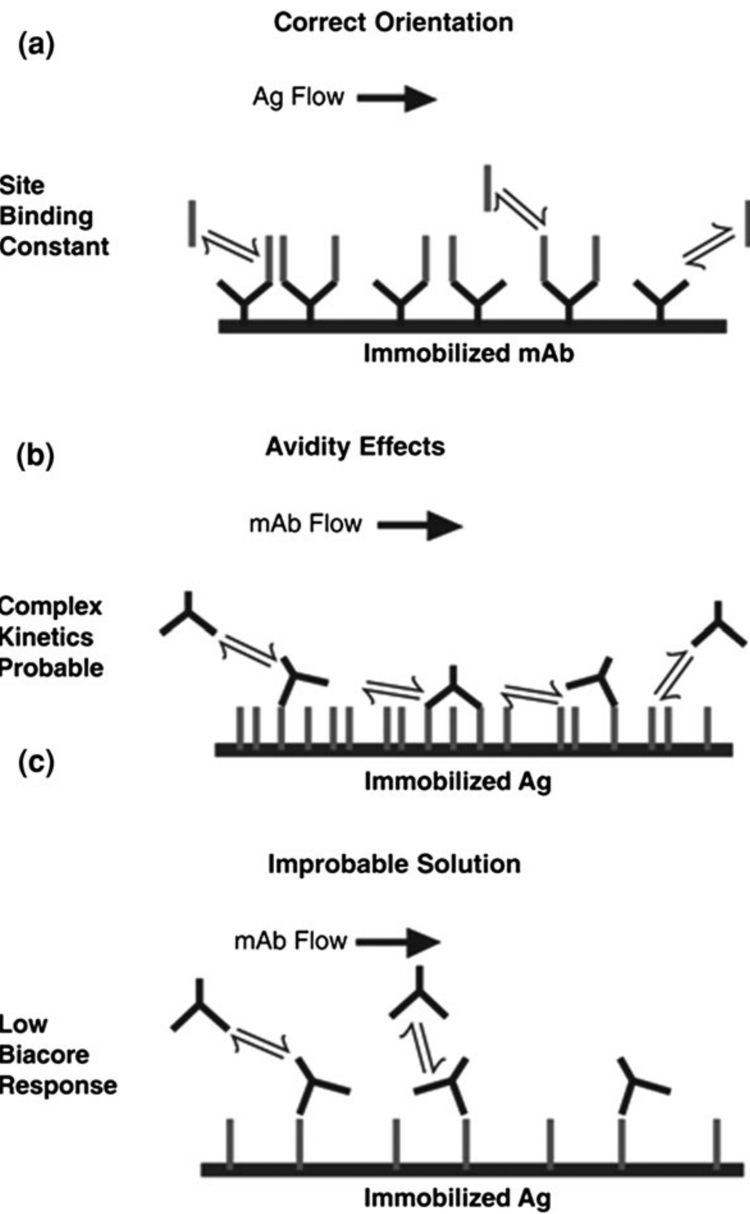

Fig. 5.2 Biacore experimental designs, both correct and incorrect, for measuring antibody–antigen binding interactions. **a** Antibody is correctly immobilized to the biosensor while antigen flows over the surface. This ensures the intrinsic site-binding K_D is being measured, especially for monovalent antigen. **b** Flowing antibody over immobilized antigen risks avidity effects from surface crosslinking which increases the probability of generating complex kinetic data and/or rate constants that may change as a function of antigen immobilization density. **c** Immobilizing a low antigen surface density more than likely does not obviate artifacts associated with flowing bivalent antibody over antigen. (Roskos et al. 2007, Fig. 7.6, p. 160, reproduced with permission.)

an immobilized bivalent antibody can fit a 1:1 interaction model, as well as a monovalent antigen binding to a single binding site of an immobilized antibody.

The drawback to the ambiguity of the nature of the Biacore affinity measurement of multivalent antigen binding to bivalent antibody is that it is always preferable, from a biophysical perspective, to measure the single site-binding equilibrium dissociation constant because it is uniquely characteristic of the bound complex. A macroscopic or stoichiometric affinity constant from a multimeric bimolecular interaction only provides information from a molecular thermodynamic perspective with no information regarding the individual binding sites that intrinsically drive the stoichiometric binding. Moreover, if a bivalent binding partner flowing over the Biacore surface does indeed crosslink two different immobilized multivalent ligands (Fig. 5.2b), then the macroscopic affinity constant should more correctly be considered an avidity constant. The obvious dilemma here is that the avidity binding constant can change as the density of immobilized ligand changes. In fact, an avidity binding "constant" that can change as a function of surface ligand density might arguably be more correctly referred to as an equilibrium dissociation "inconstant". Some scientists might claim that a Biacore-generated avidity equilibrium "constant" measured by flowing antibody over immobilized antigen is more mechanistically representative of in vivo functionality, but this claim does not hold true on further consideration. For instance, it is impossible to know if the immobilized antigen density on the biosensor surface corresponds exactly to the receptor density found on the target cells in vivo. Again, almost any antibody–antigen avidity that was desired could be measured depending on the antigen surface capacity at which the experiment was performed. Of course, if both binding sites of the flowing antibody can bind to only one immobilized multivalent antigen and no crosslinking of two antigens takes place, then the avidity constant would not change as a function of the immobilized antigen density and would be more representative of the true inherent biophysical binding constant from a macroscopic perspective, but still not from a site-binding perspective.

In summary, interpretation of Biacore data can be ambiguous when both binding partners are multivalent and the data can be described by a simple 1:1 interaction model. Does the measured binding affinity represent the intrinsic site-binding affinity or the stoichiometric affinity (avidity)? If the latter case, does the protein immobilization level dictate the measured affinity because of potential crosslinking of the flowing analyte to multiple immobilized binding partners, in which case a binding "inconstant" is being measured? Additionally, when the Biacore sensorgram data show complex kinetics, it may be advisable to instead perform equilibrium-based measurements using a different biophysical method rather than attempting to use a complex binding model without additional evidence to support the mechanism described by the complex model. Finally, no matter whether the K_D measured from data that is described well by a simple 1:1 kinetic binding model is a site or avidity K_D, researchers still consider the measurement useful during the selection stages of finding therapeutic antibody candidates

possessing the optimal affinities required to move toward further drug development stages.

Biacore Experimental Methods

In addition to the importance of deciding which binding partner in a Biacore experiment should be immobilized and which should be flowed to measure the most reliable and meaningful k_a and k_d of an antibody–antigen complex, it is equally important that the experimental design also includes techniques that have been shown to ensure the highest quality results. Myszka (1999a) has written one of the foremost primers on optimal Biacore experimental methods and biosensor processing protocols that provide the highest quality kinetic results. Highlights of the Biacore protocol suggestions from this publication are briefly summarized and discussed in more detail below. Myszka's suggestions (1999a) are especially applicable for measuring antibody–antigen kinetics assuming antigen is correctly flowed over antibody immobilized to a Biacore surface (Fig. 5.2a). It should be noted that before an experiment is even initiated, it is of utmost importance that regular cleaning and maintenance of a Biacore instrument are meticulously followed so that the microfluidics in the instrument always perform optimally.

Avoiding Mass Transport

One specific criticism of Biacore instrumentation is the technology's apparent susceptibility to collecting kinetic data under mass transport conditions. Briefly, mass transport limitations occur when the k_a at the Biacore surface is so fast ($>10^6$–10^7 M^{-1} s^{-1}), the ability of Biacore to accurately measure k_a is limited to the rate at which the flowed antigen can be delivered to the biosensor surface through bulk solution, while the ability to measure k_d is compromised by antigen rebinding effects. Rigorous theoretical considerations of mass transport in Biacore biosensors have shown when a mass transport term (k_m) describing a protein diffusion rate through bulk solution is included as a fitting parameter along with a standard 1:1 interaction model, k_m can greatly improve the theoretical fit of a kinetic Biacore data set containing some mass transport influence (Myszka 1997; Myszka et al. 1997, 1998; Karlsson 1999). In cases where the k_a is very fast ($>10^7$ M^{-1} s^{-1}), mass transport artifacts may be unavoidable, and an accurate measurement of k_a and k_d at the biosensor surface may not be possible. Karlsson (1999), however, has shown that because mass transport affects both the on-rate and the off-rate equally, one can still determine K_D from the acquired kinetic data. Briefly, Karlsson's theoretical derivation (Eq. 5.7) shows how the mass transport term (k_m) mathematically cancels, leaving the ratio k_d/k_a to calculate K_D, but not the individual values for k_a and k_d (Karlsson 1999).

$$K_D = \frac{k_d \times k_m}{k_a \times k_m} = \frac{k_d}{k_a} \tag{5.7}$$

Myszka (1999a) recommends two methods that can help obviate mass transport effects: (1) the amount of antibody immobilized should be low enough to provide a maximum antigen binding response level (R_{max}) of no more than 50–100 RU, and (2) the antigen injection flow rates should be high, preferably 100 μl/min. The optimum antibody immobilization level, of course, will theoretically depend on the molecular weights of both the antibody and the flowed antigen. An unnecessarily high antibody surface density may provide such a vast "sink" of antibody to which antigen can bind that the association reaction becomes almost entirely diffusion controlled when the k_a is extremely fast, and the dissociation reaction appears artifactually slow owing to the increased probability of antigen rebinding. A fast flow rate ensures the antigen sample is delivered to the biosensor surface as rapidly as possible, and additionally it assists in delivering a more consistent antigen plug across the surface.

Antigen Concentration

Myszka (1999a) also recommends that antigen samples be injected ideally at several concentrations ranging from at least 10- to 100-fold above and below the K_D. In cases where the binding affinity is relatively low, antigen concentrations 10- to 100-fold above the K_D may exceed high triple-digit nanomolar or single-digit micromolar levels, which often exhibit significant NSB to the biosensor surface. Conversely, when the binding affinity is very tight, antigen concentrations 10- to 100-fold below picomolar K_D's may not display enough of a detectible signal so as to provide any significant kinetic sensorgram information. Still, an antigen concentration range as wide as reasonably possible around the K_D value should always be used. In addition, the higher antigen concentrations should show enough curvature in the association phase of their sensorgrams to allow the fitting model to reliably estimate k_a, and show enough signal decay in the dissociation phase to reliably estimate k_d. In extreme cases where the on-rate is unusually slow, the necessity to inject low analyte concentrations to be near the picomolar K_D of a complex might require sample injections on the order of hours instead of the conventional 1–2 min for most antibody–antigen interactions (Navratilova et al. 2005); the long injection time requirements is beyond the capability of currently available biosensors without the use of extremely slow flow rates that should be avoided for reasons already discussed.

It is also important to remember that because association rate constants are concentration dependent, the most rigorous methods should be used to measure the antigen concentration, most notably those methods published by Pace et al. (1995) and Grimsley and Pace (2003) which describe the proper calculation of a protein extinction coefficient based on amino acid sequence and pH (if an accurate extinction coefficient is not already available), and the correct light scattering

correction when measuring protein absorbance. In fact, a Biacore instrument can determine the total active protein concentration when experimental conditions are designed to favor data generated under mass transport limited conditions. Consider when the mass transport of antigen from bulk solution is much faster than the k_a, the concentration of antigen will be the same at the biosensor surface as it is in the flowing bulk solution, and any measured binding will exclusively represent the true antibody–antigen binding kinetics. In the contrasting case where the mass transport of antigen through the bulk solution is rate limiting, any measured binding signal is directly proportional to the active antigen concentration and independent of the true binding kinetics (Christensen 1997; Van Regenmortel et al. 1998). Experimental conditions and theoretical treatment of mass transport-limited data for biosensor concentration determination methods are well documented by Karlsson et al. (1993), Christensen (1997) and Van Regenmortel et al. (1998), among other works.

Biacore Surface Regeneration

Regeneration of the biosensor binding surface between antigen injection cycles is necessary because it is normally impractical to wait for the dissociation phase of a kinetic sensorgram to decay back down to baseline RU, especially when studying highly stable antigen–antibody complexes with antibody covalently coupled to the biosensor surface. Randomly injecting antigen concentrations in duplicate or triplicate provides assurance that a sensorgram generated early in the experiment is reproduced several cycles later with the identical antigen concentration. This is only possible when the optimal surface regeneration conditions have been determined and the surface is stable for the duration of a full kinetic experiment. For capture experiments where antigen is injected over antibody captured to a high-density covalently immobilized antispecies polyclonal antibody surface, one to two short 15 s pulses of ~146 mM phosphoric acid or glycine-HCl, pH 1.7 usually works well to remove all captured antibody bound and unbound with antigen while maintaining a reproducible capture surface. There is certainly no "magic" regeneration formula that can be applied to all covalently immobilized antibody surfaces, but regeneration reagents of either low pH (i.e., phosphoric acid, glycine-HCl) or high pH (i.e., NaOH) should be injected at relatively short pulses of ~10–30 s each. Often multiple pulses of a regeneration reagent may be needed. Researchers often make the mistake of attempting to match the baseline RU before an antigen injection cycle with the postregeneration baseline RU to assess regeneration success (Andersson et al. 1999; van der Merwe 2001; Murphy et al. 2006). However, the fluid-like dextran matrix on the biosensor surface apparently can shift the baseline RU before and after an injection, which usually makes this practice futile. A successful regeneration scheme is achieved when multiple, properly referenced sensorgrams of identical antigen concentrations are reproducible. Drake and Klakamp (2011) recently detailed the Drake–Klakamp Method as a systematic, seven step experimental approach to more efficiently

determine the optimal regeneration conditions for Biacore surfaces with covalently coupled proteins. Finally, an injection of sample buffer should always be flowed over the Biacore flow cells at the start of an antigen injection cycle and immediately after the regeneration injections to wash out the microfluidics system.

Double-Referencing Data

Biacore experiments should also be designed so as to allow for double referencing of the sensorgram data. Double referencing data helps correct for artifacts such as bulk refractive index changes, NSB, systematic instrument noise, and baseline drift, all of which are common in almost every Biacore experiment (Morton and Myszka 1998). At least one flow cell of a Biacore chip should be used as a reference surface. The reference surface should either be exposed to the same chemical conditions used to covalently couple antibody on the active surfaces, or contain approximately the same amount of antibody capturing reagent as all other nonreference flow cells for a capture experiment (Morton and Myszka 1998). Also, several buffer "blank" injection cycles should be interspersed with the antigen injection cycles during the experiment. Each antigen and blank sample should be injected over the antibody surfaces and the reference surface simultaneously. To double reference sensorgram data, both the reference surface responses and the blank responses are subtracted from the analyte sensorgrams during sensorgram processing. The former corrects for refractive index shifts and NSB while the latter corrects for systematic instrument noise and baseline drift. Often the quality of sensorgram data sets cannot be assessed without double referencing, and normally data cannot be reliably fit for kinetic rate constants without double referencing. The processing power of double referencing is most effective (and impressive) when low surface capacities are used, often salvaging noisy looking, low signal raw data sets of seemingly questionable quality into surprisingly useful and reliable kinetic data. Double referencing Biacore sensorgram data is best achieved by using Scrubber biosensor data processing software (BioLogic Software, Campbell, Australia).

Global Fitting

All processed sensorgram data should be simultaneously ("globally") fit to a simple 1:1 nonlinear kinetic interaction model. Global fitting yields the most rigorous and accurate values for k_a and k_d from the entire data set, including and where appropriate, data sets collected from multiple independent flow cells in a single experiment. All sensorgrams, of course, should possess the same association and dissociation rate constants for a given interaction on multiple flow cells. However, the amount of information about each parameter will vary (Morton and Myszka 1998) for sensorgrams collected at different antigen concentrations on a particular surface for a given surface capacity of mAb (the density of mAb

immobilized). For example, a high antigen concentration might saturate the surface quickly during the association phase, thus providing minimal on-rate information but providing ample surface capacity (R_{max}) information, while lower antigen concentrations which show a slower rate of curvature in the association phase can provide a better estimate for k_a but a less than optimal estimate for the surface capacity. Globally fitting the entire data set combines all information contained in each binding response curve, thus improving the statistical power of the estimated rate constants (Morton and Myszka 1998). In addition to double referencing Biacore data, Scrubber software (BioLogic Software, Campbell, Australia) also contains global fitting functionality.

Complex Data

Complexity in sensorgram data is normally most apparent when the dissociation phase appears to be biphasic with relatively rapid signal decay over a relatively short time span immediately followed by much slower signal decay. Typical complex association data are also characterized by both fast and slow binding profiles. Of course, it is inadvisable to attempt to globally fit complex data with a simple bimolecular interaction model and assume the resulting kinetic rate constants are acceptable estimates. As discussed previously, when complex sensorgrams are observed it is also inadvisable to "surf" for a binding model that can describe the shape of the sensorgrams without any further rigorous scientific evidence to support the use of the complex model. There are several other potential artifacts besides the multivalent crosslinking issues described in Fig. 5.2b, c which can result in complex sensorgram data. These include impure antibody immobilized to the biosensor surface, impure antigen, heterogeneity of antibody binding epitopes introduced by the covalent immobilization chemistry, poor sensorgram processing, improperly referenced and subtracted NSB of the antigen, antigen forming multimers in solution, a general improper use of the Biacore instrument, or poorly performing instrumentation due to improper cleaning and maintenance. If complex data appears to be unavoidable even with proper Biacore experimental design and data processing, solution-based equilibrium methodologies independent of kinetic measurements may have to be used for a more reliable affinity measurement.

Low, Medium, and High Resolution Biacore Kinetic Experiments

One of the many advantages of using Biacore instrumentation for the development of therapeutic antibodies is its throughput flexibility. Biacore techniques can be catered to the rigor of the binding information necessary at various screening stages. Kinetic and affinity information can be measured from 1 to 3 antibodies in a single experiment to several hundred antibodies in a single experiment. Often the

progression for determining which antibody candidates have the tightest affinity to their purified antigen target begins with Biacore instrumentation performing as an invaluable high-throughput screening tool. Here “low resolution” kinetic data can allow for rapid affinity ranking of dozens to hundreds of unpurified antibodies in supernatant (Canziani et al. 2004; Säfsten et al. 2006). Low resolution approaches can also yield qualitative epitope binning information on multiple antibodies. Following a low resolution experiment, a “medium resolution” approach may be used to evaluate more rigorous kinetic information from the 4 to 8 highest affinity antibody supernatants to determine the 1 to 3 “best” candidates. These selected mAb candidates are then purified and subjected to the most rigorous “high resolution” antibody–antigen kinetics measurements. Approaches to low, medium, and high resolution Biacore experiments and the data contained therein are discussed in more detail in the sections below.

Low Resolution Screening

A typical cycle in a low resolution antibody screen includes capturing a series of mAbs and then injecting antigen at a single concentration. When antigen binding is complete, capture surfaces are regenerated so that the cycle can be repeated with a new series of antibodies, and so on. By injecting antigen at a single concentration only, the number of antibodies that can be screened is maximized. As long as the concentration of injected analyte is high enough to elicit curvature in the sensorgram, it is possible to yield reliable estimates for k_a and k_d from the injection of a single antigen concentration (Canziani et al. 2004). As discussed in “Biacore Experimental Design”, immobilizing antigen first and then injecting supernatants or mAbs is not recommended because of potential avidity effects yielding complex and/or erroneous data. This approach suffers additionally from the fact that concentrations of injected mAbs from supernatants or lysates are unknown, thus making it impossible to obtain a value for k_a. In some cases, the characteristics of an antigen (e.g., bivalency as in Fc-fusion constructs) may make this approach less desirable. Steukers et al. (2006) have proposed an alternate method where antigen is immobilized and the concentration of Fabs in periplasmic extracts is estimated by using initial binding rates of Fabs to a protein A Biacore chip under mass transport limited conditions (Karlsson et al. 1993). Although this approach is certainly valid, in the case of a very large number of Fabs, investigators should consider the time advantage of simply generating a monomeric antigen from an Fc-fusion protein. Commonly screens will rank dissociation rate constants in an attempt to correlate values of k_d with K_D. However, a tight K_D can result from a fast k_a, as well; meaning off-rate screens could miss potential high affinity mAb candidates. Plus, the K_D is normally the more interesting and useful binding parameter for pharmacokinetic evaluations under steady-state dosing conditions (discussed in Chap. 6). Hence, a full quantitative affinity screen yielding both k_a and k_d is always more preferable and informative than off-rate ranking protocols.

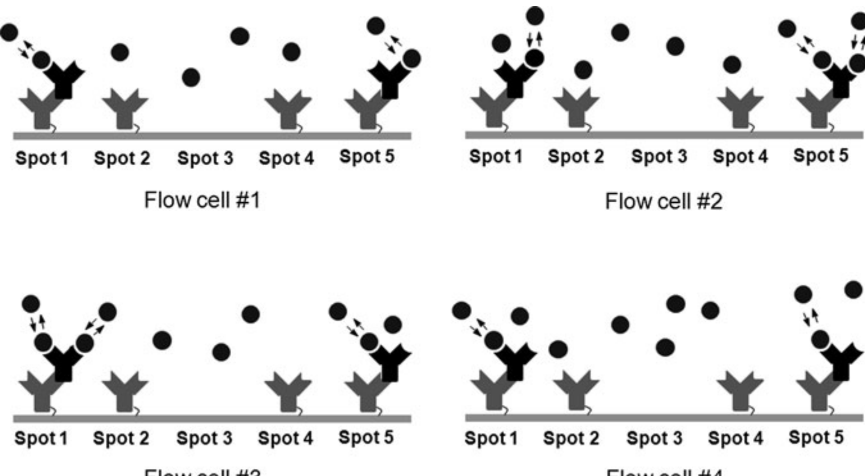

Fig. 5.3 Experimental design for a low resolution antibody screen for the Biacore 4000. Each of four flow cells is subdivided into five spots. Capturing antibody (shown in *gray*) is immobilized on spots *1*, *2*, *4*, and *5* of each flow cell. Different antibodies (shown in *black*) are then captured on spots *1* and *5* of each flow cell leaving spots *2* and *4* as reference surfaces. A single antigen concentration (*black circles*) is then injected simultaneously over all four flow cells to generate eight sensorgrams of antigen binding to each captured antibody. All capture surfaces are then regenerated so that another cycle of eight antibodies can be studied

The most recent advancements in Biacore instrumentation are specifically designed to address high-throughput low resolution screening campaigns. With only four available flow cells, traditional Biacore instruments could only capture three antibodies per cycle while leaving one flow cell as a reference surface (Canziani et al. 2004). Although promising inroads in throughput were made with Biacore's Flexchip array platform that could simultaneously analyze the interaction of antigen with as many as 96 printed mAbs (Wassaf et al. 2006), this technology has been discontinued. The new high-throughput Biacore 4000 (formerly Biacore A100) platform subdivides each of the four traditional flow cells into five individual spots which provide the capability of capturing eight different antibodies in a single cycle. The 4000 also contains a rack hotel that is capable of housing ten 96-well (or 384-well) plates. Figure 5.3 shows a schematic of the four independent flow cells and the five spots within each flow cell in the 4000 instrument.

In low resolution screening protocols, spots 1, 2, 4, and 5 of each flow cell all contain immobilized capturing antibody. In each binding cycle, four different antibodies are captured on spots 1 and 5 in all four flow cells. Spots 2 and 4 serve as internal reference surfaces for their juxtaposed spots 1 and 5, respectively. Once capturing is complete, antigen is then injected over all four flow cells simultaneously followed by surface regeneration so that another cycle can be repeated with eight more unique mAbs (Säfsten et al. 2006). The 4000 has also performed

high-throughput screens for single chain variable fragments using similar capture protocols (Leonard et al. 2007).

The ability to successfully screen numerous mAbs is dependent on capture surfaces that can be reliably and reproducibly regenerated for numerous cycles. For antibodies, this is accomplished by covalently immobilizing high density surfaces ($\sim$7,000–12,000 RU) of polyclonal anti-IgG (Fc specific) which are commercially available and are normally robust for the duration of an affinity screening experiment. Replicate antibody capture levels should ideally be reproducible within $\sim$5%. If antibody appears to dissociate from the capture surface during an antigen injection, the signal drift can be subtracted by double referencing with a cycle consisting of mAb capture followed by a buffer injection. This drift subtraction method is not technically correct since the standard kinetic fitting model assumes the surface capacity of antibody remains unchanged during the antigen injection, but it is a practical method for relatively ranking the binding affinities of several hundred mAbs. Rigorously speaking, drifting sensorgram data should be fit with a drift correction interaction model that takes into account the changing surface capacity during the association and the dissociation phase of the binding reaction as has been previously published (Joss et al. 1998). As mentioned in "Biacore Surface Regeneration", regeneration conditions for polyclonal anti-IgG capture surfaces after each mAb capture/antigen (or buffer) injection cycle typically include short pulses of phosphoric acid or glycine at low pH.

When processing sensorgrams from a low resolution screen, a large range of response levels is to be expected since antibody capture levels, activities, and affinities will vary throughout the screening samples. Of course, the reliability of the estimated rate constants will depend on how well the sensorgram profiles fit a 1:1 kinetic binding model. In cases where long off-rate candidates display virtually no dissociation phase decay, the k_d may have to be held constant at a reasonable minimum value ($\sim 10^{-5}$ s^{-1}) during sensorgram fitting. Complex sensorgram data are often observed with some antibody candidates and the biochemical significance of these complex profiles can be almost impossible to reliably evaluate. In short, affinity rankings of multiple therapeutic antibody candidates in a low resolution screen should emerge from data that yield reliable rate constants. Parameters derived from 1:1 fits of individual sensorgrams can be ranked in either tabular or graphical form. Logarithmic plots of k_d versus k_a with K_D affinity isotherms (Canziani et al. 2004) provide a straightforward visual presentation, as well as complete kinetic and equilibrium information (Canziani et al. 2004; Säfsten et al. 2006). Figure 5.4 shows an example of a kinetic isotherm plot from Säfsten et al. (2006).

Medium Resolution Experiments

A medium resolution experiment provides more rigorous kinetic data on the several (5–8) supernatants that showed the highest affinity ranking from a low resolution kinetic screen. Normally 4–6 additional antigen concentrations are

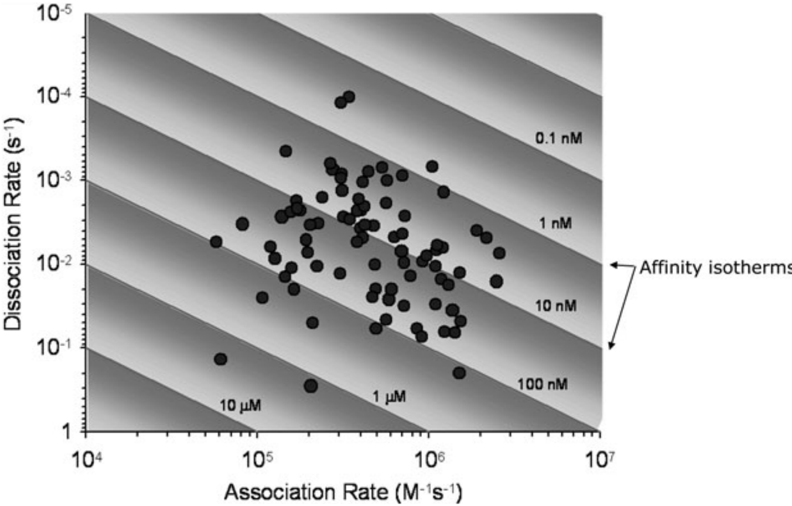

Fig. 5.4 Affinity isotherms are added to logarithmic plots of kinetic data (i.e. k_d vs. k_a) for each antibody–antigen interaction in a low resolution Biacore screen to visually assist with selecting antibodies displaying the highest affinities. (Säfsten et al. 2006, reproduced with permission.)

injected over the captured supernatants in 1–3 replicates instead of a single high antigen concentration injection as in most low resolution kinetic screens. Medium resolution experiments are limited in the amount of dissociation data that can be measured because captured antibody may begin to leach off of the capture surface after ~45 min. Any signal decay during the dissociation phase would then reflect a combination of both antigen and captured mAb dissociation and compromise the measurement of k_d. In this circumstance high resolution Biacore measurements would be required to measure long off rates, the particulars of which are discussed below. In fact, it will become apparent in the discussion of high resolution Biacore experiments that a medium resolution approach to captured purified antibodies, as opposed to antibodies in supernatant, where long off-rate data acquisition is not necessary, is actually indistinguishable from a high resolution Biacore experiment.

High Resolution Experiments

High resolution Biacore experiments are most often performed when a precise, accurate, and reliable affinity measurement for a specific purified antibody candidate is needed. Commonly this "hard number" approach assists with internal reporting, publication results, and/or Investigative New Drug applications, among other examples. A high resolution Biacore experiment involves injecting seven to eight antigen concentrations (at the appropriate range) in triplicate over the immobilized antibody in addition to buffer blanks for double referencing, and globally fitting the resulting 30–40 sensorgrams. It should be noted that when it is

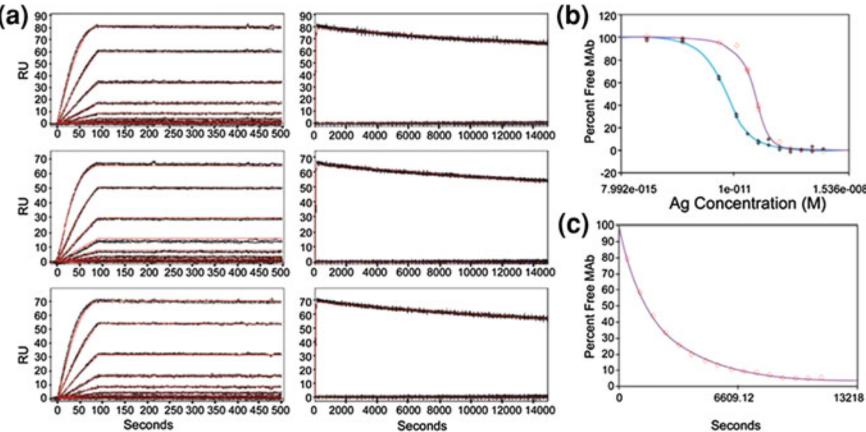

Fig. 5.5 Biacore and KinExA kinetic and equilibrium dissociation constant measurements of antigen-2′ complexing with mAb-2. **a** High resolution Biacore data of antigen-2′ binding to covalently immobilized mAb-2. Double referenced triplicate sensorgrams (*black lines*) from three independent flow cells were globally fit (*red lines*) to a simple 1:1 interaction kinetic model. On-rate data were acquired by injecting antigen-2′ at concentrations ranging from 0.18 to 23 nM (*left side*) while off-rate data were acquired by following the dissociation of additional antigen-2′ injections at 23 nM for 4 h. For this experiment, $k_a = 2.7 \times 10^6$ M^{-1} s^{-1}, $k_d = 1.6 \times 10^{-5}$ s^{-1}, and $K_D = 6.1$ pM. **b** Dual-curve equilibrium KinExA data of antigen-2′ titrated into mAb-2. The mAb-controlled curve (*top curve*) was acquired by titrating antigen-2′ at a concentration range of 40 fM–3.1 nM into 140 pM mAb-2 binding site concentration. The K_D-controlled curve (*bottom curve*) was generated with the same antigen-2′ concentration range titrated into 9.3 pM mAb-2 binding site concentration. For this replicate, $K_D = 3.8$ pM. **c** KinExA acquisition of k_a via the "kinetics direct" method with 208 pM antigen-2′ equilibrating with 86 pM mAb-2 and acquiring data points every 12 min for ~3 h. For this replicate, $k_a = 2.5 \times 10^6$ M^{-1} s^{-1}. (Drake et al. 2004, reproduced with permission.)

apparent that there is very little signal decay in the dissociation phase in all sensorgrams of a high resolution data set, it indicates the antibody–antigen complex has a very slow k_d ($<5 \times 10^{-4}$ s^{-1}) which is common in very tight complexes ($K_D < 100$ pM). Without a detectible dissociation response, the fitting model would be unable to estimate the off rate. In this case, several hours of dissociation data are recorded from several additional injections of high antigen concentrations double referenced with buffer injections (Drake et al. 2004). These additional sensorgrams are globally fit with the full antigen range response curves. Relatively slow dissociation rate constants on the order of 10^{-5} s^{-1} have been measured for single-digit picomolar K_D's using this method (Drake et al. 2004). An example of a high resolution Biacore data set using this "long off-rate" method is shown in Fig. 5.5a.

In this experiment, a monoclonal antibody was covalently coupled at various surface capacities to three flow cells of a Biacore biosensor chip with the fourth flow cell serving as a reference surface. The three panels on the left side of Fig. 5.5a show double referenced sensorgrams from several antigen concentrations

which were simultaneously injected over each flow cell. Each black line actually represents three replicate sensorgrams on top of one another, indicating a very reproducible surface. Note how there is almost no signal decay in the dissociation phase data in the left panels. The panels on the right in Fig. 5.5a show three additional double referenced replicates of the highest antigen concentration injected over the same antibody surfaces but with the dissociation followed for 4 h. With the long off-rate acquisition, enough signal decay is detected for the simple 1:1 interaction model (red lines) to estimate the k_d from a global fit of the data. There is also enough curvature in the association phase region from the higher antigen concentrations in Fig. 5.5a for the interaction model to determine a reliable estimate for k_a. The k_d/k_a ratio from this high resolution data calculated a single-digit picomolar affinity for the antigen–antibody complex which was corroborated by generating similar results using an independent solution-phase biophysical method, thus validating this novel long off-rate Biacore technique (Drake et al. 2004).

Epitope Binning

Often an important aspect of antibody characterization is to determine the region (epitope) on an antigen to which an antibody binds. The classification of antibodies into various groups based on their ability to bind to antigen in the presence or absence of each other is called epitope binning. Beyond ascribing possible functional differences between mAbs to different epitope bins, the ability to clearly distinguish antibodies based on binning can be of significant value from an intellectual property point of view. Most binning studies in some form or another rely on the sequential binding of two antibodies to antigen. Competition between the two antibodies in these experiments is commonly attributed to the two mAbs having similar or overlapping epitopes.

Epitope Binning Methods Using Biacore

Various epitope binning experiments can be performed using Biacore instrumentation. For example, antigen can be covalently immobilized on the surface followed by sequential injections of two different antibodies. A sequential additive response from both antibody injections would indicate the antibodies are binding to different antigen epitopes, and therefore should be classified as being in separate epitope bins. Binding observed with only the first antibody injection and not the second would suggest the antibodies bind to identical or overlapping epitopes, and therefore the two antibodies would be classified as being in the same epitope bin. An interesting variation on antigen immobilization experiments are those performed by Shi et al. (2006). These experiments involved structurally disrupting immobilized antigen with several proteolytic and chemical treatments and then

binning antibodies based on their ability to bind to the various surfaces. Binning protocols that use antigen immobilization are advantageous in that they require relatively minimal amounts of antigen consumption, but the potentially smaller structural motifs of some antigens may be prone to inactivation during chemical immobilization.

A more preferred epitope binning approach is to mimic a traditional "sandwich" assay on a Biacore surface. In this format, one antibody is first covalently immobilized and antigen is then injected over the surface. A second antibody is then injected and if binding is observed it is concluded the injected mAb has a unique epitope from the mAb immobilized. If no binding or a diminished amount of binding is seen, it is inferred that the injected mAb has either a similar epitope or an overlapping epitope relative to the immobilized mAb. Because relatively fast antigen dissociation from immobilized antibody does occur commonly before the second mAb can be injected, buffer injections over identical surfaces having immobilized antibody with antigen bound allow for double referencing to correct for any signal decay during the second antibody injection. This sandwich format has also been performed with a capture surface (Säfsten 2009), but a complete blocking of the capture surface is needed to prevent capturing of the second antibody, which can often be difficult. The sandwich assay approach will not work with multivalent antigen bound to the immobilized antibody since the second antibody can bind to an identical unbound epitope on the same antigen molecule. In such a case, antigen at a constant concentration can be preincubated with increasing concentrations of one antibody prior to injection over a different immobilized antibody. It can be inferred that the two antibodies bind antigen with similar epitopes when the binding of the antigen/second mAb complex to immobilized antibody decreases as antigen is equilibrated with increasing amounts of the second mAb. If the epitopes are not similar or overlapping, the binding levels observed should show an increase in response units that is directly proportional to the increasing concentration of the antigen/second antibody complex. This increased signal results from the fact that more mass would be binding to the surface immobilized mAb in the form of the antigen/second mAb complex. The Biacore 4000 instrument currently provides the highest simultaneous antibody throughput for epitope binning. Abdiche et al. (2009) have also performed epitope binning screens using ForteBio's Octet QK and Bio-Rad's ProteOn XPR36 biosensor array systems.

KinExA Technology

Another biophysical method gaining traction for measuring the affinities of antibody–antigen complexes is KinExA (kinetic exclusion assay) technology (Ohmura et al. 2001; Darling and Brault 2004) The KinExA instrument is essentially a flow spectrofluorimeter designed to measure the amount of free monoclonal antibody binding sites in equilibrated solutions of various antigen concentrations titrated into a constant monoclonal antibody binding site

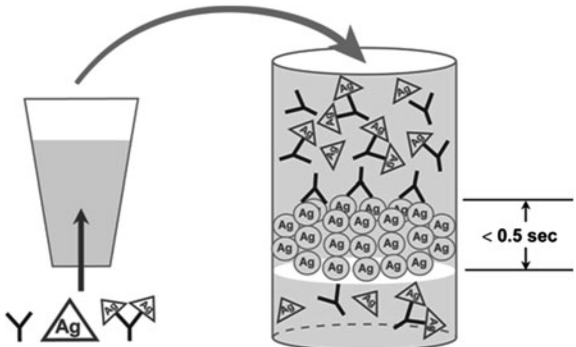

Fig. 5.6 Kinetic exclusion assay (KinExA) technology for measuring K_D. Equilibrated solutions of mAb-antigen complexes at various concentrations of antigen and a constant concentration of mAb binding sites flow through a column of beads coated with antigen. A small fraction of unbound mAb binding sites are captured to the bead resin where fluorescence from a secondary fluorescently labeled antibody is directly proportional to the concentration of free mAb binding sites in each equilibrated solution. The percent free antibody binding sites plotted as a function of antigen concentration is fit to a simple 1:1 equilibrium model to estimate the K_D of the mAb-antigen complex in solution. (Reproduced with permission from Sapidyne, Inc., Boise, ID.)

concentration (twice the molecular concentration). To accomplish this, each equilibrated antibody–antigen solution flows through a flow cell containing a small column (~7 µl) of bead resin to which antigen is adsorbed or covalently coupled. As each solution flows through the packed bead column, a portion of unbound antibody sites bind to the resin while free antigen and antibody completely bound with antigen (both binding sites complexed with antigen) flow through the column (Fig. 5.6).

A secondary fluorescently labeled polyclonal antispecies Fc specific or anti-heavy and light chain antibody is then flowed through the resin followed by a brief wash of the column. The amount of fluorescence detected from an excitation lamp directed through the bead pack is then converted to a percentage of free antibody binding sites in each equilibrated solution. A plot of the percent free antibody binding sites as a function of total antigen concentration in each solution is then fit to a simple 1:1 equilibrium binding model to estimate the K_D of the antibody–antigen complex. The abundance of choices for fluorescently labeled secondary polyclonal antibodies available commercially makes KinExA ideally suited for measuring antibody–antigen interactions. Furthermore, the fluorescence-based nature of the instrument provides a very low detection limit allowing measurement of very tight equilibrium dissociation constants.

Kinetic Exclusion

The kinetic exclusion concept behind KinExA technology assures that the amount of antibody captured on the column resin represents a true quantitative

representation of the amount of free antibody binding sites in each equilibrated solution. A measure of the antibody binding site concentration in equilibrated mixtures of antibody and monovalent antigen yields the preferred intrinsic site-binding K_D of the antibody–antigen complex instead of just a stoichiometric or macroscopic equilibrium constant. The exposure time of solution to the bead pack volume is less than half of a second, resulting in only $\sim$5% capture efficiency of any free antibody binding sites (Ohmura et al. 2001). Hence, the combination of a short contact time and low capture efficiency assures that any dissociation of antibody–antigen complexes while solution passes over the bead resin is kinetically excluded. In other words, no significant equilibrium shift of the antibody–antigen solution occurs while flowing over the bead pack, resulting in a true measurement of free binding sites in each equilibrated sample. Of course, an antibody captured to the bead pack with either one or both binding sites uncomplexed with antigen produces the same fluorescent signal. This would appear to prevent the fluorescent signal from correctly quantifying the true free antibody binding site concentration in solution. However, based on mathematical arguments antibodies with only one antigen bound are only captured with half the probability of antibodies having both binding sites free, hence the total fluorescent signal is truly representative of the free antibody binding site concentration (Lackie and Glass 2001; Ohmura et al. 2001).

K_D-Controlled and Antibody-Controlled KinExA Equilibrium Titrations

It is crucial to consider the experimental titration conditions that can affect the shape of a KinExA equilibrium titration curve and what information is available from the curve. The shape of the titration curve in KinExA, in fact, depends on the ratio of the antibody binding site concentration to the K_D of the interaction. When the antibody binding site concentration is much greater than the K_D, the curve shape is insensitive to changes in the K_D but sensitive to changes in the antibody binding site concentration. This "antibody-controlled" curve mostly contains antibody binding site concentration information and minimal affinity information. When the antibody binding site concentration is near or less than the K_D of the antibody–antigen complex, the shape of the titration curve is affected by changes in the K_D but will not change as the antibody binding site concentration changes within this concentration range. This "K_D-controlled" curve contains affinity information and very minimal information on the antibody binding site concentration. It is preferable to generate both types of equilibrium curves and then simultaneously fit both curves in a dual curve analysis to estimate the active antibody binding site concentration and the K_D. An example of a KinExA dual curve analysis for an antibody–antigen complex is shown in Fig. 5.5b and will be discussed in more detail in "KinExA and Biacore Comparison". More than two titration curves may also be used in an "*n*-curve" analysis to even more rigorously

determine the K_D and free mAb binding site. Dual and *n*-curve analyses provide more rigorously obtained values for the K_D and the antibody binding site concentration as compared to values obtained if individual curves were fit locally. Having multiple titration curves simultaneously (and successfully) fit to a simple 1:1 interaction model also provides extra assurance that a 1:1 binding model correctly describes the nature of the equilibrium binding reaction. With a well-behaved antibody–antigen system, it is likely that a single K_D-controlled curve generated under the proper titration conditions will yield a K_D value equivalent to the K_D value generated from a multicurve analysis. This can be beneficial if either the affinities of several different antibody–antigen complexes or several replicate K_D measurements of a single antibody–antigen complex need to be quickly measured. For the latter case, several measurements of course provide a 95% confidence interval based on several independent replicate experiments for a more statistically rigorous K_D value that takes into account errors from independent experiments. KinExA software, in contrast, provides a 95% confidence interval of the parameters derived from fitting a single data set and do not represent a statistical precision of several independently measured K_D values for a single antibody–antigen complex.

KinExA Kinetic Measurements

KinExA can also be used to measure the association kinetics of antibody–antigen complexes. The simplest technique is the "kinetics direct" method which measures the free antibody binding site concentration as a solution of antigen and antibody approaches equilibrium. A bimolecular rate equation is subsequently fit to the percent free antibody binding site concentration as a function of time to calculate k_a. An example of a kinetics direct association curve for an antibody–antigen complex approaching equilibrium is shown in Fig. 5.5c and will be discussed further in "KinExA and Biacore Comparison". A kinetics direct experiment follows the association kinetics for several hours in KinExA, whereas restrictions in the amount of volume that can be injected in Biacore experiments limit association data acquisition time to 2–10 min (assuming Biacore flow rates are not slow enough to induce artifacts from mass transport). Another kinetic method called the "kinetics injection" method is performed by mixing various antigen concentrations with a constant antibody concentration. A simple reversible bimolecular rate equation then estimates k_a from a plot of the percent free antibody binding site concentration measured at a fixed, calibrated time as a function of the antigen concentration. Because the time needed for collecting each data point in the direct method is normally on the order of 5–15 min, complexes that equilibrate faster than an hour or so are more suited for the kinetics injection method. The dissociation rate constant is not normally or easily measured directly with KinExA instrumentation. Unlike in a high-resolution Biacore experiment where k_a and k_d are measured to calculate K_D (Eq. 5.6), KinExA requires both an equilibrium

experiment to measure K_D and an independent kinetic experiment to measure k_a, and then k_d is calculated from multiplying K_D by k_a.

Recent modifications have been added to the KinExA control software for measuring directly the k_d of antibody–antigen complexes that are extremely slow. Here fluorescently tagged antigen is added to a solution of antibody preequilibrated with unlabeled antigen while the bead resin is coupled with an unlabeled antispecies polyclonal antibody to the mAb of interest (a "chase pulse" experiment in a sense). The experiment is run at antigen and mAb concentrations that lead to stoichiometric or quantitative binding. The rate of formation of antibody complexed with labeled antigen is monitored with KinExA by measuring the increase in fluorescence over time as more and more labeled complexes are formed from dissociation of unlabeled antigen and hence captured by the bead resin. If the dissociation rate of the unlabeled antigen is very slow, the increase in the fluorescence may only be measurable when data points are taken at intervals on the order of hours or even days, hence the flexibility in the software for the KinExA user to select an appropriate time interval between cycles. The resulting increase in fluorescence is then modeled with a kinetic algorithm to estimate the k_d of the unlabeled antibody–antigen complex. Ideally, a simultaneous fit with data from a reversed orientation KinExA experiment (a "pulse chase" experiment) measuring the decrease in fluorescence over time when unlabeled antigen is added to a solution of antibody precomplexed with labeled antigen can ensure that the dissociation rate constant is identical for antibody binding to both the labeled and unlabeled forms of the antigen, thus proving the labeling of the antigen did not artifactually alter the dissociation kinetics of the first experiment described above.

KinExA Experimental Methods

As with any biophysical technique, generating reliable high quality data using a KinExA instrument depends on correctly designing and performing equilibrium and kinetic experiments. First, for equilibrium experiments, antibody–antigen solutions must be allowed enough time to equilibrate. Time to equilibrium of any complex is driven by k_d and the reactant concentrations relative to the K_D, hence antibody–antigen complexes that have extremely slow off-rates (often corresponding to single-digit picomolar, pM, K_D values) can take days to weeks to reach equilibrium (Drake et al. 2004). For example, consider an antibody–antigen complex having a k_a and k_d of $7 \times 10^5\ M^{-1}\ s^{-1}$ and $2 \times 10^{-5}\ s^{-1}$ ($K_D = 29$ pM), respectively, and a solution containing 29 pM each of antigen and antibody binding sites. This reaction requires 35 h to reach equilibrium (Drake et al. 2004). Second, to generate a K_D-controlled curve for extremely tight antibody–antigen complexes, the antibody binding site concentration may have to be in the single-digit picomolar range or lower. Because of the low capture efficiency of free antibody binding sites to the bead pack, this could require tens of milliliters of equilibrated solution to flow through the KinExA bead pack to generate a detectible and reproducible signal.

Tens of milliliters of sample are also needed for KinExA kinetic experiments as well. Preparation of these larger volumes of samples should follow traditional analytical volumetric techniques for the most reliable kinetic and equilibrium results. For example, meticulously cleaned glass volumetric flasks should always be used, and the use of properly calibrated (and cleaned) reusable glass pipettes should always be favored over disposable plastic pipettes for sample preparation. Third, per the discussion above to generate K_D-controlled equilibrium titration curve data, the active antibody binding site concentration used should be no greater than 3-fold above the K_D, so that the titration curve possesses as much K_D information as possible. If the KinExA equilibrium data is of exceptional quality, it is possible to determine a K_D even with the antibody binding site concentration being as much as 10-fold greater than the K_D. This is helpful for measuring very tight affinities in the single-digit picomolar range (or lower) in that it allows for a lower volume of antibody–antigen sample to be flowed through the bead pack for detectible and reproducible fluorescent signals, thus decreasing experimental run time. Because of the larger sample volumes needed for very tight affinities, KinExA equilibrium experiments can require multiple days of instrument time. Fourth, when generating an antibody-controlled equilibrium curve, the antibody binding site concentration should be greater than or equal to 10-fold above the K_D. Fifth, when designing a kinetics direct experiment, always choose final binding site concentrations after mixing of antigen and antibody so that at least 80% of the total antibody binding sites will be bound at equilibrium. This can be monitored by comparing the fluorescent signal acquired at equilibrium compared to the fluorescent signals seen in equilibrium titrations at 100% free antibody and 0% free antibody. Furthermore, choose antigen and antibody binding site concentrations that will provide enough points within the 5–10 min/point time resolution of the kinetics direct method so that the fitting model has enough data points in the curved portion of the exponential to accurately estimate k_a. Sixth, for the kinetics inject method, choose a range of antigen concentrations that result in 20–100% of the chosen constant antibody binding site concentration to be bound in the short mixing time at the given flow rate for the experiment. Lastly, because the 1:1 interaction models that fit KinExA equilibrium and kinetic experiments are dependent on the hard-coded values of antigen concentration used in the assay, the most rigorous concentration measurement methods should always be used for all protein reagents (Pace et al. 1995; Grimsley and Pace 2003), keeping in mind the binding site concentrations must always be used for data analysis. Of course, measuring an accurate antigen concentration does not necessarily give any indication of the active antigen concentration. It is therefore important that careful attention is paid to the “ABC” (antibody binding site concentration) parameter returned by the KinExA “standard affinity” equilibrium model, especially if an antibody-controlled curve is globally fit in a dual curve analysis with a K_D-controlled titration curve. The standard affinity model assumes the hard-coded antigen concentrations are 100% active while the ABC is allowed to float. In a dual curve analysis, the ABC parameter should match closely with the nominal ABC used in the experiment when fitting with the standard affinity model. If the nominal ABC falls outside the lowest value of the

returned 95% confidence interval range of the calculated ABC, then it signifies the activity of the antigen is significantly lower than the hard-coded nominal antigen concentration. Here the standard affinity model is forced to increase the estimation of ABC in accordance with the discrepancy between the active antigen concentration and the input nominal antigen concentration. As a result, the calculated K_D is artifactually higher (less tight) than the true value. Barring any significant antigen concentration measurement errors or sample preparation pipetting errors, one would instead have to make the nonsensical assumption that the active ABC is greater than 100% of the nominal ABC to believe the standard affinity model. It makes more physical sense to assume a less than 100% active antigen. Therefore when the lower limit of the 95% confidence interval of the returned ABC in a dual or multicurve analysis is greater than the nominal ABC using the standard affinity model, equilibrium data should instead be analyzed using the "antigen unknown" model in the KinExA software. The antigen unknown model assumes full activity in the hard-coded ABC value(s) while floating a correction factor for the hard-coded antigen concentration values. A least common multiplier (LCM) is generated from this model which is the factor that corrects the nominal antigen concentrations to active concentrations. It should be remembered that if the antigen unknown model is used (when necessary) to measure the K_D in a KinExA equilibrium experiment, the antigen activity calculated from the LCM must be applied to the same lot of antigen when used for acquiring complementary binding data with other independent biophysical methodologies (i.e., Biacore). In other words, all Biacore measurements should use the active antigen concentration (antigen nominal concentration $\times$ LCM) determined from the KinExA experiments.

Antibody Intramolecular Binding Cooperativity

When a simple 1:1 equilibrium model fails to properly describe a KinExA equilibrium data set, cooperativity phenomena between the two antibody binding sites must be considered (Blake et al. 2003). When both binding sites of an antibody act independently with the same characteristic affinity whether one or both sites become occupied with antigen, KinExA data should follow a simple 1:1 equilibrium binding model. When it appears the percent free mAb binding site titration curve decreases more steeply than what would be predicted from an independent binding site model the only conclusion that can be drawn is that antigen binding to one antibody binding site is having a positively cooperative effect on the affinity of the second antibody binding site. In other words, after one mAb binding site is bound the remaining site has an increased affinity for antigen. At lower antigen concentrations most antibodies bind to one antigen with the same affinity. At higher antigen concentrations, more antigen is available to bind to the second unoccupied antibody binding site, therefore the effects of positive cooperativity become apparent. The "n-curve" module in the KinExA software includes the

option to fit for positive cooperativity. This positive cooperativity model should only be used with great caution and all other sources such as a large amount of experimental noise in the titration curves should be ruled out as the cause of a poor fit to a 1:1 equilibrium model. Alternatively, it is more difficult to distinguish the cause of a KinExA equilibrium curve showing a more shallow decrease in percent free antibody binding sites than expected. Such "flattening" of a KinExA curve could not only result from a singly bound antigen negatively affecting the affinity of the second antibody binding site (negative cooperativity), but also from the existence of structural variations among the antibody binding sites in solution providing multiple classes of independent antibody binding sites (Blake et al. 2003). Hence, an assumption of negative cooperativity cannot be made in this case, whereas positive cooperativity can be the only cause of a steeper decrease of percent free mAb binding site seen in a KinExA titration curve.

KinExA and Biacore Comparison

The major advantage KinExA has over Biacore for measuring affinities of antibody–antigen complexes is that binding data are collected after equilibrium has already been reached. In contrast, Biacore affinity measurements rely on real-time kinetic data which can occasionally yield sensorgrams deviating from a simple 1:1 kinetic binding model, especially if the injected antigen is multivalent. If complex kinetic binding responses are acquired using Biacore, the data cannot be described with the correct complex binding model without the assistance of a more rigorous examination of the structural biology of the binding partners. KinExA measures K_D independently of kinetics and avoids this limitation. In fact, most KinExA equilibrium binding data for antibody binding to multivalent antigen are described well by a simple 1:1 equilibrium binding model, but the same binding constant ambiguity exists as was described for Biacore: when equilibrium KinExA data of antibody complexing with multivalent antigen is described well by a simple 1:1 binding model, it is unknown without further studies beyond the scope of KinExA whether the affinity measured is the intrinsic binding-site equilibrium dissociation constant or a stoichiometric equilibrium dissociation constant.

One of the first comparisons of solution-based biophysical methods, specifically ITC and stopped-flow fluorescence, to SPR showed equivalent binding constants were obtained with small molecules binding to carbonic anhydrase II (Day et al. 2002). Drake et al. (2004) later showed solution-based KinExA and surface-based Biacore yielded similar kinetic and equilibrium binding constants for three antibody–antigen interactions having affinities ranging from single-digit nanomolar to single-digit picomolar. Panels b and c in Fig. 5.5 show, respectively, KinExA equilibrium and kinetics data sets measuring the binding constants of the same "antigen-2′/mAb 2″ complex measured using the long off-rate Biacore technique as described in "High Resolution Experiments" (Drake et al. 2004). Figure 5.5b shows a dual curve analysis of both a monoclonal antibody-controlled curve

Table 5.1 Equilibrium dissociation constants and rate constants measured for three antibody–antigen complexes with Biacore and KinExA

Interaction	Method	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (pM)
Antigen-1/mAb-1	Biacore	$4.6 (0.1) \times 10^5$	$1.2 (0.1) \times 10^{-3}$	2500 (500)
Antigen-1/mAb-1	KinExA	$3.3 (0.6) \times 10^5$	$4.3 (0.7) \times 10^{-4}$	1300 (300)
Antigen-2/mAb-2	Biacore	$6.6 (1.5) \times 10^5$	$2.1 (0.5) \times 10^{-5}$	33 (11)
Antigen-2/mAb-2	KinExA	$1.1 (0.2) \times 10^6$	$1.3 (0.3) \times 10^{-5}$	12 (1)
Antigen-2′/mAb-2	Biacore	$2.7 (0.6) \times 10^6$	$1.6 (0.2) \times 10^{-5}$	6.2 (0.8)
Antigen-2′/mAb-2	KinExA	$2.8 (0.5) \times 10^6$	$1.1 (0.2) \times 10^{-5}$	4.0 (1.9)

Errors in parentheses are 95% confidence intervals of multiple replicate measurements. (Drake et al. 2004, reproduced with permission.)

(red line) and a K_D-controlled curve (blue line). The equilibrium data fit well to a simple 1:1 interaction model. Figure 5.5c shows the percent free antibody binding site concentration measured as a function of time as a solution of antigen-2′ and mAb-2 approached equilibrium. The K_D of the antibody–antigen complex as measured in this KinExA replicate was 3.8 pM and the measured k_a was 2.5×10^6 M^{-1} s^{-1}, which results in a k_d of 9.5×10^{-6} s^{-1}. The k_a measured with KinExA for the data set shown in Fig. 5.5c (2.5×10^6 M^{-1} s^{-1}) was virtually identical to the Biacore measurement shown in Fig. 5.5a (2.5×10^6 M^{-1} s^{-1}). The calculated KinExA value for k_d was also very close to the k_d measured with Biacore (1.6×10^{-5} s^{-1}), hence the affinities measured using KinExA (3.8 pM) and Biacore (6.1 pM) were also comparable.

Table 5.1 reproduces the results of the binding constants for three antibody–antigen complexes measured in multiple replicates using Biacore and KinExA (Drake et al. 2004). Table 5.1 represents data from the first rigorous comparison study between Biacore and KinExA for the measurement of such tight antibody–antigen complexes. First, the results in Table 5.1 show the measured K_D's varied on average by ~50% between the two techniques across a wide range of affinity estimates, which is very good considering the 95% confidence intervals. Second, it is also apparent the k_d's measured by Biacore using the long off-rate method for the antigen-2′/mAb-2 complex were very precise, varying by only ~12%. Third, there was very good agreement in measuring both rate constants where the k_a varied on average by only ~33% and the k_d differed on average by only ~44% between the two technologies. Overall, the data in Table 5.1 show that similar results were obtained from two independent biophysical techniques, one surface based and the other solution based. With proper experimental design, these results highlight that two different and complementary biophysical methodologies can reliably measure binding parameters for very tight antibody–antigen interactions. Other studies have shown how solution-based and surface-based biophysical technologies can provide rigorous complementary information when studying more complex interactions between mAbs and multivalent antigen (Abdiche et al. 2008).

Certainly it is not totally correct to assume that when properly using a surface-based biosensor instrument that all kinetic rate constants and equilibrium dissociation constants measured for antibody–antigen complexes will exactly match those measured using a solution-phase technique, especially for complexes having picomolar affinities. When statistically significant differences in binding measurements do arise between the two technologies, assuming the most advanced experimental methods have been utilized to generate binding data with the most optimal data processing protocols, the absence of any "gold standard" to which results can be compared presents the dilemma of having to decide which technology is providing the most accurate antibody–antigen binding information. While both Biacore and KinExA technologies are considered to be "label free" methods, technically only KinExA can truly make this claim. Consider the fact that one of the binding partners in a typical Biacore experiment often must be coupled to a surface using covalent amine coupling chemistry similar to external fluorophore protein labeling chemistries. With KinExA, no modifications are made to the binding partners. As emphasized earlier, antigen covalently bound to the bead resin in a KinExA flow cell is used only for detection of free antibody in solution and is not involved in the binding reaction being studied. Furthermore, even if no chemical modifications are made to an antibody when captured on a Biacore surface, the entropic reduction from tethering the antibody to the solution-like dextran surface could thermodynamically alter the affinity measurement in some rare cases. Also, charge effects from the carboxyl groups on Biacore surfaces could perturb kinetic measurements in cases where an antigen of a certain pI may be attracted to or repelled from the surface. Therefore from a "purist" perspective, one may conclude that because KinExA technology requires no protein modification under normal use and both purified binding partners interact in homogeneous solution, and because there is a greater potential for multiple sources of aforementioned artifacts in Biacore technology, KinExA more than likely provides more accurate binding information for purified antibody–antigen complexes when discrepancies arise between the two methods. However, the most likely source of significant disagreements between the two methods is an improper use of one or both instruments.

Other KinExA Uses

The utility of the KinExA instrument has also been shown to extend beyond equilibrium and kinetic measurements of purified binding partners free in homogeneous solution. Sasaki et al. (2005) developed a KinExA screening method that can assess several hundred hybridomas for active antibody. In this method, supernatants are flowed through the antigen-coated bead resin and clones are deemed "positive" when a fluorescent signal is generated from the secondary antispecies fluorescently labeled polyclonal antibody. An iterative supernatant pooling method of several hundred clones proved to be more efficient using

KinExA when compared to a traditional plate-based enzyme-linked immunosorbent assay (ELISA) screen. The higher sensitivity of the KinExA screen also found more "positives" and determined fewer "false positives" when the same samples were screened using ELISA (Sasaki et al. 2005). However, unlike a Biacore "low resolution" affinity ranking kinetic screen of antibodies out of supernatant, the information generated from the KinExA hybridoma screen is strictly qualitative. Additionally, Xie et al. (2005) developed a cell-based KinExA protocol to measure the on-cell affinities of mAbs to cell surface receptors. This method will be discussed in more detail in the next section.

Cell-Based Affinity Assays

As shown in the previous two sections, Biacore and KinExA are ideally suited for measuring the equilibrium dissociation constant of mAbs binding to purified antigen. However, some cell-surface antigens possess vital transmembrane domains which may prevent purification of the receptor; particularly G protein-coupled receptors (GPCRs). Even if the extracellular domain of a transmembrane antigen can be isolated, its purified conformation may differ from the native structure found on the cell surface and therefore sacrifice native functionality. Hence, cell-based affinity determination methods overcome transmembrane protein purification limitations and provide the biophysical scientist the flexibility to be able to study mAbs binding to cell surface antigen.

Cell-Based KinExA Affinity Measurements

Recently, a method was developed to measure the affinities of antibodies binding to cell surface receptors using KinExA (Xie et al. 2005). In this method, an increasing concentration of cells expressing the antigen of interest is titrated into a constant concentration of antibody and allowed to equilibrate. The equilibrated solutions of antibody mixed with cells are then centrifuged to separate the cells from any unbound antibody in solution. The KinExA instrument then determines the percent free antibody in each solution as described in "KinExA Technology". Dual curve analysis of "K_D-controlled" and "antibody-controlled" titrations to a simple 1:1 equilibrium binding model yields the K_D of the antibody binding to the cell surface antigen. Because antigen is titrated via cells whose antigen expression levels normally cannot be definitively known, the "antigen unknown" fitting model (see "KinExA Experimental Methods") must be used (Xie et al. 2005; Rathanaswami et al. 2008). KinExA instrument limitations, however, can restrict the throughput of the number of antibodies that can be studied per experiment from a logistical viewpoint, although a recently available autosampler for KinExA experiments has improved sample capacity. A second limitation of KinExA

technology for cell-based K_D measurements is that hundreds of milliliters of cells at concentrations on the order of millions of cells per ml may be required to study multiple antibodies.

FACS Affinity Measurement

A more practical and higher throughput means to study mAbs binding to antigen expressed on cell surfaces is with flow cytometry using fluorescence activated cell sorting (FACS) instrumentation. A FACS instrument measures multiple physical and fluorescent characteristics of thousands of individual cells in a fluidic stream directed through an excitation laser (Ibrahim and van den Engh 2003). In a FACS affinity experiment, monoclonal antibody is titrated into a constant concentration of cells expressing the target antigen. The titration of antibody into cells is normally performed directly into a 96-well plate where the use of a multichannel pipette allows for a simultaneous preparation of multiple titrations. After allowing enough time for equilibration and a brief washing step, a large excess (to avoid a potential Hook effect) of fluorescently labeled antispecies polyclonal antibody is added to each equilibrated well of the plate. The mean fluorescence intensity (MFI) of the cells as a function of the titrated antibody concentration in each well is then recorded using a FACS instrument. Because FACS instrumentation can accommodate a 96-well plate format, multiple antibodies can be studied in a single experiment and the acquisition time per well is on the order of seconds to minutes owing to sample volumes of only hundreds of microliters.

FACS technology has historically been used for characterizing cell surface interactions for several different systems. FACS has been used to rank the apparent on-cell binding strength of multiple phage-generated antibodies based on relative fluorescence intensities rather than their K_D's (Geuijen et al. 2005). FACS methods have been used to measure the affinities of Concanavalin A binding to lymphocytes (Gordon 1995), of chimeric mouse monoclonal antibody (C2B8) binding to CD20 expressed on SB cells (Reff et al. 1994), of anticommon acute lymphoblastic leukemia antigen mAbs binding to NALM-6 cells (Lebien et al. 1982), and of the programmed death-1 (PD-1) receptor binding to two PD-1 ligands (B7-H1, B7-DC) (Youngnak et al. 2003).

Linear and Nonlinear FACS Data Analysis

In each of the examples above, nonlinear equilibrium FACS data underwent a linear transformation so that traditional Scatchard Plots could be used to estimate the equilibrium dissociation constants. It has been shown, however, that linearizing nonlinear isotherm data via Scatchard Plots or any other type of single or double reciprocal plots can lead to mathematically skewed or unequally compressed data, which can often lead to erroneously concluding the existence of a complex binding

mechanism (Klotz 1997; Martin 1997). Continued use of Scatchard analysis today appears to be borne more out of tradition when previous technological limitations made linear regression a more practical computational tool. However, the advent of readily available personal computers powerful enough to run numerous software packages that facilitate nonlinear regression has essentially made linear transformation of nonlinear FACS equilibrium binding data outdated and unnecessary (Klotz 1997; Martin 1997; Wilkinson 2004). Nonlinear fitting of binding isotherm data from flow cytometry measurements has given estimates for the equilibrium dissociation constants of an anti-B1 antibody binding to CD20 (Cardarelli et al. 2002), of tacrolimus binding to yeast cell surface displayed anti-tacrolimus antibodies (Siegel et al. 2008), and of variable lymphocyte receptors binding to fluorescent ligand (Tasumi et al. 2009).

K_D-Controlled and Receptor-Controlled Data

While each of the aforementioned FACS studies demonstrate the utility of flow cytometry for measuring on-cell binding affinities, it is also important to note that in each case, whether data were fit nonlinearly or with linear reciprocal plots, the assumption is made that the free ligand concentration in solution at equilibrium is equal to the total ligand concentration titrated into the cells. In fact, this assumption must also be made for linear reciprocal plots such as Scatchard Plots. This assumption is not always correct, however. As with KinExA or any other binding assay, careful consideration must be given to the conditions under which ligand, in this case mAb, is titrated into cells and how they affect the shape of the binding isotherm, and what information is contained therein. More specifically, it is important to remember the ratio of the cell surface receptor in molar concentration (calculated by converting the number of receptors into moles and dividing by the solution volume the cells are contained in; it is assumed the volume the cells occupy is negligible) to the K_D of the ligand-receptor interaction drives the shape of the titration curve. For optimal conditions to estimate the K_D, titration conditions should generate a "K_D-controlled" curve, meaning the curve's shape is sensitive to the K_D of the ligand-cell receptor interaction and not to the receptor concentration. K_D-controlled conditions dictate that the cell receptor concentration should be at or below the K_D, and usually no greater than 3-fold above the K_D. In fact, it can be shown that assuming the free ligand concentration in each equilibrated solution equals the total ligand titration concentration actually holds under highly K_D-controlled conditions. A highly K_D-controlled binding isotherm can normally be identified by its shape having ample curvature which is entirely characteristic of the K_D of the ligand-receptor binding pair. As the ratio of the cell receptor concentration to the K_D increases, the titration curve becomes more and more "receptor-controlled" where the shape of the curve is influenced more by the cell receptor concentration than the K_D of the interaction. A receptor-controlled binding isotherm is generated when the cell receptor concentration is normally 10- to 20-fold or more above the K_D. Under receptor-controlled conditions, ligand at

initially low concentrations may stoichiometrically bind to receptor, resulting in an initial linear phase to the binding curve which contains no K_D information. A receptor-controlled curve contains only minimal K_D information in the small region of curvature near the saturating plateau of the titration curve. In the extreme receptor-controlled case, the titration curve will actually show no curvature, but instead show an entirely linear approach to a sharply breaking plateau point indicative of the stoichiometry of the interaction.

A 4-Parameter Nonlinear FACS Fitting Model

In a cell-based equilibrium titration experiment designed to measure the K_D of a monoclonal antibody binding to a cell-surface receptor, the cell receptor concentration in molarity is normally unknown, making it difficult to be able to ascertain before the experiment whether the receptor concentration or the K_D will have a greater influence on the binding curve. Recently, Drake and Klakamp (2007) have developed a new 4-parameter nonlinear equation and methodology based on the traditional multiple, independent binding site (MIBS) equation which takes into account the effects of the receptor concentration on the titration curve and thus fits cell-based binding data much more rigorously than previous methods. As in any other chemical titration method that uses fluorescence detection as a spectroscopic handle for bound ligand (L_B), the 4-parameter model directly relates the fluorescence (F) from a fluorescently labeled secondary polyclonal antibody to the amount of primary monoclonal antibody bound to the cell surface and is shown in Eq. 5.8.

$$F = P \cdot \frac{(\mathrm{K_D} + L_\mathrm{T} + \mathrm{nM_C}) - \sqrt{(\mathrm{K_D} + L_\mathrm{T} + nM_\mathrm{C})^2 - 4(nM_\mathrm{C}L_\mathrm{T})}}{2} + B \qquad (5.8)$$

The derivation of Eq. 5.8 from the MIBS equation, which assumes all independent binding sites possess identical equilibrium dissociation constants, is detailed by Drake and Klakamp (2007). Equation 5.8 relates fluorescence (F) to the concentration of bound ligand in terms of two known quantities, the total ligand concentration (L_T) and the molar cell concentration (M_C), and four unknown parameters, the equilibrium dissociation constant (K_D), the number of receptors of interest per cell (n), a proportionality constant (P) that relates arbitrary fluorescence units to bound ligand concentration, and background signal B. When fitting a nonlinear binding titration curve, an estimate for K_D is obtained when L_T and M_C are hard coded into the fitting model while K_D, n, P, and B are floated freely in the nonlinear analysis. M_C is calculated based on the number of cells and the final volume of each well in the titration. Each titration experiment normally includes a "blank" well that contains only cells, with $L_T = 0$, which are exposed to the same concentration of fluorescent polyclonal labeling antibody as those wells containing the same number of cells titrated with serially diluted antibody. Any signal detected at $L_T = 0$ is considered background signal, but simply subtracting this

"blank" signal from all other data points compounds the errors inherently associated with the signals of the blank and nonblank wells (Miller and Miller 1988). Hence, rather than a direct subtraction of background, the contribution from background to the total fluorescence signal in Eq. 5.8 is fit for with the unknown parameter *B*. It should be noted that the meaning of *B* changes in Eq. 5.8 if the measured fluorescence arises from directly-labeled cell-bound ligand instead of bound unmodified primary ligand labeled with a secondary fluorescent marker. For a titration of unlabeled ligand into cells with subsequent fluorescent labeling by a pAb, *B* represents any instrument noise and NSB of the fluorescently labeled pAb to the cells and should be the same at all points since a large excess of labeling pAb is used in the procedure; therefore *B* should be a constant at each point in the titration if a significant amount of NSB is not occurring with the unlabeled ligand. In contrast, with directly labeled ligand, the parameter *B* only describes the instrument noise, and background fluorescence may vary in direct relation with the labeled ligand concentration at each data point if significant NSB is present. In this case, it is recommended that directly labeled ligand be titrated into a nonexpressing parental cell line and an antigen-expressing cell line at the same M_C and identical ligand concentrations so any NSB seen in the control titration can be properly subtracted from the titration of interest. This subtraction of control data from the titration of interest is, of course, only necessary if NSB influences adversely the titration curve describing the interaction of interest.

A Traditional 3-Parameter Model Compared to the 4-Parameter Model

Because an extremely K_D-controlled titration contains very little information to estimate any parameters related to the receptor concentration, Eq. 5.8 can be further simplified under K_D-controlled conditions by combining *n*, M_C, and *P* resulting in a proportionality constant P' in a 3-parameter fitting model shown in Eq. 5.9:

$$F = P' \cdot \frac{L_T}{K_D + L_T} + B \quad (5.9)$$

where free ligand (L_F) is known and assumed to equal L_T. P', *B*, and K_D are the three unknown fitting parameters as described for Eq. 5.9. Again, this simplification can only be done under extremely K_D-controlled conditions where it is correct to assume free ligand concentration equals total ligand concentration (Drake and Klakamp 2007). It should also be pointed out that the 3-parameter equation originally published by Drake and Klakamp (2007) is not correct. However, the corrected Eq. 5.9 above yields results that are insignificantly different from those generated with the previously published erroneous 3-parameter equation (Drake and Klakamp 2007). Because it is more difficult often to gauge the molar concentration of cell surface receptors, it is normally unknown if a cell-based titration experiment is either K_D-controlled or receptor controlled, or is

influenced partially by both. Even after a visual inspection of the resulting binding curve, the shape of the curve will rarely show obvious characteristics of extreme examples of either case and will more than likely look like a curve that is a hybrid of K_D- and receptor-controlled conditions. Therefore any cell-based titration experiment is described more rigorously and accurately with Eq. 5.8 because the model not only takes into account the K_D influence on the shape of the binding curve, but also the influence of the cell receptor concentration. It is, in fact, more reasonable to assume that most titrations actually contain both K_D- and receptor-controlled influences, which makes an even stronger case that the 4-parameter model of Eq. 5.8 is the correct nonlinear fitting model for cell-based equilibrium titration data. Actually, Eq. 5.8 can be used for any titration where a spectroscopic handle or other signal exists that is directly proportional to bound ligand. In other words, this equation holds not only for FACS titrations.

Drake and Klakamp (2007) theoretically compared the effectiveness of the 4-parameter model (Eq. 5.8) to a less rigorous 3-parameter model which only assumed a K_D-controlled titration data set. In this theoretical example, a simple 1:1 equilibrium expression (not Eq. 5.8) was used to generate simulated data sets by calculating L_B [total bound (mAb)] as a function of L_T [total (mAb)] with identical antibody titrations into three data sets with equal numbers of cells but having three different levels of receptors/cell, or n values. The K_D of the antibody-receptor interaction was held constant for all three titrations and each value for L_B was converted into an arbitrary fluorescent signal by choosing an arbitrary proportionality constant. By varying n, the molar concentration of receptors (R_T) is varied, thus changing the ratio of the receptor concentration to the K_D in each case. The simulated data sets were then fit with both the 4-parameter model and the 3-parameter model and the parameters returned with each model were compared to the theoretical parameters used to generate the data. To calculate the simulated data sets, the K_D was assumed to be 50 pM, an arbitrary P value was chosen for each data set to convert L_B into an arbitrary fluorescence signal, and the molar cell concentration was held constant at 1.107 fM (each plate well containing 200,000 cells in a 300 μl volume of buffer). B was also chosen arbitrarily in each case. The molecular mAb concentration range used for each simulated titration was 5 nM–5 pM, reflecting an ideal concentration titration range spanning 100-fold above the K_D and 100-fold below the K_D. The first data set (Case 1) assumed $n = 1.000 \times 10^3$ receptors per cell which results in $R_T = 1.107$ pM. The second simulated data set (Case 2) assumed $n = 1.000 \times 10^5$ receptors/cell which results in $R_T = 110.7$ pM. The third data set (Case 3) assumed $n = 1.000 \times 10^6$ receptors per cell giving $R_T = 1.107$ nM. Case 1 was a K_D-controlled simulation because R_T is almost 45-fold below the K_D. Case 3 was a receptor-controlled data set where R_T is approximately 22-fold above the K_D. Because R_T was only 2-fold above the K_D, the titration in Case 2 was mostly sensitive to the changes in the K_D, but was also influenced somewhat by R_T. The nonlinear fits of each simulated data set with the 4-parameter model (Eq. 5.8) and 3-parameter model are shown in Fig. 5.7 and the resulting binding parameters returned from each fit are listed in Table 5.2 (Drake and Klakamp 2007).

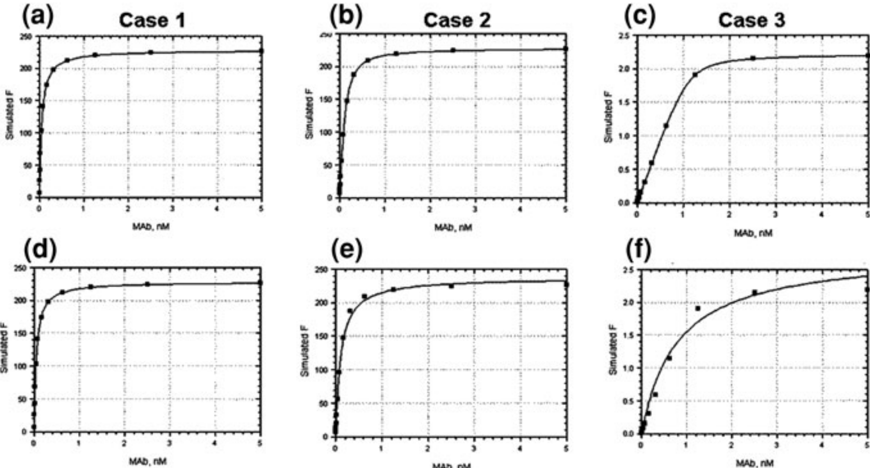

Fig. 5.7 Simulated cell-based titration curves. Fluorescence (*F*) is proportional to [mAb] bound to a cell surface as a function of total [mAb]. Case 1 data were generated under K_D-controlled conditions, Case 2 data were generated under mostly K_D-controlled conditions with some influence from the receptor concentration, and Case 3 data were generated under predominantly receptor controlled conditions. Data were generated assuming $K_D = 50.00$ pM with $R_T = 1.107$ pM in Case 1, $R_T = 110.7$ pM in Case 2, and $R_T = 1.107$ nM in Case 3. The 4-parameter model (Eq. 5.8) is used to fit data for all three cases in **a**, **c**, and **e**. A 3-parameter model is used to fit data for all three cases in panels **b**, **d**, and **f**. (Drake and Klakamp 2007, reproduced with permission.)

For Case 1, both the 4-parameter and 3-parameter models were fit to the data exceptionally well (Fig. 5.7a, b) and the fitted parameter values were almost identical to the actual parameters used to generate the data (Table 5.2). This was not unexpected since Case 1 was highly K_D-controlled and insensitive to n so either model was able to accurately describe the titration curve. With R_T being only slightly above K_D in Case 2 where the titration curve is sensitive to both K_D and n information, the 4-parameter model again fit the data well (Fig. 5.7c) and returned parameter values almost identical to the theoretical values (Table 5.2) while the parameters returned with the 3-parameter model began to deviate from their actual values (Table 5.2). In particular, the 3-parameter model estimated a K_D (105 pM) over 2-fold greater than the actual K_D (50 pM). Note, also how the 3-parameter model slightly missed several theoretical data points shown for Case 2 in Fig. 5.7d. As expected the 3-parameter model was unable to achieve a satisfactory fit to the highly receptor-controlled data set in Case 3 (Fig. 5.7f), hence the estimated binding parameters from the poor fit differed significantly from their actual values (Table 5.2). The best "fit" with the 3-parameter model returned a K_D value (787.1 pM) almost 16-fold greater than the actual 50 pM affinity. Alternatively, the 4-parameter model fit the data well in Case 3 (Fig. 5.7e) and was able to correctly estimate all theoretical parameters (Table 5.2). Figure 5.7 shows the noticeable contrast in the shapes of nonlinear binding curves generated under

Table 5.2 The resultant parameters for the affinity (K_D), proportionality constant (*P*), nonspecific binding (*B*), and (where applicable) the number of receptor binding sites per cell (*n*) when fitting the simulated data shown in Fig. 5.7 using the nonlinear 4-parameter titration model (Eq. 5.8) and a nonlinear 3-parameter titration model listed alongside the actual parameters used to generate the simulated data

Parameters	Case 1 Actual values	Case 1 4-Parameter fit values	Case 1 3-Parameter fit values	Case 2 Actual values	Case 2 4-Parameter fit values	Case 2 3-Parameter fit values	Case 3 Actual values	Case 3 4-Parameter fit values	Case 3 3-Parameter fit values
K_D (pM)	50.00	49.99	50.05	50.00	50.00	105.0	50.00	49.99	787.1
n (#rec/cell)	1.000×10^3	997.3	N/A	1.000×10^5	9.973×10^4	N/A	1.000×10^6	9.973×10^5	N/A
P (FU/M)	200.0	199.9	221.4 (221.4)	2.000	2.000	236.7 (221.4)	2.000	1.999	2778 (2214)
B (FU)	7.000	7.000	6.983	7.000	6.999	0.7587	7.000	7.000	0.0000

The numbers in parentheses are the values for P' that should have been returned from the 3-parameter fitting equation. (Drake and Klakamp 2007, reproduced with permission.)

K_D-controlled (Case 1) and receptor controlled (Case 3) conditions. The binding isotherm in Case 1 shows a K_D-controlled titration curve maintains curvature to saturation while the highly receptor-controlled data in Case 3 show a stoichiometric linear onset followed by only a small amount of curvature. These simulations served to show how the more rigorous 4-parameter model can always correctly describe a cell-based titration curve whether the titration is K_D-controlled or receptor controlled. The simulations also illustrate how the use of traditional fitting models which always assume a K_D-controlled titration and fail to consider the influence of receptor concentration can lead to erroneous cell-based affinity measurements. It should be emphasized that the values listed in Table 5.2 are the results of fitting noise- and error-free simulated data sets. In reality, binding data will most certainly contain noise and Eq. 5.8 will most likely not return a meaningful and accurate value for n when fitting a highly K_D-controlled data set. Conversely, Eq. 5.8 should not theoretically return a meaningful and accurate value for K_D when fitting an extremely receptor-controlled data set. As discussed with KinExA data analysis, a simultaneous fit of both a K_D-controlled curve and a receptor controlled curve using Eq. 5.8 would be the most ideal approach to more accurately estimate K_D and n. Here P and B would be fit locally to each curve while the values of K_D and n would be calculated from a global fit of two or more titration curves run under different controlling conditions (Drake and Klakamp 2007). Unfortunately, in cases where the K_D is extremely tight and the cells have extremely high receptor expression levels, an unfeasibly low number of cells may be required to reduce the receptor concentration below the K_D of the interaction for a K_D-controlled experiment. Hence, it is always best whenever possible to use cell lines that have lower receptor expression levels.

Experimental Methods for FACS K_D Measurements

The 4-parameter model in Eq. 5.8 will describe any titration experiment where a spectroscopic or radioactive signal is proportional to L_B as a function of L_T, which makes its application to FACS-based data an ideal method for measuring the affinities of antibodies binding to receptors expressed on a cell surface. The introduction of integrated 96-well plate-based readers in FACS instruments has made it possible to prepare and study multiple antibody titrations in a single experiment. The ideal concentration range in any titration is typically from 100-fold above the K_D to 100-fold below the K_D. When serially diluting mAb into cells, at least one well should serve as a "blank" well (no antibody added to cells) to help anchor the background parameter B in Eq. 5.8 during the nonlinear fit of the data. Ideally, titrations should be performed in duplicate or triplicate if possible. It may be more practical to perform single titrations when studying five or more antibodies in a single experiment. It is also imperative that the same number of cells is added to each well. Equilibration of the mAb cells normally occurs while the 96-well plate (or plates) is gently shaken at 4 °C to minimize antibody internalization and to prolong the viability of the live cells. The brief

postequilibration and postlabeling washing steps in the FACS affinity protocol have caused some concern in the literature that a perturbation of the equilibrium from dissociated antibody will affect the K_D measurement (Rathanaswami et al. 2008). Kinetic simulations, however, show that any postwashing dissociation of mAb results in such a low free [mAb] that effects from any antibody rebinding are minimal (on the order of 5–10%). Moreover, because the k_d is the same at all titration points since the same complex is being measured at each point, the shape of the titration curve is not affected by the mAb dissociation during the washing steps. It is important to realize that absolute bound concentrations of mAb are not being measured, but rather a signal proportional to the bound mAb is being measured, so as long as all the points relate to each other proportionally the curve describes correctly the binding reaction. Even with that said, the washing procedures should still be performed as quickly as possible to obtain the highest signal intensities for each data point. When recording the MFI using FACS instrumentation, at least 5,000–10,000 "events" (fluorescence on a live cell) should be measured for each data point. Scientist 3.0 software (MicroMath Scientific Software, St. Louis, MO) provides users with ample modeling flexibility and is highly recommended for the nonlinear analysis. Obviously, other software packages that allow the programming and fitting of Eq. 5.8 may also be used.

Interpretation of Cell-Based K_D Measurements

One of the most significant limitations of any cell-based monoclonal antibody affinity method is the interpretation of the measured K_D. Because the KinExA cell-based technique estimates on-cell affinity indirectly via free antibody in solution as a function of the cell antigen ("antigen unknown") concentration, it cannot differentiate between antibodies bound bivalently or univalently to the cell surface. In a standard solution-phase KinExA experiment, antibodies that have one or both binding sites free can bind to the detection resin, albeit the former with half the probability of the latter, where the resulting signal is directly related to the free antibody binding site concentration. In a KinExA cell-based experiment antibodies univalently bound to cells are centrifuged away from the free antibody concentration; hence, the cell-based KinExA method can only measure the free antibody molecular concentration. Because of this, the only possible interpretation of the measured K_D is that it represents the stoichiometric or molecular K_D of the antibody binding to the cell surface. In contrast to the KinExA method, the FACS affinity method detects antibody directly bound to the cell surface, but it is still impossible to discern whether the K_D measured is the site-binding or microscopic affinity or a stoichiometric or macroscopic K_D. In the former case, it is more appropriate to use the antibody binding site concentration (twice the molecular concentration) for L_T when performing nonlinear analysis of binding data with Eq. 5.8 while the whole molecular antibody concentration should be used in the latter situation. Extensive theoretical and experimental consideration has been given to the binding nature of multivalent ligand to cell surface receptors

(Reynolds 1979; Dower et al. 1984; Kaufman and Jain 1992; Ong and Mattes 1993; Vanden Broek and Thompson 1996; Hlavacek et al. 1999; Yang et al. 2002; Jung et al. 2005, 2008, 2009; Mack et al. 2008). It may be helpful to consider that exact theoretical modeling in solution (not on a cell surface) has shown that in a titration where the concentration of the bivalent ligand is in significant excess over the univalent receptor that most of the bivalent ligand has only one bound receptor (Mack et al. 2008). Considering that in a FACS titration as outlined above, the bivalent mAb is in excess at almost all the titration points except for the first one or two points, it may be reasonable to assume the mAb is binding predominately in a univalent manner. Of course, this theoretical treatment relates to univalent receptor and bivalent ligand binding in solution rather than on a cell surface where receptors may be constrained spatially near univalently bound mAb where they could more easily form a bivalent complex with mAb. Even though the above argument for monovalent binding of mAb to the cell surface antigen can be made logically, the possibility of antibody binding bivalently to the cell surface at least partially cannot be discounted until further studies with FACS-based titrations are performed to discern how mAbs bind to antigens on cell surfaces.

Normally few conclusions can be drawn by comparing Biacore or KinExA site-binding K_D measurements of purified antigen and antibody to cell-based measurements primarily for two reasons: (1) the possibility of antibody binding avidly (bivalently) to a cell surface, and (2) there is always the question as to whether purified antigen retains the same conformation and hence binding properties as native protein on a cell membrane. Additionally, most Biacore and KinExA experiments are performed at room temperature (KinExA is currently not equipped with temperature control) while FACS affinity titrations are usually equilibrated at 4 °C to avoid mAb internalization and to ensure cell viability. Measuring the affinity of a Fab fragment in a cell-based experiment could give insight to the intrinsic site-binding constant of the parent bivalent antibody, but there also may be some uncertainty as to whether even the Fab fragment retains all native binding properties of the full antibody. There is always the argument that it is irrelevant whether a cell-based experiment yields a therapeutic antibody's intrinsic single site affinity or an avidity-influenced affinity because the K_D most likely represents the more biologically relevant "functional avidity" of the antibody binding to a cell surface. But this argument quickly breaks down if the antigen expression density of the cells used in an on-cell affinity experiment does not exactly match the in vivo expression levels of antigen in the models used in animal or human preclinical and clinical studies in the latter stages of drug development. Even with cells having a perceived low antigen expression level, lipid rafts could still form concentrated microdomains of antigen on the cell surface and promote bivalent binding of antibody.

Because of the numerous limitations inherent to the interpretation of cell-based antibody affinity data, cell-based measurements are by no means meant as a far reaching substitution for other biophysical methods such as Biacore and KinExA methods which are ideal for studying purified antigen binding to antibody either immobilized to a solution-like biosensor surface or in homogeneous solution, respectively [although, it should be noted, recent advances have been made in

solubilizing stable GPCRs for Biacore studies (Rich et al. 2009)]. Instead, cell-based antibody affinity experiments using FACS or KinExA should only be performed when purified receptor is either unobtainable or known to lose native structure and/or function.

Concluding Remarks

The surface-based (Biacore), solution-based (KinExA), and cell-based (FACS, KinExA) technologies that can provide antibody–antigen affinity measurements are essentially designed for ease of use for investigators spanning several disciplines. Ease of use, of course, does not always parallel the proper use of the instrumentation. Binding estimates measured may be meaningless if data were acquired incorrectly. This chapter was designed not only to outline the proper functionality of these techniques, but also to emphasize how experimental design, data processing, and data interpretation are essential for acquiring reliable kinetic and thermodynamic binding characteristics for any therapeutic monoclonal antibody discovery program. Experimental design for any method described here must stem strictly from chemical binding principles and adhere to the demands each instrument requires for reproducible and reliable data acquisition. Optimal data processing is necessary so that an observed signal truly represents an antibody–antigen binding event in solution. Theoretical fitting models of antibody–antigen binding data must make sense within the confines of bimolecular binding phenomena in the absence of additional structural information of the complexed binding partners. In addition, experiments should be designed to obtain the intrinsic site-binding equilibrium dissociation constants of antibody–antigen complexes, which is especially challenging for cell-based affinity assays. Additionally, because there are inherently fewer sources of artifacts when purified protein binding partners interact freely in homogeneous solution, KinExA or other solution-phase technologies should always complement Biacore platforms and should yield the more reliable binding measurements when discrepancies with Biacore do occur. In summary, solid-phase (Biacore), solution-phase (KinExA), and cell-based (FACS, KinExA) biophysical methods provide rigorous and reliable kinetic and affinity data to aid in the progression of lead therapeutic antibody candidates into clinical development stages.

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Chapter 6
Considerations in Establishing Affinity Design Goals for Development of Antibody-Based Therapeutics

Mohammad Tabrizi

Abstract Establishing design goals with respect to antibody affinity is a critical consideration that should be incorporated into development strategies from the earliest stages of the discovery process for antibody-based therapeutics. Selection of the adequate affinity for a functional antibody should allow achievement of the maximum therapeutic benefit at a dose associated with a manageable cost of goods and the intended route of administration. Application of theoretical pharmacokinetic (PK) and pharmacodynamic (PD) modeling with incorporation of relevant parameters with respect to antibody PK properties and biodistribution, antigen turnover rate, and antigen concentrations under physiological and pathological conditions can facilitate determination of the optimum affinity required. This chapter will review the critical considerations necessary for the design of optimum affinity goals for antibody-based therapeutics.

Introduction

A unique feature of antibody-based therapeutics is the high specificity conferred by the antibody interaction (via the variable region i.e. paratope) with a specific region on the targeted antigen (epitope); hence, it is not surprising that efficacy and safety of this class of therapeutics is generally correlated with the extent of their interaction with their intended target antigen (Tabrizi et al. 2009a, 2010). For functional antibodies, determination of the optimum equilibrium dissociation constant of antibody-based therapeutics with their targets should allow

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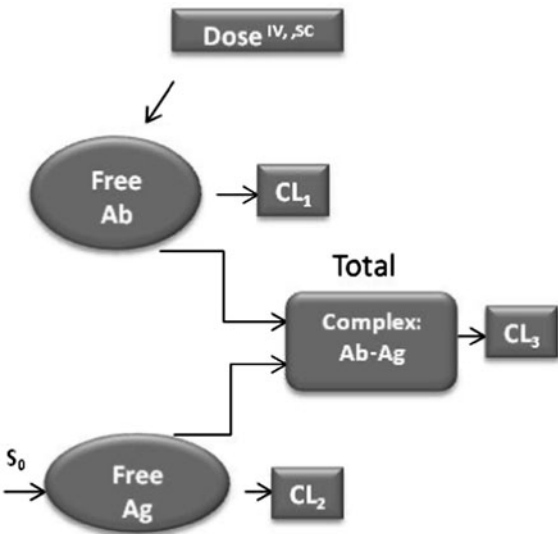


Fig. 6.1 A simple bi-molecular kinetic model of an antibody interaction in vivo with an antigen within the plasma pools. The model accounts for antibody elimination and distribution, the affinity for the interaction of the antibody with antigen, free antigen turnover rate and elimination of the antibody−antigen complex (*Ag* Antigen; *Ab* Antibody; *Ab–Ag* Antibody–Antigen Complex; *CL* Clearance, S_0 Antigen synthesis rate)

achievement of the maximum therapeutic benefit at a dose associated with a manageable cost of goods as affinity could directly impact antibody potency. As thermodynamic principles govern the bimolecular reversible interactions between antibody and antigen molecules, quantitative evaluation of this bimolecular interaction makes it possible to determine the impact of antibody affinity on the required clinical dose. Binding principles from physical chemistry can be applied to describe the association rate constant (k_a), the dissociation rate constant (k_d), and the equilibrium dissociation constant (K_D), also known as the "affinity" of the antibody−antigen interactions (discussed in Chap. 5). Knowledge of the affinity, antibody concentrations, antigen concentrations, and the fraction of the antigen bound is essential for evaluating the relationships between affinity and potency for a functional antibody. Application of theoretical and quantitative pharmacokinetic (PK) and pharmacodynamic (PD) modeling with incorporation of relevant parameters with respect to antibody PK properties and biodistribution, antigen turnover rate, and antigen concentrations under physiological and pathological conditions can facilitate determination of optimum affinity design goals for antibody-based therapeutics (Fig. 6.1; Tabrizi et al. 2009a). It is of critical importance to realize that the accuracy of theoretical predictions will be highly dependent on the underlying assumptions employed during the modeling exercise, and hence particular attention must be given in amalgamation of the relevant information with respect to the underlying biology and pharmacology, target antigen properties, and antibody characteristics as discussed in various chapters in this book.

The relationships between the clinical dose and the desired affinity for any functional antibody depend not only on antigen concentrations and antigen turnover rate, but also on antibody PK and biodistribution properties, and recruitment of effector functions as related to the antibody isotype (see Chap. 4). It is important

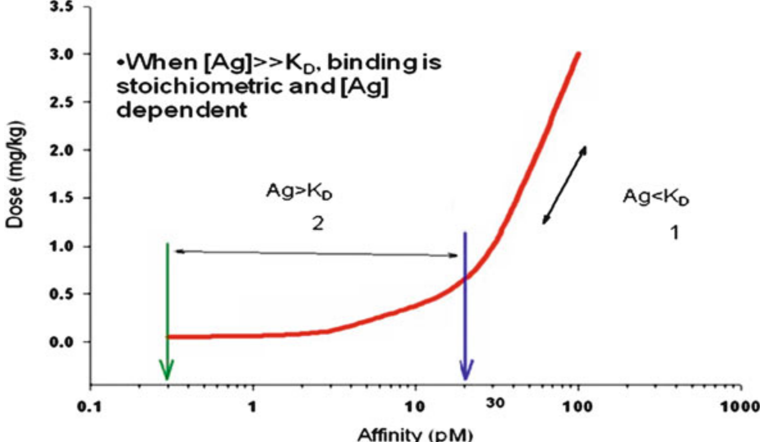


Fig. 6.2 Theoretical relationship between the antibody affinity and dose. The *curve* describes the region where improvements in affinity results in improvements in the antibody potency i.e., reduction in dose requirements (Region 1). The potency ceiling for affinity, a point where further improvements in affinity does not produce significant improvements in potency and clinical dose that occurs when affinity is reduced to about 1/10th of the antigen concentration (Region 2, Roskos et al. 2007)

to realize that improvements in antibody affinity can only reduce the clinical dose up to a certain point (Fig. 6.2); Thermodynamically, when antigen concentrations are less than the antibody affinity (Ag $<<K_D$), the binding is governed by the affinity of antibody for antigen, and an improvement in affinity can result in reductions in the required dose necessary for antigen suppression that leads to improvements in antibody potency (Fig. 6.2, Region 1); however, the potency ceiling for the affinity, a point where further improvements in affinity does not produce additional improvements in potency and clinical dose, occurs when affinity is reduced to approximately 1/10th of the antigen concentration (Fig. 6.2, Region 2). This is a critical consideration for antibody design in order to maximize potency of a therapeutic antibody in vivo (Roskos et al. 2007). This chapter will review the critical considerations necessary for the design of optimum affinity goals for antibody-based therapeutics.

Target Antigen Properties

An understanding of the target antigen properties is a critical factor for determination of the antibody affinity design goals. Antibody-based therapeutics can be designed to target either soluble or cell-associated antigens. Factors outlining the critical properties for each class of antigens are summarized in Table 6.1. In general for soluble antigens, following administration of therapeutic doses of an

Table 6.1 Considerations in the design of affinity goals with respect to antigen properties

Secreted/Soluble antigens	Cell membrane antigens
Antigen levels	Expression pattern
Serum and biophase	Location
Disease versus normal	Density/cell
Antigen serum clearance	Normal tissue expression
Antigen receptor(s)	Internalizing antigens
Affinity to antigen	Shed extracellular domain/receptors
Receptor density/cell	Serum levels
Serum antigen binding proteins	Disease versus normal
	Ligand affinity to antigen
Affinity and PK of other experimental antigen binding therapeutics showing preclinical or clinical efficacy	Affinity and PK of other experimental Ag binding products showing preclinical or clinical efficacy

antibody, it is anticipated that the free concentrations of the soluble antigen are suppressed (Tabrizi et al. 2009a, b). However, while the elimination rate of small antigens is reduced following binding to the antibody, a simultaneous increase in the antibody−antigen complex is observed (Chap. 13). The magnitude of the in vivo increases in antibody−antigen complex concentrations will be dependent on the turnover rate of the antigen (i.e. antigen synthesis and clearance rate) relative to that observed for the antibody and the elimination rate of the antibody−antigen complex (Tabrizi et al. 2009a, b). Unlike soluble antigens, interaction of antibodies with cell-associated internalizing antigens can greatly impact their PK. In contrast to soluble antigens, membrane-associated antigens that internalize can greatly enhance the antibody clearance through a target-mediated, specific process (Tabrizi et al. 2009a, b; Roskos et al. 2007). The antigen-mediated clearance pathway takes place through binding of the antibody to the antigen and subsequent internalization of the antibody–antigen complex, which is followed by degradation of the internalized antibody and antigen (Tabrizi et al. 2009a, b; Roskos et al. 2007). Under certain conditions, high affinity antibodies can be cleared at a faster rate relative to low-affinity antibodies (Roskos et al. 2007). This property can be highly beneficial for development of antibody–drug conjugates (ADC's, see Chap. 16) where a more rapid internalization rate can be beneficial in delivering ultra-potent toxins into cancerous cells more efficiently.

Considerations with respect to antigen concentrations in serum or biophase (effect compartment) are critical for the design of optimum affinity design goals for antibody-based therapeutics aimed at maximizing their in vivo potency. As shown in Fig. 6.3, the potency ceiling for affinity is highly dependent on the antigen concentrations. In general, when antigen concentrations are lower than antibody affinity (Fig. 6.3), higher affinity antibodies may be more efficacious.

In the presence of a large gradient between antigen concentrations in plasma and the effect compartment(s) such as lungs, synovial fluid, and lymph nodes, integration of information regarding antibody PK and biodistribution, and antigen concentrations in the biophase is highly critical for determination of antibody

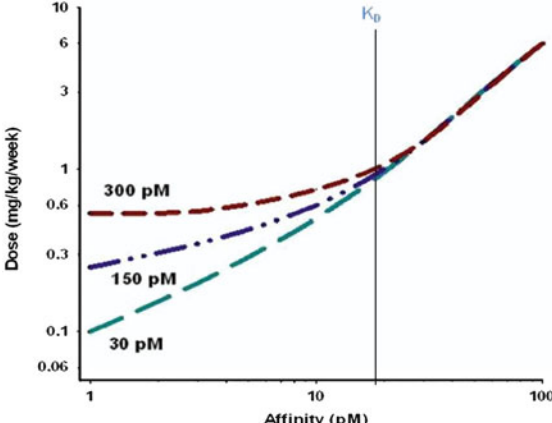


Fig. 6.3 Theoretical relationship between the antibody affinity and dose at three different antigen concentrations

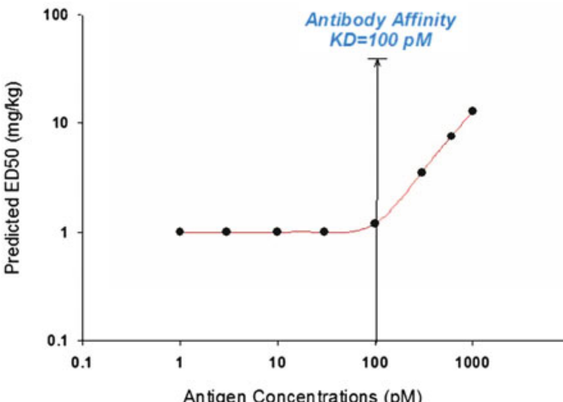
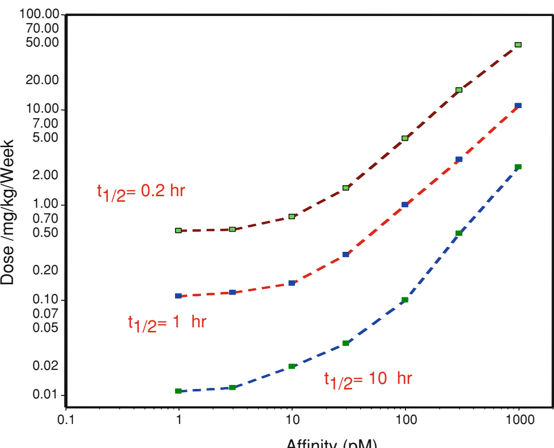


Fig. 6.4 Theoretical relationship between the biophase antigen concentrations and antibody neutralization potency (ED50) for an antibody with KD of 100 pM. Understanding of this simple property is of critical importance in translation of in vivo preclinical efficacy and safety data in prediction of dose requirements in human clinical studies

affinity design goals. In instances where antibody penetration in a particular organ can be limiting (see "Design Goal Considerations: Antibody Biodistribution"), particular considerations with respect to antigen concentrations, antibody affinity, and antibody penetration (ratio between antibody concentrations in the biophase relative to that in serum; *K*p) as well as biodistribution properties (linear vs. nonlinear distribution) will be critical for understanding of the dose requirements and establishing antibody affinity design goals (Tabrizi et al. 2009a, b). Figure 6.4 highlights the impact of the biophase antigen concentrations on the predicted antigen neutralization potency for an antibody with a K_D of 100 pM. Where baseline antigen concentrations are below the antibody affinity, as the binding is K_D-dependent, no impact on antigen neutralization potency (and hence antibody dose) is observed. When baseline antigen concentrations are above the antibody affinity, an increase in the antibody dose is predicted as the neutralization potency of the antigen by antibody is now antigen dependent. An understanding of this simple property is of critical importance in translation of in vivo preclinical

Fig. 6.5 Theoretical relationship among the antibody affinity, dose, and antigen turnover rates (antigen clearance). Increases in antigen turnover rates will have a direct impact on antibody dose requirements for suppression of >98% antigen in serum or plasma at a given antibody affinity



efficacy and safety data for prediction of the dose requirements in human clinical studies.

In addition, the turnover rate for the target antigen is a critical consideration for determination of the optimum affinity for antibody-based therapeutics. Figure 6.5 depicts the theoretical relationships between antibody dose, antibody affinity, and antigen turnover rates. For soluble antigens with rapid turnover rates (as reflected by a short elimination half-life: 0.2 h), a larger dose is required to suppress the antigen in serum relative to the dose required for suppression of the same antigen with a slower turnover rate (as reflected by a longer elimination half-life: 10 h).

Design Goal Considerations: Antibody Biodistribution

Similar to their small molecule counterparts, biodistribution of antibody-based therapeutics is a key consideration that can be modulated to impact the ensuing in vivo pharmacological effect(s) (Tabrizi et al. 2009b). As the concentration of drug within the proximity of the biological receptor determines the magnitude of the observed pharmacological responses, the optimum balance between the antigen concentrations and the antibody affinity can directly impact the requirements for dose and dosing frequency for antibody-based therapeutics. For example, it is established that under steady-state conditions, 500- to 1,000-fold lower antibody concentrations in lungs are achieved relative to the antibody concentrations in serum (Tabrizi et al. 2009b). Hence, in instances where lungs are the target organs for antigen neutralization, evaluation of the balance between antibody biodistribution across species, antibody affinity for the orthologous antigens, and antigen concentrations are critical components in determination of antibody design goals and translational considerations. Similarly, antibody-based therapeutics have been

utilized successfully in the management of various inflammatory diseases such as RA, and psoriasis. As inhibition of the target antigen in synovium or psoriatic skin is necessary for induction of the pharmacological effect in these indications, antibody penetration into these compartments is a critical requirement for therapeutic efficacy. For example, previous studies reported approximately >5-fold lower concentrations of IgG antibodies in synovial fluid in human RA patients (Tabrizi et al. 2009a, b). Due to lower synovial concentrations relative to serum following systemic administration of antibodies, both affinity and PK half-life are among the critical factors that could impact the clinical dose, dosing frequency, and the extent and duration of synovial antigen suppression (Tabrizi et al. 2009b). Under these conditions, application of theoretical PK–PD modeling can be useful in demonstrating the impact of improvements in antibody affinity on the suppression profile of a circulating antigen- this can be evaluated using a bimolecular interaction PK–PD model as shown in Fig. 6.1 (Tabrizi et al. 2009b).

Additionally, distribution of antibody-based therapeutics from the vascular space to the target tumor compartment and optimum tumor exposure are important considerations in designing antibody-based oncology drugs (see Chap. 9 for a detail discussion). In addition to tumor properties, penetration of antibodies into tumors can be influenced by factors such as antibody affinity, antigen density, and internalization, as well as antibody metabolism by the tumor. A number of comprehensive reviews have recently addressed this topic in detail (Tabrizi et al. 2009b; Thurber et al. 2008a, b, 2007; Graff and Wittrup 2003). Under non-steady-state conditions, an inverse relationship between antibody affinity and tumor penetration has been predicted. This inverse relationship is termed the "binding site-barrier" hypothesis and can be offset by factors such as dose and antibody elimination half-life. For antibody fragments with rapid clearance rates ($t_{1/2} \approx$ minutes for scFv; hours for Fabs), and under non-steady-state conditions, tumor penetration is predicted to be highly influenced by antibody affinity. However, full length antibodies generally have a long elimination half-life and are administered frequently, (i.e. weekly, biweekly, or monthly) where steady-state serum concentrations are achieved (Chap. 9). Under steady-state conditions, which is a condition generally met in clinical practice, binding equilibrium between antibody and antigen is achieved rapidly and the movement of antibody through the tumor can be governed by antigen turnover rate, antigen density, as well as the antibody concentration gradient. In general, cell-associated antigens undergo internalization at constitutive rates, ranging from minutes to days. After binding to cell-associated antigens, antibodies are internalized at a similar rate as the antigen. The internalized complex then is transferred to the lysosome where it undergoes degradation. The newly synthesized antigen following resurfacing can then interact with the unbound antibody. Again under non-steady-state conditions, it is predicted that tumor exposure can be reduced by the rate of antigen recycling.

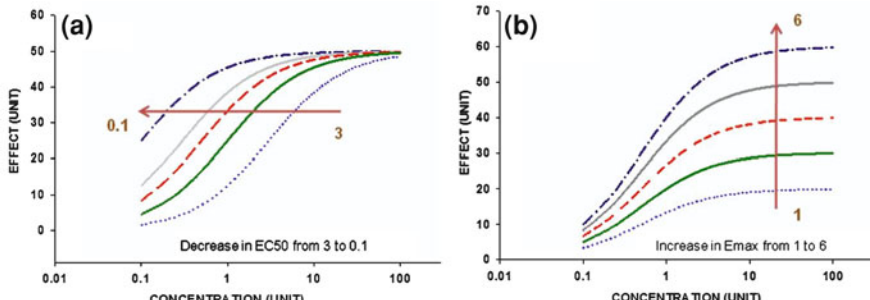


Fig. 6.6 **a** Modification of antibodies to increase activation of complement by enhancing affinity for C1q results in an increase in complement recruitment and target cell lysis in vitro with a direct impact on antibody potency (EC_{50} is increased by 30-fold). **b** Higher affinities for Fcγ receptors impacts the maximum efficiency of cell killing (E_{Max} is increased by sixfold) via the ADCC pathway (Moore et al. 2010)

Design Goal Considerations: Effector Function Recruitments

Interaction of human IgG antibodies with Fcγ receptors and the complement pathway (C1q) is of critical importance for antibody function as the PD and safety profiles of therapeutic antibodies in vivo can be partly regulated by these interactions (Desjarlais et al. 2007; Bornstein et al. 2009). Engagement of antibody with various immune cells and complement proteins can result in activation of effector functions like antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) respectively. Optimization of antibody interactions with immune effector cells or complement proteins has favorably impacted the clinical efficacy profile of therapeutic antibodies (discussed in Chap. 4). Modification of antibodies to increase activation of complement by enhancing affinity for C1q results in an increase in complement recruitment and target cell lysis in vitro with a direct impact on antibody potency (EC_{50}; Fig. 6.6a). Engineered antibodies with higher affinities for Fcγ receptors have directly impacted the maximum efficiency of cell killing (E_{Max}) via the ADCC pathway (Fig. 6.6b). When the in vivo potency of therapeutic antibodies is modulated to enhance ADCC and CDC activity, many important factors are critical for effective determination of antibody design goals. These factors are: (1) antibody affinity for the target antigen, (2) antibody affinity for effector cells, (3) antibody distribution into the biophase, (4) target and effector cell concentrations (ratio) in the biophase, and (5) cross-reactivity of the lead candidate to target and effector cells across species.

Table 6.2 Summary of selected examples where subcutaneous injection is the preferred route of administration at market entry for the final product

Drug	Dose	Route	Frequency	Formulation	Delivery
Simponi	50 mg fixed dose	SC	Once a month	50 mg/0.5 ml	Pre-filled syringe
Humira	40 mg fixed dose	SC	Once every 2 weeks (+MTX) Once weekly (−MTX)	40 mg/0.8 ml	Pre-filled syringe
EnbreL	25 mg fixed dose 50 mg fixed dose	SC	Once every 2 weeks (Psoriasis) Once weekly (RA)	25 mg/1.0 ml after reconstitution	Sterile powder to be reconstituted with 1 ml of sterile water for injection (physician supervision)
Stelera	45 mg (≤100 kg) or 90 mg (>100 kg) fixed dose	SC	45 mg or 90 mg weeks 0 and 4 and repeated every 12 weeks	45 mg/0.5 ml or 90 mg/ 1.0 ml	Pre-filled syringe or vial (physician supervision)
Prolia	60 mg	SC	60 mg once every 6 months	60 mg/1.0 ml	Pre-filled syringe or vial

Information was extracted from the product package inserts as published by the Food and Drug Administration

Design Goal Considerations: Administration Route

A final critical consideration for establishing the optimum affinity design goal for antibody-based therapeutics is the desired route of administration in the target population. A review of the currently marketed antibodies in the USA indicates that the subcutaneous (SC) route is a preferred method of administration for antibodies employed in the treatment of various inflammatory diseases (See Table 6.2 for selected examples). Administration of the antibody-based therapeutic via the SC route generally requires that the final efficacious dose be delivered as a single fixed dose based on the desired administration frequency (biweekly or monthly). Delivery of fixed doses for antibody-based therapeutics via the SC route is limited generally by an acceptable delivery volume of approximately 1 ml per injection due to ease of application for patients and the maximum final formulation concentrations currently achievable at 100–120 mg/ml. Based on these considerations, it is not generally possible to deliver fixed doses of greater than 100–120 mg per injection (Table 6.2).

As evident from Table 6.2, an integrated approach via application of theoretical PK and PD modeling with incorporation of relevant parameters as described previously can facilitate determination of the optimum affinity requirements when SC injection is the desired route at market entry. The theoretical relationships among antibody affinity (2–300 pM), antibody dose (1–10 mg/kg; fixed doses

Table 6.3 Relationships among antibody affinity (2–300 pM), antibody dose (1–10 mg/kg), and predicted antigen (50 pM) suppression following administration of 4 monthly doses of antibody (steady-state serum concentrations of the antibody were achieved) via a subcutaneous route

Dose (mg/kg)	1	2	3	4	5	6	8	10
Fixed dose (mg)	60	120	180	240	300	360	480	600
KD (pM)	Predicted antigen suppression (%) Ag = [50 pM]							
2	98	>99	>99	>99	>99	>99	>99	>99
10	94	97	98	99	99	99	>99	>99
20	90	94	96	97	98	98	99	99
30	84	91	94	96	96	97	98	98
70	68	81	87	90	92	93	95	96
100	56	76	83	82	85	90	92	94
300	30	44	56	63	68	74	80	92

fixed doses were estimated from the weight-adjusted doses assuming human body weight of 60 kg

(60–600 mg)), and predicted antigen suppression following administration of 4 monthly doses of the antibody via a SC route are summarized in Table 6.3. A high degree of theoretical suppression of the antigen in serum (>95%) can be achieved at various antibody affinities and doses. However, when delivery volume and the maximum concentration of antibody-based therapeutics are limited, antigen/antibody affinities of <10 pM are required under the simulation conditions employed.

Concluding Remarks

Understanding of the relationships between the clinical dose and the required affinity for any functional antibody should allow achieving the maximum therapeutic benefit at a dose with a manageable cost of goods. Considerations with respect to antibody PK properties and biodistribution, antigen turnover rate, and antigen properties, recruitment of effector functions, and the final intended route of administration are crucial information for estimation of the optimal affinity design goals. Therefore, particular attention must be given to amalgamating the relevant information with respect to the underlying biology and pharmacology, target antigen properties, and antibody characteristics.

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Chapter 7
Bioanalytical Considerations for Development of Antibody-Based Therapeutics: Pharmacokinetics and Immunogenicity

Cherryl B. Funelas and Scott L. Klakamp

Abstract The drug development process for therapeutic proteins requires a plethora of supporting data prior to market entry and approval. Effective strategies for successful translation of information into the later phases of antibody development require the use of relevant bioanalytical (BA) methodologies from early preclinical stages. Here, we discuss a suite of BA methodologies that can be used to enable pharmacokinetics, immunogenicity, and biomarker evaluation. The results of these assays should provide insight into the mechanism(s) of drug efficacy that can be utilized for translation of relevant information across species.

Introduction

Establishing relevant bioanalytical (BA) methodologies from early preclinical stages is critical for implementation of effective strategies necessary for successful translation of information into the later drug development phases. Robust and effective BA methodologies assist in addressing important questions regarding pharmacokinetics (PK), immunogenicity (IM), and pharmacodynamics (PD) of drug candidates. Moreover, BA methodologies are critical for translation of exposure–response data from preclinical efficacy and nonclinical safety studies in

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support of the effective design of First-In-Human (FIH) clinical programs. In order to achieve these objectives, BA methods must be well characterized and provide a certain degree of robustness even at early stages of preclinical development. In line with the model of continuity, whereby a continuous flow of information from early discovery to the clinic is maintained, BA methods are critical for successful development of antibody-based therapeutics (Tabrizi et al. 2009). The methods employed for generation of data throughout various development phases are crucial for understanding the underlying system pharmacology, mechanisms of action, and the effect of therapeutic drugs once administered to animal models and translated to non-human primates (NHP), and ultimately, to human subjects.

An effective translational strategy must be employed from the early stages of antibody development (Tabrizi et al. 2009). Well-defined design goals for a drug candidate should be established prior to initiation of an antibody therapeutic program (Chap. 6). Biophysical characterization must be performed to ensure that the drug candidates selected have the appropriate physicochemical properties for binding to the relevant target which then allows lead selection (Chap. 5). During lead selection, relevant biomarker (BM) assays should be developed to further understand the mechanisms of action of an antibody drug candidate in relevant biological systems (Chap. 13). As an antibody therapeutic program progresses into early preclinical and clinical phases, pertinent BA methods to support PK, PD, preclinical safety, IM, and BMs (where feasible) must be developed. This chapter will review critical considerations necessary for development of PK and IM methods for effective development of antibody-based therapeutics.

Bioanalytical Considerations

Platforms

Over the years, immunoassays have been an essential tool in various research and development laboratories. Immunoassay methods have been exploited for several decades and the underlying principles relevant to these methods have been employed in diagnostic test kits as well as in proteomic research. With advances in science and the development of novel technologies, simple immunoassays have evolved in complexity. Immunoassays are no longer limited to a common 96-well plate format and have advanced into miniaturized (384-well) or super-miniaturized (1536-well) formats. In addition to increases in capacity, new innovations including the ability to multiplex and to utilize compact disc (CD) platforms have increased assay throughput and efficiency as well as the assay detection limit (commonly referred to in BA studies as "sensitivity", see "Cut Point, Limit of Detection, and Limit of Quantitation" for further details).

There are several immunoassay platforms that have emerged over the years, ranging from basic colorimetric methods to luminescence formats. The platform

choices include, but are not limited to, enzyme-linked immunosorbent assay (ELISA), electrochemiluminescence (ECL), and fluorescence-based AlphaLISA®. These platforms offer assays with low detection limits and quantitative determination of analytes in various complex biological matrices. The choice for the use of the appropriate platform depends on several factors like the detection limit required, cost-effectiveness, ease of use, as well as adaptability to automation and integration with other laboratory systems.

Enzyme Linked Immunosorbent Assay

Over the years, analytical laboratories have employed the ELISA assay format as an integral component of drug development programs. ELISA is defined as a platform that uses enzyme proteins that catalyze a specific reaction with a substrate. These labeled enzymes, when exposed to a substrate, can generate a response such as color or luminescence that is read by an optical reader. Each enzyme molecule converts several substrate molecules to generate signal. ELISA reagents are more affordable in comparison to other immunoassay platforms and are easily accessible as these reagents are considered to be universal. A typical ELISA assay is best described by having a capture reagent immobilized on a plastic surface (e.g. a polystyrene immunoplate). After a blocking step, test samples are then added and analytes are allowed to bind over a period of time under controlled conditions. After equilibrium is achieved, unbound analytes are washed away and a detection reagent conjugated to an enzyme is subsequently added.

As with most technologies, the ELISA assay has advantages and disadvantages when compared to other immunoassay platforms. Because the ELISA platform has been in use for many years, it has garnered loyal followers who believe in its utility and reliability in many applications. In addition, availability of commercial reagents for ELISA is facile. The vessel for the platform is an immunoplate that is available in various sizes, forms, binding capacity, and can offer flexibility in the number of wells. The secondary reagents are also readily available to customize an assay to the analyte of interest. The substrate is quite inexpensive and is provided in various modes depending on the detection procedures employed. Furthermore, there are a variety of manufacturers that specialize in ELISA plate readers; these readers are easy to use and are in compliance with current Good Laboratory Practice (GLP) regulations.

Although the ELISA assay has been essential in analytical laboratories due to its ease of application and access to reagents, the platform is challenged by other technologies partly because it requires a higher sample volume. An ELISA assay can use from 50 to 200 μl of sample. This volume requirement is a critical challenge for small animal studies. In addition, the ELISA assay requires several wash steps which is an issue for low affinity analytes. Furthermore, the overall assay time and analysis efficiency (for high-throughput operations) to run an

ELISA assay becomes a limitation in the drug development timeline when compared to other technologies. Immunoplates can either be coated a day before (overnight at 2–8 °C), or the same day (higher temperature, 37 °C for at least 1 h), and the actual assay time spans about 4–6 h depending on the optimized conditions. Hence, due to the ELISA assay time and numerous wash step requirements, efforts have focused on the development of new and improved technologies.

Electrochemiluminescence

Electrochemiluminescence, commonly referred to as the ECL platform, is a type of immunoassay developed by IGEN International (which became Bioveris, Inc.). In its first introduction, this platform was developed based on the use of paramagnetic streptavidin (SA) coated beads as a capture vehicle. Antibodies that captured the analyte were labeled with biotin which bound to the SA beads. The captured antibody (analyte) was then detected by another antibody labeled by ruthenium. The bead-based format of the ECL technology has now been replaced by a plate-based ECL format offered by Meso Scale Discovery (MSD; www.meso-scale.com).

The ECL signal results from luminescence of a ruthenium label owing to an electron transfer reaction at an oxidizing electrode in the presence of a tertiary amine. The ECL methodology was initially reported in the early 1960s (Kuwana et al. 1964). Nevertheless, its application in BA laboratories only became commonplace in the 1990s. This technology has found its way into many analytical laboratories with applications relevant to both biopharmaceutical and diagnostic arenas. In most cases, the ECL technology has become a replacement for the ELISA assay platform; compared to ELISA, ECL offers a lower detection limit, minimum assay development time, less sample volume, a wide dynamic range, and equally reproducible or precise data. With the ECL platform, tris(2,2′-bipyridyl) ruthenium(II) ($Ru(bpy)_3^{2+}$), an ECL labeling agent, undergoes a reduction/oxidation reaction in the presence of a tertiary amine to form a luminescent excited state; emission is captured by a charge coupled device (CCD) camera. Specifically, $Ru(bpy)_3^{2+}$ is oxidized to $Ru(bpy)_3^{3+}$ near the electrode surface whereby the $Ru(bpy)^{3+}$ complex is reduced to an excited-state $Ru(bpy)_3^{2+}$ complex by a co-reactant, tripropyl amine (TPA) radical. To perform ECL experiments, MSD-TAG or MSD-SULFO TAG™ labels (ruthenium label) are attached to antibody or another protein for detection. Once a potential is applied to the surface electrode of the plate, the $Ru(bpy)^{2+}$/TPA oxidation–reduction reaction generates luminescence that is read by the MSD Sector Imager. The labels that are bound to the complex on the surface electrodes are the only entities detected. This special feature provides specificity even with minimal or no wash steps, which proves to be an advantage in detecting low affinity antibodies. The emission is read at 620 nm, thereby eliminating issues with quenching as observed in some cases with ELISA. Since a single ruthenium complex undergoes many oxidation/reduction reactions,

this greatly amplifies the luminescent signal and therefore leads to assays with low detection limits (MSD Technology Platform Product Brochure). ECL immunoplates, called Multi-Array Plates, are carbon-surfaced and efficiently bind antibodies or other proteins via passive adsorption. The plates are available in either “standard bind” or “high bind”. The “high bind” plates have a textured surface that allows a greater surface area for protein binding.

The ECL platform has been well adopted by analytical laboratories. Assay development approaches utilizing ECL assays are similar in concept to ELISA assays. The ECL platform offers an assay with a wider dynamic range of more than 2-logs compared to its ELISA counterpart. This flexibility in range is useful for samples at varying concentrations, thereby minimizing the need for dilution. ECL-based assays have lower detection limits as compared to other plate-based assays, which makes it favorable over the ELISA platform. This plate-based platform works well even with small volumes of sample. For example, one can easily evaluate less than 25 μl per well, which proves advantageous with limited sample volumes. Moreover, the platform has an advantage over ELISA in its flexibility in minimizing the number of wash steps. The washing step, which is inherently common in immunoassays, contributes to loss of low affinity antibodies. It is believed that low affinity antibodies may have clinical relevance and therefore must be assessed and characterized. Assay development for the ECL platform is performed sequentially (also common with ELISA) or with a homogeneous solution-based approach. The ECL technology, compared to ELISA, offers multiplexing capability to detect several analytes in one sampling time. This additional feature has positioned the technology to compete with other instruments with multiplexing capability, such as the microsphere bead-based platform from Luminex. There are a variety of plates that are offered by MSD that are either uncoated or pre-coated with avidin/SA, glutathione, or proteins such as anti-species antibodies. MSD also offers various services such as labeling of reagents, plate coating, spotting (for multiplex), and even assay development assistance. Assays that are developed for an ECL platform are as easy to validate as those for the ELISA platform.

As more and more analytical laboratories adopt the ECL platform, there are several issues that arise and require consideration when developing assays using this platform. As with any immunoassay, a homogenous approach makes it prone to the hook or prozone effect. This phenomenon is a result of having excess amounts of therapeutic antibodies present in solution with limiting reagents. This effect is addressed through appropriate assay optimization with reagents, sample dilutions, or addition of wash steps. When developing assays with minimum wash steps, high background, especially in high matrix concentrations, becomes an issue. Therefore, it is recommended to add a wash step prior to addition of labeled detection antibody to remove other non-relevant proteins, thereby minimizing non-specific binding. Although ECL plates are similar to a plastic immunoplate in structure, the bottom of the well is different. As shown in Fig. 7.1, the non-smooth high binding surface of the carbon electrode allows maximum binding of proteins;

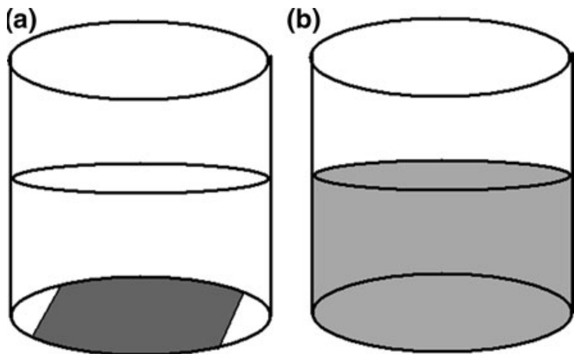

Fig. 7.1 Different surface areas where proteins bind for both platforms are illustrated. A single well of an MSD plate where the *dark gray* area represents the area of binding (**a**). A single well of an ELISA plate where the *light gray* area represents the area of binding (**b**)

the multi-array plate warrants a special technique for the addition of reagents since each reagent needs to be placed in a specific area of the well bottom.

One other possible disadvantage with the ECL platform is the limitation of being supported by only one manufacturer, namely MSD. Unlike the ELISA platform, whereby several manufacturers provide flexibility in terms of choice of reagents and assay design, as well as inexpensive alternatives, the multi-array plates are sold exclusively by MSD. ECL detection buffers that contain the co-reactant TPA are reasonably affordable and can be purchased in bulk at a discounted rate. The main financial burden in employing the use of the ECL platform is associated with the initial purchase of the plate reader. The MSD Sector Imager is available in several models and can cost over a hundred thousand dollars depending on the capacity required. Due to the limitation that only one manufacturer supports the ECL platform, a highly skilled technician is required for troubleshooting, instrument repairs, and preventive maintenance. Therefore, it is recommended to purchase a reasonable service package to ensure the integrity of the equipment, specifically for the CCD camera, which is costly to replace.

Gyros Technology

Gyros® is another emerging technology that offers a nanoliter scale platform. The major advantage is its capability to miniaturize an immunoassay using a CD during the entire assay method. This platform employs a sandwich immunoassay format for the detection of an analyte. The technology is a semi-automated system whereby samples are pre-prepared (test samples diluted to appropriate target concentrations within a standard curve) and then loaded onto the CD. This is performed with the instrument referred to as a Gyrolab workstation. The CD platform, or the Gyrolab Bioaffy CD, contains several microstructures which correspond to single wells in a plate-based assay. An individual microstructure has an affinity capture column that contains SA-coated particles. The analyte is captured by an antibody (or protein) labeled with biotin and detected by an antibody

(or protein), coupled to a fluorophore. The CD is spun at optimized velocities for each assay step, generating a centrifugal force to drive the liquid through the channels of the column. The laser-induced fluorescence is measured by a scanner with the Gyrolab workstation. The amount of fluorescence measured is directly proportional to the amount of protein captured (www.gyros.com).

The major advantage of the Gyros® system over other platforms is its capacity to test samples with limited volumes, i.e., nanoliter scale. Assay development for this platform is very similar to what would be done for a sandwich ELISA; therefore, familiarity with the principles of immunoassays should facilitate method development. Moreover, the built-in robotic system is semi-automated and thus minimizes any variability that may be introduced during manual sample and reagent loading. Also, overall assay (sample and reagent loading) time is reduced. A carousel inside the Gyrolab workstation holds the microplates containing the samples and necessary reagents for the assay. A robotic arm then performs the transfer of samples and reagents into the CD. All the reactions occur in the CD as it spins; centrifugal forces drive the reactions to an optimal flow rate to maximize the binding of a specific protein while reducing assay time. These properties make the Gyros® platform advantageous, specifically for high throughput applications.

The Gyros® system is considered to be a new technology platform that is gaining popularity among BA laboratories. However, due to its complex mechanics and its semi-automation features, the workstation carries an expensive price tag. Similar to other technologies, after the initial purchase of the equipment, the consumables for use in the platform must be considered. The cost and use of the Gyrolab Bioaffay CDs can accumulate; thus, careful planning and tracking of the use of columns must be noted to maximize the use of a single disc. For small studies that are typical in the early preclinical development phase, this platform may not be of optimal value. Nevertheless, for reasonably large studies, such as clinical studies that may require frequent sampling, the Gyros® platform may prove advantageous. Just like the ECL platform from MSD, the Gyrolab workstation requires a highly skilled technician for operation, maintenance, and troubleshooting.

Surface Plasmon Resonance

Biacore instrumentation is the most ubiquitous platform utilized in surface plasmon resonance (SPR) assays. The Biacore instrument is a surface-based optical biosensor that uses SPR to measure antibodies that bind to antigen immobilized to a non-crosslinked carboxymethyl dextran matrix bonded to a gold surface (Chap. 5). Unlike other platforms, Biacore does not require conjugated detection reagents. The system detects antibody association and dissociation rates and is applicable across species. SPR is routinely used for IM testing to support clinical trials (see "Surface Plasmon Resonance in Clinical Immunology"). Nevertheless, factors such as low throughput and the requirement for highly specialized technical expertise and equipment must be considered prior to the use of SPR for IM

applications. Additionally, other issues may arise, especially if the sample matrix involves a soluble ligand that interferes with the detection of an anti-drug antibody (ADA). Overall, the Biacore platform provides valuable data for IM testing and readily detects various isotype responses compared to other IM assay platforms.

Other Platforms

Other immunoassay platforms are available for use in the industry. The top four immunoassay platforms that are commonly used in BA laboratories have already been described above. However, Perkin Elmer has introduced an alternative to conventional ELISA that is referred to as AlphaLISA® (www.perkinelmer.com). This immunoassay utilizes the AlphaScreen technology using a bead-based platform. AlphaLISA technology consists of binding a biotinylated anti-analyte antibody to an SA-coated donor bead and coupling another anti-analyte antibody with a different epitope (noncompetitive epitope to the antibody on the donor bead) to an acceptor bead. When analyte is present, the donor and acceptor beads are brought in close proximity to one another. Upon excitation of the donor bead with laser light at 680 nm, singlet oxygen ($^1O_2$) is generated that diffuses no more than 200 nm from the donor bead. Any acceptor beads within the 200 nm distance are excited and emit fluorescence at 615 nm. Obviously, only acceptor beads bound in a sandwich complex with analyte and donor beads are excited, hence the amount of fluorescence observed is directly proportional to the amount of analyte.

Considerations for Assay Development

Development of an assay will inherently possess deliverables and limitations. A well-characterized assay will provide useful information that can support successful development of antibody-based therapeutic programs. During assay development, there are several factors that need to be considered which include, but are not limited to the following parameters: (a) the nature of the analyte, (b) the matrix of the assay and potential assay interferences, (c) required detection limit, (d) choice of reagents and availability, (e) the cost of materials, (f) the type of controls (positive control/reference standards), and (g) the ease of validation.

Nature of the Analyte

It is important to understand the nature of the analyte in question when developing assays. For antibody-based therapeutics, the analyte is an antibody or an antibody-derived molecule. Antibodies (immunoglobulins) are grouped into different

isotypes (IgG, IgM, IgA, IgE, and IgD); however, the most common class currently in use is the IgG isotype. There are four different subclasses of IgGs: IgG1, IgG2, IgG3, and IgG4. The most common of these that are developed as therapeutics are IgG1 or IgG2 isotypes. In BA assay development, the isotype of an IgG antibody becomes important as it dictates the assay configuration, choice of reagents, and detection system. In assays where antibodies are not the analytes, such as BM assays, similar principles apply and characterization of the analyte must be considered.

Matrix and Assay Interferences

Matrix effects and potential sources of interference that may be present in complex biological matrices are critical considerations during assay development. Analytical assays that can assess the analyte in a buffer system are easy to develop due to the lack of interferences in the buffer. Once the analyte becomes part of a complex biological matrix, such as serum, plasma, urine, etc., the effects of the matrix become a critical consideration and can significantly impact the detection limit of the assay. BA assays are developed to support studies performed in animals and humans, where the samples collected are in biological matrices. Therefore, it is crucial to differentiate the analyte from other background proteins that are inherently present in biological matrices. In a biological matrix, an interfering molecule may compete with the analyte in the capture or detection step; hence, this type of interference must be addressed and differentiated from the analyte. To address such interference, there are various techniques that have been employed to minimize the matrix effects on the assay. These techniques include dilution of the samples prior to loading, sample treatment (i.e., heat inactivation, pH, etc. to inactivate endogenous protein that can interfere with the assay), platform/technology assessment, and addition of other reagents (i.e., other serum IgGs from a different species to remove non-specific binding proteins).

Sensitivity[1] of the Assay

Sensitivity in a quantitative assay is based on a determination of the lowest concentration of the analyte that can be measured with acceptable precision and

[1] It should be noted that "sensitivity" in the context discussed in this section and as used in BA assay development is poor terminology at best. For a rigorous discussion of the correct terminology that should be used in BA assays see "Cut Point, Limit of Detection, and Limit of Quantitation". We only adhere to the less rigorous terminology in the current section because the term "sensitivity" is so engrained in the BA literature and psyche. To change terminology here might confuse the reader and obscure the important concepts presented within.

accuracy. In a quantitative assay, the target sensitivity plays an important role in assessing pharmacokinetic parameters. For dose finding studies, where animals can be given a low dose of the antibody, the importance of detecting the low circulating analyte has a biologically relevant impact. Therefore, designing an assay that can detect up to low nanomolar to picomolar levels may be needed. There are various ways of ensuring the sensitivity of the assays; however, this task is not straightforward and will depend on the assay configuration. Minimizing the background noise in order to increase the signal-to-noise ratio (S/N ratio) between the lowest concentration level and the background noise is one of the ways to achieve better sensitivity. In an ELISA platform, additional wash steps in between addition of reagents may minimize the background and ensure removal of any unbound materials that may still be present in the well. A blocking agent that contains casein, bovine serum albumin, or other protein carriers may aid in minimizing background noise and may be used as assay diluents. Another way of increasing the sensitivity of an assay is using capture and detection antibodies that are highly specific. However, finding a reagent with specificity to the analyte can prove challenging. It is prudent to initially invest time in evaluating the specificity of a panel of antibodies to potentially increase the sensitivity of the assay. Minimizing the dilution of samples may also help in increasing the sensitivity of the assay; however, employing this approach may contribute to higher background noise and a lower S/N ratio. There are few technologies that are available which claim to deliver highly sensitive assays. The ECL platform offers more sensitive assays based on signal amplification whereby a series of excitation cycles of the detection antibody label results in enhancement of signal. Overall, in developing BA assays, it is important to assess the relationships of all the parameters to determine the intrinsic limitations of the method under study.

Choice of Reagents and Availability

Once the need for an assay arises, a schematic of the assay design and experiments are planned accordingly. Probable schematic configurations are useful to assess and possibly foresee any issues that may arise in the initial planning stages before selecting reagents. Having well-characterized reagents will be critical in the initial stages of assay development. Unlike other analytical assays, where analytes are tested in their purified state in buffer, the choice of reagents in BA assays is not as straightforward. Some reagents may require extra purification steps, such as affinity purified secondary antibodies, adsorption against species, and labeled or tagged antibodies. Moreover, assay requirements are determined depending on the stage of drug development. For preclinical programs, an assay that can be used early on during preclinical development may prove useful. These assays are sometimes referred to as General Assays or Universal Assays (UA). UA use reagents that support quantitation of human therapeutics in various animal species such as rodent and non-human primate biological matrices. The application of the

UA can be highly effective in minimizing the assay development time and, ultimately, is a more cost effective approach. Reagents may be available in several forms, concentrations, and purity. There are several commercially available reagents that are well suited for ELISA, ECL, Gyros, and other platforms that can be used for UA development. Unlabeled antibodies are available for laboratories that prefer the flexibility of labeling these reagents in-house. There are also pre-labeled antibodies or proteins that are commercially available. Most commercially available reagents have reasonable quality control systems in place that allow minimum variability between batches. However, it is a good practice to always qualify the reagents prior to use in an established method. Once the appropriate reagents are tested for use in an assay, most of the commercial manufacturers offer bulk purchases at a discounted price. In many cases, when reasonably sized studies are planned, as in the case of IND-enabling studies and for clinical studies, laboratories tend to order larger quantities of a specific lot from a commercial source to minimize lot-to-lot variability and ensure availability of such reagents during the course of preclinical development. This strategy minimizes the potential for variation in the manufacturing process of the reagents in question and is a critical factor when dealing with validated methods necessary for the support of GLP-compliant studies.

Cost of Materials

The cost of materials for the overall assay must also be considered. The driving force behind many therapeutic programs in a highly competitive landscape is the financial burden associated with the cost of development. It may take years to develop a biotherapeutic program from inception to commercialization. Thus, BA assays are critical in supporting drug development from early preclinical to clinical stages. Each drug development phase requires development of methods to support relevant studies. The cost of method development includes reagents, platforms (ELISA, ECL, Gyros, etc.), buffers, and time. All these aforementioned factors contribute to the financial burden of a program.

Reference Standards/Positive Controls

The source of reference standards and controls is another factor that should be considered during assay development. Anti-idiotypic (anti-ID) antibodies have been commonly used as positive controls for IM assays to qualitatively detect ADA responses that develop as a result of biotherapeutic drug treatment; anti-ID antibodies are directed against the antigen binding site of the therapeutic antibody. This approach has been widely accepted but the current challenge that BA testing laboratories face is selecting the appropriate anti-ID antibodies for use in ADA

assays. Monoclonal anti-ID antibodies are derived from a mouse that is hyper-immunized with the therapeutic antibody. A specific clone is selected from the antibody responses that are generated from the mouse and further amplified. Monoclonal anti-ID antibodies used as positive controls have high specificity and affinity against the therapeutic antibody drug. The use of monoclonal anti-ID antibodies as positive controls becomes an issue when compared to an expected immune response from a human subject. If an ADA were to be observed in humans, the response will likely produce antibodies that are polyclonal. Hence, a polyclonal anti-ID positive control should best represent the true ADA response of the study samples. However, alternative approaches to selection of positive controls are possible. The first solution is to use a mixture of monoclonal anti-ID antibodies where the affinities are known. The mixture can serve as a representation of an ADA response with various affinities. Another alternative is to use separately three or more anti-ID mAbs of known affinity to establish a detection limit for each positive control mAb in the IM assay. From the three or more detection limits determined for each anti-ID mAb, it would be determined how the assay performs for mAbs of differing affinities. These data would allow one to "bracket" what detection limits would be expected from a polyclonal anti-ID mixture composed of mAbs with varying equilibrium dissociation constants (K_D).

Ease of Validation

The last factor to consider when developing immunoassay methodologies is the ease of validation. Not all assays can be validated, and this is attributed to several factors including the choice of assay reagents and assay platforms. In validating an assay, it is important to consider the robustness of the approach. For example, the availability of reagents is a key consideration. For the sake of comparability and as discussed above, having assay reagents that will be accessible to support the entire preclinical development phase is an important consideration. A method developed for supporting IND-enabling studies that also has a high likelihood of further continuation into the clinical stage favors validated assays and reagents. It is recommended to avoid method revalidation due to changes in assay reagents. In these instances, consistency becomes a critical consideration. In addition, having to retest all the samples tested previously while trying to bridge the data collected on various assays may pose a problem. Comparability studies can become time-consuming and can have a significant financial consequence. Therefore, it is crucial to plan for acquisition of reagent supplies early on to avoid assay variability due to availability, or lack thereof, of critical reagents. However, reagent supplies are not the only factor that should be considered during validation activities; the platform of choice must also be well thought out. Not all platform technologies are easily validated. It is advisable to favor a technology that is proven and has a higher probability of being available during the development phases of the clinical candidate.

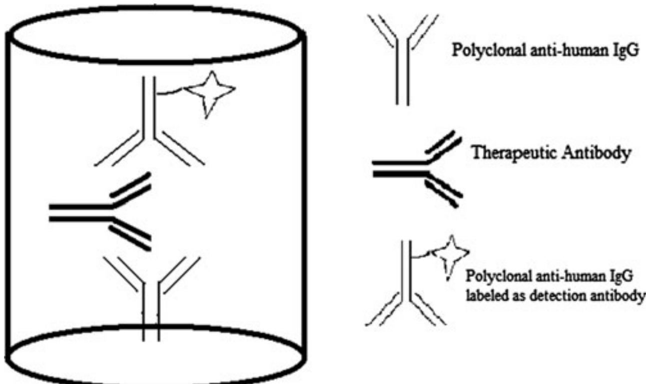

Fig. 7.2 A schematic diagram of the configuration of a UA in a single well

Bioanalytical Methodologies

Pharmacokinetics

As highlighted, development of robust and well-characterized BA methodologies is critical for implementation of effective translational programs in antibody drug development. Several types of PK assays can be employed to generate and provide reliable data during the course of development of antibody-based therapeutics (Tabrizi et al. 2010). The PK assays listed below are referred to as quantitative BA assays generally used for determination of therapeutic antibody concentrations in non-clinical and clinical studies.

Universal Assays

UA utilize a capture and detection system that consists of two non-competing reagents/antibodies that bind to humanized or fully human therapeutic antibodies. The configuration of this assay is shown in Fig. 7.2 and requires an anti-human IgG antibody to be immobilized on the surface of an immunoplate; the biological matrix containing the therapeutic antibody is then added to the well and following equilibration and binding, a detection antibody (labeled anti-human IgG) is added. The assay configuration allows detection of therapeutic antibodies in a complex biological matrix such as serum and/or plasma. The UA measures the total (free and bound to target) antibody concentrations in the matrix. However, utility of the UA is limited only to samples obtained from animal studies, such as rodents and NHP. The UA in general cannot be applied to clinical studies due to the presence of endogenous IgG in a human matrix which interferes with the capture and detection of antibodies. Even in non-human primate matrices, secondary antibody reagents that have undergone adsorption against monkey IgGs must be used to

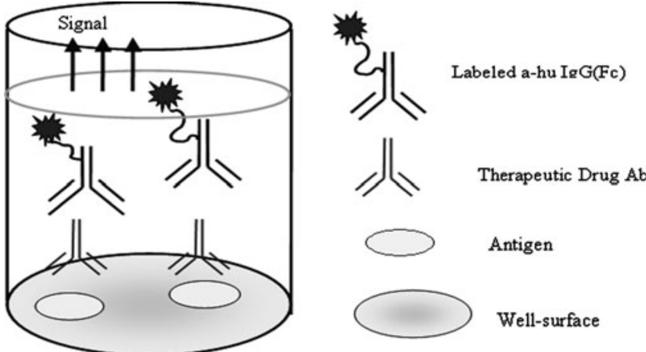

Fig. 7.3 A schematic diagram of the configuration of an ACA in a single well

minimize non-specific binding due to the high homology between monkey and human IgGs. The advantage of the UA is its universal application and high efficiency in supporting various preclinical studies of human-derived therapeutic antibody programs. In general, UA can be applied across species and across a variety of therapeutic antibody programs, dramatically decreasing the need for developing specific assays for each antibody candidate. As a result, the assay development time and cost are greatly reduced. UA are readily cross-qualified between matrices and their application is straightforward. These assays are commonly used in rodent and monkey studies that are in early preclinical stages, where compliance to GLP is not necessary. Secondary antibodies that are used in UA for rodent matrices do not require adsorption treatment due to the lack of homology between human and rodent immunoglobulin; therefore, non-specific binding to IgG is minimal. A well developed UA assay configuration provides a minimal background to noise signal. An ELISA, ECL assay, or other platforms can be used to develop this type of assay.

Antigen-Capture Assay

Antigen-capture assays (ACA) utilize a configuration where the therapeutic antibody is captured by the target antigen and detected by an anti-human IgG antibody. The ACA configuration allows for enhanced specific detection of free (unbound to target) therapeutic antibody in a complex biological matrix. ACA are frequently used for detection of therapeutic antibodies in human matrices. In this assay format, the target antigen is immobilized to an immunoplate, and after a blocking step, the biological matrix containing the therapeutic antibody is added to the well and further detected by a labeled anti-human IgG reporter (Fig. 7.3).

Unlike the universal assay, the ACA format can be applied across species, including humans, and because of improved specificity, the interference imposed by the endogenous IgG in this assay is minimal. The ACA, similar to UA, also

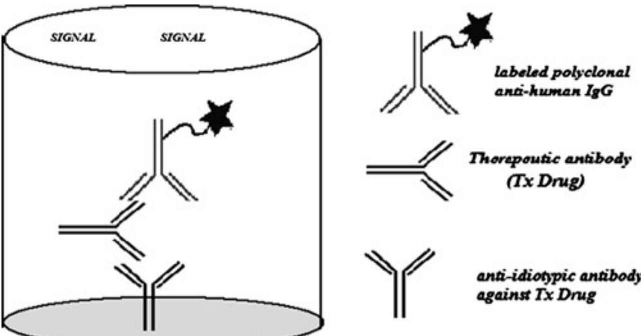

Fig. 7.4 A schematic diagram of an AIA that uses α-ID as a capture reagent and polyclonal anti-human IgG as the detection reagent for therapeutic drug

provides reliable data. However, ACA can be costly due to potentially expensive reagents (i.e., the target antigen). Most of the antigens (i.e., recombinant proteins) are derived from cell lines that express the antigen of interest and are purified to ensure quality. Moreover, reagent availability is a critical consideration in order to avoid variability across reagent lots, and most importantly, potential differences in the affinities of the therapeutic antibody drugs against different sources or lots of antigen. Binding activity of the antigen must be tested due to differences in commercially available sources. Due to the specificity of the assay, the format is not able to detect therapeutic antibodies bound to the target in the matrix. Such an assay format may appear to show nonlinear pharmacokinetic profiles due to the competition between the therapeutic antibody bound to the antigen in the sample and the capture reagent. This nonlinear PK profile may be erroneously interpreted as target-mediated clearance, when in fact the observed profile is the result of an assay artifact (Tabrizi et al. 2010).

Anti-Idiotypic Assays

The third type of quantitative assay is the Anti-Idiotypic Assay (AIA). This system utilizes anti-ID antibodies that have been generated against the therapeutic antibody. Generally, the AIA requires a more customized approach in developing assays. It can be costly to generate an anti-ID antibody that is specific to the therapeutic drug; however, if available, it is a useful tool for development of PK assays and as a positive control in IM assays for late stage preclinical and clinical studies. The use of anti-ID antibodies in quantitative assays can be configured in two formats. In one approach, the anti-ID antibody is used to capture the therapeutic antibody drug in conjunction with a polyclonal anti-human IgG antibody for detection of the therapeutic antibody drug (Fig. 7.4). Alternatively, the anti-ID antibody can be used as both a capture and detection reagent. Similar to the ACA, anti-ID assays can only detect free unbound therapeutic antibodies. The dual

anti-ID assay system works when both binding arms are available for one anti-ID antibody to capture and another anti-idiotype antibody to detect the therapeutic antibody drug.

Immunogenicity

Immunogenicity of therapeutic antibodies containing xenogeneic protein sequences may be a significant problem in the clinical setting. Marketed antibodies have shown some level of IM (Tabrizi and Roskos 2007). Although evolution in the generation of monoclonal antibodies, i.e., from murine antibodies to humanized or human antibodies, has been crucial in reducing the IM profiles of marketed antibodies, the ADA response to therapeutic antibodies is still of clinical relevance (Chap. 2). An ADA response may not only alter PK by impacting clearance, but can also reduce efficacy (via anti-ID neutralizing antibodies) and may have potential safety risks (Ponce et al. 2009). In general, the predictability of non-clinical in vivo studies for evaluation of IM does not necessarily translate into humans.

Assessment of IM is important due to the high risk for therapeutic proteins to be immunogenic. Since immune reactions mounted against biotherapeutics can be minimal or acute in nature, it is important to establish well-characterized BA methodologies that reliably detect immunogenic responses. In the draft guideline published by the United States Food and Drug Administration (FDA) for assay development titled IM Testing of Therapeutic Proteins, it is stated that "Immunogenicity in animal models is not predictive of immunogenicity in humans." Nevertheless, the information derived from IM testing of biotherapeutics in animal studies provides insight into assessing "potential antibody related toxicities" [Assay Development for Immunogenicity Testing for Therapeutic Proteins, Guidance for the Industry (DRAFT) 12/2009]. It is also vital that BA methods that are developed for purposes of detecting IM be optimized for sensitivity (or more rigorously put, detection limit; see "Cut Point, Limit of Detection, and Limit of Quantitation"), specificity, precision and robustness. Similarly, the European Medicines Agency (EMEA) guidelines on IM assessment on biotechnology-derived therapeutic proteins have highlighted parallel views and recommendations (EMEA Guideline on Immunogenicity Assessment of Biotechnology-Derived Therapeutic Proteins 2007). Thus, assessing IM early on during the preclinical development stages of a candidate drug program may facilitate development of strategies to minimize adverse immune responses in patients (Chirino et al. 2004).

Various methods and assay platforms have been employed for detection of ADA responses, however, these assays can vary widely in their specificities and detection limitations (Wolbink et al. 2009). Detection limit and specificity are important components of an IM assay. The detection limits can vary depending on the affinities of the antibodies present in the sample, the platform used and the method, the presence of endogenous proteins, the therapeutic target, and the

antibody concentrations measured by the assay (Thorpe and Swanson 2005). A recent white paper publication addresses some recommendations on IM assay design that has paved the way for laboratories in attempting to standardize assay development approaches (Mire-Sluis et al. 2004). There are several factors that should be considered when developing BA methods for IM assays as discussed below.

Positive Controls

An important factor to consider during IM method development is the availability of a suitable positive control(s) that is a relevant representation of an immune response to the therapeutic antibody. Initiating an anti-ID antibody campaign can be costly, but the resulting reagent that exhibits specificity to the therapeutic antibody can prove to be highly useful in supporting assay development efforts. There are two (2) types of anti-ID antibodies that may be utilized for this purpose: monoclonal and polyclonal antibodies. The differences between these two types depend on how they are generated. Monoclonal antibodies are derived from a single B cell lymphocyte clone while polyclonal antibodies are derived from different B lymphocytes that recognize various epitopes of a given antigen (in this case the therapeutic antibody can be the antigen for developing anti-ID antibodies). Both approaches have their own advantages and disadvantages depending on the specific application (Lipman et al. 2005). Recently, monoclonal positive control antibodies have been used by analytical laboratories due to their high specificity and affinity. Unlike monoclonal anti-ID positive controls, where affinity to the therapeutic antibody drug may be measured, polyclonal anti-ID positive controls have varied affinities, thereby not allowing affinity measurement due to their heterogeneity. Nevertheless, analytical laboratories attempt to comply with current regulatory recommendations in generating relevant controls that best represent the "varied avidities" against the therapeutic product.

Immunogenicity Assay Configuration

IM assays use therapeutic antibody drugs as assay reagents. IM methods are designed to detect antibodies against the therapeutic antibody drug that are present in the biological sample. These assays can be a ligand-binding assay or a bioassay. Ligand binding assays (LBA) detect any ADA that binds to a therapeutic antibody. Therefore, an LBA detects both non-neutralizing and neutralizing ADAs. Bioassays on the other hand only detect neutralizing ADAs. Bioassays are cell-based assays that evaluate the neutralizing capacity of an ADA. An LBA measurement is determined qualitatively by either being positive or negative against an assay threshold or cut point (CP). A bioassay measurement is based on the ability of a neutralizing antibody (nAb) to inhibit a cellular response mediated by a therapeutic antibody drug.

Immunoassay Platforms

Ligand Binding Assays

In an ELISA format, an unlabeled therapeutic (Tx) antibody is immobilized to an immunoplate. Unknown samples that may contain ADA are then added to a blocked plate and allowed to bind. A labeled therapeutic antibody, as the detection reagent, is then added to form a bridge to detect the ADA-therapeutic antibody drug complex. A colorimetric readout is produced and compared against the assay CP or threshold (discussed in "Assay Cut Point" and "Cut Point, Limit of Detection, and Limit of Quantitation"). Samples that yield a signal equal to or above the assay threshold are deemed positive for ADA, and samples where the signal falls below the threshold are deemed negative for ADA. Similarly, an ECL-based assay format uses the same configuration as ELISA; however, the label on the therapeutic antibody for detection is ruthenium. An alternative configuration can also be performed in ELISA where a biotinylated therapeutic antibody drug is used as a detection reagent in conjunction with SA-horseradish peroxidase. All assay configurations are tested and are optimized to produce the lowest detection limit. In general, ELISA assays are well established technology and are reasonably inexpensive and give low detection limits. However, due to the number of wash steps in this assay format, low affinity ADA may be lost. Alternatively, ECL technologies can be used due to their outstanding detection limits and broader dynamic ranges. In addition, due to the flexibility of ECL-based methods, this technology has the ability to detect low affinity antibodies. Based on this premise, it is important to consider all factors prior to execution of validation activities when developing IM assays of any format.

Radioimmunoprecipitation

Radioimmunoprecipitation (RIP) can also be used in assessing IM; this format is used as a confirmatory assay where therapeutic mAb drug is radioactively labeled. Upon binding of ADA to the radioactively labeled mAb drug, the immune complexes are precipitated using Protein A and analyzed by gel electrophoresis followed by autoradiography (Gulig et al. 1984). RIP assays usually have very low detection limits, but tend to be time-consuming, tedious, and exhibit limitations in throughput (i.e., handling requires use of tubes and liquid scintillation counters). There are, however, some RIP-based assays that have been adapted to reduce assay time through a semi-automated system using a 96-well microplate format (Fida and Rowley 1998).

Surface Plasmon Resonance in Clinical Immunology

SPR spectroscopy is often used in IM assays (Mason et al. 2003; Swanson et al. 2002; Swanson 2003; Wong et al. 1997). Specifically, Biacore® instrumentation is the most commonly used SPR biosensor employed for clinical immunology

(see Chap. 5 for a detailed explanation of SPR). IM assays that use SPR can determine whether the binding response to the antibody drug is the result of a true ADA or whether it is a nonspecific interaction from a serum component. Additional information that can be obtained from SPR IM tests include determination of the isotype of the ADA and the relative concentration and affinity of any ADA present in the matrix.

In a Biosensor IM assay, the therapeutic monoclonal antibody (drug) is immobilized to a high surface capacity on a biosensor chip, and patient serum samples (usually varying in concentration from 10 to 25% serum) are injected across the biosensor surface. Any binding to the mAb therapeutic is observed by a positive resonance unit response at the beginning of the dissociation phase relative to the baseline signal. A reference surface is generally not used in IM biosensor assays, in contrast to what is done for rigorous kinetic characterization of antibodies (Chap. 5), since construction of a relevant high capacity reference surface is not straightforward. When considering the complexity of a 10–25 % serum sample flowing across a high density surface of immobilized antibody drug, and a bare or an irrelevant protein-immobilized reference surface, the bulk refractive index change (and any nonspecific binding characteristics of the serum sample) on the reference surface may be very different from that of the antibody therapeutic surface. This caveat invalidates the utility of a reference surface in the first place. In addition, a reference surface is not required since any binding events that indicate an interaction with the immobilized antibody drug can usually be seen at the start of the dissociation phase of the sensorgram. The only exception is that for an ADA with a fast dissociation rate, it can be difficult to measure the response immediately after dissociation begins. This is the region of the dissociation phase where many molecules (other than ADA) that are nonspecifically bound to the immobilized antibody are dissociating and where the resonance unit signal may be decreasing very rapidly, which can lead to irreproducibility of replicates. It can be a fine line between choosing the correct time interval after dissociation begins to monitor for any ADA responses resulting from low affinity ADA interactions and achieving the precision desired among replicates. If it is observed that most test samples result in dissociation phases that take several minutes or longer to decay back to baseline, it would be advisable to choose a time interval of 30 s to minutes after dissociation begins since this region of the dissociation phase of the sensorgram should be devoid of nonspecific, low affinity interactions of serum components (and low affinity ADA so care here is needed). Hence, it is important to ensure that most samples still demonstrate dissociation decay several minutes after the end of the injection if this recommendation is to be followed.

To confirm the SPR response is due to ADA, an anti-human antibody (monoclonal or polyclonal) is injected across the immobilized antibody surface after injecting a serum sample containing purportedly ADA to observe whether additional binding occurs. If so, the binding response can be attributed to the presence of an ADA. It is also commonplace to inject soluble antibody drug if an ADA signal is detected (for final confirmation of ADA) as increased binding should be observed if an ADA is bound to the immobilized antibody drug. However, if

antigen (the target molecule in vivo for the therapeutic mAb) is also present in the serum, it can also lead to a false confirmatory signal when soluble antibody drug is injected. Even if the antigen is monomeric, the antigen may be slightly aggregated and may therefore result in a misleading confirmatory signal. To confirm the presence of ADA, soluble drug is also spiked commonly into test samples in a depletion assay before injection into the Biacore instrument. If a decrease in SPR signal is observed relative to the primary screening signal, the presence of ADA in the sample is confirmed. The same caution is also warranted with this type of confirmatory assay as already discussed above; the presence of soluble antigen will also lead to depletion of spiked antibody drug, resulting in a decrease in binding signal relative to the primary screen and in turn, a false confirmation. Klakamp et al. (2007) have demonstrated how the presence of soluble antigen can be addressed in a confirmatory Biacore IM screen by using a non-competitive antibody to the antigen.

ADA isotyping and subtyping experiments can also be performed with a Biacore biosensor more easily than with other platforms (Swanson et al. 2002). To determine the isotype of the ADA, different anti-isotype antibodies (either mono or polyclonal), like α-IgG_1, α-IgG_2, α-IgG_3, α-IgG_4, α-IgM, α-IgA, and α-IgE can be injected across the surface of the biosensor chip after ADA from serum has bound to the immobilized antibody drug. When the SPR signal increases for a specific anti-isotype or anti-subtype antibody, one can conclude that the ADA belongs to the corresponding isotype or subtype class. If the antibody drug has been immobilized to the sensor surface, which is usually the case, then ADA belonging to the same isotype and subtype class of the drug cannot be identified in this type of SPR experiment. Obviously, if the anti-isotype antibody is flowed across the surface of the biosensor chip, it will bind to the immobilized antibody and confound identification of the isotype or subtype of any ADA that may be bound. One way to circumvent this problem is to generate Fab fragments of the therapeutic antibody and to immobilize these instead of the whole mAb. When this is done however, any ADA against the Fc portion of the drug is not observed.

As with other IM assays, drug tolerance in SPR assays is a major issue (possibly larger than in ELISA and ECL assays owing to the generally higher limit of detection, or LOD, with Biacore) that must be overcome to have confidence in the data. To tackle the drug tolerance problem, a protocol has been devised wherein serum samples are pre-treated with acid (pH 2.5) prior to injection into the Biacore (Sickert et al. 2008) instrument. This acid dissociation method appears to improve the drug tolerance of a Biacore IM assay by 10- to 200-fold.

Two investigations have been performed that compared the detection limits achieved with IM assays using Biacore, ECL, or ELISA formats (Liang et al. 2007; Lofgren et al. 2007). Lofgren et al. (2007) have shown that Biacore has a better limit of detection (LOD) for ADA with weaker affinities, whereas ELISA has much better detection limits for tighter binding ADAs. Interestingly, they showed that the detection limit did not correlate with the affinity of the ADA for the antibody drug, but rather directly varied with the association rate constant, k_a. In other words, if the ADA had a faster k_a, the detection limit in the Biacore assay

was lower. A second comparative study by Liang et al. (2007) also demonstrated that Biacore exhibited a better detection limit than ELISA for one low affinity ADA, but for the lowest affinity ADA in the study, the LOD was comparable to that seen in ELISA, which was surprising. One explanation for this is that the time at which data was collected in the Biacore experiments, 3 min after the end of the injection of a 10% serum sample, was too far past the end of the injection to observe weakly bound ADA. As discussed above, this 3-min report point was chosen so the SPR signal would not be decreasing exceedingly fast in order to enter more precision into replicate measurements. Considering the fact that the LOD for both ELISA and Biacore was similar for the weakest affinity ADA ($K_D = 340$ nM), one can probably conclude safely that even if the SPR signal had been measured closer to the end of the injection, the Biacore LOD still would not have been exceptionally better, if at all, as compared to the ELISA LOD. A partial explanation for this observation is that even though much ADA has been washed away in an ELISA, this type of assay uses an enzymatic detection system that produces a chromophoric product that greatly amplifies the signal for any remaining ADA. Biacore has no signal amplification in its detection. Hence, the main conclusion of the study by Liang et al. (2007) is that electrochemiluminescent (ECL) methods provided a superior detection limit over ELISA and Biacore for ADA even with K_D values varying over three orders of magnitude. In summary, SPR is an invaluable tool for IM assay development.

Immunogenicity Testing

In the draft guidance for IM assessment, the FDA recommends a multi-tiered testing approach for clinical samples [Assay Development for Immunogenicity Testing for Therapeutic Proteins, Guidance for the Industry (DRAFT) 12/2009]. Initally, all samples are tested in a screening assay. ADA-positive samples are tested in confirmatory and titration assays (if needed), and further tested to characterize the ADA once it is detected with neutralizing capability (Fig. 7.5). The level of testing required is dictated by the degree of the safety risk of the therapeutic antibody drug to patients. Depending on the factors that contribute to the potential immunogenic response, a risk-based method must be employed during the antibody drug development program (Koren et al. 2008).

Screening Assay

The initial step in assessing IM is screening for the presence of ADA in a given sample. Therefore, it is important to develop a screening assay that has a reasonable detection limit, often referred to incorrectly as sensitivity. The draft FDA guideline recommends assessing sensitivity using affinity purified antibodies that are either monoclonal or polyclonal and reporting results in mass units/ml of the

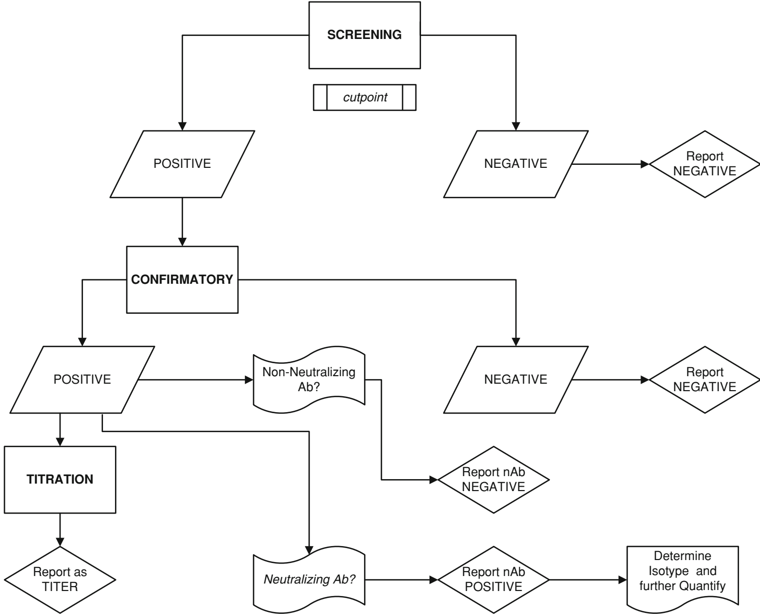

Fig. 7.5 Workflow strategy of assessing and reporting IM

matrix [Assay Development for Immunogenicity Testing for Therapeutic Proteins, Guidance for the Industry (DRAFT) 12/2009]. The reported LOD of the assay represents the lowest concentration where the antibody (positive control in this case) consistently produces a positive readout against a CP (discussed in "Assay Cut Point"). It is also recommended to have assays that can detect approximately 250–500 ng/ml of antibodies against a therapeutic antibody drug, as these concentrations are relevant based on the data compiled from historical clinical trials. There is a major weakness in this definition of the detection limit in that it only takes into account the false positive error rate and not the false negative error rate ("Cut Point, Limit of Detection, and Limit of Quantitation" addresses this problem in more detail). Specificity is one parameter that must be tested during IM method development to ensure that the assay specifically detects the ADA and not the therapeutic antibody drug, endogenous antibodies, or other reagent materials in the assay. It is also noted in the draft guideline that if the therapeutic target population has Rheumatoid Factors (RF), the assay must be tested for RF interference. Screening assay results are reported as positive or negative using an assay CP as a threshold.

Confirmatory Assay

Confirmatory assays utilize the same format as the screening assays. However, these assays are designed to confirm any samples that tested positive during the screening phase. In confirmatory assays, therapeutic antibody drugs are added to

the samples, where a decrease in signal (calculated as a percent inhibition) implies a confirmed positive result. If the signal did not significantly change against the validated inhibition value, the samples are reported as negative. The acceptable percent inhibition is established during the qualification or validation of the assay.

Titration Assay

A titration assay also uses the same method as the screening assay. Once a sample is confirmed positive, samples are tested in a titration assay. Samples are diluted to a point where the signal is closer to the assay CP signal while staying slightly above the CP. The titer value is the reciprocal of the dilution factor where the response is just above the assay CP.

Bioassay

Bioassays are cell-based assays that are used to further characterize ADA in samples that were confirmed positive. Bioassays determine whether the ADA has neutralizing properties (i.e., if the ADA can block the biological effect of a therapeutic antibody drug). Such anti-drug antibodies are referred to as neutralizing antibodies or nAbs. Cell-based assays require a longer assay time when compared to LBA. Cell-based assays can be a functional assay, target binding assay, or receptor binding assay. Functional assays are cell-based assays that assess the cellular effects of therapeutic antibody drugs. Depending on the functional endpoint, the duration of the stimulation can be on the order of minutes to overnight treatment. Proximal endpoint bioassays assess events such as receptor phosphorylation, mRNA expression, gene and enzyme activation, and intracellular trafficking. Distal endpoint cell-based assays assess cellular events such as proliferation, release of cytokines, and apoptotic events. Target and receptor binding cell-based assays evaluate the neutralizing ability of an ADA against the therapeutic drug and the resulting effect. For example, using receptor expressing reporter cells, the ligand-receptor binding can be accessed.

Considerations for Immunogenicity Assay Development

Assay Cut Point

An assay CP is a signal threshold level that is used to report samples as positive or negative to ADA. It is defined in the FDA draft guideline as "the level of response of the assay at or above which a sample is defined to be positive and below which it is defined to be negative". Assessment of the initial CP is done during assay

optimization, where the final assay CP is established during the assay validation process. There are two ways to determine assay CP: the parametric or non-parametric approach. The approach chosen depends on the data distribution of the population being assessed during the validation process. Therefore, it is important to ensure that a significant number of samples from various subjects are tested to obtain a statistical determination of population distribution. Population distribution can be either normal or non-normal. The parametric approach uses the mean of the negative population (blank samples) and adds 1.645 standard deviations (SD). The 1.645 represents the 95th percentile of a normally distributed population. The non-parametric approach applies to a population that is not normally distributed. These approaches are not limiting and other relevant methods for CP determination can be employed as long as they are statistically valid. One such alternative technique for determining CP is the Hubaux–Vos method (discussed further in the following section) (Klakamp et al. 2007).

Cut Point, Limit of Detection, and Limit of Quantitation

The CP, LOD, and limit of quantitation (LOQ) are three of the most important concepts to define and truly understand for any BA assay. The definition of the CP (synonyms include terms such as threshold, critical value, and limit of blank) is taken as the signal at or above which analyte is declared to be present in the test sample (Currie 1968, 1995, 1997, 2004; Tholen et al. 2004; Mire-Sluis et al. 2004). It is important to realize that CP is an assay signal and its corresponding analyte concentration is not the LOD (also known by "detection limit") for the assay. The CP is calculated with only consideration of the type I (α or false positive error rate). Because type II (β) error rates (false negative error rate) are not taken into account in the CP, there is in effect a 50% false negative error rate at the CP. Many analytical scientists have failed to realize this fact, and whether they acknowledge it or not, a 50% false negative error rate exists in any assay at the analyte concentration corresponding to the CP. Logically, it then follows that the CP should not be used to define the detection limit of an assay. Rather, the CP should be used as the signal at which a decision is made on whether analyte is present or not. No wise analytical scientist would ever want their assay to be judged for detection capability at a concentration of analyte (corresponding to the CP signal) that is not detected 50% of the time. Quite often the BA and even the analytical chemistry literature take the "sensitivity" of an assay to be the concentration of analyte that corresponds to the CP signal. In this context, the "sensitivity" of the assay is being used to signify the LOD, which is absolutely incorrect for the reasons discussed above. The most rigorous definition of "sensitivity" is the slope of a calibration curve for the analyte as defined by the International Union of Pure and Applied Chemistry (IUPAC) and the International Organization for Standardization (ISO) (Currie 1995). We recommend the term "sensitivity" not be used to denote any detection parameter of an assay since it is

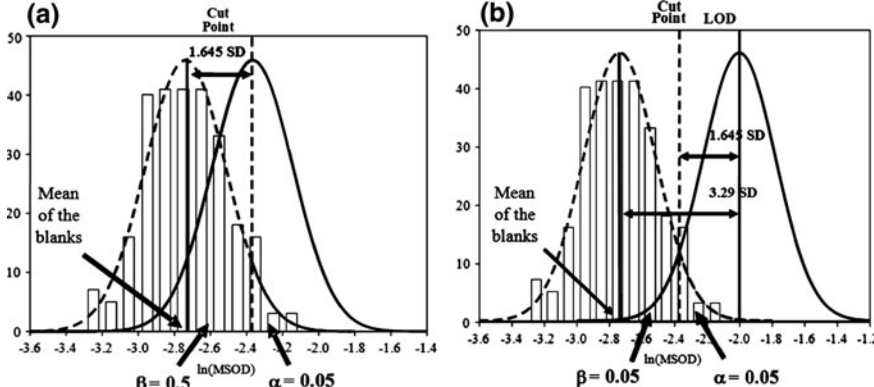

Fig. 7.6 **a** Histogram and frequency distribution function (*dashed line*) for blanks and theoretical frequency distribution function (*solid line*) for ADA at a level equal to the CP ($\alpha = 0.05$). **b** Histogram and frequency distribution function (*dashed line*) for blanks and theoretical frequency distribution function (*solid line*) for ADA at a level equal to the LOD ($\alpha = 0.05$, $\beta = 0.05$). Reprinted with permission from Klakamp et al. (2007). Copyright 2007 American Chemical Society

used in so many ambiguous and incorrect ways depending upon the scientific discipline. However, we are also aware that "sensitivity" is so engrained in BA nomenclature that this is most likely wishful thinking on our part that the term not be used at all.

The definition of the detection limit is the lowest concentration of analyte that can be detected with given type I and II (α and β) error rates with both commonly set to 0.05. The concentration of the analyte at the LOD is not quantitated accurately but is detected reliably at the given type I and II error rates set by the analyst. Figure 7.6 illustrates schematically the concepts of CP and detection limit in terms of normal distributions from an ADA assay for an anti IL-8 antibody (Klakamp et al. 2007). First notice the data in Fig. 7.6 had to be log-transformed to fit a Gaussian distribution. More times than not raw BA data is not normal and needs to be log-transformed, or more yet, requires some other mathematical operation to be performed to allow the data to conform to a normal distribution. With a type I error rate of 0.05 the CP is calculated by multiplying the mean sample optical density (MSOD) of the blanks by 1.645 (one-tailed t-value for the 95% confidence limit for a normal distribution) to arrive at the signal corresponding to the CP. It is important to understand that even if the α error rate had been set to 0.01 or 0.1 or any other value the β error rate will always be 50%—half the MSOD values will be above the CP and half below as shown in Fig. 7.6a. To calculate the detection limit with a β error rate of 0.05—meaning the ADA will be detected 95% ($1-\beta$) of the time at that signal (really the concentration of analyte corresponding to the LOD signal to follow our definition above rigorously)—the MSOD of the blanks is multiplied by 3.29, see Fig. 7.6b. If different α and β error

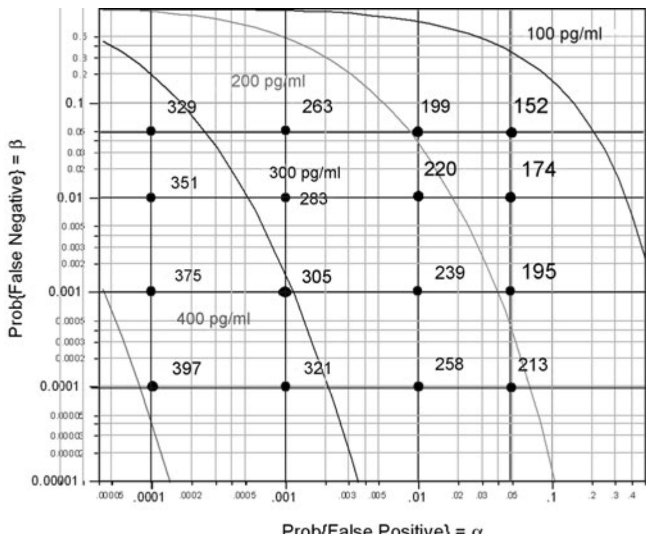

Fig. 7.7 ROC curves and LOD values generated from Hubaux-Vos analysis of an antibody calibration curve in 10% serum. The ROC *curves* (*solid lines*) illustrate corresponding false positive and false negative error rates needed to maintain LOD values equal to 100, 200, 300, or 400 pg/ml. The *points* represent calculations of LOD for various fixed values of α and β. Reprinted with permission from Klakamp et al. (2007). Copyright 2007 American Chemical Society

rates are chosen, then obviously different t values have to be used and can be easily found in statistical tables for one-tailed Student t distributions.

One of the most rigorous ways of calculating CPs and LODs from an analyte calibration curve is by the Hubaux-Vos method (Hubaux and Vos 1970). In this technique, prediction intervals are calculated about the calibration curve at desired α and β error rates from which the CP and LOD are derived (Klakamp et al. 2007). Construction of receiver-operator curves or ROCs can be a very powerful statistical tool for determining the best LOD possible from an experimental data set with given α and β error rates. Figure 7.7 shows ROCs for the ADA assay alluded to in Fig. 7.6. The four curved lines spanning the entire graph correspond to different LOD values of 100, 200, 300, and 400 pg/ml, where any combination of α and β error rates (shown on the x and y axes, respectively) falling on the curved lines would maintain the given LOD. For example, if we set α and β error rates each to 0.05, it is observed that the best LOD possible for this ADA assay is 152 pg/ml. Of course, all of the discussion above presumes the data can be described rigorously by a normal distribution. If the data cannot be made normal through some type of transformation, it is possible to perform non-parametric statistical analyses to calculate the CP and LOD. Non-parametric methods will not be discussed here and the reader is referred to articles by Klakamp et al. (2007) and Liang et al. (2007).

In contrast to the rigid definitions for CP and LOD, the LOQ is a less well-defined term, and more freedom is given to the analyst in defining the term depending upon the demands of their assay. In BA settings, the LOQ is usually taken as the concentration where analyte can be quantitatively determined with acceptable accuracy and precision as required for a particular assay. In other words, the LOQ is the concentration of analyte that can be determined with a satisfactory relative standard deviation (RSD) or coefficient of variation (%CV) for replicate measurements (Tholen et al. 2004). For example, quite often in analytical chemistry the concentration of analyte that can be determined with an RSD of 10% is defined as the LOQ (Currie 1968, 1995, 1997, 2004). Obviously, to interpret BA data with the highest rigor, the CP, LOD, and LOQ must be defined, understood, and reported correctly.

Drug Tolerance

Drug Tolerance is another parameter that poses a challenge in developing an IM assay with an acceptable detection limit. The FDA draft guideline recommends that antibody samples are tested at various concentrations of drug. A favorable IM assay has the ability to detect ADA even in the presence of therapeutic antibody, which can potentially interfere with the detection, especially in a bridging assay format. Some assay formats tolerate the presence of mAb drug better than others, so it is recommended to assess various assay formats. There are several ways to overcome drug interference. One way to approach drug interference is to allow samples to incubate for long periods of time. This process relies on the kinetics of the ADA-therapeutic drug association and dissociation properties. Once the ADA complex is dissociated, the capture and/or detection antibody binds to ADA, and eventually it is detected in the assay. Another approach that is employed to improve drug tolerance is the Acid Dissociation Method. In this protocol, samples are treated with acid to induce the ADA-drug complex to dissociate with immediate neutralization while adding an excess amount of labeled therapeutic antibody drug. This treatment increases the chance that dissociated ADA will bind to the labeled therapeutic antibody drug and be detected by the assay.

Precision, Robustness, and Stability

IM assay precision is tested by evaluation of the intra- (repeatability) and inter- (intermediate) assay variability. The FDA draft guideline recommends evaluation of the inter-assay precision by testing samples at a minimum of three (3) assay days and a minimum of three (3) replicates. Intra-assay precision is tested with a minimum of six replicates per assay plate. Moreover, operator precision and positional effects are incorporated in the assay validation activities. Robustness of the assay is also assessed to ensure assay reliability under possible changes in

assay conditions such as pH, buffer, temperature, incubation times, and temperature. Stability in the IM assay is important, and it is recommended to determine a sample storage condition that ensures the antibody reactivity is not compromised during the testing period. Freeze/thaw cycles are assessed using the reagents, such as positive controls, to support the stability studies. Although this testing may not reflect the stability of the actual samples, it will provide assurance that an assessment is performed and the closest representation is used to support the study. Additional parameters for assessment also include the state of sample hemolysis and anti-coagulants that are used during sample collection.

Concluding Remarks

BA techniques are crucial in establishing a translational strategy for progressing mAb therapeutics from preclinical to FIH clinical studies and to marketing approval. Several BA methodologies exist and are commonly used for investigating the PK, PD, and IM of mAb drugs. The understanding of the terminology of BA chemistry is also vital for reporting results that are valid and substantiated based on scientific principles. It is important to define and calculate the CP, LOD (incorrectly stated as "sensitivity" quite often in BA settings), and the LOQ with logical and statistically based methods.

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Chapter 8
Preclinical Considerations for Development of Antibody-Based Therapeutics in Oncology

Gregory Landes and Kathleen Elias

Abstract In this chapter the preclinical considerations for antibody target discovery, validation, and in vitro and in vivo pharmacology will be discussed. A deep understanding of both the biology and the pathology of disease is essential for target identification and validation. This knowledge enables the rationale design of antibody therapeutic attributes including mechanism of action (MOA), specificity, potency, isotype subclass, affinity, and half-life. Subsequently, consideration and utilization of the appropriate animal models will enable optimal translation to the clinic and assist in clinical trial design. Additionally, preclinical considerations for the successful HER2 and CD20 antibody drug therapeutics will be presented as case studies.

Introduction

What makes a good antibody target is central to any antibody development program in oncology. As effective treatment of tumors with antibody therapy must dramatically impact tumor growth and survival, it follows that the viable antigen targets must either be directly involved in the growth and/or survival pathways of tumors or enable selective targeting of cancerous cells with cytotoxic mechanisms. Direct involvement of viable antigen targets whose biology is critical for growth and/or survival requires that the therapeutic antibody affect the function of the

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antigen. Alternatively, selective targeting of cancerous cells requires that viable antigen targets or their neo-epitopes be overexpressed in disease tissue relative to normal tissue counterparts, enabling the use of indirect cytotoxic mechanisms including effector function and antibody drug conjugates. The specificity, tunable effector function, and amenability to payload additions provide many options for arming therapeutic antibodies with multiple mechanisms of action.

Antigen Targets Directly Involved in Tumor Growth and/or Survival

Cancer is a polygenic disorder that is propelled by mutations that select for growth and survival of aberrant cells. The genetic alterations that affect the growth and survival attributes of cancer cells occur progressively during the transition from normal to cancer cells. These genetic lesions or "hallmarks of cancer" allow the resulting cells to become self-sufficient in growth signals, insensitive to antigrowth signals, evasive of apoptosis, unregulated in their replicative potential, self-sustained in vascular access, and highly tissue invasive and ultimately, metastatic (Hanahan et al. 2000). The absolute number of mutations required for cells to become cancerous is unknown, but experimental studies and estimates based on incidence of occurrence of mutations suggests that 6–20 mutations are required (Sjoblom et al. 2006; Wood et al. 2007; Ding et al. 2008; Jones et al. 2008; Cancer Genome Atlas Research Network 2008; Parsons et al. 2008; Stratton et al. 2009; Bell 2010). These mutations are often referred to as "driver" mutations as they confer cellular growth advantages that have been causally implicated in cancer and have consequently been selected for in tumors. The constellation of genes harboring driver mutations varies from tumor type to tumor type and patient to patient. Current estimates suggest that there are greater than 200 genes that can be mutated and result in "driver" mutations (Greenman et al. 2007; Velculescu 2008). Additional mutations that are not critical for the "hallmarks of cancer" also occur during the transition from normal to cancer cells and are referred to as "passenger" mutations. These mutations are biologically silent as they provide no cellular growth advantage.

Drug modalities that favorably alter the function of targets and their pathways with driver mutations will provide some therapeutic benefit to patients. However, with the exception of chronic myeloid leukemia and its association with the bcr-abl oncogene, the polygenic nature of cancer obviates singular or even simple broad-based therapeutic interventions as curative treatments. Moreover, effective cancer treatments will require customized, "precision medicines" that personalize treatment to affect the relevant pathways that are driving the growth and survival of the tumor cell population(s) (von Hoff 2010). Consequently, effective treatments will confront the "hallmarks of cancer" and therapeutically alter tumor growth and survival. It is obvious that affecting the function of a target and its pathway that represent passenger mutations will have little or no therapeutic consequence.

Antibody Drug Target Expression

The development of the initial wave of targeted antibody cancer therapies relied on targets that were identified as either cell specific markers like CD20 or receptors like EGFR and HER2.[1] In the former case, antibodies to CD20 allowed the selective targeting of B cells and B cell malignancies. In contrast, growth factor receptors EGFR and HER2, whose intrinsic biology conferred growth benefit, were often overexpressed in carcinomas as determined by classical methods like northern blots, RT-PCR, FACS, and IHC. As discussed further in the case study section, HER2 is overexpressed in 20–30% of breast cancers with over 1 million receptors per cell. The success of this discovery paradigm coupled with advancements towards global gene expression analysis accelerated the molecular characterization of tumors and tumor cell lines by differential expression analysis. Similar expression efforts have been carried out using proteomics platforms, although the throughput availability and cost have biased platform utilization predominantly toward the use of transcript levels as a surrogate for gene product expression. Hundreds of published studies on global gene expression differences between tumors, tumor cell lines, and their corresponding normal cell and tissue counterparts have been performed and have resulted in the identification of differentially expressed genes and their respective pathways that influence tumor growth and survival. Most of these studies were performed without in-depth information about the genetic and epigenetic genotypes of tumors, so driver and passenger information could only be inferred from which pathways appeared to be more or less active based on gene expression. Expression methods have continued to evolve from cataloging these transcript expression differences using microarrays, sequencing-based digital gene expression by serial analysis of gene expression (SAGE), exome sequencing by Sanger dideoxy terminator chemistry, and more recently, exome sequencing by RNA-Seq, a high-throughput next generation sequencing technology to sequence cDNA. The expression profiles using both chip-based methods and SAGE have been sufficiently robust and comprehensive to illuminate biological processes that tumors have co-opted to achieve uncontrolled growth and unmonitored survival. Interestingly, the combination of gene expression analysis and genetic analysis of mutations from several platforms, including DNA copy number determination by hybridization and exome sequencing by Sanger or RNA-Seq, has provided for the first time a strong correlation between pathways altered by driver mutations and their overall gene expression differences (Jones et al. 2008; Parsons et al. 2008).

Several excellent examples of the relationship between pathways altered by driver mutations and overall expression differences exist from the Vogelstein and Kinzler laboratories, including analyses of pancreatic and brain tumors

[1] For this chapter, gene names will be italicized, e.g.,*HER2*, while the use of gene name or an accepted protein name without italics or other qualifier, will refer to the gene product, the HER2 protein.

(Jones et al. 2008; Parsons et al. 2008). The study of Jones et al. examined pancreatic cancer by global genome analysis (Jones et al. 2008). The methods used included expressed gene mutation analysis by Sanger exome sequencing, gene expression by digital sequencing using SAGE, and deletion and amplification determination by analysis using SNP microarrays with <1 million SNPs. Their results identified 12 core pathways and processes that were genetically altered with driver mutations in 67–100% of 24 advanced pancreatic tumors. Interestingly, the 31 gene sets comprising these core signaling pathways and processes were more highly enriched for differentially expressed genes than the remaining 3041 gene sets. The 12 core pathways and processes altered in pancreatic cancer include apoptosis, DNA damage control, regulation of G1/S phase transition, Hedgehog signaling, homophilic cell adhesion, integrin signaling, c-Jun N-terminal kinase signaling, k-Ras signaling, regulation of invasion, small GTPase-dependent signaling, TGF-β signaling, and Wnt/Notch signaling. Examination of these 12 core pathways for those that could be sensitive to an antibody therapeutic based on antigen localization in the extracellular and plasma membrane compartments, the results of Jones et al. indicate that about one-half of the pathways and processes can be affected by antibody drugs including Hedgehog signaling, homophilic cell adhesion, integrin signaling, regulation of invasion, TGF-β signaling, and Wnt/Notch signaling.

A similar study was described by Parsons et al. in their integrated genomic analysis of a discovery set of 22 glioblastoma multiforme tumors, comprising 7 primary tumors and 15 primary tumor xenografts (Parsons et al. 2008). An additional 83 glioblastomas were analyzed for the presence of point mutations. Using the same methodological approach as described by Jones et al. 9 core pathways and processes were identified that were genetically altered in at least 10% of the glioblastoma tumors analyzed. Some of the core pathways identified were the same ones that were also identified in pancreatic, breast, and colorectal tumors, e.g., cellular growth, apoptosis, and adhesion. This intersection of common core cancer pathways across disparate tumor types may very well reflect essential growth and survival pathways for tumorigenesis. Not surprisingly, the investigators identified several core pathways that were unique to glioblastoma, including neural signaling functions, ion transport, synaptic transmission, and axon guidance. The core pathways that are genetically altered in glioblastoma predict that targeted therapy with an antibody to either the integrin or EGFR pathways should directly affect tumor growth and survival.

Validation of Antibody Drug Target Expression

The identification of differentially expressed genes in tumors and the correlation observed between differential expression and core pathways and processes altered with driver mutations is just the first step for target identification and validation. Additional confirmatory activities of global gene expression differences are

required including qRT-PCR (quantitative real-time PCR) and IHC of tumor and normal samples; this strategy ensures that differential gene expression is confirmed using orthogonal methods such as qRT-PCR and IHC. In the case of qRT-PCR, it is recognized as the gold standard for quantitative gene expression measurements because of its large dynamic range and sensitivity. Ideally, RNAs from clinical specimens used in the microarray studies that exhibit transcript overexpression when compared to normal counterparts can be confirmed by qRT-PCR. Parallel assessments of transcript levels of housekeeping genes, e.g., GAPDH, β-actin, rRNA, etc., will provide additional quality measures in the analysis. A point worth mentioning is that the expression level of housekeeping genes is not always static; (Selvey et al. 2001; Solanas et al. 2001; Zhu et al. 2001) consequently, several housekeeping genes can and should be monitored for comparison between disease and normal tissues (Vandesompele et al. 2002).

The identification and confirmation of overexpressed cancer transcripts still requires a determination that the encoded protein is overexpressed and an assessment of the role of the gene product in tumor growth and survival. The former activity will establish that the actual antigen is overexpressed, which is not the case 10–20% of the time by qRT-PCR. The latter activity will determine the critical design goals that will be necessary for an antibody drug to affect antigen function therapeutically. These activities can be performed sequentially or in parallel. If commercial antibodies are available, the conditions will need to be established for using one or more of these antibodies to interrogate tissue microarrays (TMAs). Once IHC conditions are optimized, the expression of antigen can be surveyed across a collection of normal human tissues including critical organs as well as tumor specimens. Critical parameters in evaluating antigen expression include staining intensity of tumor, homogeneity of tumor staining, and the cellular topology of staining (Tennstedt et al. 2010). As most cancer targets for antibody therapeutics will be membrane associated, the desired outcome of an IHC validation study is that most tumor cells will be stained, the staining intensity will range from moderate to strong, and the staining will be localized to the plasma membrane. Under the same staining conditions, normal tissue staining, particularly in critical organs, will be of lower intensity (Hewitt 2009; Simon 2010), providing a tumor tissue/normal tissue differential. As with a tissue cross-reactivity protocol, it is often advantageous to survey the staining at the identified optimal concentration and at a higher concentration (e.g., 2 and 10 μg/ml). Although there will be additional background staining, this procedure will also allow the localization of targets with low expression in tissue as well as further define the tumor/normal differential.

Functional Genomics with RNAi

RNAi screens can be used for target identification or to make a preliminary assessment of the role of a gene product in tumor growth and survival without

having to generate antibodies to all targets of interest (Westbrook et al. 2005; Moffat et al. 2006; Luo et al. 2008; Silva et al. 2008; Luo et al. 2009; Quon et al. 2009). Furthermore, the technology provides a means to query target biology at multiple levels including oncogene addiction, synthetic lethality, sensitization to standard of care therapy, or combination with other drugs including those in development. The platform is scalable with commercial knockdown reagents and RNAi screening services are available from multiple vendors. This approach requires one or more relevant cancer cell line models that express the target of interest and can be readily transfected, electroporated, or transduced with the knockdown construct(s) for assessment of the target's role in cellular growth and proliferation.

While RNAi can make a substantial contribution to target discovery and validation, it does have limitations that still require additional antibody-based validation studies to be performed. One such limitation concerns the frequency of false positives. RNAi can have off-target effects from partial complementarity with other transcripts, resulting in a microRNA-like activity as evidenced by inhibition of translation and a corresponding reduction in specific gene product protein synthesis (Seinen et al. 2010; Sigoillot et al. 2010). This possibility can be minimized by demonstrating that several RNAi constructs against different regions of the same transcript result in the same biological consequence. Alternatively, demonstrating decreased transcript levels after RNAi treatment using qRT-PCR would support that the observed effect was due to that RNAi.

Another limitation of the RNAi knockdown method is that the observed cellular response in vitro may differ from the effect seen following targeted drug treatment. For example, knockdown of Aurora B kinase gene expression leads to the absence of all of its biological functions, resulting in a broad and dramatic mitotic dysfunction (Moffat et al. 2006). However, pharmacological inhibition of Aurora B kinase activity only alters its enzymatic activity and produces a readily detectable but less severe mitotic effect. It has been speculated that the more severe phenotype resulting from RNAi treatment may be due to the loss of Aurora B′s scaffolding functions as well as kinase activity.

In Vitro and In Vivo Validation with Biologic Tool Reagents

After an RNAi knockdown effect is observed in one or more assays, the next step is using a tool-grade biologic to validate the target as antibody druggable. Depending on the putative MOA of the target, several options are available for carrying out these essential validation studies. If the target is believed to act as a receptor, its quaternary structure is a monomer or homomultimer (preferably a dimer), the ligand is known, and the knockdown yields a therapeutic phenotype, then a dominant negative strategy using a soluble receptor of a Type I or II membrane protein is generally effective and efficient. As inferred, Type III membrane proteins are not as amenable to this approach. For the above example

and in the absence of a commercially available neutralizing antibody, a dominant negative strategy can be performed with the entire ectodomain of the target. Alternatively, if the critical portion of the receptor required for ligand binding is known, then a truncated isoform can be used. For in vitro validation, the dominant negative-protein construct can be just the ectodomain or designed as an ectodomain-Fc fusion protein. In the latter format, the receptor ectodomain is often expressed and secreted more efficiently as an Fc domain, while facilitating purification on a Protein A resin and ultimately, providing advantages for subsequent in vivo validation. The main attraction of the dominant negative approach in vitro is the biochemical simplicity that the soluble receptor will compete for ligand with its cellular counterpart without having to generate and characterize antibody reagents that neutralize ligand binding and downstream signaling. Also, advancing the validation studies from in vitro to in vivo is more readily accomplished using Fc fusion constructs rather than the ectodomain alone as the improved half-life of the Fc construct reduces the amount of recombinant protein required, favorably affecting both protein production costs and pharmacology expenses related to dosing frequency. In vivo validation studies with soluble receptor Fc constructs may be done with weekly or twice weekly dosing at a single high concentration, e.g., 20 mg/kg, over a 3–5 week-period based on the tumor model. At this validation stage, high dosing paradigms and less stringent efficacy limits are often utilized, e.g., Tumor Growth Inhibition (TGI) approximating 60%, as this is not the therapeutic candidate. Preferentially, additional insight can be obtained by performing a dose–response study with an index of activity obtained with the soluble receptor that will help define the design goals for the subsequent drug discovery program, assuming that target validation is successful.

Exceptions for using a dominant negative validation approach exist and include Type III membrane proteins, single-pass membrane receptors of high molecular weight, and single-pass membrane receptors that are poorly expressed for unknown technical reasons. In these instances, functional validation requires an alternative strategy using tool-grade antibodies against the antigen. The activities required for the creation and identification of tool-grade antibodies are identical to those for antibody drugs and, in fact, the tool antibody may become the therapeutic candidate, or at least a starting point for molecular engineering to a therapeutic candidate. However, the specifications of tool-grade antibodies are less stringent, particularly with respect to potency, as this attribute can be overcome in vitro and in vivo by using dose levels beyond what would be clinically and commercially viable, e.g., 30 mg/kg, once a week to obtain in vivo validation. In addition, while monoclonal antibodies derived from hybridoma or phage display provide a reliable solution for validation, affinity purified polyclonal antibodies can also be used for some applications.

Although antibodies to targets that directly affect tumor growth and signaling are important therapeutics in oncology, targets that show significant disease association by protein expression analysis, but do not appear to be integrally involved in tumor growth and survival, may still provide a therapeutic opportunity by indirect cellular cytotoxicity mechanisms (Fig. 8.1). Antibodies to tumor-associated antigens can exploit Fc effector functions, antibody-dependent cellular

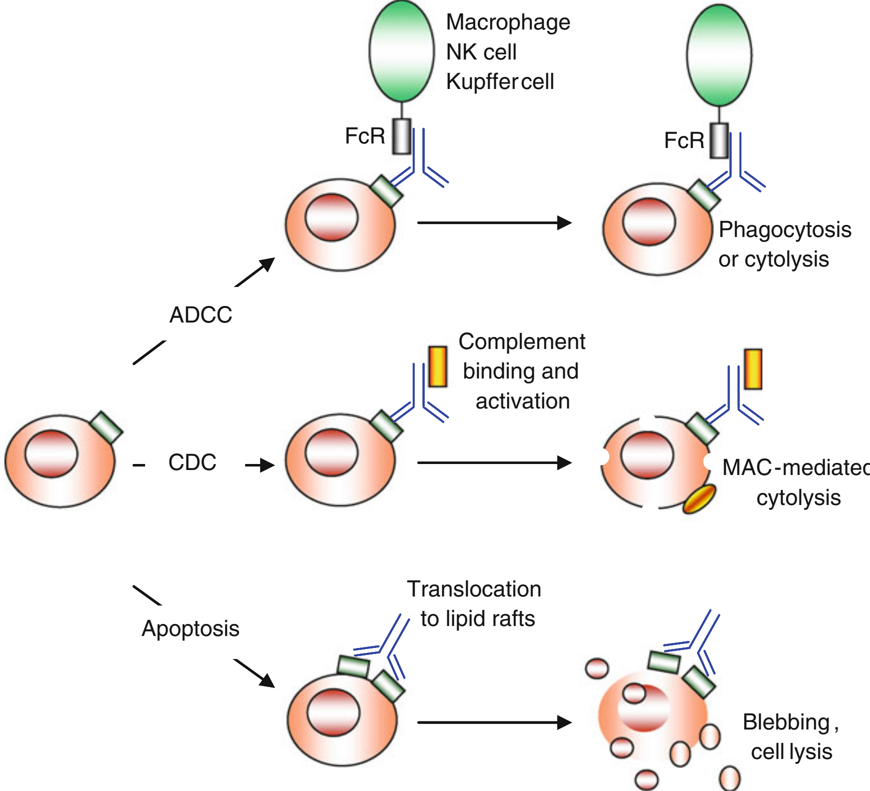


Fig. 8.1 Antibody mechanisms of action. Antibodies to tumor associated antigens can exploit Fc effector functions including antibody-dependent cellular cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) to reduce tumor cell burden. The mechanisms of action (MOA) for the hematological cancer antibody, Rituxan, include the effector functions ADCC and CDC. Additional antibody MOAs may include effects on growth and survival, e.g., apoptosis, through modulation of signaling activity

cytotoxicity (ADCC), and/or complement dependent cytotoxicity (CDC), to reduce tumor cell burden. Effector function is particularly effective for the treatment of hematological tumors and may be sufficient for tumor regression as a single agent, or even eradication, when used in combination with small molecule cytotoxic drugs.

The anti-CD20 antibody Rituxan is a compelling example of the potency of effector function for the treatment of non-Hodgkins lymphoma and chronic lymphocytic leukemia, although this drug and other anti-CD20 drug candidates may have additional functional activities, which are independent of effector function (see case study in Anti-CD20). The potency of effector function for the treatment of hematological cancers is not too surprising as both ADCC and CDC require plasma components including macrophages, NK cells, and complement factors, respectively, that are abundant in vascular and lymphatic circulation but present at

lower amounts in large solid tumor masses. Nevertheless, effector function activity also has a role in the treatment of solid tumors based on the studies of several investigators demonstrating that the IgG1 anti-HER2 antibody trastuzumab, also known as Herceptin, was more effective in treating patients that express the FcγRIIIa 158V/V variant as opposed to the FcγRIIIa 158F/F variant (Tamura et al. 2011; Musolino et al. 2008). Generally, therapeutic antibodies to solid tumor antigens that rely solely on ADCC or CDC are not expected to have sufficient efficacy without additional MOAs, including in combination with chemotherapy. Additionally, some antibodies induce apoptosis, resulting in cytotoxicity, but those with this sole MOA, in the absence of effector function, may not be sufficiently efficacious in solid tumors.

Recent clinical progress with antibody drug conjugates (ADCs) provides encouraging support for this new MOA for treating cancer. With respect to ADC targets, the membrane antigen must be overexpressed in tumor cells and absent or expressed at low levels on normal tissue counterparts, particularly on critical organs such as the heart or kidneys. In addition, the antigen must internalize upon antibody binding and be appropriately trafficked intracellularly to release the small molecule cytotoxic payload present on the ADC. A variety of target attributes are important for qualifying ADC druggable targets including moderate to high antigen density, moderate to high internalization rate, low levels of shed antigen, potent small molecule payloads with GI50 values (the concentration of a drug required to inhibit the growth of treated cells by 50%) of <1 nM, etc. However, the complexity of factors is such that there can be compensation by one or more factors, e.g., moderate antigen density of 50,000 antigen molecules per cell with a rapid internalization rate may give the same ADC-dependent cytotoxicity as a high antigen density of 500,000 antigen molecules per cell with a slower internalization rate.

Target Epitope Selection

Rarely are the epitopes of a cancer antigen preselected for targeted antibody therapy. In fact, all therapeutic antibodies that have been commercialized to date were initially identified based on binding specificity, desirable pharmacological effect on a biological process (antagonism, agonism, internalization, etc.), and/or eliciting effector function. However, recent advances made in the genetic analysis of tumors including identification of prevalent chromosomal translocation breakpoints and alternative spliced transcripts may provide new opportunities to target disease-specific antigen variants. The best known example of an antibody druggable cancer target that can be selectively targeted by epitope for pharmacological effect is EGFRvIII. EGFRvIII is expressed in glioblastoma, particularly high grade tumors, and accounts for approximately 60% of all known EGFR mutations in tumors. This variant is a consequence of an in-frame deletion of 801 base pairs. The resulting protein lacks amino acids 6–73 of wild-type EGFR, contains a glycine at the novel junction, and no longer encodes the ligand binding site.

Surprisingly, EGFRvIII is constitutively activated. Antibodies like mAb 806 have been made that selectively target the novel epitope encoded by EGFRvIII (Mishima et al. 2001). When mAb 806 is used in glioblastoma xenograft studies, it has a profound effect leading to tumor growth inhibition, increased tumor cell apoptosis, and animal survival.

Next generation sequencing of cDNA from diverse human tissues and epithelial cell lines has shown that more than 90% of human genes undergo alternative splicing (Wang et al. 2008) suggesting that additional disease-associated targets may be available. Generally, alternative splicing manifests itself as exon skipping (Sultan et al. 2008). Alternative splicing is influenced by cell type, developmental stage, external conditions, and even pathological conditions (Venables 2006). In fact, aberrantly spliced variants have been shown to be actively involved in the initiation and/or progression of many types of cancer (Kalnina et al. 2005). The alternative splicing observed in cancers are often the result of splice-site mutations, dysregulation of splicing regulatory factors, or both (Grosso et al. 2008). Splice variants that are specific to tumor cells and affect coding regions will result in new epitopes that can serve as an important starting point for immune therapy or targeted delivery, as well as for the development of new diagnostic or prognostic tools (Kalnina et al. 2005).

Pio et al. examined NSCLC tumor specimens for the presence of tumor specific splice junction variants using custom microarrays (Pio et al. 2010). Two-hundred and sixty potential splice variants were identified of which 4 of the top 10 splice variants were validated by qRT-PCR of tumor and normal lung RNAs and included CEACAM-1, FHL1, MLPH, and SUSD2. For CEACAM-1, lung tumors frequently overexpress three splice isoforms: CEACAM1-1, CEACAM1-3, and CEACAM1-3A. The alternative use of these exons affects different Ig-like structural domains in the extracellular portion of the respective proteins.

Target Validation Impact on Antibody Design Criteria

Validation methods with tool-grade biologics not only demonstrate the tractability of a target in vitro and in vivo but also provide an empirical means of defining the minimum drug design goals for biologics drug discovery. Correlating the effects of tool-grade biologics on in vitro target activities with the extent of efficacy in relevant animal models of established disease can identify the requirement for antagonism or agonism while approximating the magnitude of the minimum specific activity or activities of the drug candidate. For oncology drugs, multiple MOAs are often required. When possible, the use of tool grade reagents for in vitro and in vivo validation with different combinations of activities, e.g., antagonist with or without effector function or antagonist with or without antigen internalization, can provide a more comprehensive picture of the essential activities required for antibody leads generated during a drug discovery campaign.

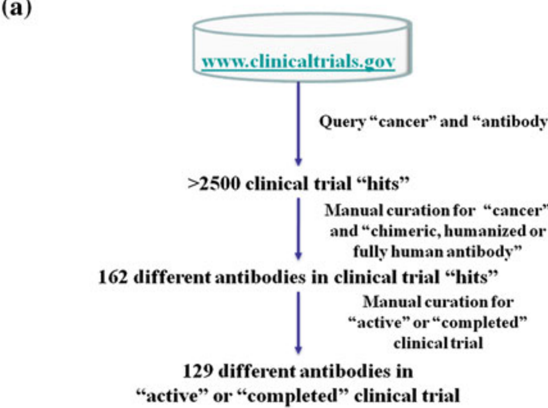
Pharmacology and Antibody Drug Mechanisms of Action

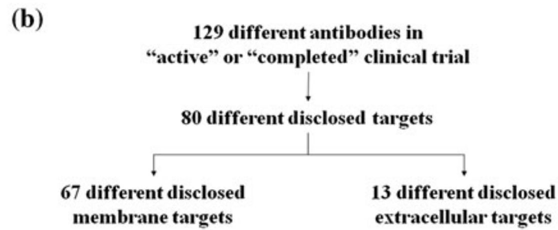
As described previously, rigorous target validation with tool-grade biologics can quickly catapult an early discovery biology program to a full therapeutic drug discovery program. When done systematically, drug design goals can be readily formulated and the existing tool-grade biologics can be used as starting drug scaffolds for humanization and affinity maturation. Alternatively, tool-grade biologics can be repurposed for refining in vitro and in vivo assays, and as benchmarking standards for de novo antibody drug discovery campaigns. Validation studies can establish the functional role of the target in the biology of disease, define a disease pathway, and help to determine the mechanism of action. But such studies can also uncover additional questions concerning the biology, including consequences of downstream signaling, identification of redundant pathways, and disadvantages of pharmacologically affecting a ligand or receptor target.

Insight into antibody drug design in oncology can be gleaned from analyzing recent clinical trial activities involving antibody drugs and drug candidates (Fig. 8.2). While this type of analysis is not comprehensive and only provides a snapshot in clinical development time, this fact does paint a picture of the actual distribution of target diversity, target topology, and target MOAs in clinical oncology. The clinicaltrials.gov database was queried using only the terms “cancer AND antibody” and yielded over 2,500 trial hits. Several observations from this simple query were made. First, the search was not comprehensive as some drug modalities were incomplete or underrepresented, such as ADCs. Second, the database search was heavily biased toward several well-known antibody drugs that were being used in many trials, often in combination with other drugs and drug candidates. For example, Rituxan is listed in 930 trials, Avastin in 1,150 trials, Herceptin in 466 trials, Erbitux in 442 trials, and Panitumumab in 105 trials. Manual curation of these hits to ensure that each trial was an oncology indication and that the antibody drugs were either chimeric antibodies, humanized antibodies, or fully human antibodies (no murine antibodies were included) reduced the oncology clinical trial set to 162 different antibody drugs and drug candidates. A further reduction in antibody drug complexity to 129 was achieved by focusing on only those molecules that were either in “active” or “completed” trials (Fig. 8.2a).

The 129 different oncology antibody drugs and drug candidates in clinical trials are against 80 different disclosed targets (4 targets were not disclosed). Eighty-five percent of the targets are plasma membrane targets (Fig. 8.2b). As shown in Fig. 8.2c, a closer inspection of public information at clinicaltrials.gov, published literature, or the drug developer’s homepage revealed that 78 antibodies (60%) are predicted to alter signaling, 54 antibodies (42%) are capable of eliciting effector function, and 14 antibodies are antibody–drug conjugates (11%). The remaining 9 antibody drug candidates in oncology trials are comprise immunotoxins (4%), radioimmunotherapeutics (2%), and antibody-cytokine fusions (1%).

As the sum of MOAs is greater than 100%, it is apparent that several of the antibodies in the clinic have multiple activities (Fig. 8.2c). In fact, approximately





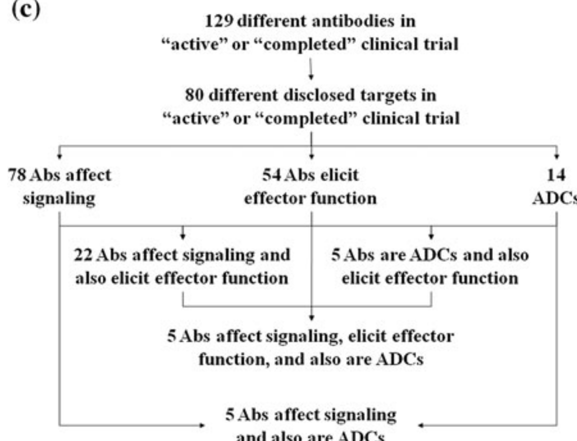


Fig. 8.2 Active or completed clinical trials with antibody drug candidates. Query description of the clinicaltrial.gov database is detailed in panel (**a**). The number of antibody trials is further categorized to target type, membrane or extracellular, in panel (**b**). **c** Details the individual as well as combination mechanisms of action for 129 different antibodies

20% of the drug candidates have two or more MOAs: 19 affect signaling and also elicit effector function, 2 affect signaling and are also ADCs, 2 elicit effector function and are also ADCs and finally, 3 antibody drugs in the clinic not only affect signaling and elicit effector function but also are ADCs. Not surprisingly, all

Table 8.1 Marketed oncology monoclonal antibody therapeutics

Target	Monoclonal antibody	Company/Molecule	Type of antibody	Indication
CD20	Rituxan/ Mabthera	Biogen Idec/Chugai/Genentech/ Zenyaku Kogyo's rituximab	Chimeric IgG1 Fc	NHL
CD20	Arzerra	GlaxoSmithKline ofatumumab	Fully human IgG1	CLL
CD52	Campath	Takeda/Genzyme/Bayer Schering's alemtuzumab	Humanized IgG1	CLL
EGFR	Erbitux	Bristol-Myers Squibb/Imclone/Merck Serono/Eli Lilly's Cetuximab	Chimeric IgG1 Fc	CRC, SCCHN
EGFR	Vectibix	Amgen/Takeda's Panitumumab	Fully human IgG2	CRC
HER2	Herceptin	Genentech/Roche/Chugai's trastuzumab	Humanized IgG1	BC
VEGF	Avastin	Genentech/Roche/Chugai's bevacizumab	Humanized IgG1	CRC, NSCLC, BC, RCC
CD20	Bexxar	Glaxo/Smithkline/GE Healthcare's tositumomab	Iodine-131 radiolabeled murine IgG2a	NHL
CD20	Zevalin	Biogen Idec/Spectrum/Bayer Schering's ibritummob tiuxetan	Yttrium-90 radiolabeled murine IgG1	NHL

Currently marketed monoclonal antibody oncology products listed with the company and molecule names, antibody type and indication. *NHL* Non-hodgkin lymphoma, *CLL* Chronic lymphocytic leukemia, *CRC* Colon Rectal Cancer, *SCCHN* Squamous Cell Carcinoma of the Head and Neck, *BC* Breast Cancer, *RCC* Renal Cell Carcinoma

antibody drugs that have 2 or more MOAs are mediating their activities through plasma membrane targets. This retrospective analysis supports the advantages of targeting membrane antigens and also the importance of leveraging multiple MOAs. Approved antibody therapeutics for cancer are listed in Table 8.1.

Antibody Drug Candidates that Affect Signaling

In active and completed clinical trials with antibodies, there are 51 drugs and drug candidates that rely solely on affecting signaling pathways as their MOA (Table 8.2). Of these 51 drugs and drug candidates, 37 are directed against 28 different membrane antigens. Thirty-four of the 37 drugs and drug candidates against membrane antigens are antagonizing the activity of 26 different membrane antigens; the only exceptions being the 3 agonizing antibodies against the 2 TRAIL receptors, TNFSFR10A and 10B. In contrast, there are 14 drugs and drug candidates that neutralize 12 extracellular antigens that are involved in cell signaling.

For signaling pathways that are validated to play a role in cancer, the biologics drug discovery question becomes "which is the best target, the ligand (particularly the soluble ligand) or the receptor?" Considerations that need to be addressed to answer this question include the promiscuity of the ligand and receptor, the ligand concentration in disease, the tissue distribution of the receptor in normal tissue, the need for additional mechanisms to increase overall potency, and as always, the intellectual property landscape. In the current clinical trials setting, four different pathways are being addressed with drugs/drug candidates to both the receptor(s) and ligand(s): MET/HGF, CCR2/CCL2, IGF1R/IGF1 and IGF2, and VEGFR1, VEGFR2, and NRP1/VEGF. For MET/HGF, there is no promiscuity of the ligand or receptor and the preclinical data supports the tractability of both the secreted and membrane-bound binding partners. Interestingly, for the other receptor ligand pairs, there is some promiscuity that may need to be taken into account. These ligand-receptor binding pairs will be discussed in the following section.

Redundancies in Ligand and Receptor Mediated Signaling

Different CCR2 ligands have been described in the literature including CCL2, CCL7, CCL8, CCL12, CCL13, CCL14, and CCL16 (Viola et al. 2008; Le et al. 2004; Hembruff and Cheng 2009). However, scientific support for cancer-associated CCR2 ligands is strongest for CCL2 at this time. CCL2 is expressed by a variety of normal cells including epithelial cells, macrophages, fibroblasts, and endothelial cells (Biswas et al. 1998; Buettner et al. 2007) as well as human tumors, particularly prostate and breast cancer. Furthermore, expression of CCL2 in cancer has effects on the host, leading to enhanced macrophage infiltration, increased angiogenesis, elevated osteoclast differentiation, and recruitment of regulatory T cells (Tregs) to dampen the local anti-tumor immune response. Similarly, CCL2 affects the tumor as evidenced by increased prostate cancer proliferation, survival and metastasis. Inhibition of either CCL2 or CCR2 will give the same pharmacological effect, although therapy directed at CCL2 will achieve similar efficacy but better safety because other CCR2 ligands can still recruit relevant immune cells to sites of inflammation.

Bevacizumab has become a blockbuster drug while validating anti-angiogenesis as an important means of affecting the growth and survival of solid tumors. Its clinical success has spurred multiple fast follower antibody and antibody-like drugs that affect tumor vasculature through identical or similar mechanisms (aflibercept, IMC-18F1, ramucirumab, and MNRP1685A). VEGF, the antigen recognized by bevacizumab, is one of five ligands in the VEGF family (VEGF1-4 and PIGF) and appears to be the most important angiogenic factor for tumor vasculature within the VEGF family (Ferrara 2004; Hoeben et al. 2004). VEGF binds to VEGFR1, VEGFR2, and the non-catalytic co-receptors Nrp1 and Nrp2. The most closely related drug candidate to bevacizumab is aflibercept, which is a fusion protein comprising selected VEGFR1 and VEGFR2 domains and an Fc

Table 8.2 Active or completed clinical trials with antibodies that affect signaling

Antibody drug candidates that affect signaling (http://www.clinicaltrials.gov on 7/1/2010)

Drug name	Gene name of target	Target topology	Drug name	Gene name of target	Target topology
MLN1202	CCR2	Membrane	CNTO 888	CCL2	Extracellular
KW-0761	CCR4	Membrane			
MORAb-004	CD248	Membrane			
			ACE-041	ACVRL1	Extracellular
MetMAb	MET	Membrane	SCH900105	HGF	Extracellular
			AMG102	HGF	Extracellular
APG101	CD95	Membrane			
Ipilimumab or MDX-010	CTLA4	Membrane			
Tremelimumab	CTLA4	Membrane			
Nimotuzumab	EGFR	Membrane			
Panitumumab	EGFR	Membrane			
Pertuzumab	ERBB2	Membrane			
MM-111	ERBB2 and ERBB3	Membrane			
MM-121	ERBB3	Membrane			
			CNTO 328	IL6	Extracellular
APO010	FAS	Membrane			
AMG 479	IGF1R	Membrane	MEDI-573	IGF1 and IGF2	Extracellular
Dalotuzumab or MK-0646	IGF1R	Membrane			
CP-751, 871	IGF1R	Membrane			
Ganitumab	IGF1R	Membrane			
BIIB022	IGF1R	Membrane			
Cixutumumab or IMC-A12	IGF1R	Membrane			
Figitumumab	IGF1R	Membrane			
			AMG 386	ANG1 and ANG2	Extracellular

(continued)

Table 8.2 (continued)

Antibody drug candidates that affect signaling (http://www.clinicaltrials.gov on 7/1/2010)

Drug name	Gene name of target	Target topology	Drug name	Gene name of target	Target topology
Mik-beta-1	IL2RB	Membrane			
CSL-360	IL3RA	Membrane			
			tanezumab	NGF	Extracellular
Natalizumab	ITGA4 and ITGB7	Membrane			
PF-04605412	ITGA5 and ITGB1	Membrane			
EMD 525797	ITGAV	Membrane			
CNTO 95	ITGAV	Membrane			
			CAL	PTHLH	Extracellular
AMG531	MPL	Membrane			
			denosumab	RANKL	Extracellular
IMC-3G3	PDGFRA	Membrane			
MEDI-575	PDGFRA	Membrane			
			fresolimumab	TGFB1, 2, 3	Extracellular
Mapatumumab	TNFRSF10A	Membrane			
Conatumumab	TNFRSF10B	Membrane			
CS-1008	TNFRSF10B	Membrane			
			belimumab	TNFSF13B	Extracellular
PDL192	TWEAKR	Membrane			
IMC-18F1	VEGFR1	Membrane	bevacizumab	VEGF	Extracellular
Ramucirumab or IMC-1121B	VEGFR2	Membrane	aflibercept	VEGF	Extracellular
MNRP1685A	NRP1	Membrane			
IMC-EB10	FLT3	Membrane			

Table detailing the target gene and description of antibody topology (i.e. membrane or extracellular) for the target. In many cases, e.g. CCR2, there are clinical antibodies both to the membrane-bound receptor (CCR2) as well as to the ligand (CCL2) of a specific pathway

domain for improved half-life (Holash et al. 2002). VEGFR1 is the high affinity VEGF receptor although it has weak kinase signaling activity when compared to VEGFR2. Aflibercept, like bevacizumab, can bind VEGF and prevent signaling through VEGFR1 and VEGFR2. The pharmacological activity of aflibercept is similar to that of bevacizumab.

VEGFR2, the receptor that is most critical for tumor angiogenesis (Terman et al. 1992; Millauer et al. 1993; Quinn et al. 1993) is the target for the neutralizing antibody ramucirumab (Zhu et al. 2003; Miao et al. 2006). It has the advantage over bevacizumab in that it will not only block VEGF mediated signaling but also prevent minor signaling events that may occur through other family members like VEGF-C and -D. VEGFR2 blockade leaves VEGFR1 available but because of VEGFR1′s weak kinase activity, it behaves almost like a decoy receptor, providing additional potency to ramucirumab's inhibition of VEGFR2. Unfortunately, the soluble form of VEGFR2, which has an inhibitory function on lymphangiogenesis, is also neutralized by ramucirumab. A consequence of this activity may be increased lymphangiogenesis leading to elevated metastases. VEGFR2 is also expressed on macrophages and when inhibited by ramucirumab, immune cell infiltration to the tumor will be reduced, decreasing cytokine and chemokine release with corresponding reductions in tumor growth and proliferation. VEGFR2 is also expressed on some tumors and in these instances may have an additional role in tumor growth and proliferation, while being sensitive to the actions of ramucirumab. It is unclear at this time whether there is any additional benefit offered by ramucirumab over that provided by bevacizumab.

Inhibition of VEGFR1 in animal models has been shown to have distinct advantages in reducing tumor metastases over that of VEGFR2 neutralization (Kaplan et al. 2005). The activity of anti-VEGFR1 is claimed to be mediated through VEGFR1-expressing tumors rather than effects on the neovasculature and may involve additional ligands beyond VEGF, e.g., VEGF-B and PIGF. If so, then the anti-VEGFR1 antibody IMC-18F1 will block signaling through VEGFR1 on tumor cells (Wu et al. 2006a; Wu et al. 2006b). Unfortunately, IMC-18F1 may also repress the decoy activity hypothesized for VEGFR1 on tumor neovasculature and thereby enhance VEGFR2 signaling, leading to increased neovascularization, tumor growth, and proliferation. If true, this could be a pathologically expensive consequence. Clinical studies will be necessary to assess the utility of VEGFR1 blockade for inhibition of tumor signaling versus increased VEGFR2 signaling in the tumor vasculature.

Nrp-1 and -2 are co-receptors for VEGFR family members. Nrp-1 is believed to be the most important co-receptor for angiogenesis and also has a role in lymphangiogenesis. The details of Nrp-1′s role in VEGFR2 signaling are not complete but the collective data are consistent with Nrp-1 stabilizing the complex of VEGF-VEGFR2, which may improve the extent or efficiency of VEGFR2 signaling. At the biochemical level, antibody blockade of VEGF-Nrp-1 complex formation in endothelial cells decreases co-immunoprecipitation of VEGFR2-NRP-1 but does not dramatically alter activation of VEGFR2, phospholipase C $-\gamma$, Erk1, and Akt (Pan et al. 2007). At the cellular level, antibody blockade of VEGF-Nrp-1 had no

effect on VEGF-induced permeability, only a modest effect on VEGF-dependent cell proliferation, but a marked reduction in VEGF-induced cell motility. Given these results, it appears that Nrp-1 modulates VEGR2 signaling and if so, antibodies to Nrp-1 like MNRP1685A may offer a modest therapeutic effect on neovascularization when used alone but are unlikely to surpass the activity of standard monotherapies that act directly on VEGF or VEGFR2. However, the combination of anti-VEGF and neutralizing anti-Nrp-1 enhances vascular regression (Pan et al. 2007).

Ligands as Antibody Drug Targets

Antibody inhibition of ligand interactions with signaling receptors is dependent on two factors: (1) the relative location of the antibody epitope compared to the critical ligand residues involved in the binding interface with the receptor and (2) the relative affinity of the antibody-ligand interaction versus the ligand-receptor interaction. Antibody drugs are of sufficient size and volume that they can inhibit ligand-receptor interactions sterically by either binding to or even near the critical ligand residues needed for interacting with the receptor. For example, the epitope on VEGF recognized by bevacizumab comprises 19 residues for which 12 alanine mutants within this epitope displayed at least a fivefold reduction in Ab binding (Muller et al. 1998). Six of the 12 potent mutants encoded dominant effects as they exhibited markedly reduced binding of antigen to antibody by 22–107-fold. Interestingly, 9 of these 19 amino acid residues in the epitope are also involved in VEGF binding to the high affinity receptor VEGFR1 (Muller et al. 1998). VEGFR1 and VEGFR2 bind to partially overlapping regions of VEGF based on mutational analyses. However, only Ile83 of VEGF is an important binding determinant for both bevacizumab and VEGFR1. The neutralizing effect of bevacizumab is consistent with steric hindrance of ligand-receptor interactions.

While the above example illustrates that the location of the epitope is crucial for blocking ligand-receptor interactions, the MOA also involves competitive binding such that the antibody drug must effectively compete with the pathological ligand-receptor interaction. Detailed pharmacology studies have not established absolute minimum affinities for antibody drugs to effectively inhibit ligand-receptor interactions. However, equilibrium estimates would predict that if the K_D of a neutralizing anti-ligand antibody drug is at least 10-fold less than that of the K_D of the ligand for its cognate receptor, then the antibody drug would compete effectively with the cognate receptor for ligand binding so long as the local concentration of antibody exceeds its K_D by a factor of 10 or more. For example, the approximate K_D of CCL2 for CCR2 is 0.6 nM by Scatchard analysis (Monteclaro et al. 1997). In contrast, the K_D of CNTO 888 for CCL2 is 22 pM, or almost 30-fold higher affinity. Assuming local concentrations of CCL2 do not exceed those of CNTO 888, and that CNTO 888 concentrations are 10-fold higher than its K_D for antigen, then CNTO 888 will bind to $<90\%$ of the available CCL2, leaving insufficient amounts of ligand to bind to CCR2 and elicit significant signaling.

Receptors and Non-Signaling Membrane Proteins as Antibody Drug Targets

Antibody drugs and drug candidates to plasma membrane targets, both receptors and non-signaling membrane antigens, can affect tumor growth and proliferation by one or more mechanisms including inhibition of ligand binding, blockade of hetero/homo receptor dimerization, elicitation of effector function, and targeting of toxic payloads. Both cetuximab and panitumumab directly block ligand binding to EGFR as well as decrease surface expression through antibody-mediated receptor internalization (Kim et al. 2001; Yang et al. 2001; Lynch et al. 2002). EGF exhibits sequential bipartite binding to EGFR through domains III and then domain I. The K_D of cetuximab and panitumumab for EGFR is 200 pm and 50 pM respectively, (Goldstein et al. 1995; Yang et al. 2001) and bind substantially more tightly to the receptor than do EGF (1.9 nM), HB-EGF (7.1 nM), and TGFα (9.2 nM) (Jones et al. 1999).

An alternative mechanism to disrupt ligand-dependent RTK signaling is to prevent dimerization of receptors. This means of inhibition has been exploited for several different ErbB family members including EGFR and HER2 (Albanell et al. 2003; Schmiedel et al. 2008). For EGFR, Schmiedel et al. described a novel mechanism for inhibition using the antibody EMD72000, also known as matuzumab. Matuzumab binds to its epitope located in domain III of EGFR, the same domain that encodes the epitope for cetuximab. The Biacore-determined site binding constant of the immobilized Fab isoform of matuzumab for the EGFR ectodomain is only 113 nM, an affinity that is <1–10th that of the known receptor-ligand interaction. In contrast, using a radioligand binding assay on intact cancer cell lines, the authors state that the K_D of matuzumab for EGFR is 1–10 nM, depending on the cell line. While matuzumab binds to the same domain as cetuximab, it does not compete for binding with cetuximab and it does not completely disrupt ligand binding like cetuximab. In fact, matuzumab is only able to block about 55% of ligand binding to the EGFR ectodomain. However, once matuzumab binds to EGFR, it sterically prevents ligand-dependent receptor rearrangement that is required for high affinity ligand bipartite binding to the remaining binding site on domain I. Ultimately, matuzumab blocks the formation of an extended and stabilized EGFR conformer capable of receptor dimerization. While the MOA of matuzumab is novel, it is unclear whether its kinetic binding properties are strong enough to compete favorably with local ligand concentrations within the tumor microenvironment and provide efficacy that would exceed that seen with direct ligand blocking antibodies like cetuximab and panitumumab.

Recruitment of Effector Function as a Mechanism of Action

Effector function is becoming an increasingly important mechanism for tumor-specific cytotoxicity. In the current active and completed clinical trials (Fig. 8.2c),

30 antibody drugs and drug candidates rely solely on this MOA while an additional 19 antibody drugs and drug candidates utilize both effector function and inhibition of signaling to affect tumor growth and proliferation. The combination of multiple MOAs for 19 antibody drugs and drug candidates highlights the need to increase the potency of antagonistic antibody drugs and the reality that the plasticity of the cancer genome is such that inhibition of growth and survival pathways is a moving target as tumors evolve to evade treatment regimens. Accordingly, additional non-overlapping cytotoxic mechanisms are needed to improve drug potency and therapeutic efficacy. It is no coincidence that blockbuster antibody drugs that act directly on the tumor such as rituximab, cetuximab, and trastuzumab leverage effector function as well as alter growth and survival pathways.

The fact that 30 antibody drugs and drug candidates in clinical development rely solely on effector function makes a strong statement about tumor targets, antibody drug mechanisms, and technology advancements. First, antibody druggable tumor targets that have profound effects on tumor growth and survival are rare with respect to drug discovery and development, and if not already, they very well may become extinct. Second, analysis of overall response rate for ritumixmab, trastuzumab, and cetuximab clearly shows that amino acid polymorphisms in FcγRIIIa, which directly affects ADCC activity, can have a strong influence on patient outcome in some hematological and solid tumors (Cartron et al. 2002; Musolino et al. 2008; Tamura et al. 2011). Similar genetic correlations have been shown for complement although there are fewer examples to date (Racila et al. 2006; Racila et al. 2008). Third, protein and carbohydrate engineering strategies on IgG Fc can improve the affinity of the interaction of IgG variants with ADCC activating receptors or critical components of the complement cascade, and result in significantly enhanced effector function as measured both in vitro and in animal xenograft models. Clinical verification of these preclinical demonstrations is ongoing and will be critical to confirm the importance of this mechanism for improved patient care.

For the treatment of hematological malignancies, several groups have examined the relationship between a patient's *FCGR3A* genotype and the observed efficacy of an antibody drug utilizing ADCC as an MOA. For this discussion, *FCGR3A* denotes the gene that encodes the protein product FcγRIIIa. Cartron et al. hypothesized that because the 158V amino acid variant of FcγRIIIa, the sole activating Fc receptor on NK cells, has a higher affinity for IgG1 than its 158F counterpart, there may be improved clinical benefit through enhanced ADCC activity for those rituximab-treated patients expressing the FcγRIIIa 158V variant (Cartron et al. 2002). Forty-nine follicular lymphoma patients were evaluated at 2 and 12 months. At both time points used for patient monitoring, patients homozygous for the 158V allele showed superior overall response rates (100 and 90%, respectively) compared to the patients that were either homozygous or heterozygous for the 158F allele. In addition, the non-responding patient segment was only 10% for the 158V homozygotes compared to 51% for those patients with either one or two copies of the 158F allele. Finally, progression-free survival at

36 months was 56% for the patients expressing the FcγRIIIa 158/158V variants compared to 35% in the patients that were 158F carriers.

Cartron et al. performed additional analyses on the same patient population and in the same manner but focused on the FcγRIIa 131 arginine and histidine allelic variants (Cartron et al. 2002). FcγRIIa is another FcγR that can mediate ADCC. FcγRIIa is expressed only on macrophages and not on NK cells. These investigators were unable to demonstrate any statistically significant correlation between the clinical responses and the *FCGR2A* genotypes.

Weng and Levy (2003) also analyzed follicular lymphoma patients treated with rituximab and were able to confirm the allelic *FCGR3A* correlations with overall patient responses and progression-free survival previously described by Cartron et al. In addition to the confirmatory studies of Cartron et al. Weng and Levy assessed the ex vivo sensitivity of patient-derived lymphoma cells to rituximab-dependent ADCC using PBMCs from healthy donors that only expressed FcγRIIIa 158V. These studies were performed prior to patients receiving rituximab treatment. The range and mean ADCC activity observed was compared to the responses observed in patients that had undergone treatment. Surprisingly, there was no correlation with the ex vivo responses and the extent of responses observed in treated patients after 6, 9, and 12 months, or even in their durations of remission. These results extend those of Cartron et al. by showing that the genotype of *FCGR3A* is important but other factors also play a role in response rates to rituximab. Additional studies of Weng and Levy (2003) and Kim et al. provide further clinical support for the role of *FCGR3A* genotype in patient response to anti-CD20 therapy for some B cell malignancies (Kim et al. 2006). Evidently, not all B cell malignancies respond as Farag et al. were unable to observe a relationship between *FCGR3A* genotype and an objective response in B-cell chronic lymphocytic leukemia patients treated with rituximab (Farag et al. 2004).

In their initial study, Weng and Levy were unable to confirm the findings of Cartron et al. when they analyzed the same patient population for correlation of FcγRIIA 131R and 131H allelic variants with patient responses to rituximab (Cartron et al. 2002; Weng et al. 2003). In their study, clinical objective responses in patients that were 131H homozygotes were approximately twice that seen for 131R carriers for evaluations at 6, 9, and 12 months. IgG2 and 1 Fc variant of IgG1 have been reported to have improved binding to FcγRIIa. Unfortunately, based on the isotype subclass and Fc sequence of rituximab, these reports do not explain the results of Weng and Levy. Additional studies will be needed to understand the relationship between *FCGR2A* genotype and rituximab efficacy in patient populations.

The importance of the *FCGR3A* genotype has also been reported in several clinical studies using antibodies for the treatment of solid tumors. The first correlative study was reported by Musolino et al. using trastuzumab for the treatment of HER2-positive breast cancer patients (Musolino et al. 2008). The overall median progression-free survival (PFS) independent of patient's *FCGR3A* genotype was 16.8 months. For patients that were FcγRIIIa 158F

carriers, their median PFS was reduced to 12.9 months. In striking contrast, the homozygous 158V patient population did not reach a median PFS over a period of about 67 months, with <55% of this genetically defined patient population being progression-free.

Similar studies have been carried out using cetuximab, the chimeric antibody against EGFR. However, the correlations appear to be more variable than for rituximab and trastuzumab and the lengthening of the median PFS is much less dramatic. These differences may reflect greater genetic heterogeneity downstream of EGFR in colorectal tumors with mutations encoding k-Ras, b-Raf, and PIK3CA, possibly negating some of the efficacy provided by ADCC. Nevertheless, the findings of Bibeau et al. are an example of effects on median PFS that are most aligned with previous studies described above for trastuzumab and rituximab (Bibeau et al. 2009). In their study of cetuximab-treated patients with metastatic colorectal cancer that were irinotecan-refractory and predominantly k-Ras wild-type, Bibeau et al. showed that patients homozygous for the FcγRIIIa 158V variant had a statistically significant difference in PFS when compared to the 158F carriers (6.9 vs. 3.2 months). This observed improvement was evident even in patients with activating k-Ras mutations (5.5 vs. 2.8 months). These authors failed to observe a significant difference in response to cetuximab or PFS based on the FcγRIIa amino acid polymorphism. Patients that were homozygous for *FCGR2A* and *FCGR3A* alleles encoding the 131H and 158V variants, respectively, had significantly longer median PFS than other genetic combinations of these alleles.

The importance of the FcγRIIIa amino acid polymorphism described by Bibeau et al. were opposite from that shown by Zhang et al. (2007). There may be several explanations including the type and level of previous treatments as these pre-treatments may result in selective loss of different immune cell populations and their corresponding ADCC activities that are mediated and regulated through activating and inhibitory Fcγ receptors. Collectively, using first-generation antibodies with native Fc domains, ADCC function is important in the treatment of some B cell malignancies and breast cancer but may be less impactful for colorectal cancer.

Racila et al. (2008) examined the effect of genetic variation of complement C1q on the overall response rate and duration of response in rituximab treated patients (Racila et al. 2008). While C1q amino acid polymorphisms did not affect overall response rate in the 133 patient trial, patients that were carriers for the C1q 276A polymorphism remained in remission significantly longer than those that were C1q 276G homozygotes. Interestingly, expression of the G allele resulted in greater complement activity leading to enhanced phagocytosis and clearance of apoptotic tumor cells. At first this correlation seems counterintuitive. However, less efficient opsonization of tumor cell fragments may enable more effective processing and presentation by dendritic cells, ultimately resulting in a stronger adaptive immune response. The authors caution that the trial size was small and that additional results are needed to substantiate their findings.

The clinical support for the role for ADCC in the efficacy of antibody drugs has prompted numerous groups to devise molecular and cellular platforms to improve

antibody effector function. The platforms have focused on engineering strategies that alter the Fc domain of the antibody to improve Fc binding affinity to the activating Fcγ receptors, namely FcγRIIIa and its allelic variants. Molecular platforms have identified amino acid residues within the IgG1 Fc domain that when mutated, lead to improvements in FcγRIIIa binding, in vitro ADCC activity and in vivo activity. The pioneering efforts of Shields et al. described the systematic evaluation of the in vitro consequences of alanine mutations to the exposed amino acids within the Fc domain of trastuzumab (Shields et al. 2001). Ten different classes of mutations were identified based on their FcγR binding effects. Of importance for this discussion is that Class 3 variants showed improved binding to FcγRIIIa and FcγRIIb, Class 7 variants exhibited improved binding to FcγRIIIa and reduced binding to FcγRIIb, and lastly, Class 9 variants demonstrated improved binding to FcγRIIIa and no effect on FcγRIIb. The Class 7 variants appeared to have the most interesting properties as they would be predicted to show improved ADCC activity with NK cells, macrophages, and neutrophils while eliciting reduced inhibitory effects from FcγRIIb binding on macrophages and neutrophils. Shields et al. were able to show that improvements in FcγR binding as determined by ELISA, also mediated enhanced ADCC activity in vitro. These ADCC improvements were evident using PBMCs expressing either the FcγRIIIa 158F or the 158V allotypes, although the largest effect was seen with the 158F allotype.

The studies of Lazar et al. extended those of Shields et al. by illuminating additional combinations of amino acid mutations that could improve FcγRIIIa binding (Lazar et al. 2006). These authors demonstrated that S239D/I332E and S239D/I332E/A330L mutations improved binding to FcγRIIIa 158V and 158F by 1–2 logs over wild-type Fc domains when incorporated into alemtuzumab and trastuzumab. Furthermore, enhanced FcγRIIIa binding was accompanied by comparable levels of improvement of in vitro ADCC activity.

In addition to the molecular engineering strategies just described, several cellular platforms have been developed to enhance ADCC. Glycart has created a manufacturing CHO cell line that overexpresses both β-1, 4-N-acetylglucosaminyltransferase III (GnT-III) and α-mannosidase II, which results in the production of N-linked glycoproteins that are enriched in bisected oligosaccharides and increased in non-fucosylated and hybrid oligosaccharides. Antibody drug candidates made using this production system have increased ADCC activity (Mossner et al. 2010). For example, when recombinant GA101, a CD20 antibody with an epitope that partially overlaps with that of rituximab, is expressed using this production system, the resulting antibody showed a 35-fold improvement in ADCC activity in vitro over that of rituximab. Mossner et al. extended their in vitro findings to animal models using established lymphomas of approximately 250 mm^3 at the time of treatment. Improved tumor growth inhibition was seen for GA101 using weekly doses of 1, 3, or 10 mg/kg when compared to rituximab at the same doses and schedule.

Biowa, now part of Kyowa Hakko Kogyo, created a genetic null of FUT8 in CHO cells to express only non-fucosylated antibody drugs. The studies of Niwa et al. showed that rituximab produced in a CHO cell line lacking FUT8,

referred to as KM3065, bound to FcγRIIIa 158F or 158V about 100 times more strongly than authentic rituximab when measured by ELISA (Niwa et al. 2004). The tighter binding to FcγRIIIa by KM3065 also resulted in a corresponding 100-fold increase in ADCC activity in vitro when compared to that of authentic rituximab.

While Niwa et al. did not follow up their in vitro assessment with in vivo studies, Junttila et al. did use the same production system to express afucosylated trastuzumab (2010) and evaluate its efficacy in a novel transgenic model (Junttila et al. 2010). An orthotopic model was employed and consisted of the human breast cancer KPL-4 cell line implanted into the mammary fat pad of the novel mouse strain *FCGR1*$^{-/-}$, *FCGR3*$^{-/-}$, *RAG*$^{-/-}$, Tg *FCGR3A*. This strain lacks murine *FCGR1* and *FCGR3* genes but contains human *FCGR3A* gene controlled by the human *FCGR3A* promoter. Expression of human FcγRIIIa protein in this strain occurs in NK cells and macrophages. Treatment of 125 mm^3 tumors by both trastuzumab and afucosylated trastuzumab resulted in significant activity in this model, but the median PFS for the afucosylated isoform was only twice that of trastuzumab (48 vs. 23.4 days). Similar studies were carried out using the recombinant cell line MCF7-neo/HER2 instead of KPL-4. Trastuzumab treatment resulted in no complete responses and 1 partial response. Afucosylated trastuzumab showed superior efficacy with 4 complete responses and 5 partial responses.

The studies described above indicated that protein and carbohydrate engineering can improve antibody affinity for FcγRIIIa with corresponding improvements in ADCC activity in vitro and enhanced tumor growth inhibition in vivo. Studies by Masuda et al. were conducted to determine whether one approach was better than the other and whether they could be combined for additional ADCC improvement (Masuda et al. 2007). Using rituximab again as a model for evaluating engineered isoforms, a 2 by 3 matrix of glycan and polypeptide isoforms of rituximab was compared. The glycan isoforms were either fucosylated or non-fucosylated while the polypeptide variants used encoded either the wild-type Fc protein sequence or alternatively, an Fc variant with enhanced FcγRIIIa binding, namely S239D/S298A/I332E or S239D/A330L/I332E (Shields et al. 2001). Biacore-based binding studies of antibody variants that contained the triple amino acid mutations and were non-fucosylated showed higher affinity interactions with FcγRIIIa. Similar studies performed using ELISA were not as easily interpreted. Surprisingly, when these same isoforms were compared for their ADCC activity in vitro using PBMCs from healthy donors, all variants showed the same extent of enhanced ADCC compared to fucosylated rituximab even though the non-fucosylated triple amino acid mutants exhibited markedly higher NK cell binding by FACS than the other isoforms. Collectively, these results suggest that there may be a ceiling for enhancing ADCC activity through FcγRIIIa and that either protein or carbohydrate engineering can provide a means to achieve improved effector function. It is important to emphasize that the clinical benefit of ADCC-enhanced antibodies remains to be determined.

Antibody Drug Conjugates

The promise for developing ADCs is that they combine the specificity of an antibody drug, which minimizes off-target effects, with the cell killing potency of small molecule cytotoxics, creating the elusive magic bullet. This approach provides a means to enhance the potency of antibody drugs through an additional mechanism beyond direct effects on tumor growth and survival or eliciting effector function.

ADCs or immunoconjugates comprise three components: a tumor-specific antibody to a cell surface antigen, a linker that couples the antibody to the toxic payload, and lastly, a payload which is a potent small molecule cytotoxic drug. While the antibody to the tumor-specific antigen is important, its antigen is even more important. The antigen must be differentially expressed on tumor cells and absent or expressed at low levels on surrounding normal cells as well as critical organs. The tumor antigen on the plasma membrane must be present at a sufficiently high surface density to facilitate effective cell killing upon antibody-mediated antigen internalization and trafficking to the appropriate intracellular compartment. It should be pointed out that tumor antigens that are differentially expressed, albeit at low membrane densities, can still be excellent ADC targets so long as they are rapidly internalized and efficiently trafficked intracellularly to the lysosomal compartment to release the small molecule payload. In contrast, tumor antigens that are differentially expressed and present on tumor cells at high membrane densities may not be useful as ADC targets if they are poorly internalized or are rapidly recycled to the membrane without release of the small molecule payload. Finally, if the antigen has a role in tumor growth and survival and the immunoconjugate is capable of affecting antigen function therapeutically, then the ADC will exhibit multiple MOAs, although this benefit is not an antigen requirement for an immunoconjugate. In fact, the ADC platform may enable new targets to be considered for drug development that were discounted previously because they were of insufficient functional relevance in tumor biology to merit pharmacological intervention.

There are several antibody characteristics for ADCs that are shared with other MOAs. First, the antibody isotype is not fixed, although IgG1 antibodies offer the opportunity for effector function along with payload delivery, which would be lost with either IgG2 or IgG4 isotypes. Second, the affinity of the antibody for its membrane tumor antigen will be another factor that will determine the amount of payload that can be delivered. Studies performed by Weiner et al. described the relationship between antibody affinity and effector function (Tang et al. 2007). Their studies indicate that higher affinity antibodies more effectively bind to antigen expressing cells, particularly tumor cells with lower antigen densities, leading to greater ADCC. It is very likely that this correlation can be extended to ADCs in that higher affinity antibodies will bind to the tumor cells more effectively and for a longer duration, resulting in a greater likelihood of being internalized rather than dissociating. The third characteristic of the antibody has to do with the

type of chemistry that will be used to add the linker to the antibody. If active ester chemistry on primary amines is to be employed and the paratope contains one or more critical lysine residues that are highly reactive with the chosen conjugation chemistry, then considerations may need to be given to creating paratope variants with alternative amino acids refractory to the coupling chemistry while maintaining antigen specificity and affinity. Alternatively, a different linker chemistry could be used assuming plug and play alternatives are available.

The linker chemistry used in ADCs is crucial for the successful development of this class of drugs. The linker chemistry must provide a stable covalent connection between the tumor-specific antibody and the small molecule drug during storage and administration. While in circulation, the linker must be resistant to the extracellular environment and accompanying enzymatic activities that can release the small molecule payload from the antibody. Early generation linker chemistries were less stable and not only released the payload in the extracellular environment causing unwarranted toxicities, but also created an unarmed antibody with reduced or inadequate cytotoxicity that could compete with the intact ADC for tumor binding, possibly leading to a further decline in ADC-mediated tumor cell killing. Also, it should be pointed out that ADC linker chemistry of greater stability enables the use of more potent small molecule payloads with correspondingly enhanced cellular cytotoxicity.

The most advanced ADC linker chemistries are those from ImmunoGen and Seattle Genetics. The ImmunoGen linker series is attached to the antibody through the ε amino group of lysines using a linker containing a terminal N-hydroxysuccinimidyl ester (Chari 2008; Lewis Phillips et al. 2008). In contrast, for coupling the linker to the antibody scaffold, Seattle Genetics creates antibody-derived thiols using a partial reduction strategy (Doronina et al. 2003). The resulting thiols can react with the terminal N-hydroxymaleimidyl moiety of the peptide linker. The antibody sites of conjugation, the amide bonds of ImmunoGen and the thioether bonds of Seattle Genetics, are stable in the extracellular environment of plasma and lymph.

Both Immunogen and Seattle Genetics as well as other companies have developed cleavable and non-cleavable linker chemistries. In the case of the scissile linkers, these are designed to be much more stable in the extracellular environment than in the relevant intracellular compartment of targeted cells. However, once an ADC containing a cleavable linker is internalized, the respective linker becomes sensitive to chemical or enzymatic cleavage and releases the small molecule drug. The Immunogen cleavable linker technology incorporates a sterically hindered disulfide linker that protects the disulfide from reduction or disulfide exchange in the extracellular compartment. In contrast, the scissile linker system of Seattle Genetics utilizes a peptide linker that is resistant to most of the extracellular proteases but sensitive to the lysosomal proteases, particularly members of the cathepsin family.

While improvements have been made in the cleavable linker chemistry, the stability of these linkers is not equivalent to that observed for their non-cleavable counterparts. Recent clinical progress of trastuzumab-DM1 using the non-cleavable

SMCC linker has highlighted the potential of ADCs with non-cleavable linkers. The stability advantage of ADCs using this linker chemistry is likely to result in reduced off-target toxicities due to inappropriately released drug, thereby enabling extended periods of dosing and a higher likelihood of tumor eradication. The mechanism of intracellular payload release from the tumor specific antibody relies entirely on lysosomal degradation of the antibody rather than reduction or enzymatic hydrolysis of the linker. The resulting lysosomal protease digestion product comprises the lysine amino acid used for linker attachment, the linker, and the small molecule drug. The protease digestion product can diffuse from the lysosome into other cellular compartments and elicit its cytotoxic activity. However, the presence of a charged amino acid in the protease digestion product prevents the diffusion across the plasma membranes and eliminates the potential for bystander effects. In contrast, some versions of ADCs with a scissile linker facilitate a bystander effect that occurs upon intracellular activation and release of an uncharged small molecule drug that is capable of diffusing across intracellular membranes as well as plasma membranes.

As the ADC technology has evolved, small molecule drugs of increasing cytotoxicity have become part of the ADC armamentarium. However, as the cytotoxicity of the small molecule drugs has increased from paclitaxel and doxorubicin with GI50 values of 24 and 66 nM to maytansine and auristatin with GI50 values <1 nM, the requirement for stable linker chemistry in the extracellular environment has become paramount. The recent clinical successes of SGN-35 (Younes et al. 2010; Ansell 2011) and T-DM1 (Burris et al. 2010) demonstrate that linkers of sufficient stability exist and can be used to arm antibodies with extremely potent small molecule payloads. Furthermore, this improvement in linker stability in the extracellular space creates an opportunity to examine new and old compounds alike that were too toxic on their own but their full potency may be realized in a targeted manner as the warhead of an ADC. Furthermore, the stability of the leading linker systems as well as those that are in development will enable a more systematic examination of additional payloads with MOAs different from those currently in the clinic or in late-stage development.

The future is bright for ADCs as the chemistries become more robust, the MOAs of the small molecule payload diversify and in some cases, the MOAs are selected for based on tumor sensitivity. While the field took a setback in July of 2010 with the voluntary withdrawal by Pfizer of Mylotarg from the US market, recent clinical progress with Trastuzumab-DM1 and SGN-35 indicates that this modality has finally arrived.

Antibody-Related Isotype Considerations

The selection of the IgG isotype subclass for antibody drugs for the treatment of cancers is relatively straightforward in contrast to their use for inflammation or immunological indications. The approved antibody drugs for cancer are IgG1s, with only one current commercial exception, panitumumab, a fully human IgG2.

The only IgG4 member, Myoltarg, was recently taken off the market. There are several reasons why IgG1s predominate for oncology. First, most tumor antigens that are being exploited for targeted therapy by antibody drugs are membrane associated and accordingly, utilization of the IgG1 subclass in drug design enables effector function as a MOA to increase drug cytotoxicity. As human IgG2 and IgG4 have little to no effector function respectively, the choice of IgG1 is obvious. Second, for PK considerations, IgG1, IgG2 and IgG4 are preferred as this set of subclasses has a mean serum half-life in man of approximately 3 weeks. IgG3 is not recommended as it has the shortest IgG half-life in man of only about 1 week (Morell et al. 1970). The intrinsic PK properties of IgG1 drugs have a positive impact on both cost of goods and dosing convenience compared to IgG3. Third, while antibodies are structurally stable in general, IgG1, IgG2, and IgG3 are the most stable. The IgG4 hinge is less stable than that of the other IgG subclasses. The reduced stability of the IgG4 hinge can result in the presence in serum of IgG4 antibodies that are essentially monovalent due to half-dimer exchange mediated bispecificity (Aalberse et al. 1999; Schuurman et al. 2001). For most antibody drugs, there is no apparent advantage of losing bivalency, particularly for membrane antigens. The stability of the IgG4 hinge can be improved by converting the serine within its hinge sequence C–P-S–C into an additional cysteine or alternatively, converting the entire hinge into that of the IgG1 hinge C–P–P–C (Angal et al. 1993; Bloom et al. 1997). Mylotarg, a calicheamicin-based ADC, was the first and only IgG4 antibody drug in oncology. It was taken off the market due to poor efficacy in July of 2010. The insufficient efficacy of this ADC was not due to hinge-related IgG4 instability as a hinge stabilized IgG4 isoform was used in creating the corresponding ADC (Labrijn et al. 2009).

Immunogenicity Evaluation of Antibody Drugs

Methods to evaluate the immunogenicity of antibody drugs during drug discovery have evolved with the clinical success of antibody therapies. The clinical success of antibody therapies was enabled by recognizing the immunological limitations of using rodent antibodies as therapies and switching to antibody formats that were more human in primary sequence—starting with mouse-human chimeric antibodies and progressing to humanized antibodies and then, fully human antibodies. Nevertheless, anti-drug antibody (ADA) responses of antibody-treated patients still occur to varying extents even when using fully human antibody drugs. While much of the emphasis over the past decade has been on developing robust methods for assessing patients' ADA responses to meet industry-based and regulatory guidelines, antibody drug developers are now entering into a new era that offers tools for evaluating the immunogenicity of antibody drug candidates during discovery.

Tools have been developed that can evaluate the intrinsic immunogenicity potential of biologics based on the existence of immunostimulatory linear T cell epitopes in the primary sequence of protein therapeutics. The presence of T cell epitopes in biotherapeutics is correlated with their ADA titer and duration of response (Barbosa et al. 2007; De Groot et al. 2007; Koren et al. 2007; Tatarewicz et al. 2007). T cells recognize specific T cell epitopes when displayed on antigen presenting cells (APCs) by allelic variants of MHC. A critical determinant of T cell-dependent ADA response is the strength of the binding of the T cell epitope to MHC class II molecules present on APCs.

Computer algorithms have been developed to survey and predict putative T cell epitopes within the primary sequence of a series of drug discovery candidates (Brusic et al. 2004; De Groot et al. 2007; Zhang et al. 2008). This bioinformatic assessment can reduce the epitope complexity by a factor of 20. Some of these programs such as EpiMatrix, can also measure epitope density for each drug candidate and provide a ranking from low to high of potential T cell-dependent immunogenicity.

Once putative T cell epitopes have been identified, several different assays can be performed to provide empirical support for the bioinformatic predictions. One such assay uses a competitive binding format that utilizes recombinant MHC class II receptors, corresponding fluorescently labeled T cell epitopes, and increasing amounts of unlabeled individual T cell epitopes derived from the bioinformatic prediction. Confirmation of a T cell epitope occurs when individual unlabeled T cell epitopes compete effectively with the binding of one or more of the labeled T cell epitopes to their corresponding MHC class II receptor (Steere et al. 2006; McMurry et al. 2007).

An alternative assay design assesses T cell responses in the presence of individual T cell epitope-MHC class II complexes. In this format, parallel assays are performed by culturing human T cells in the presence of MHC class II recombinant tetramers loaded with individual putative T cell epitopes. Recognition of the MHC class II-T cell epitope complex by specific T cells can be monitored by either following DNA replication (labeled thymidine or unlabeled BrdU incorporation) or using ELISpot to detect increased production and secretion of IL-2 or IFN-γ. In this format, increases in DNA replication or cytokine production correlate with the presence of a T cell epitope.

If several antibody drug candidates of similar potency were evaluated for immunogenicity as described above, then any candidate lacking T cell epitopes can be considered for advancement towards IND enabling activities. In those cases where a drug candidate or all candidates encode an in vitro verified T cell epitope or epitopes, then the drug candidate can be re-engineered to remove this liability. The previously described computer algorithms can be useful for prioritizing candidates for re-engineering as well as molecularly defining the primary sequence changes needed to remove T cell epitopes. Re-engineered constructs can be assessed in vitro as described above to ensure that the molecular engineering was successful.

Table 8.3 Xenograft testing variables for evaluation of oncology antibodies

Variable	Comment
Tumor origin	Cell line, engineered cell line, human primary tumor sample
Target status in tumor	Receptor/antigen density
Mouse strain	Examples include SCID, Nude, SCID.bg, NOD, transgenic or KO
Implantation site	sc, orthotopic, injection
Size of tumor at dosing onset	Subcutaneous established tumors (>150 mm^3) recommended
Tumor growth properties	Doubling time, degree of necrosis, stromal compartment
Dosing	i.v., i.p, < 20 mg/kg BIW recommended
Endpoints	%TGI, T/C, growth delay, survival, imaging techniques

The most common variables and that need to be addressed when establishing a xenograft model for efficacy studies in oncology. Recommendations and examples are provided

Introduction to Animal Models for Evaluation of Antibody Drug Candidates

Animal models are central in preclinical development to validate a target, determine efficacy, establish dose response relationships, define drug interactions, and optimize dosing schedules (Gerber et al. 2005; Pegram et al. 2006; Damia et al. 2009). As these models also serve as surrogates for patients in the evaluation of novel therapeutics, in vivo studies ideally should exhibit high fidelity to human tumors and the endpoints from the preclinical studies should translate to the clinic. However, there is considerable debate over the fidelity with which animal tumor models recapitulate the clinical setting. Typically, these comparisons have focused on small molecule drug preclinical endpoints to assess their value in predicting clinical efficacy (Boven et al. 1992; Voskoglou-Nomikos et al. 2003; Fiebig et al. 2004). Obviously, there are many different animal models and multiple variables must be taken into account when choosing a relevant model for preclinical studies. In this section, we will highlight the importance of animal models in preclinical development noting not only their contribution to drug development but also their limitations in their translation to the clinic.

Xenograft Models

Human tumor xenografts established in immunodeficient mice are the mainstay of preclinical proof of concept testing in oncology. Moreover, the choice of in vivo model is critical for addressing specific hypothesis-driven scientific questions and ensuring improved translation to a clinical setting (Firestone 2010). Multiple factors dictate which in vivo model to utilize and a deep understanding of the target biology is central to deciding on a model and its utility. As reviewed by Kelland (2004) and modified in Table 8.3, numerous critical variables must be

considered when designing animal models including tissue or cell source, target status in tumor, mouse strain, site of implantation, growth characteristics, and appropriate endpoints.

Cell and Primary Tumor Sources for Xenografts

For all xenograft models, it is critical for the antibody target or antigen to be expressed and accessible in sufficient amounts by the cell line or primary tumor to be examined. As described earlier in this chapter, extensive in vitro analysis of the cell lines by biochemical, FACS, IHC, and genetic techniques to support the advancement of the cell lines or primary tumors for in vivo analysis is required. While xenografts derived from continuous cell lines typically exhibit a more homogeneous, undifferentiated histology, those derived directly from patient biopsies appear to retain better the morphological and molecular characteristics of source tumors. In fact, due to likely selection pressure in vitro during extensive culturing, continuous cell lines often lose expression of target receptors and proteins (Staroselsky et al. 1992; De Both et al. 1997). It is also very likely that new biochemical pathways are induced under the selective tissue culture conditions used to screen for immortalized cell lines.

As aforementioned, solid tumor xenografts are still the most commonly used models for preclinical proof-of-concept. This is because xenograft models are widely available, reproducible, straightforward to manipulate, and allow for follow-up of tumor over time by serial tumor size measurements or repeated imaging. A major criticism of the subcutaneous (s.c.) xenograft model is the lack of an appropriate tumor microenvironment and the absence of spontaneous metastases to clinically relevant sites. On the other hand, the orthotopic model implants the tumor into the correct anatomical site to provide a more appropriate stromal environment (Bibby 2004). However, the microenvironment around the tumor is still provided by the animal host cells.

Due to concern about how readily cell lines recapitulate human tumors, xenografts derived from primary human tumors are also extensively used. In this labor-intensive process, primary tumors from biopsy are cut into fragments and serially passaged through animals. In early studies, a total of 329 primary human tumors were investigated; 34 (52% success rate) of colorectal xenografts were established, 9 (60%) of melanomas, 11 (23%) of ovarian cancers, 8 (10%) of breast cancers, and 14 (30%) of testicular tumors (Steel et al. 1983). Other reports suggest the overall take rate varies from 40 to 60% for non-small-cell lung cancer and colon cancer, with melanomas down to 12, 20% for breast cancer and only 3% for prostate cancer (Garber 2009). While more morphologically similar to the original tumors, these models can be plagued by slow growth, are labor intensive, and also difficult to use in large efficacy studies. Similar to xenografts derived from cell lines, these primary tumors can be implanted s.c., under the renal capsule, or orthotopically. There are now multiple contract research organizations that will

perform studies on their established primary models in the US, Europe, and Asia. Importantly, in many cases the molecular attributes of the primary model are available including gene expression profiles.

Target Status of Tumor

As described in an earlier section, cell lines and primary tumors should be extensively profiled to assess levels of target expression prior to establishing a xenograft model. In practical application, a panel of cell lines can be analyzed by flow cytometry to demonstrate binding of the antibody to the target on the cell surface. Optimally, for a receptor target, there will be multiple cell lines that differ from each other in the level of membrane displayed target. In this scenario, cell collections such as these may enable a target dose–response relationship to be established in vitro and in vivo, although this will depend on the MOA of the antibody candidate and the intrinsic biology of the target. Unfortunately, not all human tumors or cell lines with antibody tractable targets grow readily in immunodeficient mice. In the preclinical development of trastuzumab for example, no HER2-overexpressing cancer cell lines were available for in vivo studies. Consequently, a HER2-overexpressing cell line that was also tumorigenic in the mouse was engineered and used for preclinical testing. When this is required it is essential that the level of antigen target expression is similar, and does not exceed the levels observed in actual human tumors (Pegram et al. 2006).

Mouse Strains for In Vivo Studies

The strain of mouse selected for in vivo studies has critical implications for xenograft growth as well as MOA studies. For many xenograft models, athymic nude mice are utilized because their T lymphocytes are present in reduced numbers, allowing for engraftment of human cells and tissue. Although these animals maintain intact NK cells, they still require care appropriate for their immunocompromised state. For more difficult to grow tumors, or for primary human tumors, severe combined immune deficiency (SCID) mouse strains are commonly used. These animals are further immunocompromised but it is only the SCID/beige strain that also does not possess functional NK cells. Both NSG (NOD scid gamma) and NOG mice combine multiple immune deficits from the NOD/ShiLtJ background, the severe combined immune scid mutation, and genetic inactivation of the interleukin-2 receptor gamma chain. The NOG mouse has a truncated interleukin-2 receptor gamma chain (Ohbo et al. 1996), whereas the NSG mouse carries a null interleukin-2 receptor gamma chain mutation (Shultz et al. 2005). As a result, both NSG and NOG mice lack mature T, B, and NK immune cells, resulting in elimination of adaptive immunity, functional deficiencies in innate

immunity, and reduced complement activity. Direct comparisons have shown that human hematopoietic stem cells and peripheral blood mononuclear cells engraft more efficiently in these animals than in any other immunodeficient mouse strain (Shultz et al. 2005; Shultz et al. 2007).

All these strains can be effectively used to demonstrate the specific MOA of antibody therapeutics. For example, an antibody that only elicits ADCC would demonstrate activity in nude and SCID mice models but not in models lacking NK function, such as the NOG and SCID/beige strains.

The use of knockout animal models has been central for academics and industry to validate the target protein and for MOA studies. While knockout models are fundamental tools, the issues with embryonic knockouts concern the total inactivation of a single protein from the earliest stages of embryonic development; this is not always the same as a reduction of a protein in an adult or in a pathological setting. Additionally, compensatory signaling redundancies during development may not result in an effect following the inactivation of a single protein—in other words, knockout mice may develop in the absence of the gene or protein under investigation.

The evolution of cancer xenograft models includes using genetically engineered mice (GEM) that accurately mimic the pathophysiological and molecular features of human malignancies (Hansen et al. 2004). GEM can be classified as either transgenic or endogenous (Frese et al. 2007). Transgenic GEM are mutant mice that express either an oncogene or a dominant-negative gene construct in a non-physiological manner, owing to ectopic promoter and enhancer elements. Endogenous GEM represent mutant mice that: (a) lose the expression of tissue-specific genes or, (b) express dominant-negative tumor-suppressor genes or, (c) express oncogenes from their native promoters through the use of knockout and knock-in technology. These models develop cancers with specific features relevant to human disease and may fill a gap between simple xenografts and translation to clinical trials.

Sites of Tumor Xenograft Implantation

Subcutaneous implantation (s.c.) is the mainstay of xenograft models in immunocompromised mice. This route of implantation is favored because manipulations are easy and facilitates the straightforward use of calipers to determine tumor diameter/volume for serial measurements. Most commonly, 1–5 million cells are injected by trochar into the flank of an immunocompromised mouse. Often, a substrate like Matrigel™ (BD Biosciences) is used to achieve a better tumor take rate.

There are many reports of differences in biological behavior, e.g., the ability to metastasize and receptor/target status, when tumors are grown s.c. relative to orthotopic models (Eccles 2002), and it is now recognized that orthotopically transplanted xenografts maintain some of the biological properties of the original human tumors. This is particularly the case for difficult to grow xenograft models of prostate and cervical cancers. As described previously, it is proposed that orthotopically implanted tumors more closely mimic the progression of tumorigenesis specifically as it relates to metastasis to clinically relevant sites (Bibby 2004).

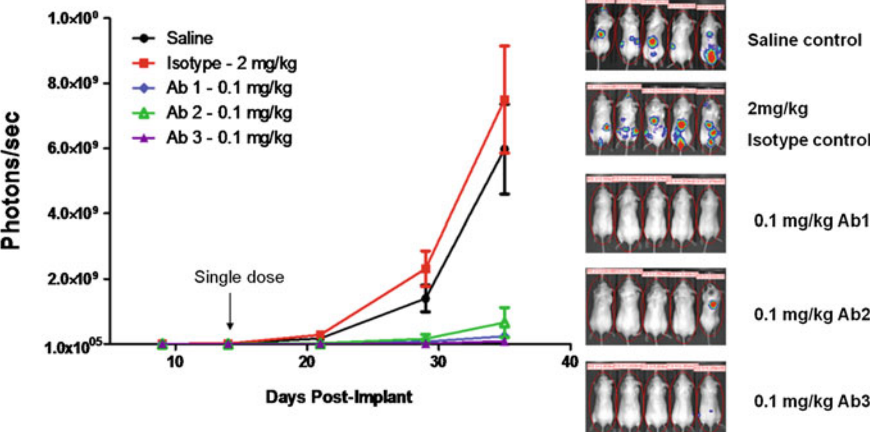


Fig. 8.3 Daudi luciferase disseminated xenograft model with bioluminescent imaging. In this multiple myeloma disseminated xenograft model, Daudi-luciferase cells are injected via the tail vein into scid mice. Increasing tumor cell growth can be observed in the isotype-matched antibody control and vehicle control both visually and by photons/sec measurements throughout the 35 day study. In contrast, a single injection of three different target-specific antibodies showed efficacy in this disseminated model as demonstrated by a marked reduction in luminescence relative to control animals

For hematological cancers or metastasis studies, disseminated models are often utilized as they more closely mimic the clinical setting. In these models, cells are injected via the tail vein (cells lodging in the lungs) or via cardiac puncture to disseminate cells throughout the animal. Current protocols utilize ultrasound techniques to enable successful cardiac punctures. In disseminated models, bioluminescence imaging technology (Xenogen Corporation, now Caliper Life Sciences) is commonly employed to monitor tumor growth serially. For these studies, genes encoding luciferase or a fluorescent protein are genetically engineered into one or more relevant cancer cell lines. The resulting recombinant cancer cells are then used in disseminated disease models. In this format, tumor burden can be visualized optically through the tissues of a live animal using a CCD camera and specialized software. The dual bioluminescence and fluorescence imaging systems are highly sensitive and exhibit a broad dynamic range over 3–4 logs of photon detection. Consequently, this technology enables exquisite non-invasive longitudinal monitoring of disease progression. As shown in Fig. 8.3, when Daudi-Luciferase cells are injected via the tail vein into SCID mice, increasing tumor cell growth can be observed in the isotype matched Ab control and vehicle control both visually and by photons/s measurements throughout the 35 day study. In contrast, a single injection of three different target-specific antibodies showed efficacy in this disseminated model as demonstrated by a marked reduction in luminescence relative to control animals (Fig. 8.3).

Tumor Size at Dosing Onset

Depending on the biology of the tumor antigen, as will be detailed below, dosing is often initiated when an established tumor size of >180–200 mm^3 is reached for s.c. tumor models. This experimental design philosophy increases data robustness by utilizing only mice with well engrafted tumors of similar size and growth properties for randomization into study groups. Consequently, this design strategy sets a more stringent and relevant standard for demonstration of efficacy. In contrast, if dosing is started too early when tumors are small, then false positives can arise due to insufficient engraftment, cellular necrosis, etc., all of which can occur independent of pharmacological treatments and can be misinterpreted as efficacy.

Importantly, the biology of the target and the potency of the antibody candidate dictate the appropriate size of the tumor at study initiation. Some antibodies work optimally on smaller tumors such as anti-VEGF antibody drugs. This is not surprising given that the angiogenesis component is most relevant and accessible for intervention early in tumor development. Also, if an antibody's only mechanism of action is effector function, it typically needs to be administered when tumors are small or in metastasis models. As discussed previously, although there also may be an effector function component, most clinically validated antibodies developed for treatment of solid tumors require multiple MOAs as effector function alone is insufficient for significant efficacy in larger established solid tumors.

Tumor Growth Properties

For the development of s.c. xenograft models, the tumor xenograft must exhibit a reasonable take rate to allow appropriate cohort sizes upon randomization and grow at a uniform rate throughout the study, optimally reaching the endpoint (e.g., tumor volume of 1,000 mm^3) approximately 30 days into the dosing period. These studies are more representative of how the antibody will be applied in the clinic, but one needs to evaluate the possibility that either target expression may decrease in larger tumors, or that antibody penetration may be compromised due to poor vasculaturization or increased areas of necrosis within the tumor. It is also vital to consider animal health and not allow tumors to exceed ethically acceptable limits. Tumors that only reach 300–400 mm^3 volume and then regress are suspect as implanted cells can necrose, fill with infiltrating cells, and "regress" as the inflammation resolves. One should be be aware of the possibility of the residual immune system of the host (principally NK cell activity) mediating tumor regression or cures. Also, it is important that the developing tumor not develop excessive necrosis along the tumor periphery or generate cysts that can complicate analysis of tumor growth and thus lead to erroneous results.

Antibody Drug Dosing

In preclinical testing, there are multiple dosing strategies that can be used to evaluate the activity of antibody candidates. Initially, if there are multiple candidate antibodies against the same target, it may be more efficient to eliminate those antibody candidates with only modest or low activity by comparing their efficacy at a single antibody concentration in one established tumor xenograft model. For example, a common high dose scheme for evaluating the efficacy of a series of antibody drug candidates would be 20 mg/kg, twice a week dosing (i.v. or i.p.) into mice with established tumors of about 200 mm^3. Generally, this type of dosing regimen is sufficiently tolerant to accommodate the individual PK properties of most antibody candidates, such that PK studies can be deferred until only 2–3 antibody candidates are under consideration. Although most antibodies are efficacious at much lower concentrations than 20 mg/kg, a high concentration such as this remains clinically feasible from a cost of goods perspective and for further advancement into development. Appropriate negative controls for efficacy studies include both a vehicle control as well as an isotype-matched antibody to an irrelevant antigen, which is not expressed in either the tumor cell line or murine host. Antibody candidates with TGI (tumor growth inhibition) of less than 60% should be eliminated or engineered to improve potency.

After eliminating antibody candidates with insufficient efficacy using the single high dose scheme described above, dose response studies should be performed in several models ideally to further prioritize lead antibodies. At least three different antibody concentrations should be used and the incremental decrease in antibody concentration should be in one log or half log increments, e.g., 20, 2 and 0.2 mg/kg or 20, 7 and 2 mg/kg, respectively. For optimal translation to the clinic, i.v. dosing is the more appropriate route although both i.v. and i.p. routes often demonstrate similar efficacy.

Experimental Endpoints

For s.c. xenograft studies, the most commonly used measurement is comparing tumor volumes of an irrelevant antibody of the same isotype (control) to the proposed antibody therapeutic candidate. The formula for tumor growth inhibition (%TGI) is: %TGI = (Δ control average volume −Δ treated average volume) × 100/(Δ control average volume) (Manfredi et al. 2007). The T/C measurement (% of control for Δ growth) is calculated from the formula (Δ T/Δ C) × 100, where Δ T and Δ C are changes in tumor volume (Δ growth) for each treated and vehicle control group. In the case of reduction of tumor volume, Δ T/C values are calculated according to the following formula, Δ T/C (%) = (TV*n*-TV1)/TV1 × 100, where TV*n* is the tumor volume of treated mice on day *n* (Semba et al. 2004). While a s.c. tumor volume measurement is technically easy to perform, it is

not a translatable endpoint to the clinic. Consequently, imaging is an important and growing component of xenograft monitoring. Imaging can also be used for orthotopic xenografts and monitoring metastasis. Many investigators are now combining multiple imaging techniques to monitor the tumor, with bioluminescence analysis for tumor growth and examining the tumor vasculature by Doppler flow using ultrasound (Qayum et al. 2009) being one example. Other endpoint methods routinely used to monitor the pharmacological antibody response in the xenograft include examining the target or pathway by histochemical methods or molecular techniques (e.g., pERK). Ideally, a PK/pharmacodynamic profile is obtained to enable a complete evaluation of the therapeutic.

Disadvantages of Tumor Xenograft Models for Evaluating Antibody Drug Candidates

While the use of animal models is essential for antibody programs, there are also limitations in the translation of preclinical data to the clinic. For example, if the specificity of the antibody candidate is such that its epitope is not conserved between human and rodent, then some antibody candidates may only recognize their cognate human antigen and fail to react with the corresponding rodent ortholog. Extrapolating from experience with leukocyte antigens, human epitopes are well conserved in apes (e.g., chimpanzees), usually in old world monkeys (e.g., macaques), sometimes conserved in New World monkeys (e.g., marmosets), and less conserved in rodents (Loisel et al. 2007). There can be several consequences when using antibody candidates in preclinical efficacy studies that only bind to their cognate human antigen and not their rodent counterpart. First, the potency of human antigen-specific antibodies may be overestimated because their pharmacokinetics (PK) in the rodent model is not influenced by the PK of the rodent ortholog like it can be when the antibody is used to treat patients. In general, this phenomenon is not a major problem for two reasons. First, most short-lived antigens are soluble proteins that are cleared by the kidney. When complexed with an antibody, the half-life of the antigen is increased as the size of the antibody: antigen complex exceeds the filtration limits for renal clearance while the half-life of the free antibody is unaffected. Second, the half-life of IgG in primates is approximately 10–14 days, about 3–5 days longer than the half-life measured in rodents. Therefore, the increase in half-life in primates over that in rodents can often compensate for unaccounted antigen-related PK effects of non-cross-reactive antibody candidates used in preclinical disease models. Nevertheless, some antigens have specific, non-renal, clearance mechanisms that can dramatically alter the half-life of antibodies when bound to antigen (GML unpublished observations). These rare PK anomalies in rodents will not be detected using a non-cross-reactive antibody but can be revealed during non-GLP studies in non-human primates as long as the antibody candidate cross-reacts with the non-human primate ortholog. Program results such as these will generally require further characterization of

backup antibody candidates to rescue the program. If the PK liability is due to a specific epitope(s) and other pharmacologically active epitopes exist, then the program can continue with the corresponding antibody candidate(s) that recognize the alternative active epitope.

Another weakness of animal models related to antibody drug candidates that do not cross-react with the murine ortholog is that potential toxicology issues will not be identified early during the preclinical efficacy evaluation. This risk can be mitigated by using a surrogate antibody specific to the rodent ortholog. However, the investment of resources to this end equates to another antibody drug discovery program. Instead, this type of liability is examined during non-GLP toxicology studies so long as the antibody candidate(s) cross-reacts with the relevant non-human primate ortholog used in the study. If the need for a surrogate Ab outweighs the expense of an additional antibody drug discovery campaign, there are several examples where surrogate antibody programs, such as those for anti-CD20 and -CD19, have demonstrated efficacy based on effector function and safety using mouse models (Tedder et al. 2006).

Trastuzumab is an example where preclinical xenograft studies in rodents demonstrated drug efficacy but failed to predict potential toxicity issues. As predicted from preclinical models, trastuzumab has proven to be beneficial in metastatic as well as early invasive breast cancer in patients whose tumors overexpress the HER2 protein. However, trials of the combination of trastuzumab plus chemotherapy with anthracyclines have revealed an elevated incidence of cardiotoxicity greater than that predicted by preclinical models or even early clinical trials. In part, this is because trastuzumab does not cross react with murine HER2 so no cardiac side effects were observed. This will be discussed more fully in the HER2 case study section.

Other issues include the preclinical difficulty of mirroring a complex disease like cancer which is heterogeneous at both the genetic and cellular level. Furthermore, for reasons of economy and efficiency only, a limited number of mouse strains can be examined and even then, the differences in size and physiology between man and mouse, as well as animal handling and anesthesia concerns, often complicate the interpretation of preclinical data (de Jong et al. 2010). Nevertheless, animal models remain an essential tool for translating target biology into novel drug candidates for clinical evaluation.

Case Studies

In the first portion of this chapter, we discussed a preclinical paradigm that was neatly segmented into target identification, pharmacology, MOA, and animal models. While the actual steps along the drug discovery path do not change, they are not always sequential and in fact, can be quite convoluted. Nevertheless, a fundamental understanding of the critical biology of the disease and the target enables the generation of potent and novel therapeutics with the desired MOAs that can be translated into significant clinical efficacy.

For example, the target HER2 was initially discovered because the rat homolog *Neu* oncogene was found in chemically induced rat neuroectodermal tumors. It was an interesting target for oncology and the human *HER2* gene, was eventually understood to be amplified and overexpressed in breast cancer. Due to this disease correlation with a set of patients with a poor prognosis, antibodies were made to HER2 and evaluated in engineered cells and animal models. Using the in vitro and in vivo results, an antibody was selected for humanization and developed for the treatment of breast cancer. A more detailed study of this antibody, Trastuzumab, is described below.

The other case example concerns the preclinical development of the CD20 antibody Rituxan. The target CD20 was already well established as a cell-specific marker for B cells in the immune system. The compelling cellular association of CD20 with B cells was extended to B cell malignancies, which catalyzed the directed therapy approach for antibody-based treatment of B cell leukemias and lymphomas even with limited overexpression of CD20. Although efforts to determine the function of CD20 were investigated in knockout mice the function did not appear critical for survival. Other studies were able to demonstrate that the lack of internalization, long surface residence time, and minimal shedding attributed to maximizing effector function. As described previously, while effector mechanisms are of limited efficacy in the eradication of solid tumors, this is not necessarily the case for hematological malignancies. Antibodies to CD20 have been described as Type I or Type II based, in part, on their ability to redistribute CD20 into lipid rafts. Rituximab, a CD20-specific chimeric monoclonal antibody, is considered the first effective targeted therapy approved by the US Food and Drug Administration (FDA) for the treatment of relapsed or refractory low-grade or follicular B-cell non-Hodgkin's lymphoma. Although rituximab exhibits significant antitumor activity in patients, there is a need to further improve the efficacy of antibody agents for the treatment of B-cell malignancies. A more detailed account of the preclinical development of Rituxan and of efforts to develop improved agents is detailed in Anti-CD20.

Anti-HER2

Trastuzumab is a monoclonal antibody drug to the gene product of *HER2,* now named *ERBB2*. Trastuzumab was approved by the Food and Drug Administration in 1998 for the treatment of advanced breast carcinomas with overexpression of the HER2 protein. Trastuzumab is now the standard of care for the treatment of patients with HER2-positive breast cancer. The preclinical development of trastuzumab began with the discovery that *ERBB2* was a putative oncogene, based on an in vivo rat study. The exposure of perinatal BDIX rats to a single dose of ethylenitrosourea leads to a high incidence of neuroectodermal tumors. Robert Weinberg's group at M.I.T. (Massachusetts Institute of Technology) showed that when DNA from a ethylnitrosourea-induced rat neuroglioblastoma cell line was

transfected into NIH 3T3 cells, a transformed phenotype resulted based on the ability of transfected cells to grow in soft agar and generate tumors when the transfected cells were implanted into mice (Shih et al. 1981). Later studies established that independent ethylnitrosourea-induced rat neuroglioblastomas cell lines all contained the same transforming gene coined *neu*. *Neu*, the rat ortholog of *ERBB*2, was discovered to be mutated in transforming cDNAs derived from ethylnitrosourea-induced rat neuroglioblastomas tumors. In fact, the transforming mutation is a single point mutation in the neu transmembrane region that leads to constitutive kinase activation and oncogenic activity (Bargmann et al. 1986). The *neu* gene encodes a tumor antigen of approximately 185,000 daltons and shares homology but not perfect identify with what was known as the c-Erb-B protein and now referred to as ErbB1 (Schechter et al. 1984). It merits mentioning that c-Erb-B shares sequence homology with the avian erythroblastosis virus gene v-ErbB, also shown to be a viral oncogene.

It was not until 1992 that the pathogenic role of HER2 in breast cancer was demonstrated in vivo. In these studies, *neu* transgenic mice were created with the *neu* gene under the transcriptional control of the mouse mammary tumor virus promoter (Guy et al. 1992). The resulting transgenic female mice showed high penetrance with tumors occurring in the mammary glands, with some primary tumors giving rise to metastasis to other organs, similar to the human disease. These animal model studies were essential in demonstrating a role of HER2/neu in the pathogenesis of breast cancer in vivo.

The seminal studies of Weinberg and colleagues demonstrated that HER2 mutations, which led to constitutive signaling through the neu signal transduction pathway, acted as a driver of cellular transformation and tumorigenesis in the rodent disease model. Slamon et al. extended these observations by establishing human disease relevance when they examined breast cancer patient samples and demonstrated that *HER2/neu* was genetically amplified in 20–30% of these patients (Slamon et al. 1987; Slamon et al. 1989; Plosker et al. 2006). Genetic amplification of *HER2* in this subset of patients led to overexpression of transcript and protein as measured by Northern, Western and immunohistochemistry methods. Furthermore, performing multivariate analysis of breast cancer patients using a variety of prognostic parameters including node status, ER status, PR status, tumor size, and patient age revealed that *HER2* amplification and node positivity were the two most significant predictors of disease-free survival and overall survival. Slamon and colleagues echoed the opinion of Muller et al. (1988) that *HER2* should be "a focus of attention for diagnosis and therapy in breast cancer."

As the HER2 target now had a significant disease correlation, a collection of antibodies to the human ortholog of neu were made by immunizing BALB/c mice with two different HER2 immunogens and then immortalizing the harvested splenocytes by PEG fusion using hybridoma technology (Hudziak et al. 1989; Fendly et al. 1990). The two HER2 immunogens were administered in series and consisted of a recombinant cell line consisting of methotrexate-amplified HER2 in NIH3T3 cells (Hudziak et al. 1987), and a wheat germ agglutinin-purified, plasma membrane enriched preparation derived from NIH3T3/HER2-3_{400} transfectants.

The cell-based immunogen was given as 4 i.p. injections over 7 weeks. Immunized mice that exhibited a serum titer to HER2 were boosted with a WGA-purified plasma membrane preparation that was administered twice by i.p. over 4 weeks. Immunization was terminated with a single i.v. injection of the WGA-purified plasma membrane preparation. Approximately 100 hybridomas were generated of which 10 hybridomas expressed antibodies that had readily detectable HER2 binding by ELISA and immunoprecipitation. None of the anti-HER2 antibodies bound to EGFR family members by ELISA. By competition binding studies, there appeared to be at least 4 epitope bins (2 antibodies were not evaluated).

For antibody design goals, the studies of Slamon et al. suggested that a therapeutic anti-HER2 antibody should inhibit the in vitro growth of breast cancer cell lines containing an amplified *HER2* locus (Slamon et al. 1987; Slamon et al. 1989). Hudziak et al. conducted proliferation studies using the SK-BR-3 cell line, which expresses approximately 1 million HER2 receptor sites per cell (Hudziak et al. 1989). This goal was met as most of the anti-HER2 antibodies, when used at ~33 nM, showed some inhibitory activity on the proliferation of SK-BR-3 cells. The antibody 4D5 exhibited the most potent activity with 56% growth inhibition at concentrations ranging from 5 to 33 nM. The growth inhibitory effect of 4D5 was cytostatic as removal of the antibody from treated SK-BR-3 cells restored their proliferative capacity. Additional studies were performed on other breast cancer cell lines including MDA-MB-157, MDA-MB-361, MDA-MB-231, MDA-MB-175, and MCF-7 (Hudziak et al. 1989). Only MDA-MB-361 and MDA-MB-175 exhibited growth inhibition in the presence of 4D5, although the extent of inhibition was not as great as for SK-BR-3. Based on FACS characterization of most of these cell lines, there appears to be a minimum threshold of HER2 expression required for 4D5 to inhibit cell growth, as resistant cell lines MDA-MB-231 and MCF-7 have reduced expression of HER2 (Hudziak et al. 1989; Lewis et al. 1993). Repeated evaluation of a set of 10 anti-HER2 antibodies consistently demonstrated that 4D5 was the most potent antibody with respect to growth inhibition of breast cancer cell lines that overexpress HER2. These studies were essential to reveal the biology of HER2 and the 4D5 antibody.

The MOA of 4D5 was examined in a series of in vitro studies. In the original studies of Hudziak et al. it was shown by a pulse-chase experiment that 4D5 decreases the half-life of HER2 in SK-BR-3 cells by about 28% (Hudziak et al. 1989). In the same publication, 4D5-treated SK-BR-3 cells were substantially more sensitive to macrophage derived TNF-α than untreated counterparts. When breast cancer cell lines like SK-BR-3 were exposed to 4D5 for 1 h or more, decreases in HER2 phosphorylation occured as well as a reduction of downstream signaling molecules including DAG (diacylglycerol), a cofactor for activation of protein kinase C (Sarup et al. 1991). DAG is a necessary cofactor for protein kinase C activity and while the effects of decreased PKC activity were not fully appreciated when 4D5 was discovered, it is now known that decreased PKC activity, particularly the α and β isoenzymes, leads to reduced cellular proliferation, increased apoptosis, maintenance of cell junctions, reduced cell motility, and increased drug sensitivity (Koivunen et al. 2006). Surprisingly, acute treatment

with 4D5 for 5–15 min actually stimulated HER2 signaling, but this effect was short-lived.

The MOA studies of Hudziak et al. (1989) were extended by Mendelsohn et al. wherein they established that 15 h of exposure to 4D5 at 30, 150 or 300 nM caused a dose-dependent decrease in HER2 phosphorylation of 44, 49, and 80%, respectively (Kumar et al. 1991). Interestingly, this marked inhibition of HER2 activation could also be achieved with 4D5 in the Fab format (400 nM). Subsequent studies using different metabolic labeling strategies with either ^{32}P-orthophosphate or ^{35}S-cysteine established that 45% inhibition of HER2 activation by 4D5 over the course of 11 h of treatment could only be partially explained by the 14% down-regulation of total cellular HER2. Under the same conditions, a 400 nM concentration of 4D5 Fab abolished HER2 phosphorylation but had no effect on HER2 levels. Additional studies performed at 4 and 37° C extended previous results to show that 4D5 inhibition of HER2 activation cannot be completely accounted for by plasma membrane down-regulation of HER2.

Collectively, the inhibitory effect of 4D5 on in vitro growth of sensitive breast cancer cell lines was consistent with the HER2 overexpression in disease initiation and progression of HER2-positive breast cancers. The growth inhibition observed in vitro with the 4D5 antibody indicated that HER2 was pharmacologically tractable in a simple in vitro system. The MOAs of the 4D5 antibody observed in vitro included receptor down-regulation and decreased signaling based on a reduction in both HER2 phosphorylation and diacyglycerol, a critical cofactor for protein kinase C signaling.

The in vitro validation studies described above were followed by a series of efficacy studies using several different types of xenograft models. Unfortunately, most of the HER2-amplified human breast cancer cell lines do not grow subcutaneously in nude mice. Consequently, the original preclinical in vivo validation studies were performed using recombinant cancer cell lines that overexpressed HER2 (Chazin et al. 1992) or fresh HER2-positive primary patient tumor samples implanted into the renal capsule. In the former case, either NR6 or MCF7 cells were transfected with a HER2 expression construct. The resulting high expressing clones derived from the respective parental hosts were evaluated in vitro and in vivo for their sensitivity to 4D5 mediated growth inhibition. Neither of the parental lines was sensitive to the presence of 4D5 in vitro while their respective high HER2-expressing cell counterparts became markedly growth sensitive. The 4D5 sensitivity that was established in vitro was also observed in mouse xenografts derived from recombinant NR6 and MCF7 cell lines.

As described in elegant detail by Pegram and Ngo, the development of humanized 4D5 or trastuzumab relied heavily on the use of cancer animal models to validate HER2 in vivo as an antibody target and to test the efficacy of anti-HER2 antibodies, develop dose–response relationships, and optimize dosing schedules for combination with chemotherapy (Pegram et al. 2006). One of the most challenging issues encountered in this drug discovery campaign was the limited number of primary human tumor passages available and their intrinsic low tumor take rate of 60–85%. Consequently, few animals were used per group for

each in vivo study making the experimental results more qualitative than quantitative. In this in vivo assay, fresh HER2-positive patient tumor samples were implanted into the renal capsule and then the animals were treated with the 4D5 antibody. Tumor growth inhibition was observed relative to control treated animals, thus validating the target for antibody treatment. Based on these in vivo studies, in combination with the in vitro proliferation data, the 4D5 antibody was selected for humanization.

Interestingly, a number of questions were posed about the use of an anti-HER2 therapeutic antibody that prompted the initiation of a Phase I clinical study with the murine antibody 4D5. The objective of this early study was to assess safety, pharmacokinetics, and tumor localization Shepherd et al. 2008). The study enrolled 12 breast and ovarian cancer patients that overexpressed HER2. The clinical study design was an open-label dose escalation with patients receiving a single dose from 3 to 500 mg/kg. In addition, prior to dosing, the 4D5 antibody was mixed with 1–5 mCi of ^{131}I-labeled 4D5 antibody to enable tumor localization through external gamma scintigraphy. The 4D5 antibody drug was well tolerated at all doses tested. Imaging revealed the location of the tumor. PK analyses showed a small initial volume of distribution, dose-dependent clearance, and a long terminal half-life. Not surprisingly, a HAMA response was readily detected. These encouraging drug tolerability and PK results supported the proposed targeted therapy for treatment of HER2-positive breast cancer patients and confirmed the need to humanize the 4D5 antibody to eliminate, or at least minimize, the HAMA response.

Based on its preclinical in vitro and in vivo activity, antibody 4D5, was engineered for clinical use by simultaneous humanization of the heavy and light chains by gene conversion (Carter et al. 1992). Eight different IgG1 variants, humAb4D5-1 through humAb4D5-8, and the mouse-human chimera of humAb4D5 were analyzed for affinity and their effect on cell proliferation. Seven of the eight antibody variants contained one or more amino acid sequence changes back to the original murine 4D5 variable domain. The K_Ds of the humanized antibodies ranged from 0.1 to 25 nM using solution-based equilibrium measurements. Two of the eight antibodies (K_Ds of 25 and 4.7 nM) failed to inhibit proliferation of SK-BR-3 cells at saturating antibody concentrations (16 μg/mL). Interestingly, one of these antibodies was the most human based on primary sequence. The other 6 humanized variants exhibited 34–52% growth inhibition compared to the 63% growth inhibition of chimeric 4D5. A dose–response curve revealed that humanized antibody 4D5-8 was the most potent inhibitor of proliferation of SK-BR-3 cells, although it was not as potent as the parental murine antibody 4D5. Additionally, Carter and colleagues showed that humanized antibody 4D5-8 could elicit ADCC activity in vitro against SK-BR-3 cells in the presence of human peripheral mononuclear cells as effectors. In contrast, murine 4D5 showed very low effector function consistent with the murine IgG1 isotype subclass. The humanization of 4D5 yielded an antibody that maintained most of its growth inhibitory activity in vitro while potentially gaining an additional MOA as a consequence of creating a humanized IgG1 with effector function.

Subsequently, HER2-overexpressing cell lines were generated that were tumorigenic and the xenografts were used in tumor localization and efficacy studies. Radiolabeled studies (Shalaby et al. 1995) provided compelling evidence of anti-HER2 antibodies localized to known metastatic tumors. Clear in vivo dose response relationships were defined (Pietras et al. 1994) and the minimum target serum concentration for trastuzumab was predicted prior to Phase 1 initiation. Multiple models from different laboratories demonstrated that the effect was general and not restricted to a single model. The humanized 4D5 antibody 4D5-8 advanced to the clinic and became known as trastuzumab.

The clinical trials with trastuzumab highlight one of the disadvantages of xenografts models. While xenograft models demonstrated efficacy and helped to define the clinical strategy, trastuzumab does not cross react with the mouse neu protein and therefore it was not possible to anticipate other toxicities such as the now well-known cardiac side effects. The association between trastuzumab and cardiac dysfunction was not described in the early clinical development process because HER2 is not overexpressed in the heart and no evidence of this effect was observed in preclinical (Klein et al. 2003) or even early clinical studies. In the preclinical safety studies for example, HER2 expression in human or monkey cardiac myocytes was not detected by IHC methods. However, subsequent clinical trials demonstrated an unexpectedly high incidence of cardiac side effects. In most cases the cardiotoxicity observed is an exacerbation of the anthracycline-induced cardiotoxicity and has been the subject of multiple reviews and studies (Seidman et al. 2002; Suter et al. 2004; Guglin et al. 2008; Walker et al. 2009).

The exact mechanism of trastuzumab-induced cardiac dysfunction is not well understood. The target receptor, HER2, is important in both the development and function of the heart. HER2 can heterodimerize with HER3 and HER4 and form a receptor for neuregulins, including heregulins. Neuregulins have distinct effects, such as growth, differentiation, and activation of survival pathways in epithelial cells, neurons, and muscle cells; moreover, neuregulin signaling is essential in the developing heart. Murine experiments have shown that the HER2 receptor plays a crucial role in cardiogenesis, and when not present in the heart, the resulting lack of signaling leads to death in utero (Lee et al. 1995). Using HER2-deficient conditional knockout mice, animals were viable and displayed no overt phenotype (Crone et al. 2002). However, over time these animals revealed the onset of multiple independent parameters of dilated cardiomyopathy, including chamber dilation, wall thinning, and decreased contractility. Furthermore, when these animals were subjected to increased cardiac stress, such as aortic banding, mortality was significantly higher in HER2-deficient mice than in control animals. Additionally, cardiomyocytes isolated from these conditional mutants were more susceptible to anthracycline toxicity. These data indicate that HER2 signaling is important for the maintenance of cardiac contractile function and structure, and that HER2 might be a protective factor in the stressed heart (Suter et al. 2004). The use of trastuzumab and the resultant loss of HER2-dependent cardiac myocyte survival pathways may make patients more susceptible to cardiac damage (Chien 2000) perhaps by inhibiting the gp130 signal cascade (Grant et al. 2002). It should

be noted that the resulting effects of trastuzumab on the heart have been shown to differ from those of the anthracyclines. Anthracyclines can cause myocardial structural abnormalities and apoptosis, while trastuzumab causes cardiac dysfunction through alterations in signaling without apoptosis. Additionally, trastuzumab-induced cardiotoxicity is generally reversible and can be managed with standard medical treatment.

Despite the clinical success of breast cancer treatment with trastuzumab, a significant proportion of patients either does not respond or eventually relapses (Slamon et al. 2001; Nahta et al. 2006). The proposed next generation treatment now utilizes transtuzumab to deliver a cytotoxic maytansinoid, a potent microtubule-depolymerizing agent, specifically to the antigen expressing tumors. Because HER2 is highly expressed with 1–2 million copies per cell compared to normal cells, it is an ideal target for ADC therapy. Available data suggest that cancer cells do not lose HER2 expression when they become refractory (Nahta et al. 2005; Ritter et al. 2007). Studies with different linker combinations with in vitro and in vivo analysis have identified trastuzumab-MCC-DM1 (Lewis Phillips et al. 2008) as the clinical candidate, which is now in Phase 2 clinical trials for HER2-positive metastatic breast cancer.

Anti-CD20

Rituximab, a chimeric monoclonal antibody targeted to the pan-B-marker CD20, was the first monoclonal antibody to be approved for oncology (US approval 1997; Cartron 2004; Smith 2003). Rituximab is currently indicated in both follicular and aggressive B-cell non-Hodgkin lymphomas (NHL). The preclinical development of CD20 antibodies is detailed below.

CD20 is expressed on all stages of B cell development, from pre-B cells through memory cells, but not on either pro-B cells or plasma cells. In normal healthy individuals, CD20 does not appear to be expressed at measurable levels in non-B cells. CD20 has no known natural ligands and its function is unclear, although there are data to suggest that it acts as a calcium channel (see below).

In addition to its expression pattern in healthy individuals, CD20 is also expressed and even overexpressed on some B cell malignancies such as NHL. However, at the B cell level, the disease association of the target in normal and diseased patients is not as remarkable as it is for other targets, e.g., HER2 in breast cancer. Nevertheless, as a cell type specific marker for B cells, pharmacological targeting of CD20-positive cells enables directed therapy for B cell malignancies. Moreover, CD20 does not appear to be shed into plasma at any measureable level nor does it undergo readily detectable internalization upon antibody binding. The lack of antibody-dependent internalization of CD20 and the corresponding long cell surface membrane residence time is postulated to be an important target attribute for maximizing the effector function of anti-CD20 antibodies.

CD20 is a 297 amino acid non-glycosylated Type III plasma membrane phosphoprotein that plays a role in the development and differentiation of B-cells.

Topologically, the termini of CD20 are intracellular with two extracellular loops of 12 and 43 amino acids. The *MS4A1* gene encodes CD20 and is the founding member of the 16-member membrane-spanning 4A gene family located on chromosome 11q12 (Liang et al. 2001; Zuccolo et al. 2010). At the protein level, CD20 exhibits between 15 and 23% sequence identity with other MS4A family members and 73% sequence identity with its mouse ortholog. In contrast to other members of the MS4A family, there is a single CD20 isoform.

To determine the function of CD20, mouse knockouts were created by several groups (O'Keefe et al. 1998; Uchida et al. 2004). The resulting knockouts from both research groups were categorized as normal with respect to anatomical or morphological defects as well as susceptibility to infections. O'Keefe et al. did not observe any effects of CD20 genetic inactivation on B cell differentiation and function based on surface marker expression, antigen receptor signaling, proliferative responses, or calcium uptake. Interestingly, the $CD20^{-/-}$mice described by Uchida et al. (2004) showed a 20–30% lower IgM expression in immature and mature B cells relative to B cells from wild-type littermates. $CD20^{-/-}$ B cells of these mice also showed significantly reduced intracellular calcium responses following antibody-mediated ligation of either CD19 and to a lesser extent, IgM. These results suggested a role for CD20 in transmembrane Ca^{+2} movement in mouse primary B cells, which complemented previous results obtained using human CD20 cDNA-transfected cell lines (Bubien et al. 1993), but this function was not critical for B cell survival.

The 12 and 43 amino acid extracellular loops of CD20 would be expected to have few epitopes. Consistent with these predictions are the results of competition binding studies which show that binding of any single CD20 antibody such as 2H7, 1F5, B1, HI47, Rituximab, AT80, LT20, and 11B8 to CD20 expressing cells block the subsequent binding of other fluorescently labeled anti-CD20 monoclonal antibodies (Clark et al. 1985; Tedder et al. 1989; Chan et al. 2003). From a biochemical perspective, this is a standard approach to assess the epitope landscape.

However, a more detailed understanding of the epitope space can be appreciated by examining the qualitative and quantitative effects that result from antigen engagement using a collection of different anti-CD20 antibodies. A series of studies performed over the past decade using hybridoma-derived mouse and human antibodies to CD20 have shown a larger number of epitopes based on different B cell effects in vitro upon CD20 engagement. For example, the 1F5 anti-CD20 antibody, when used alone, stimulates resting B cells to progress through the cell cycle. In contrast, another anti-CD20 antibody, B1, significantly inhibits B cell proliferation that is normally induced by anti-IgM antibodies (Tedder et al. 1985). In this example, B1 inhibited both RNA synthesis (37–80%) and progression through the cell cycle following activation. Additional in vitro studies revealed that hypercrosslinking of 1F5 when bound to Ramos cells, a human B cell lymphoma line, inhibited cellular proliferation and induced apoptosis (Shan et al. 1998). This hypercrosslinking effect could be achieved with a goat anti-mouse antibody or with transfected fibroblasts overexpressing FcγRIIa. The molecular mechanism for CD20-mediated apoptosis is calcium dependent and may be a

consequence of either sustained calcium channel conductance or possibly, release of intracellular calcium stores. Collectively, these findings indicate that even though 1F5 and B1 bind to CD20 in a competitive manner, the mode in which they interact with the antigen differs and results in distinct cellular consequences, several of which could be therapeutic in the context of B cell depletion.

Anti-CD20 antibodies have been grouped into two major types based on biological activities. Type I anti-CD20 antibodies activate complement but are generally poor at mediating apoptosis in the absence of cross-linking. Type II anti-CD20 antibodies exhibit minimal CDC activity but demonstrate substantial pro-apoptotic activity. Rituxan is a prototypic example of a Type I antibody while Bexxar, a radioimmunotherapeutic version of the antibody known as B1, is an example of the much more rare, Type II antibody. Both Type I and II antibodies support significant ADCC activity. The differences in the activities of Type I and II antibodies correspond to the differential effects that each antibody type has on CD20 membrane re-organization. When Type I antibodies engage CD20, the antigen: antibody complex is re-localized to Triton X-100 insoluble lipid rafts or microdomains. The potent CDC activity of Type I antibodies appears to be dependent upon coalescing CD20 complexes into lipid rafts, whereby the corresponding assembly of Fc domains enables efficient C1q engagement and maximal CDC activity. While these in vitro differences are reproducible from lab to lab, the importance of CDC activity for drug efficacy remains controversial. Interestingly, most mouse antibodies to human CD20 have limited epitope diversity with the majority, including Rituxan, recognizing a single linear epitope within the second extracellular loop that encompasses A170 and P172 (Teeling et al. 2006).

In contrast to Type I antibodies, Type II antibodies are unable to re-localize CD20 into lipid rafts. However, Type II antibodies induce homotypic interactions when assayed using cell aggregation assays. Another apparent difference between Type I and II antibodies is that in antibody excess, Type I antibodies exhibit twice the extent of binding to CD20 cell lines as do the Type II antibodies. A simple explanation for this observation, although without support at this time, is that the number of available CD20 epitopes for Type II antibody binding is reduced because of steric hindrance from the CD20 quaternary structure.

In 1994, Reff and colleagues described a new mouse monoclonal anti-CD20 antibody named 2B8 (Reff et al. 1994). This antibody was re-engineered to create a mouse-human chimeric antibody called C2B8, which consisted of mouse 2B8 heavy and light chain variable regions and human IgG1 constant regions. C2B8 is now known as Rituxan, Rituximab, and Mabthera. Much of the initial C2B8 characterization focused on assessing various aspects of CDC. For example, when C2B8 was incubated with the human lymphoblastoid cell line SB in the presence of fluorescently labeled human C1q, SB cells became fluorescently labeled in an antibody-dependent manner. Neither mouse 2B8 nor an irrelevant antibody enabled fluorescent labeling of SB cells with human C1q.

These C1q cell binding studies were extended to examine the extent of C2B8-mediated CDC activity in lymphoid cell lines that expressed or did not express CD20. Using a ^{51}Cr release assay format, approximately 14.6 nM C2B8 was

demonstrated to lyse 50% of the SB cells in a 4-h incubation with a 1:4 dilution of human serum as a source of complement. Under the same conditions, C2B8 did not lyse CD20-negative HSB cells and the parental antibody 2B8 failed to show significant CDC activity in CD20-positive SB cells.

In a similar assay format and duration as that described for CDC activity, C2B8 ADCC activity was also evaluated. Using C2B8 at a concentration of 26 nM and an effector:target ratio of 100, Reff et al. showed that about 50% of the CD20-positive SB cells were specifically lysed by ADCC while CD20-negative HSB cells were refractory to this mechanism. As described previously for the creation of trastuzumab from mouse antibody 4D5, engineering of the mouse antibody 2B8 to an antibody containing a human IgG1 Fc conferred CDC and ADCC activity as an additional MOA, which was lacking in the original mouse parental antibody.

As the C2B8 antibody did not cross react with the rodent, C2B8 was subsequently evaluated for its ability to deplete peripheral blood B cells in cynomolgus monkeys. Animals were intravenously dosed daily for 4 days at antibody concentrations of 0.01, 0.1, 0.4 and 1.6 mg/kg, respectively. The three highest doses depleted >95% of the circulating B cells for as long as 8 days post-infusion while the lowest dose depleted >50% of the circulating B cells. In contrast, when the same type of study was performed using C2B8 as an IgG4 isotype subclass rather than an IgG1, no B cell depletion was observed in monkeys (Anderson et al. 1997). These findings clearly demonstrated that effector function is the major and possibly sole MOA of C2B8 in vivo and highlighted the importance of the IgG1 isotype for therapeutic antibodies to membrane-bound tumor antigens. Follow-on studies were performed to study the effect of C2B8 treatment on B cells within the lymph node compartment. Using cumulative doses of 1.6 or 6.4 mg/kg from either daily or a single bolus injection of C2B8, respectively, the B cell population in the lymph node was reduced by 34–78% of that observed in vehicle-treated animals at 15–29 days after dosing. These studies were consistent with effector function and Fc cross-linking being critical for B cell depletion by C2B8, which ultimately became Rituxan. Unfortunately, the relative importance of different effector functions and IgG1 Fc crosslinking through FcγRIIA and FcγRIIIA could not be discerned from these results.

Toxicity studies in cynomolgus monkeys were performed at a dose of 16.8 mg/kg administered weekly for 4 consecutive weeks. At 22 days post-infusion, the lymph node compartment was reduced by >84% while the bone marrow compartment was depleted by >73%. At 36 days post-infusion, B cells in the lymph node compartment were reduced in the two treated animals by 87 and 69% while the treatment effect decreased B cells in the bone marrow by 95 and 73% in the same two animals. The treated monkeys did not exhibit any adverse effects and the cell number alterations appeared to be restricted to the B cell population. Finally, the treated animals that were not sacrificed recovered their B cell population, providing additional support that pluripotent hematopoietic stem cells do not express CD20 and thus are refractory to anti-CD20 therapy.

The successful clinical progression and commercialization of Rituxan in 1997 has spurred over a decade of studies to create more potent anti-CD20 antibody drugs.

The simplicity of this goal has been confounded by our lack of understanding of the relative importance of ADCC or CDC as the MOA of Rituxan. Nevertheless, the quest to build a better Rituxan has uncovered a series of correlations that may yield one or more fast follower agents with improved or even new pharmacological activities.

Attempts to improve on Rituxan were examined by Beers et al. (2008) using a humanized CD20 mouse model. Murine IgG2a subtypes, which have the greatest intrinsic effector function of the rodent isotype subclasses, were used for both Rituxan (Type I) and Tositumomab or Bexxar (Type II), formerly known as B1 (Beers et al. 2008). The in vitro activities of IgG2a murinized Rituxan and Tositumomab held true with Rituxan showing potent CDC activity that was ~3-fold that of Tositumomab, which correlated with antibody-dependent CD20 relocalization to lipid rafts. In contrast to the readily apparent differences in CDC, both antibodies mediated equivalent in vitro phagocytosis of humanized mouse splenocytes using thioglycollate-induced mouse macrophages.

In vivo comparisons were performed using humanized CD20 mice in both C57Bl/6 and BALB/c genetic backgrounds. Expression of human CD20 was detected only on CD19-positive B cells and was expressed at higher levels in BALB/c mice than in C57Bl/6 mice. Using a single dose of 250 μg, Tositumomab provided enhanced depletion of peripheral B cells in both mouse strains over that observed with murinized Rituxan based on when 50% of the B cells returned. The greater B cell depletion activity of Type II antibodies was also extended to other lymphoid compartments including bone marrow, spleen, and lymph nodes. This enhanced activity did not correlate with antibody affinity or PK (determined with radiolabeled antibody). These findings show that while Rituxan is a very effective means of B cell depletion in the treatment of B cell malignancies, Type II anti-CD20 antibodies that induce both apoptosis and elicit ADCC activity with minimal CDC activity may be even more potent than Rituxan. These results also indicate that CDC may not play a significant role in rodent disease models.

Beers et al. extended their in vivo studies using the same murinized Tositimomab and Rituxan with additional genetically engineered mouse strains that were unable to support ADCC or CDC, respectively, to elucidate which effector function was most important for anti-CD20 dependent depletion of B cells (Beers et al. 2008). When fluorescently-labeled murine B cells expressing human CD20 were transferred to either WT (wild-type) mice, $C1q^{-/-}$ or $C3^{-/-}$ mice, both murinized Rituxan and Tositimomab exhibited the same extent of B cell depletion in the knockout mice as observed in WT mice. These results showed that CDC activity has little B cell depleting activity in the syngeneic model. In a similarly designed study, fluorescently labeled humanized CD20 murine B cells were transferred to either WT or Fcγ chain knockout mice and then treated with murinized Rituxan and Tositimomab. No B cell depleting activity was observed in the absence of the Fcγ chain for either Type I or II antibodies in this syngeneic B cell depletion model. This series of studies clearly illustrated the importance of ADCC as an MOA for both Type I and II anti-CD20 antibodies using rodent disease models.

While the results described above support ADCC as the major MOA for anti-CD20 antibodies and diminish the importance of CDC activity, these studies did not explain their previous finding that Type II antibodies are more potent at B cell depletion in syngeneic models than Type I antibodies. However, the use of the Fcγ chain null mouse strain, allowed Beers and colleagues to examine the effect of Type I and Type II antibodies on humanized CD20 levels in murine B cells when transferred to knockout mice. Surprisingly, Beers et al. showed that murinized Rituxan but not Tositimomab reduced surface exposed human CD20 on B cells by 80–90% in 16 h (Beers et al. 2008). ADCC activity studies were performed in vitro after a 16 h pre-incubation with murinized antibodies and the Rituxan isoform showed a 50% decline in ADCC activity while the ADCC activity of Tositimomab was unaffected. These results are consistent with Rituxan mediating internalization of human CD20 when overexpressed in murine B cells, which decreased the levels of antigen displayed on B cells, reduced ADCC activity and would be expected to reduce antibody half-life. A bioactive FACS assay confirmed that murinized Rituxan has a 6–7-day half-life in humanized CD20 mice while murinized Tositimomab has a 14 day half-life. Both murinized antibodies have a 14-day half-life in WT mice.

As Rituxan is arguably the most successful antibody cancer therapeutic ever commercialized, efforts to further optimize and engineer better CD20 antibodies are continuing. The preceding results make a strong case for improving the potency of anti-CD20 antibodies by enhancing effector function, specifically ADCC activity. Five of the six second-generation antibody candidates are Type I antibodies and have improved effector function relative to Rituxan; 4 of 6 exhibit enhanced ADCC and two have improved CDC activity (Robak et al. 2011). Ofatumumab, a Type I antibody with improved CDC activity, has been approved for patients with CLL refractory to fludarabine and alemtuzumab. This approval implies that CDC remains an important MOA for anti-CD20 antibodies. The phase III clinical findings for ocrelizumab, a Type I antibody with enhanced ADCC and decreased CDC activity, were disappointing because of an unsatisfactory risk/benefit ratio. The remaining drugs under clinical evaluation will test the importance of different effector function activities as well as therapeutic benefit of Type I versus Type II drug candidates. The results from these trials should provide critical insight into the mechanistic design features of antibody drugs to treat hematological malignancies, some of which will be applicable to solid tumor applications.

Concluding Remarks

The preclinical considerations for antibody therapeutics require a comprehensive understanding of the target and its biology to initiate an antibody drug development program. Subsequently, careful in vitro and in vivo evaluations are required to optimize the antibody therapeutic for oncology indications. The antibody drug

development landscape is rapidly evolving, with the first oncology antibodies approved only in 1997 and now second-generation antibodies, including ADCs, are entering or nearing the market. Emerging technologies that enable the discovery of new targets and advances such as antibody drug conjugates will enable even better antibody therapeutics for the treatment of cancer patients.

Note The antibody drug conjugate SGN-35 (cAC10-vcMMAE), now known as Brentuximab vedotin, was granted accelerated approval to treat anaplastic large cell lymphoma (ALCL) and Hodgkin lymphoma by the U.S. Food and Drug Administration on August 19, 2011. It is marketed as Adcetris and is currently the only approved ADC on the market.

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Chapter 9
Factors Impacting the Tumor Localization and Distribution of Antibody-Based Therapeutics in Oncology

David C. Blakey

Abstract Distribution of antibody-based therapeutics from the vascular space to the target tumor compartment is an important consideration in designing antibody-based oncology drugs. Mouse tumor models represent a reasonable approach for exploring antibody biodistribution. In general, a number of factors such as molecular size, antibody dose, and the length of in vivo tumor exposure can influence antibody localization and tumor penetration. With a few exceptions, the current available data indicate that at clinically relevant doses (ranging from 1 to 10 mg/kg) and over a reasonable clinical exposure time (days rather than hours), antibody biodistribution into tumors is unlikely to be the most significant factor hindering the clinical efficacy of antibody-based therapeutics. Other factors such as antigenic heterogeneity leading to variable distribution of antibody drugs into the tumor, or intrinsic resistance to antibody-mediated effects, may play a far greater role in the resistance properties impacting the antibody efficacy profiles.

Introduction

The distribution of antibody-based therapeutics from the vasculature to the target tumor cells can represent a significant barrier in achieving therapeutic efficacy. As a tumor grows, it develops new blood supply to provide nutrients and removes waste products through a process termed angiogenesis. The vessels that are formed during angiogenesis are typically immature, have poorly defined basement

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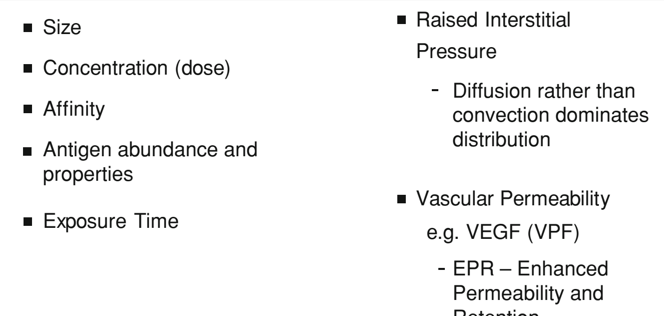

Fig. 9.1 Key factors that influence the localization of therapeutic antibodies used for therapy of solid tumors

membranes, and are often highly permeable to macromolecules. In part, these properties can be attributed to Vascular Endothelial Growth Factor (VEGF), which in addition to its ability to stimulate proliferation and migration of endothelial cells during the angiogenic process, is also a highly potent agent at enhancing vascular permeability; VEGF was originally called Vascular Permeability Factor (VPF). The increased permeability of tumors has been exploited for therapeutic purposes since it leads to the preferential accumulation of large macromolecules in tumors through the Enhanced Permeability and Retention (EPR) effect. As described recently, injection of non-targeted polymer-based drug conjugates exploited the EPR effect to achieve selective localization within tumors (Duncan 2009). In contrast to the EPR effect which favors selective localization of macromolecules in tumors, a barrier to macromolecule localization is the high interstitial fluid pressure (IFP) within solid tumors (Jain and Baxter 1988) that inhibits movement through the tumor structure by convection (a process that depends on the pressure gradient). Consequently, in the tumor microenvironment, diffusion is likely the key driver for movement of antibody-based therapeutics from the vasculature to the distal tumor cells. Diffusion is impacted by factors such as molecular size, shape, and concentration gradients. Without additional barriers, a molecule of the size and shape of an antibody should be able to diffuse the distance from a blood vessel to tumor cells—approximately 100–200 μm—in about 60 min (Jain and Baxter 1988). Typically, distribution of antibodies within tumors takes much longer and there are clearly other barriers, such as the complex stromal matrix of the tumor microenvironment that can influence antibody biodistribution. Binding of the antibody molecule to the target antigen will also slow the passage of the antibody through the tumor mass, and properties such as antibody affinity for the target antigen and the antigen internalization rate can impact tumor distribution. Some of the factors that influence tumor localization and distribution are summarized in Fig. 9.1. This short review will focus on evaluating the relevant literature regarding biodistribution of antibody-based therapeutics in tumor tissues.

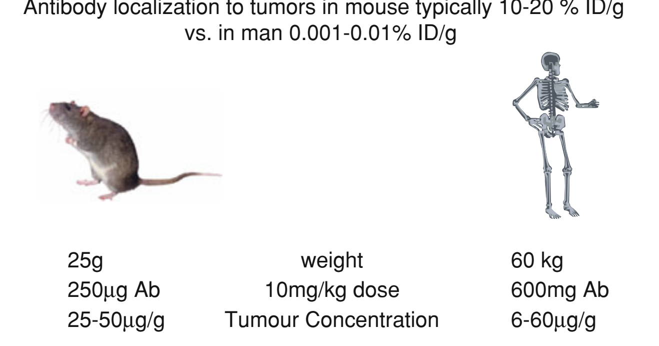

Fig. 9.2 Comparison of localization of antibodies to mouse and human tumors

Tumor Models: Are They Relevant to Man?

Before discussing distribution of antibodies, it is worth considering whether biodistribution of antibodies in preclinical tumor models parallels that observed in clinical studies in patients. Generally, the very low percentage of administered dose, typically between 0.01 and 0.001% injected dose/g (ID/g) in man (Estaban et al. 1987; Scott et al. 2005) compared to typical levels in human tumor xenografts implanted in immunocompromised mice (where it can reach 10–20% injected dose/g) is often used as evidence for a major discrepancy that invalidates these mouse models. However, as Fig. 9.2 illustrates, this estimate is really only a reflection of the much larger size of a human in comparison to a mouse and so if equivalent doses are given on the "mg/kg" basis, a typical human would receive 2–3,000 times the dose given to a mouse. Consequently, the absolute amount and thus the concentration of the antibody in the mouse and human tumor are broadly similar based on the percent injected dose (Fig. 9.2). Since it is the concentration of the antibody per unit tissue that will drive efficacy, these data suggest that mouse models can reflect the human situation. It is not really surprising that the percent injected dose is much lower in a 1 g tumor in a 60 kg patient than in a 1 g tumor in a 25 g mouse. Hence, the number of times and the likelihood that each injected antibody molecule passes through the blood vessel, perfusing the tumor microenvironment, will clearly be much lower in the human setting.

In terms of preclinical rodent models, the most widely used models for exploring antibody biodistribution are either syngeneic tumor models or human tumor xenografts in immunocompromised mice. In both cases, tumor cells are typically implanted subcutaneously under the skin of mice. They provide a useful initial model to investigate the activity of antibody therapeutics where tumors typically are generated within 1 to 2 weeks following tumor cell implantation, allowing for rapid screening. However, a challenge for such models is that the histology of such tumors can often bear little resemblance to human tumors,

particularly with respect to stromal components. Also, the lack of tumor cell heterogeneity in murine tumor models can be a significant issue in interpreting the results. Transgenic tumor models are generally considered more representative of human disease, especially if the genetic alteration mimics a key driving mutation in the disease setting (Carver and Pandolfi 2006). However, a challenge with such models is that often tumor development takes many months; thus, carrying out a series of consecutive experiments can be a major limiting factor. Using primary tumor models in which patient tumor samples are implanted in mice is an additional alternative that represents an emerging approach more suitable for exploring antitumor activity, biodistribution, and tumor penetration for antibodies. The histology of these primary tumor explants more closely resembles the clinical situation both in terms of overall histology and heterogeneity. There appears to be little published data on the distribution of therapeutic antibodies in such models to judge whether these models offer a significant advantage over the human tumor xenograft models; hence, the data discussed in this review are mostly from either syngeneic mouse models or human tumor xenograft studies.

The Impact of Drug Size on Tumor Localization

The size of the macromolecule influences both the rate and extent of distribution into tumors (Schmidt and Wittrup 2009). Figure 9.3 illustrates the relationship between the molecular size and antibody distribution. Small macromolecules (<10 kDa) localize rapidly in the tumor but also typically clear rapidly from the circulation as they are subject to renal clearance (smaller than the kidneys' glomerular filtration cutoff point of ~50 kDa). Additionally, to maintain good tumor localization, the small macromolecules must have high affinity for the target antigen so that following antigen binding they are retained within the tumor compartment. Small macromolecules with low affinity will be quickly removed from the tumor due to the rapid decrease in blood concentrations leading to a negative concentration gradient. Macromolecules of intermediate size that fall below the kidneys' filtration size (i.e., the widely used scFv, single-chain variable fragments) localize slower to the tumor. Additionally, as these molecules still clear rapidly from the circulation, localization of intermediate size molecules is inferior to high affinity small macromolecules. Macromolecules larger than ~75 kDa (Berndorff et al. 2005) or full length antibodies (150 kDa) are cleared much slower from the circulation, allowing time for the antibody to accumulate within the tumor compartment. Hence, a higher affinity is generally not required to maintain tumor concentrations due to the prolonged presence of the macromolecule in the blood compartment. Further data to support these conclusions were published recently using a series of DARPins (Designed Ankyrin Repeat Proteins) with differing molecular size and affinity profiles (Zahnd et al. 2010).

There are a number of published studies investigating the impact of antibody size on tumor penetration. Different types of antibody or antibody-like fragments

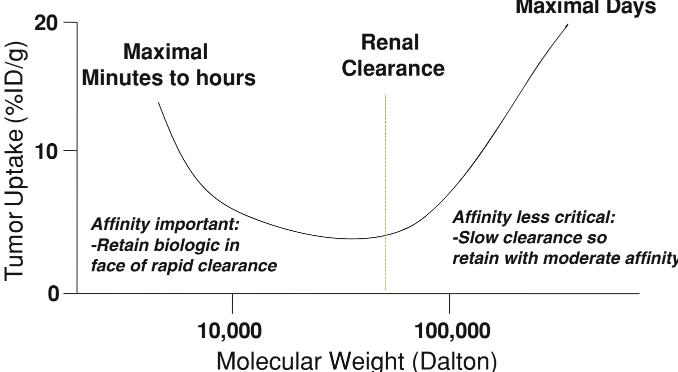

Fig. 9.3 Schematic relationship between tumor localization and size of antibody fragment. The figure is based on data from Zahnd et al. (2010) and Schmidt and Wittrup (2009)

have been generated ranging in size from 10 to 150 kDa (full IgG). In an early study by Yokota et al. (1992), localization of radiolabelled intact CC49 antibody that binds to the TAG-72 antigen, its $F(ab')_2$ fragment (100 kDa), Fab fragment (50 kDa), or scFV (25 kDa) were studied in LS174T colorectal tumor xenograft models in mice. Quantitative autoradiography analysis of surgically excised tumors at various time intervals was carried out with grain density quantified at 10 μm intervals from blood vessels within the tumor. The scFv fragment showed extremely rapid tumor penetration while achieving maximal penetration through the tumor mass within 30 min. In contrast, the intact antibody required 48–96 h to reach an equivalent level of tumor penetration. The Fab and $F(ab')_2$ showed intermediate rates of penetration. Thus, while the scFV showed rapid penetration, the intact antibody was able to distribute through the tumor mass, similar to the scFV fragment, albeit at a slower rate. In a study by Dennis et al. (2007), intact trastuzumab (Herceptin®) was compared to its Fab fragment (Fab4D5) in an orthotropic breast tumor model. In this model, fragments from spontaneous tumors generated in MMTV/HER2 transgenic mice were implanted in the mammary fat pad of the mice. The antibody and the Fab fragments were labelled with a fluorescent cyanine compound, Cy3, to enable fluorescence detection of the antibody in tumor sections after antibody administration. The Fab fragment showed strong staining homogeneously across the tumor by 2 h, with complete loss of signal by 48 h. In contrast, intact trastuzumab showed little penetration at 2 h, but by 24 and 48 h, a more pronounced penetration was observed with the majority of the cells stained with trastuzumab, consistent with the study of Yokota et al. (1992).

The time-dependent penetration of the intact antibody in tumors has also been shown in a range of other studies. For example, in a study by Baker et al. (2008), following administration of single doses of trastuzumab, the distribution of unlabelled antibody was examined in MDA435/LCC6 Her2-transfected tumor xenografts in mice. Animals were treated with 20 mg/kg trastuzumab and tumors were

excised at 3 or 26 h post antibody administration. A heterogeneous antibody distribution with trastuzumab, predominantly adjacent to the perfused blood vessels, was observed 3 h post antibody dosing. By 26 h, a more substantial distribution of the antibody throughout the tumor was observed although a few sections remained unstained even in the perfused areas. In a recent study by Lee and Tannock (2010), distribution of trastuzumab and cetuximab was examined in 231-H2N Her2- and A431 EGFR-expressing tumors, respectively. Similar to the studies described above, at early time points (30 min and 4 h post dose administration) there was a gradient of decreasing antibody concentrations at increasing distances from the blood vessels, but at 24 and 48 h, both trastuzumab and cetuximab were distributed relatively uniformly within the tumor at a dose of 1 mg/kg. Additionally, there was relatively poor distribution of both antibodies in hypoxic regions as detected by cyanine-5-conjugated mouse anti-EF5 antibody.

The Binding-Site Barrier: Impact of Dose

The binding-site barrier involves the ability of the target antigen to impede tumor penetration. It was first postulated by John Weinstein in the early 1990s and the data to support this hypothesis were published in a guinea pig tumor model (Juweid et al. 1992). In this study, either a tumor reactive antibody "D3" that bound to an antigen present on L10 guinea pig cholangiocarcinoma cells, or a non-binding isotype control antibody, were injected into guinea pigs bearing established intradermally implanted L10 tumors. Distribution within the tumor was measured using a combination of autoradiography and immunohistochemistry (IHC). At a very low antibody dose of <0.1 mg/kg, antigen binding adjacent to the blood vessels significantly impacted tumor penetration 6 h post antibody administration. While some improvement was seen by 72 h, the penetration was far from uniform and large numbers of antigen positive cells remained unstained. An equivalent dose of the non-binding isotype control antibody homogeneously diffused throughout the tumor in both antigen-positive and antigen-negative areas, indicating that simple physical barriers were not preventing the control antibody from entering the central regions of tumor where the D3 antibody did not reach. These findings supported the view that binding to the antigen by D3 hindered its penetration into the tumor. However, a higher dose of the D3 antibody (i.e. ~2–3 mg/kg) demonstrated a better penetration after 6 h, and by 72 h, there was homogeneous staining of the D3 antibody in the antigen-positive regions—demonstrating that at least in this model, with relatively high antigen density (i.e. 355,000 antigens/cell), the binding-site barrier could be overcome by increasing the antibody dose. The remaining heterogeneity in D3 antibody distribution within the tumors was attributable to heterogeneous tumor antigen expression and density. Thus, in this study, increasing the antibody dose to levels of 2–3 mg/kg completely overcame the binding-site barrier phenomenon. Therapeutic antibodies such as trastuzumab and cetuximab are given at far higher doses to cancer patients,

typically 300–600 mg per dose (equivalent to or >10 mg/kg). Interestingly, the results obtained for the control antibody in this study suggested that, at least in this model, there were no other major impediments to diffusion of the antibody to cells distant from the blood supply. Further supportive data for this observation have been reported in studies where antibody or $F(ab')_2$ fragments, targeting nuclear histone proteins released in necrotic regions of tumors, were shown to penetrate the necrotic regions of animal tumor models distant from the blood supply (Epstein et al. 1988). Additionally, clinical data demonstrating the ability of such antibodies to image tumors have been reported, supporting the fact that antibodies can penetrate through a tumor mass if administered at an appropriate dose and if sufficient time for diffusion through the tumor mass is allowed.

The impact of increasing dose on improving antibody tumor penetration has also been demonstrated in a number of other experimental studies. For example, Blumenthal et al. (1991) demonstrated, using either anti-CEA or anti-mucin antibodies administered in a number of tumor models in mice, that at low doses of 10 μg (corresponding to approximately 0.4 mg/kg) distribution was heterogeneous but at doses of 400 μg (corresponding to approximately 16 mg/kg), where saturation of the target antigen was anticipated, significantly enhanced antibody distribution was observed across the tumor mass. This study also demonstrated that increased tumor penetration was a function of time, consistent with the studies of Baker et al. (2008) and Lee and Tannock (2010). The impact of time on tumor penetration has also been demonstrated by Fidarova et al. (2008), using a fluorescently labelled A5B7 antibody that binds the carcinoembryonic antigen (CEA) in an orthotropic model of colorectal cancer. Localization was investigated in colorectal liver metastases of differing size at 10 min, 1, and 24 h post antibody administration, respectively. While at the early time points the antibody was localized at the periphery of the tumor in medium- and large-sized metastatic deposits, in some of the small deposits (≤125 μm), the antibody had already diffused fully across the tumor. By 24 h, increased penetration of the antibody was seen in the larger tumor deposits, including some diffusion into necrotic regions in the larger tumors, and by 24 h, antibody penetration appeared homogeneous over the viable regions of the tumor.

The properties of the target antigen might influence antibody distribution as well; for example, high antigen density can provide a greater barrier that may impact the dose required to saturate the target antigen and to achieve efficient distribution. Additionally, internalization rate (turnover rate) of the antigen can also impact antibody tumor penetration (Rudnick and Adams 2009). Rapid internalization of the target antigen can act as a potential sink leading to the metabolism of bound antibody and a reduction of tumor penetration (Thurber et al. 2008). For example, Ackerman et al. (2008) examined the impact of antigen turnover rates and antigen expression level on antibody penetration using tumor spheroids to represent a three-dimensional environment. These authors evaluated the penetration of antibodies binding either to the CEA or the A33 antigens. At a low antibody concentration of 1.5 nM, corresponding to approximately 0.23 μg/ml, the penetration of CEA antibodies that internalized at different rates was

examined. The CEA antibody with more rapid internalization (3-fold higher rate) penetrated the tumor spheroid to only about half the depth of the slower internalizing antibody after 48 h. Increasing the concentration by 10-fold to 15 nM completely overcame this barrier, suggesting that at therapeutic antibody plasma levels, typically in the 10–100 μg/mL range, antigen internalization rate may not be a major issue hindering antibody penetration. Additionally, in these studies, the impact of antigen density was also examined and higher antigen density was shown to slow penetration of the A33 reactive antibody through the spheroid. Comparing the CEA and A33 antibodies in LS174T colorectal cancer cell spheroids, low concentrations of the CEA antibody (1.5–3 nM) failed to fully penetrate the spheroid even after 48 h, but at high antibody concentrations (15–150 nM), full penetration of the antibody to a depth of 250 μm by 48 h was observed. A33 antibodies at lower concentrations (1.5–3 nM) achieved complete penetration after 48 h and even at very low doses (70–150 pM) penetrated 150–200 μm after 72 h. The difference in ability of the antibodies to penetrate was linked to the much slower antigen turnover of A33 relative to the internalization rate observed with the CEA antigen. Interestingly, with both antibodies at concentrations that should be achievable in clinical studies in cancer patients, complete penetration was seen to depths equivalent to the oxygen diffusion distance from a blood vessel in a tumor and thus represents the distance an antibody is likely required to penetrate to reach all viable tumor cells. These results highlight that, for high density targets that undergo rapid turnover rates, application of low therapeutic doses of the antibody may result in poor tumor penetration.

Increasing the delivery of the antibody into the tumor compartment from the bloodstream and thus increasing the concentration of antibody in the tumor microenvironment may be another approach for improving tumor penetration. In a recent report, Sugahara et al. (2009) discussed an approach where attachment of the tumor homing peptide, iRGD, to nanoparticles (iron oxide for imaging or albumin embedded paclitaxel for therapy) improved the delivery of nanoparticles to the tumors, resulting in improved imaging and efficacy. In a follow-up study (Sugahara et al. 2010) it was found that the iRGD, when co-administered with a range of agents such as small molecule drugs, antibodies, and nanoparticles could enhance tumor localization. It is believed that the iRGD localizes to the tumor vasculature by binding to integrins and then is enzymatically cleaved to reveal a CendR motif; the binding of this motif to neuropilin then triggers the endocytic uptake. This uptake is believed to help deliver the antibody that is in the vicinity through the endothelial barrier and into the tumor microenvironment. Similarly, co-injection of iRGD with trastuzumab in Her2 expressing orthotopic BT474 human breast tumor xenografts led to a 40-fold increase in the accumulation of the antibody into the tumor 3 h post antibody administration. In the absence of the iRGD, trastuzumab was primarily localized adjacent to the blood vessels, while in the presence of the iRGD, a 14-fold increase in trastuzumab positive areas within the tumor was detected. In efficacy studies, combining trastuzumab with iRGD also led to significantly improved tumor efficacy at equivalent doses of the antibody.

Impact of Tumor Heterogeneity

Heterogeneity of the target antigen within the tumor can have a major impact on therapeutic approaches for antibodies that rely on delivery of a cytotoxic agent or recruitment of effector functions for killing tumor cells. Most tumor antigens have a heterogeneous distribution and expression in primary human tumors. However, tumor models often used for evaluation of antibody efficacy contain homogeneous antigen profiles as generally a single cell line that homogeneously expresses high levels of the target antigen is used in generation of these cell lines. In general, improving penetration may have little impact when heterogeneous tumor expression is observed. In these instances, the ability of an agent to induce cell killing of the adjacent cell lacking the target antigen (bystander activity) can be a much more important consideration. As an example, antibody C242 which targets the "CanAg" antigen has been used as an antibody to deliver either toxins (Debinski et al. 1992; Calvete et al. 1994) or cytotoxic drugs (Liu et al. 1996). In both studies (Debinski et al. and Calvete et al.), either a C242-Pseudomonas exotoxin or a C242-ricin A chain immunotoxin resulted in substantial antitumor activity in Colo 205 human tumor xenografts where the antigen was present homogeneously throughout the tumor. Since the toxin moiety had to be directly delivered to the tumor cell to exert its activity, these antitumor studies supported a rather effective penetration profile of the immunotoxins throughout the tumor mass. Similarly, the C242-DM1 maytansinoid drug conjugate also resulted in effective antitumor activity in Colo 205 tumor xenografts where homogeneous expression of the target antigen was reported (Liu et al. 1996). Additionally, the C242-DM1 drug conjugate resulted in excellent antitumor activity (tumor regressions) in both LoVo and HT29 tumors where, similar to CanAg, heterogeneous antigen expression was observed (Liu et al. 1996). The drug conjugate contained a cleavable disulphide bond, allowing release of free drug following localization in the tumor (bystander activity). In contrast, if the C242 antibody was attached to ricin A-chain and used to treat HT29 tumor xenografts, which heterogeneously express the target antigen, no significant antitumor effect was observed (Blakey et al. unpublished results). These studies illustrate the importance of considering the properties of the target antigen not only in terms of tumor distribution but also in terms of the therapeutic approach employed to achieve antitumor activity.

Clinical Data on Tumor Localization

As discussed in "Tumor Models: Are They Relevant to Man?", a low percentage of administered antibody dose, typically between 0.01 and 0.001% per ID/g in man, localizes to human tumors based on imaging studies. This is comparable to the amount of antibody localized to human tumor xenografts implanted in immunocompromised mice when the concentration of antibody is considered

rather than the percentage of injected dose (Fig. 9.2). There are numerous studies demonstrating penetration of a wide range of antibodies and fragments into tumors in patients (Welt et al. 1990), but there are limited studies which have explored the distribution time-course of the antibody to target antigen within tumors due to the challenges of obtaining frequent tumor biopsies post treatment. Where data are available, due to limited sample collection and dose range studies, the impact of dose and time on tumor penetration cannot be determined.

Studies with either murine (Welt et al. 1990) or humanized (Scott et al. 2005) radiolabelled A33 antibody have been reported. These antibodies recognized the A33 antigen expressed on >95% of colorectal tumors. Both antibodies localized to colorectal liver metastases and biopsies confirmed positive tumor-to-normal tissue ratios typically in the range of 5–10-fold higher than the normal tissues for the majority of the patients. Autoradiography of biopsies with either the murine or humanized antibody showed good antibody distribution within the tumor mass. In the case of the human antibody which had a longer half-life (80–90 vs. 38 h for the murine A33), distribution of the antibody into the tumor demonstrated penetration to central portions of the tumor despite the presence of bulky disease and in some cases extensive necrosis.

Studies with the murine radio-iodinated monoclonal antibody B72.3 recognizing the TAG-72 antigen examined factors which impacted localization of antibody in 20 surgical specimens taken from patients with colorectal cancer following administration of the labelled antibody (Esteban et al. 1987). The most significant factor was the percentage of tumor cells in the specimen. Autoradiography was used to examine the penetration and distribution of the antibody, and although the distribution of the radioactivity was heterogeneous, it was equally distributed in the medial and peripheral regions of the tumor, indicating good penetration of the labelled antibody throughout the tumor mass despite the relatively low doses of antibody in this study (0.3–20 mg per patient). The distribution was consistent with the heterogeneous distribution of the target antigen in the samples although dual labelling studies were not used to confirm this correlation. These studies suggest that heterogeneity of target antigen within the tumor mass probably had a much greater impact on localization rather than penetration of the antibody.

In clinical trials with the BR-96 antibody that recognized the LewisY antigen and was conjugated to doxorubicin, efficacy was limited due to normal tissue (GI tract) localization and toxicity. However, in a few patients where biopsies were obtained and the distribution of the BR-96-Doxorubicin drug conjugate was examined, good tumor distribution was seen with the antibody localized to antigen positive tumor cells. Confocal microscopy revealed intra-nuclear deposition of doxorubicin corresponding to localization of BR96 to the tumor tissue, confirming targeted delivery of the drug (Saleh et al. 2000). The BR-96 antibody is rapidly internalized and so at least in this case, rapid internalization had not prevented tumor penetration. Patients received between 66 and 875 mg/m^2 in this trial, corresponding to approximately 1–10 mg/kg, supporting the fact that at reasonable

dose levels, and even in the face of rapid antigen internalization, good penetration can still be achieved in human tumors.

In another therapeutic approach involving Antibody Directed Enzyme Prodrug Therapy (ADEPT), delivery of an antibody–enzyme fusion protein (MFE-CPG2) was followed by an alkylating agent prodrug; in this study, localization of the fusion protein was examined. Clinical trial biopsies confirmed localization of the CPG2 enzyme to the tumor and co-localization of the MFE-CPG2 with CEA antigen in a liver metastases biopsy just 4 h after drug administration (Mayer et al. 2006). As the molecular weight of the MFE-CPG2 fusion protein was similar to an intact antibody, this example demonstrates that localization and penetration of the drug was not a factor limiting therapeutic efficacy. Finally, in a limited study with the fully human antibody (CNTO 95) which recognizes αv integrin, a single post treatment biopsy from a patient treated with 10 mg/kg of the antibody was obtained (Mullamitha et al. 2007). Evidence of tumor penetration of the CNTO 95 antibody was seen by immunohistochemistry and linked with a reduction of a biomarker (bcl-2) as compared to a pretreatment biopsy from the same patient.

Concluding Remarks

As ultimately, drug concentrations per unit tissue will drive the efficacy profile, it is important to consider localization and penetration of antibody-based therapeutics both preclinically and in clinical studies as part of their development programs. In light of the data that highlight the impact of antibody dose on tumor distribution and penetration, a key factor that needs to be considered in the design of effective translational strategies for development of antibody-based therapeutics is specific considerations with respect to the antibody dose and dosing schedule that should parallel the expected dose to be used in Phase II and III clinical trials. When repeated administration of the therapeutic antibody in the clinic is likely, the impact of maintaining antibody concentrations in the plasma for prolonged periods and under steady-state conditions should be examined before concluding that drug penetration might limit therapeutic efficacy. Similarly, the sampling time will need to be effectively designed and optimized with particular considerations given to the drug pharmacokinetic half-life and the likely dosing schedule in the clinical setting. Properties of the target antigen may impact tumor localization and penetration but there exist limited examples where this has been directly demonstrated in either tumor models or in the clinic to be a limiting factor. The majority of studies which have examined tumor penetration of antibodies and which have used reasonable doses and schedules do not indicate that tumor penetration is a major limitation hindering the clinical efficacy for antibody therapeutics. However, intrinsic antigenic heterogeneity may play a far greater role in the initial resistance to antibody-based therapeutics and this factor is a critical consideration in selecting both the target and the therapeutic approach relevant for development of effective therapeutics for application in oncology.

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Chapter 10
Preclinical Safety Considerations for the Development of Antibody-Based Therapeutics

Lolke de Haan

Abstract It is now clear that the preclinical safety assessment of biologics is a holistic approach that first and foremost takes into account species relevance and requires an in-depth scientific understanding of the in vitro and in vivo properties of the antibody. An in-depth understanding of these properties may allow for the prediction of safe starting doses for clinical first in human trials and for continued patient safety during subsequent clinical development phases. In this chapter, considerations for preclinical safety testing of monoclonal antibodies as well as the relevant regulatory guidelines are described.

Introduction

In the last decade there has been exponential growth in the number of monoclonal antibodies (mAbs) in development for therapeutic use in man. Monoclonal antibodies represent a unique drug class that combines exquisite target specificity and consequent reduced risk of off-target effects with a long half-life, and the opportunity to target mechanisms that cannot easily be addressed by conventional small molecule (or New Chemical Entity, NCE) approaches. In addition, the increased interest in therapeutic mAbs has been fuelled by significant advances in: the discovery, isolation, and production of fully human mAbs, the reduced number of regulatory approvals of NCEs combined with the clinical success of a considerable number of mAbs, the perceived shortened timelines,

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improved chances of success, and as a consequence, reduced cost for the development of mAb-based therapeutics.

This chapter focuses on the considerations for preclinical safety testing of mAbs, and describes the relevant regulatory guidelines as harmonized globally by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). In addition, key considerations for conducting preclinical safety studies (more commonly referred to as toxicology studies) will be discussed and examples to illustrate these considerations are presented. Finally, the utility of preclinical safety data in the selection of starting doses for first-time-in-man studies is discussed.

The key relevant regulatory guidelines for the preclinical safety testing of mAbs are summarized in Table 10.1, and the guidelines themselves can be found on the ICH web site (http://www.ich.org/products/guidelines/safety/article/safety-guidelines.html). It is important to note that what these guidelines provide is indeed guidance, and that regulatory agencies may request for additional studies and/or studies with a different scope to be conducted by the drug sponsor. This may be dependent on the molecule, its mode of action, toxicological or pharmacological findings, disease indication, and other relevant factors. Key guidance for the development of biologics is contained in ICH S6 and the draft ICH S6 Addendum, with additional relevant guidelines from the ICH, European Medicines Agency (EMA), and Food and Drug Administration (FDA) being cited throughout. As the focus of this chapter is on preclinical safety testing of mAbs, specific attention will be given to issues arising in the safety testing of these molecules. However, many of the principles outlined below apply to biologics in general.

The standard for the preclinical safety testing of mAbs has been derived from the existing paradigm for NCEs. The latter involves preclinical testing of a drug substance in two animal species—a rodent and a non-rodent species—to determine preclinical drug-mediated adverse events and toxicity. The use of both a rodent and non-rodent species for toxicity studies—for NCEs, typically the rat and the dog for general toxicity and safety pharmacology studies, and the rat and rabbit for reproductive toxicology studies—have been shown to have considerable predictive value for safety and tolerability in man. Indeed, the results of a multinational pharmaceutical company survey and the outcome of an International Life Sciences Institute (ILSI) workshop investigating the concordance of the toxicity of pharmaceuticals with known human toxicity with data from animal studies, showed a true positive human toxicity concordance rate of 71% for rodent and non-rodent species (Olson et al. 2000). Non-rodents alone were predictive for 63% of human toxicities, while rodents alone were predictive for 43% (Olson et al. 2000). Interestingly, when animal models predicted human toxicity, 94% of these toxicities were observed in studies of 1-month duration or less, regardless of the number of species used (Olson et al. 2000). Together, these data provide compelling evidence for the predictive value, as well as a justification for the use of animals for the prediction of human safety.

While the predictive value of animal models for human safety of mAbs and biologics in general has not been systematically reviewed, a similar preclinical

Table 10.1 Key relevant regulatory guidelines for the preclinical safety testing of mAbs

Guideline	Issuing authority	Key topics
S6: preclinical safety evaluation of biotechnology-derived pharmaceuticals	ICH[a]	Biologics-specific: species selection, study design, duration of studies
S6(R1) Addendum: preclinical safety evaluation of biotechnology-derived pharmaceuticals	ICH	Biologics-specific: Species selection, dose setting, study duration, recovery
S8: immunotoxicity studies for human pharmaceuticals	ICH	Immunotoxicity testing, case-by-case approach
S9: nonclinical evaluation for anticancer pharmaceuticals	ICH	Timing and duration of studies (including reproductive toxicology studies) for oncology drugs
M3(R2): non-clinical safety studies for the conduct of human clinical trials and marketing authorisation for pharmaceuticals	ICH	Timing and duration of preclinical studies to support clinical development
Guideline on strategies to identify and mitigate risks for first-in-human clinical trials with investigational medicinal products	CHMP[b]	Dose setting for first-time-in-human trials
Guideline on similar biological products containing monoclonal antibodies	CHMP	Approach for preclinical development of biosimilar mAbs
Points to consider in the manufacture and testing of monoclonal antibody products for human use	FDA[c]	Tissue cross-reactivity requirements
Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers	FDA	Dose setting for first-time-in-human trials based on MRSD[d] and PAD[e]

[a] *ICH* International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use
[b] *CHMP* Committee for Medicinal Products for Human Use
[c] *FDA* Food and Drug Administration
[d] *MRSD* maximum recommended starting dose
[e] *PAD* pharmacologically active dose

safety paradigm has been implemented for this different class, with a number of key differences. For example, central to preclinical safety testing of mAbs is species selection, which involves the identification and use of pharmacologically relevant species for in vivo studies. Because of the high selectivity of mAbs, achieving cross-reactivity against the target antigen in multiple species is challenging, and therefore preclinical safety studies with mAbs are often conducted in a single species only, most often the non-human primate (NHP). NHP in the context of this chapter refers to the monkey species which is most commonly used in preclinical safety testing, i.e., the cynomolgus monkey (crab-eating macaque—*Macaca fascicularis*). It should be recognized, however, that other NHP species are used for preclinical safety testing—such as the rhesus monkey

(rhesus macaque—*Macaca mulatta*), and the marmoset (common marmoset—*Callithrix jacchus*), which each present with their own benefits and limitations. Given that conducting preclinical studies in NHPs presents with a number of ethical, logistical, and scientific issues, implications of using NHPs for preclinical safety studies with mAbs will be given specific attention throughout.

ICH S6: The Regulatory Framework for Preclinical Safety Testing of Biologics

Since guideline ICH S6 (Preclinical Safety Evaluation of Biotechnology-derived Pharmaceuticals) was adopted in 1997, a dedicated regulatory guideline has been in place that recognizes the distinct properties and issues arising in the preclinical development of biologics. The guideline is broadly applicable to all biologics, defined as products derived from characterized cells through the use of a variety of expression systems including bacteria, yeast, insect, plant, and mammalian cells. This includes but is not limited to cytokines, recombinant blood products, growth factors, fusion proteins, enzymes, receptors, hormones, mAbs, and antibody fragments. However, in this chapter the focus will be on the implications of the guideline for the preclinical development of mAbs; in the context of this chapter mAbs and biologics can generally be used interchangeably. It should be recognized, however, that mAbs only represent a subclass of therapeutics that are referred to as biologics, and that they have their own unique properties that impact preclinical safety testing. An Addendum to the ICH S6 guideline is reaching the final stages of approval. The draft ICH S6 Addendum is reflective of the significant improvement in our understanding of the pharmacokinetic (PK) and pharmacodynamic (PD) properties of mAbs/biologics, and the consequent exaggerated pharmacology and toxicity that may arise upon dosing in preclinical species and man. Key topics of ICH S6 are species selection, study design, duration, and scope. A unique requirement for mAbs also described in ICH S6 is tissue cross-reactivity studies. Each of these topics is described below.

Species Selection

As indicated above, for NCEs in general, two animal species—one rodent and one non-rodent—are used for preclinical safety testing. Important considerations for species selection for NCEs include PK, metabolism (including the formation of reactive and genotoxic metabolites, bioavailability, interaction with metabolizing enzymes, etc.), and sensitivity to specific target organ toxicity of the selected species. As toxicity seen with NCEs is not usually mediated by pharmacologically mediated effects but rather due to off-target effects, affinity of the drug for the

target and consequent pharmacological activity is generally considered to be of lesser importance for toxicity. The rat typically is the preferred rodent species for general toxicology studies with NCEs, with the dog generally being the non-rodent species of choice, unless there is a scientific justification for using NHPs (or other species) as the non-rodent species. This justification could be based on tolerability, species sensitivity, and metabolic considerations.

In contrast to NCEs, the ICH S6 guideline stipulates that for mAbs the use of pharmacologically relevant species for toxicology studies is central to the preclinical safety assessment, as also reviewed by Chapman et al. (2007) and Bussiere (2008). The reason for this is that the toxicity of mAbs (and biologics in general) is mediated by their pharmacological activity, and manifests itself as exaggerated pharmacology in the setting of toxicology studies. A relevant species is defined in ICH S6 as a species in which the mAb is pharmacologically active due to the expression of the target or epitope. ICH S6 advocates the use of two relevant species (one rodent and one non-rodent) for preclinical safety studies with mAbs. However, due to their exquisite specificity, mAbs are often highly species selective and cross-reactivity to multiple species is not commonly achieved. Generally, a higher level of protein sequence identity is observed between humans and NHPs than between humans and rodents or the dog, which is a direct consequence of the greater evolutionary distance between humans and rodents and the dog. This increases the likelihood of the NHP being a relevant species for preclinical safety testing of a mAb. Moreover, due to the high specificity of mAbs, often cross-reactivity to only one single species is obtained, with the most likely species to which this cross-reactivity is achieved being the NHP. According to ICH S6, in case cross-reactivity against only one single species is observed, a single species preclinical safety program is justified. However, ICH S6 does not explicitly rule out the use of species other than NHP for single species safety programs, and single species preclinical safety programs in a rodent species or the dog should be permissible (if unlikely). Also, there is historical precedent for using the chimpanzee (*Pan troglodytes*) as a relevant species, as in the case of infliximab (Remicade®, anti-TNF-α), efalizumab (Raptiva®, anti-LFA-1), and keliximab (anti-CD4) (Anderson et al. 1997; Treacy 2000; Newman et al. 2001; Clarke et al. 2004; Wu et al. 2006). It should be noted however, that in case any of the great apes [e.g. chimpanzee, gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus* or *Pongo abelii*)] are relevant species for preclinical safety testing, significant restrictions are in place for using these species for research. In Europe, according to European Directive 2010/63/EU, the use of the great apes for research purposes is no longer permitted and in the US there are ongoing efforts to ban use of great apes in research (through the Great Ape Protection Act). Furthermore, the scope of the studies that can be conducted in these animal species is also extremely limited and only PK and PD endpoints can be evaluated; high, toxicologically relevant doses or terminal endpoints cannot be evaluated for humane reasons. Therefore, the territory in which studies involving great apes can be conducted as well as the scope of these studies is severely restricted.

The above may suggest that sequence identity alone is sufficient for assessing species relevance or indeed concluding non-relevance of a species. However, basing species relevance on the basis of sequence identity alone can be misleading. For example, it is possible that the overall sequence identity is not representative of sequence identity at the epitope level (see also the example below). Therefore, as a minimum, it is recommended that both sequence identity and binding to the target is assessed when determining species relevance. If binding is detected, the relative binding affinity to the human target and the target in the preclinical species is an important factor in determining suitability of a preclinical species. In case a significant drop-off in binding affinity is observed, it is important to use PK/PD modeling simulations to determine whether or not sufficient suppression or activation of the target can be achieved in the preclinical species in vivo. Finally, it is important to assess the functional consequences of binding, as binding alone does not guarantee functional activity. Ideally, this functional activity is demonstrated both in vitro in cell-based potency assays as well as in vivo, for example in PK/PD studies and/or in pharmacology models.

Recently published data on the IL-15-specific DISCO280 mAb provide an example of the challenges that may arise in determining species relevance (Finch et al. 2011; Lowe et al. 2011). Protein sequence data revealed an overall sequence identity of >95% between human and cynomolgus monkey IL-15, suggesting a high likelihood that cynomolgus monkey cross-reactivity would be obtained during the phage display-based mAb isolation process. However, in in vitro assays the lead mAb DISCO280 demonstrated a $\sim$100-fold drop-off in affinity for human IL-15 (hIL-15) compared to the cynomolgus monkey IL-15 (cIL-15) ortholog. Furthermore, in in vitro biochemical competition binding experiments and CTLL2 and Kit225 cell line-based potency assays, DISCO280 showed an even greater drop-off in potency against cIL-15. The X-ray crystallography data generated for the DISCO280 Fab complexed to hIL-15 showed that Leu52 in hIL-15 is involved in the epitope of DISCO280 on hIL-15 (Lowe et al. 2011); in cIL-15, Leu52 is replaced with His, and it is highly likely that this explains the drop-off in binding and potency against cIL-15. Finally, in a murine pharmacology model involving hIL-15-stimulated increases in the number of splenic and peripheral blood NK1.1^{+} and CD3^{+} cells, DISCO280 augmented rather than suppressed the hIL-15-mediated effects (Finch et al. 2011). Together, these findings illustrate that species relevance is defined by a combination of sequence identity, in vitro binding and potency, and in vivo pharmacodynamics or pharmacological activity. Moreover, basing conclusions on only one of these properties can be misleading.

An additional consideration for determining suitability of a toxicology species is the potential for immunogenicity of the mAb. Given that mAbs are protein molecules that can be recognized as foreign by the immune system of the preclinical species, they can therefore be processed and presented by antigen presenting cells to B and T cells in the context of major histocompatibility (MHC) molecules, potentially eliciting anti-drug antibody responses (ADAs). Anti-drug antibodies can mediate a range of different effects (De Groot and Scott 2007; Swann et al. 2008). They can be binding, and as such have no effect on exposure,

activity, or potential toxicity of the mAb. ADAs can also affect clearance and either reduce or sustain exposure to the mAb. Finally, ADAs can also neutralize the activity of the mAb or mediate toxicity due to immune complex deposition. Importantly, preclinical immunogenicity is not generally predictive of immunogenicity in man. However, ADAs impact on the relevance and validity of the preclinical studies for safety in man. Immunogenicity testing therefore aids significantly in the interpretation of preclinical safety studies. As outlined in ICH S6, the propensity of a preclinical species to mount antibodies to the therapeutic mAb has significant impact on species selection. As discussed above, rodents and dogs share lower proteome sequence identity with man, and therefore mAbs are generally more immunogenic in rodents and dogs than in the NHP. This may in itself prevent the use of rodents and dogs for repeat dose studies with fully human mAbs. It should be noted however, that mAbs are not always immunogenic in rodents, and there are examples of fully human mAbs that have shown little evidence of immunogenicity in rodents upon subchronic or chronic dosing. Rodents can therefore not be excluded from preclinical safety studies solely on this basis. Monoclonal antibodies generally exhibit a longer half-life in NHPs compared to dogs, with the consequent exposure and prolonged PD effects being more similar to man. Furthermore, NHPs demonstrate greater tolerability to mAbs, with the dog being more prone to raising ADAs and anaphylactoid responses. Therefore, the NHP is generally the preferred non-rodent species for preclinical studies with mAbs. However, while mAbs tend to be less immunogenic in NHPs, primate species can also mount significant antibody responses against mAbs that may lead to the effects described above.

As already outlined above, another key consideration in assessing species relevance is the ability to demonstrate PD effects in vivo. PD effects can be determined in various ways, for example by demonstrating target engagement by measuring free and total (free + mAb complexed) soluble target or by receptor occupancy assays, or measurement of downstream markers of target engagement (Tabrizi et al. 2009). PD effects can often most easily be demonstrated in pharmacology models. However, the availability of such models in non-rodent species is restricted, which limits the ability to demonstrate PD effects. Pharmacodynamic endpoints in NHPs are therefore often restricted to markers of target engagement, which may not always represent true markers of pharmacology. If a target is not expressed or only expressed at a very low level in naive animals, the utility of conducting long-term toxicology studies in two, or even one species is questionable as the likelihood of the mAb to induce exaggerated pharmacology is low. ICH S6 states that it may be possible to justify the use of only one species for long-term toxicity studies if the toxicity profile of the drug in two species is comparable in the short term. The ICH S6 Addendum stresses this point (see also below), and in the case of targets that are expressed at a low or undetectable level, a similar argument could possibly be made. If a target is only expressed under disease conditions, ICH S6 suggests preclinical studies may be performed in animal models of disease as an acceptable alternative to standard toxicity studies in naive

animals. However, many of these models are often acute in nature and have not been fully characterized, and therefore this is generally not considered an attractive path forward for preclinical development.

Pharmacodynamic endpoints can also be used as an indirect way of demonstrating meaningful exposure. In the absence of immunogenicity testing, if the anticipated PK profile is obtained and PD is maintained during dosing, this would represent evidence that full pharmacology was maintained. This approach can have particular impact on study duration, as many study designs for preclinical safety studies with mAbs include long recovery periods to allow for drug washout. In addition to assessing recovery from drug-mediated effects, these long recovery periods are often included to assess immunogenicity with assays that are intolerant to high levels of drug. However, if there is no evidence of abnormal PK and PD is maintained, this observation could be sufficient justification for terminating the study. This is further emphasized in the ICH S6 Addendum and discussed below.

Finally, species differences in target biology, background pathology, and other factors also need to be taken into consideration when selecting a species. If the observed mAb-mediated effects are relevant to man, the most sensitive preclinical species may provide a more accurate assessment of safety.

Preclinical Safety Testing Approaches for mAbs Where No Pharmacologically Relevant Species can be Identified

In case no cross-reactivity to any preclinical species is obtained using methodologies outlined above, ICH S6 outlines that consideration should be given to the use of transgenic animals or preclinical studies with a homologous protein or surrogate antibody, as reviewed by Bussiere et al. (2009). Given that toxicity mediated by mAbs is directly linked to their pharmacology, preclinical safety studies in non-pharmacologically relevant species are discouraged and are unlikely to provide relevant data for human safety.

A number of options remain for preclinical testing of mAbs for which no relevant preclinical species exist, including the use of surrogate mAbs and transgenic animals; however, these options present a number of important challenges. The surrogate antibody approach involves the use of a mAb to the same target as the clinical candidate in the preclinical species. This often requires the isolation and in depth in vitro characterisation of the surrogate mAb, in order to ensure that the surrogate is truly representative of the clinical candidate in terms of affinity, specificity, potency, epitope, in vitro PD, etc. The surrogate can also undergo in vivo testing in pharmacology models, which may predict the potential pharmacology of the clinical candidate in man. At the same time, given that the clinical candidate cannot be tested in vivo other than in the clinical setting, and that subtle changes in affinity, potency, mode of binding, etc. can affect the mode of action of a mAb, the safety evaluation conducted with the surrogate is often considered for

hazard identification only. With regard to selection of a species for a surrogate, generally the preference would be to use a surrogate selective for the same target in the rodent. However, if there is a scientific justification for using a surrogate mAb in a higher species, a higher species can be used, and there are reports on the use of surrogates in NHPs (Bussiere et al. 2009). An added complexity that should be considered is the mAb isotype and antibody effector function if this biological activity is key to the mode of action of the clinical candidate. It is important to establish whether or not the Fc domain of the surrogate mAb can fix complement and mediate complement-dependent cytotoxicity (CDC), or bind Fc receptors and mediate antibody-dependent cellular cytotoxicity (ADCC) in a manner comparable to the clinical candidate. For example, the IgG2a isotype in the mouse matches the effector function of human IgG1, while mouse IgG1 does not mediate effector function (Hulett et al. 1994).

Some regulatory success has been obtained using the surrogate antibody approach, with surrogate mAbs muM17 and cV1q having successfully supported licensing of infliximab and efalizumab (Treacy 2000; Clarke et al. 2004; Wu et al. 2006). It is important to note that in both cases the respective drugs were also evaluated in the chimpanzee at various doses and study durations (for up to 5 days' duration for infliximab, and studies involving weekly dosing for up to 26 weeks for efalizumab). However, the preclinical package in support of licensure of the human selective anti-complement factor 5 (C5) mAb eculizumab (Soliris®) was solely based on studies with the mouse BB5.1 surrogate mAb (Frei et al. 1987), which was used in two repeat dose toxicity (4 and 26 weeks in duration, weekly dosing) and three reproductive toxicity studies (see also below) (FDA eculizumab pharmacology review, http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm). These cases highlight the regulatory acceptance for the use of surrogate approaches during the preclinical development of mAbs.

If the use of a surrogate mAb is not preferred, transgenic mouse models can be used to assess preclinical safety. These transgenic animals could be knock-outs (KOs) or knock-ins (KIs), (i.e. mice in which the target of interest is genetically deleted or overexpressed), or so-called knock-out/knock-in (KOKI) models, in which the mouse target has been replaced at the genetic level with the gene of the human ortholog. While these options are specified in ICH S6, not many sponsors have used this approach as it is fraught with a number of issues. For all these transgenic models, it is essential that the biology and background pathology of the transgenic is well understood. In particular, in KO models and overexpressing transgenic strains, there are risks around adaptive responses and the emergence of specific histopathological background findings. These models may also not always be representative of mAb-mediated effects, as in KO models the gene of interest is usually deleted from all tissues, including from tissues that a mAb may not normally penetrate. Furthermore, mouse KO models have the added complication that they can be associated with embryonic lethality. It may be possible to avoid embryonic lethality by using conditional KOs, in which targeted disruption is limited to a specific developmental stage or restricted to certain tissues (Guan et al. 2010). However, whether or not this approach impacts the relevance of the safety

assessment must be considered. In overexpressing transgenic strains, the level and site of production may not recapitulate the normal in vivo situation and lead to deleterious effects. For example, human TNF-α overexpressing transgenic mouse strains have been developed, with some of these developing a lethal wasting syndrome resulting in 80–100% mortality at 10–18 weeks after birth (Probert et al. 1993; Douni et al. 1995). Again, some of these effects may be circumvented by limiting the level of expression and/or by tissue or cell type-specific expression of the target—as with conditional KOs, the latter may impact the relevance of the safety assessment.

The mouse KO/KI models represent the greatest level of complexity, both from a generation and characterization perspective, but have the benefit of having the ability to be used for testing the clinical candidate in vivo. However, these models must also be characterized from a biological and histopathological perspective as abnormalities may occur. The most important aspect to consider is the interaction of the transgene with ligands and/or receptors in the preclinical species. For example, for some biological systems multiple ligands signal through the same receptor, and in those cases it is very important to assess whether the ligands from the preclinical species signal through the human receptor. It is also important to establish whether the spectrum of ligands is identical to those in man, and verify whether any downstream consequences that may arise following ligand–receptor interaction are similar to those seen after interaction between the native ligand and receptor. Should this not be the case, a double KO/KI model could be considered in which both the human ligand(s) and receptor are knocked in, but this obviously adds further layers of complexity. One of the most widely published and successful examples of the use of a transgenic species for preclinical safety testing is the human CD4 transgenic mouse model that supported the development of the human/chimpanzee cross-reactive anti-CD4 mAbs keliximab (IgG1—CD4 cell depleting) and clenoliximab (IgG4—not depleting CD4 cells) (Reddy et al. 2000; Sharma et al. 2000; Newman et al. 2001). While the acute effects of clenoliximab and keliximab were studied in chimpanzees, the transgenic mouse model was employed to study the effects of comparative PD, single and repeat dose toxicity, reproductive toxicity, and also to assess potential effects on host defense (Chirmule et al. 1999; Bugelski et al. 2000; Podolin et al. 2000; Reddy et al. 2000; Sharma et al. 2000; Herzyk et al. 2001; Newman et al. 2001; Herzyk et al. 2002). The CD4 transgenic mouse model was successfully used to support safety and to predict PK/PD relationships in clinical trials (Kon et al. 1998, 2001; Mould et al. 1999).

From a drug discovery perspective both surrogate and transgenic approaches require significant effort, both in time and cost. For surrogate mAbs, consideration needs to be given to the time and cost associated with the isolation and assessing in vitro and in vivo properties of the mAb, assay development (PK, PD, and ADA assays), as well as the significant efforts required to manufacture the surrogate mAb, including the physicochemical characterization (e.g. glycosylation, stability, aggregate formation, deamidation, etc.), and formulation development that follows. Therefore, this means two mAb molecules (i.e. the surrogate and clinical

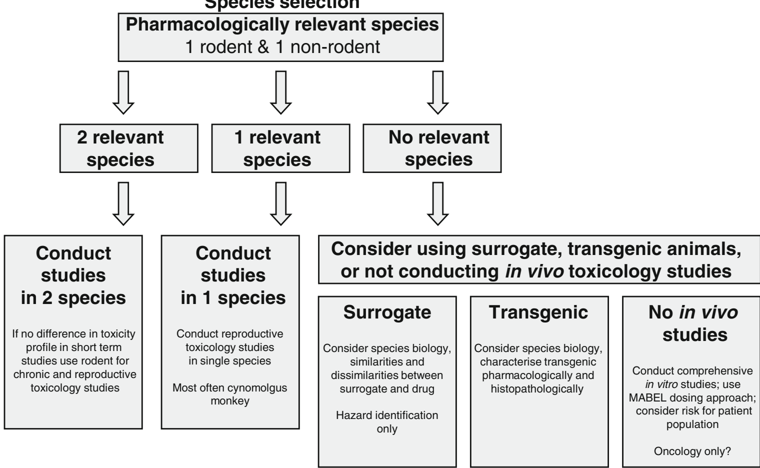

Fig. 10.1 Summarises species selection for preclinical safety studies

candidate) will essentially have to be co-developed. With regard to transgenic approaches, the main drawbacks are time and cost required to generate and characterize these models as outlined above. At the same time, for both surrogate and transgenic approaches there are risks around acceptability of these models by regulatory agencies. Careful consideration to species cross-reactivity should therefore be given during the lead isolation stage in the mAb discovery phase, to ensure the appropriate species cross-reactivity is built in, and that acceptable models for preclinical safety testing are available.

If no relevant species can be identified and none of the above approaches can be followed, it is possible to support clinical dosing based on in vitro data with the clinical candidate only. This approach carries the most risks and can often only be justified in lethal indications (e.g. in oncology), or for preclinically and clinically well-established biological mechanisms. This approach involves an in-depth characterization on the in vitro properties of the candidate, and using the most sensitive in vitro marker of mAb activity as a basis for determining a Minimum Anticipated Biological Effect Level (MABEL) dose that can be safely administered to humans. The MABEL concept is further explained in the section describing considerations for first-time-in-man (FTIM) dosing.

The flow diagram in Fig. 10.1 summarizes species selection for preclinical safety studies. As outlined above, ICH S6 outlines the use of two pharmacologically relevant species (one rodent and one non-rodent species); however, if only one relevant species is identified, a single species preclinical program is acceptable. If no relevant species are identified, the options that remain include the use of a surrogate mAb, transgenic animals, or an in vitro-based approach as outlined above. In the event no relevant species are identified, alternative approaches to

provide safety data must be considered—such approaches are highly dependent on an understanding of the target biology, availability of tools and reagents, as well as time and resource constraints.

Study Design, Duration, and Scope

ICH S6 contains guidance on study design, duration, and scope. With regard to duration and timing of studies, these typically do not differ from the ICH M3 guidance (Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorisation for Pharmaceuticals). The overall goal of preclinical safety or toxicity studies should be to characterize toxic effects with respect to target organs, and establish dose dependence and relationship to exposure. Information from initial studies will be used to estimate a safe starting dose and dose range for clinical trials and identify parameters for clinical monitoring for potential adverse effects. With regard to study scope, ICH S6 provides specific guidance on the conduct and design of single and repeat dose studies, safety pharmacology, immunotoxicity, developmental and reproductive toxicity studies, genotoxicity and carcinogenicity studies, as well as overall relevance to biologics. This is further detailed below.

Study Design and Duration

ICH S6 recognizes that the number of animals used per dose group is directly related to the ability to detect toxicity, and that studies involving small group sizes can be misleading. However, it is also recognized that NHPs are often the only relevant species for preclinical safety evaluation of mAbs and generally speaking, group sizes of three males and three females each for short term subchronic and chronic studies are acceptable. For rodent studies, group sizes of 10 males and 10 females each are often used (excluding satellite animals for toxicokinetic sampling). The route of administration should mirror the clinical route, while for the frequency of administration, consideration should be given to PK and bioavailability of the mAb in the preclinical species. Given the faster clearance rates of human mAbs in the preclinical species, this often means the frequency of administration is increased to weekly for studies in rodents and NHPs. As mAbs are typically administered via intravenous or subcutaneous routes, local tolerance should be assessed in single dose and on an ongoing basis in repeated dose studies using dermal Draize scoring (Draize 1959) as well as histopathological evaluation at termination. Generally, histopathological changes associated with injection site reactions are most apparent within 2–3 days of dosing, and typically resolve within a week. Therefore, in case local tolerance assessment is included in single and/or repeat dose toxicology studies, this should be taken into consideration and animals should be terminated within 2–3 days after the final dose.

Especially in early toxicology studies, dose levels should be selected such that a pharmacological dose response as well as a relationship between pharmacology and toxicity (exaggerated pharmacology) can be obtained. Depending on the pharmacological activity of the mAb in naive animals, it may be desirable to select a dose level that does not fully saturate the target as the low dose in early preclinical studies, with the mid and high dose groups representing multiples of the dose that mediates full pharmacology and/or provides a significant multiple of the clinical exposure, in an attempt to identify a toxic dose and a no-observed-adverse-effect-level (NOAEL). It is recognized that mAbs in particular are not often associated with acute toxicity, and a maximum tolerated dose (MTD) is not often identified. A justification for high dose selection in those cases is provided by projected multiples of the human dose combined with the practical limitations of the available formulation. The relative affinity and potency of the mAb for the human target and the target in the preclinical species should also be taken into consideration when selecting dose levels, as a drop off in affinity or potency for the target in the preclinical species may mean that higher doses are required to achieve target saturation relative to man.

There is no formal regulatory requirement for single dose toxicity studies with mAbs. This is consistent with the updated ICH M3 guideline, which, following a drive from European pharmaceutical industry experts (Robinson et al. 2008), now states single-dose toxicity information can be obtained from appropriately conducted dose-escalation studies or short-duration dose-ranging studies that define an MTD in the preclinical species used in general toxicity studies. However, single-dose studies can be extremely useful for assessing PK/PD relationships. For repeated dose toxicity studies, ICH S6 states that typically these should include an assessment of toxicokinetics and reflect the intended clinical use or exposure. Repeat dose toxicity studies should also assess recovery, which is defined as an assessment of the reversal or potential worsening of pharmacologically mediated or toxicological effects, and/or potential delayed toxicity due to prolonged target suppression during the recovery phase due to the long mAb half life. The ICH S6 Addendum contains further useful guidance on the utility of recovery and is discussed below.

ICH S6 states that the route and frequency of administration should be as close as possible to that proposed for clinical use. Whilst this provides some guidance with regard to timing of studies, ICH M3 in principle applies to both NCEs and biologics and specifies that the timing and duration of preclinical safety studies should mirror or exceed the frequency of dosing and duration of exposure in human clinical trials. For mAbs this means that typically up to 4 weeks dosing supports a single dose in man, 3-month studies support 3 monthly doses in the clinic, and so forth. To support chronic dosing (i.e. >6 months) in the clinic, studies of 6 months' duration have generally provided sufficient preclinical cover. Indeed, a retrospective analysis of chronic toxicology studies conducted with 23 biotechnology-derived products showed that for only two of these products, new findings emerged in studies of greater than 6 months' duration (Clarke et al. 2008).

Of these, one was associated with a well-established risk of dosing human mAbs to animals (immune complex deposition), while the other case was associated with tumor formation after 12 months' dosing (Clarke et al. 2008). As determination of carcinogenic potential is not a goal of chronic toxicity studies, this was not considered relevant. In light of the mounting evidence that chronic studies of 6 months' duration are sufficient to capture all potential preclinical safety findings and support chronic dosing in man, the Expert Working Group drafting the ICH S6 Addendum sought to clarify this point further (see below). For mAbs developed in an oncology indication, ICH S9 (Nonclinical Evaluation for Anticancer Pharmaceuticals) applies. To support continued development in patients with advanced cancer, results from repeat dose studies of 3 months' duration should be provided prior to initiating Phase III studies and should also be sufficient for registration.

Study Scope: Safety Pharmacology, Immunotoxicity, Developmental and Reproductive Toxicity, Genotoxicity, and Carcinogenicity Assessment

Pharmacology

Safety pharmacology studies are a regulatory requirement and conducted routinely to support development of NCEs according to guidelines ICH S7A (Safety Pharmacology Studies For Human Pharmaceuticals) and ICH S7B [The Non-Clinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals]. However, as stated above, the risk for off-target effects of mAbs on the major organ systems is much lower than that for NCEs, due to their much greater specificity and selectivity, size, and reduced ability to penetrate tissues. For example, it is recognized that mAbs do not interact with the hERG (human ether-à-go–go related gene) channel to induce QT interval (QTc) prolongation (Vargas et al. 2008). Therefore, for mAbs and biologics in general, stand-alone safety pharmacology studies are not usually conducted unless there is a cause for concern, either based on the mode of action, tissue binding, etc. However, it is recommended that safety pharmacology endpoints such as electrocardiogram (ECG), blood pressure and respiratory rate measurements are routinely included in repeat dose toxicity studies in NHPs (Vargas et al. 2008), while, for example, limited functional observational tests can be included in repeat dose toxicity studies in rodents.

Immunotoxicity

ICH S6 acknowledges that many biologics including mAbs are intended to modulate the immune system and therefore may affect not only humoral but also cell-mediated immunity. As a consequence, potential effects on the immune system should be carefully evaluated in preclinical safety studies to assess potential risk of immune-mediated events that are, or could become adverse.

This includes adverse events such as infusion reactions, cytokine storm, immuno-suppression or -stimulation and autoimmune reactions. Typically, a tiered approach should be followed to assess potential immunotoxicity, starting with assessment of the literature and in vitro studies, to preliminary in vivo assessments and dedicated immunotoxicity studies if warranted. While guideline ICH S8 'Immunotoxicity Studies for Human Pharmaceuticals' states that it does not apply to biotechnology-derived pharmaceutical products, the guiding principles as outlined in ICH S8 would appear to also apply to mAbs. ICH S8 suggests that preliminary evidence for immuno-toxicity should be gained from standard toxicity studies. Endpoints that should be evaluated in standard toxicity studies include standard hematology, organ weights, and/or histology of immune organs (lymph nodes, spleen, bone marrow, etc.), serum globulins, incidence of infections and tumor incidence. Based on the findings in these studies and a review that takes into consideration the biology of the target (based on current knowledge from the literature), the effects seen in standard toxicity studies, the potential clinical risk/benefit profile, intended duration of clinical exposure, clinical precedence and so forth, additional dedicated immuno-toxicity studies may be conducted, or inclusion of additional immuno-toxicity endpoints in standard toxicity studies should be considered. Additional immuno-toxicity endpoints that could be included in standard toxicity studies include flow cytometry to identify potential changes in leukocyte subsets (in the circulation and/or of lymphoid organs), and assessments of immune system function, e.g. by evaluating immune responses to T cell-dependent antibody responses or by determining NK cell activity (Muller and Brennan 2009; Brennan et al. 2010). A well-known effect of prolonged immuno-suppression mediated by mAb therapies is an increase in opportunistic infections, such as the well documented increase in *Mycobacterium tuberculosis* infections associated with anti-TNF-α therapies (Wallis 2009). Preclinical studies to predict these infectious events, for example by conducting host defence studies, are not routinely conducted within the industry as their predictive value for humans is unclear. However, for some mAbs such studies have been conducted (Herzyk et al. 2001; Burleson and Burleson 2008). The utility of these models is restricted to rodent cross-reactive mAbs as host defence models have not been qualified in NHPs.

Developmental and Reproductive Toxicity

Developmental and reproductive toxicity (DART) studies typically assess all stages of the reproductive cycle, including fertility, embryo-fetal development (EFD), and peri- and post-natal development (PPND), historically referred to as Segment I, II, and III studies. An excellent recent review by Martin and colleagues describes the considerations and provides clear recommendations for assessing developmental and reproductive toxicity with biologics (Martin et al. 2009). According to ICH S6, M3, and S5 (Detection of Toxicity to Reproduction for

Medicinal Products & Toxicity to Male Fertility), the requirement for DART studies is dependent upon the product, clinical indication, and intended patient population. The draft ICH S6 Addendum gives additional guidance for DART studies, in particular with regard to the use of the NHP for DART studies (outlined below). As for general toxicity studies, DART studies with biologics in general should be conducted in pharmacologically relevant species and specific consideration needs to be given to immunogenicity, biological activity, and/or elimination half-life. In case only one species is pharmacologically relevant, DART studies in a single species should be sufficient to address the reproductive hazard. ICH S5 Note 5 (2.1) also suggests that if the species selected for the reproductive toxicity assessment is a relevant model for man, a single species is sufficient. Furthermore, it is stressed that there is little value in using a second species if it does not show the same similarities to man.

For only two licensed mAbs, two relevant species for reproductive toxicity studies were identified—the guinea pig (male and female fertility and EFD) and NHP (EFD and PPND) for natalizumab (Tysabri®, anti-α4 integrin), and the rabbit (EFD) and NHP for bevacizumab (Avastin®, anti-VEGF) (Martin et al. 2009; Wehner et al. 2009a, b, c, d). Frequently, for mAbs the traditional rodent and non-rodent species used for DART studies—the rat and the rabbit—cannot be used as they are not pharmacologically relevant. In case there are no pharmacologically relevant preclinical species, alternatives, including the use of surrogates or transgenic animals, need to be considered taking into account all the limitations described in the species selection section above. There are a number of examples where alternatives have proved successful in addressing the reproductive hazards associated with mAbs. With efalizumab and infliximab, both of which are human/chimpanzee cross-reactive, fertility, EFD, and PPND studies were completed in the mouse with surrogate mAbs cV1q and muM17, respectively (Treacy 2000; Clarke et al. 2004). Also, in support of the eculizumab preclinical package, male and female fertility, EFD and PPND assessments were conducted with surrogate mAb BB5.1 (FDA eculizumab pharmacology review on the Drugs@FDA web site, http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm). Finally, with respect to transgenic animals, reproductive toxicity studies in human CD4 transgenic mice with keliximab have been completed and published (Herzyk et al. 2002).

When considering species relevance for DART studies, specific attention should be given to exposure to the mAb during the embryonic, fetal, and postnatal phase. Antibodies are transported across the placenta through the neonatal Fc receptor (FcRn) by receptor mediated endocytosis (Simister and Story 1997; Simister 2003; Chucri et al. 2010). In humans, placental transfer of maternal antibodies, and presumably also therapeutic mAbs, during the first trimester—which represents the period of organogenesis—is minimal, with exposure peaking during the third trimester (Palfi and Selbing 1998; Jauniaux and Gulbis 2000). In preclinical species, there are marked differences in placentation, and these impact the transfer of antibodies. In rabbits and NHPs, transfer of antibodies to the fetus appears to follow a similar pattern to man, and hence exposure during

organogenesis is limited (Fujimoto et al. 1983; Coe et al. 1993; Pentsuk and van der Laan 2009; Martin et al. 2010). However, in rodents there is evidence of transfer of antibodies through the visceral yolk sack early in gestation, and hence organogenesis may be influenced by mAb exposure (Gitlin and Morphis 1969; Masters et al. 1969; Morphis and Gitlin 1970). Therefore, when employing rodents for reproductive toxicity testing with mAbs, teratogenic effects may be observed that may not be relevant to man.

As NHPs are often the only relevant species for the safety testing of mAbs, reproductive toxicity studies may often use the NHP. Given the limited exposure during organogenesis and ethical considerations in using these species, a single reproductive toxicology study design—referred to as an enhanced PPND (ePPND)—has been proposed to assess reproductive risk (Stewart 2009). This study design involves: dosing dams from the time when pregnancy is established [~gestational day 20 (GD20)] until natural birth (around Day 160), monitoring organogenesis/fetal growth using noninvasive techniques (e.g. ultrasound), and assessing the infants for potential mAb-mediated effects from day 30, e.g., by assessing development of the immune system and/or immune function. Similar to stand-alone EFD studies in the traditional species (rat and rabbit), in EFD study designs in NHPs, animals are only dosed during the period of organogenesis (GD20–GD50), followed by cesarean section (C-section) at GD100. However, given the limited exposure during organogenesis, the information that can be obtained at C-section would appear to be limited, and a single combined study would appear to be more relevant and scientifically robust. This approach is consistent with ICH S6 and S5, which state that combining studies is acceptable.

For reproductive studies with NHPs, specific consideration needs to be given to the number of animals per group as spontaneous fertility rates are low (Chellman et al. 2009; Martin and Weinbauer 2010); pre-implantation loss is estimated to be ~25% (Hendrickx and Binkerd 1990), and, in addition, spontaneous post-implantation loss is significant (Small 1982). In an attempt to provide further justification for group size for developmental toxicity studies, Jarvis et al. conducted a post hoc analysis of pregnancy and infant losses until postnatal day 78 from 93 in cynomolgus monkey studies. By using a combined approach of survival analysis and simulation experiments, the influence of group size on the ability to predict adverse pregnancy outcome was explored (Jarvis et al. 2010). This study concluded that a group size of 20 would be required to detect a 3-fold increase in test item-related pregnancy or infant loss (Jarvis et al. 2010). Although the ePPND study design would appear to provide a scientifically sound approach, for mAbs developed for the treatment of patients with advanced cancer, according to ICH S9, PPND studies are not generally required to support clinical trials or marketing. It is possible, therefore, that EFD studies in NHPs will continue to be conducted despite the anticipated low exposure during organogenesis.

Finally, with regard to timing of DART studies, ICH M3 states that for molecules for which embryo–fetal exposure during organogenesis in man is understood to be low, such as mAbs, developmental toxicity studies can be conducted during Phase III clinical studies, with completed reports submitted with the

marketing application. While this may suggest that all reproductive toxicity studies with mAbs can be deferred and conducted in parallel with Phase III clinical studies, it seems likely that the timing of these studies will be dependent on the number of women of child bearing potential exposed in early phase trials, and the consequent perceived overall clinical risk.

Genotoxicity and Carcinogenicity

Similar to safety pharmacology studies, genotoxicity studies are a regulatory requirement and are conducted routinely to support development of NCEs [according to guideline ICH S2A (Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals) and ICH S2B (Genotoxicity: A Standard Battery for Genotoxicity Testing for Pharmaceuticals0)], but are not applicable to mAbs and biologics in general. This is based on the fact that mAbs are large protein molecules that are not expected to cross the nuclear or mitochondrial membrane and interact directly with DNA or other chromosomal material. Furthermore, mAbs do not form (reactive) metabolites and are eliminated mainly via the reticuloendothelial system (RES) and subsequent catabolism, the end products of which are amino acids which enter the existing amino acid pool (Tabrizi et al. 2006). However, in case a drug (e.g. a toxic payload) or another protein is chemically linked to a mAb, such as in an antibody–drug conjugate (ADC), then genotoxicity testing is warranted.

Carcinogenicity testing for biologics has been debated considerably over the last decade. ICH S6 states that standard carcinogenicity bioassays—i.e., 2-year bioassays in rodents—are generally inappropriate for biotechnology-derived pharmaceuticals. Two-year bioassays are routinely performed for NCEs; however, as mAbs and biologics in general are not intrinsically genotoxic or carcinogenic, 2-year bioassays would not seem appropriate. There are also a number of practical considerations that make the 2-year bioassay challenging or even unsuitable for carcinogenicity assessment with mAbs. As also outlined above, mAbs are highly species selective and may not have cross-reactivity to the rodent target. In addition, mAbs have a greater tendency to be immunogenic in rodents, which may preclude chronic dosing. As outlined above, transgenic animals or surrogate mAbs could be considered for use in carcinogenicity studies. However, transgenic models and surrogate mAbs have their own caveats and should not be generated solely for this purpose, as the outcome of such studies may be misleading.

Despite the fact that ICH S6 acknowledges that limitations with respect to assessment of carcinogenicity exist and that 2-year rodent bioassays are not appropriate for biologics, it does suggest that product-specific assessment of carcinogenic potential may still be needed depending upon duration of clinical dosing, patient population, and/or biological activity. A recent collaborative publication by industry toxicologists reviews past and current practice with regard to carcinogenicity testing of biologics (Vahle et al. 2010). The review is based on

publicly available information on 80 marketed protein biotherapeutics, 14 of which are therapeutic mAbs (excluding mAbs not intended for subchronic or chronic use, ADCs, radiolabeled mAbs for imaging, and mAb fragments or Fc fusion proteins). For 51 of the 80 biotherapeutics, a 2-year bioassay in rodents was not conducted (Vahle et al. 2010), and this included the 14 therapeutic mAbs. However, for three mAbs (infliximab, efalizumab, and natalizumab), it is cited that alternative carcinogenicity assessments were conducted. For infliximab, which is human/chimpanzee cross-reactive, a 26-week chronic toxicity study with surrogate mAb cV1q in CD-1 mice was conducted, and no findings relevant to carcinogenicity or tumor promotion were observed (Treacy 2000; Clarke et al. 2004). However, as studies with infliximab in chimpanzees were maximally of 5 days' duration, the main purpose of this 26-week study was likely to support chronic dosing in the clinic. In support of licensing efalizumab, a 26-week study in TGS-p53 wild-type mice with surrogate mAb muM17 was conducted. In this study, hypercellularity of the splenic white pulp and decreased lymphocytic infiltration of various organs (pancreas mandibular salivary gland, and kidney) was noted, but there were no findings relevant to carcinogenicity or tumor formation (efalizumab pharmacology review: http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm). Finally, in support of licensing of natalizumab, two alternative approaches to carcinogenicity testing were employed. In the first approach, the in vitro proliferative and cytotoxic effects of natalizumab on a series of tumor cell lines were investigated. Tumor cell lines were also tested for natalizumab binding. These assays showed no effects of natalizumab on cytotoxicity or tumor cell proliferation. The second approach involved an assessment of the effects of natalizumab on tumor promotion in a mouse xenograft tumor model in severe combined immunodeficiency (SCID) mice and/or the NCR-NU nude mouse model. In neither of these models was there evidence of natalizumab exacerbating primary tumor growth or increasing metastatic tumor formation (natalizumab pharmacology review: http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm).

Based on the above, to assess carcinogenicity of mAbs, alternative investigations such as those conducted with natalizumab could be considered. However, generic guidance for alternative carcinogenicity assessment of mAbs (and biologics in general) cannot be given, and any assessment should be based on a weight of evidence-based approach for which the scientific knowledge of the mAb target, antibody mode of action, duration and extent of target suppression, and clinical precedence should be taken into consideration. For example, mAbs that induce cellular proliferation are more likely to be associated with an increased risk of tumor development, and in vitro studies combined with an assessment of proliferative lesions in chronic toxicity studies may provide an adequate assessment of risk. Similarly, for mAbs that are immuno-suppressive, susceptibility to certain tumor types such as lymphomas and melanomas can be increased. Characterization of the extent of immuno-suppression might in this case provide valuable information for risk assessment. In the end, it is important that

preclinical data provide a rational risk assessment that will translate into meaningful and useful product labeling.

Tissue Cross-Reactivity

According to ICH S6, tissue cross-reactivity (TXR) studies should be conducted with antibody and antibody-like molecules (e.g. Fabs, single chain antibodies, etc.) to identify target and off-target binding. Such studies may also identify sites of on-target binding in tissues that were not previously identified as expressing the target, and thereby identify potential target organs for toxicity. In the FDA's 'Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use' from 1997, a list of 32 tissues is specified that should be investigated for tissue cross-reactivity. This list comprises the following cells and tissues: adrenal, bladder, blood cells, bone marrow, breast, cerebellum, cerebral cortex, colon, endothelium, eye, fallopian tube, gastrointestinal tract, heart, kidney (glomerulus, tubule), liver, lung, lymph node, ovary, pancreas, parathyroid, pituitary, placenta, prostate, skin, spinal cord, spleen, striated muscle, testis, thymus, thyroid, ureter, and uterus (cervix, endometrium). As outlined in a recent review (Leach et al. 2010), current Good Laboratory Practise (GLP) TXR studies involve immunohistochemical (IHC) staining of a panel of frozen tissues from three different unrelated human donors and from two different unrelated donors for tissues from relevant preclinical species—these stipulations are also a regulatory requirement prior to dosing humans. However, while in the S6 guideline it is suggested that the TXR profile may be used as an indicator of species relevance, the draft ICH S6 Addendum de-emphasizes the use of TXR for this purpose and recognizes that this technique should not be employed to demonstrate species relevance. Nevertheless, when no relevant species can be identified for preclinical safety testing, and alternative approaches such as the use of surrogate mAbs or transgenic animals are being considered, comparing the TXR profile for the surrogate mAb on tissues with that of the candidate mAb on human tissues is important; alternatively, comparing the TXR profile of the mAb on tissues from the transgenic species and human tissues is also imperative.

TXR studies may present with considerable challenges. In many instances the mAb target is expressed at very low levels and thus is undetectable by IHC staining, the epitope and/or the mAb is unsuitable for use in IHC methodology, or unexpected staining is observed, which may call into question the specificity of the staining method and/or the relevance of the study. Furthermore, in many instances differences in staining are observed across human tissues and tissues from the relevant preclinical species, which could call into question species relevance. Finally, in only a few cases has unexpected binding in a TXR study translated into toxicity—this may however increasingly be the case for ADCs delivering a toxic payload (Leach et al. 2010).

The Draft ICH S6 Addendum

The ICH S6 guideline was originally adopted in 1997 and since then there have been significant improvements in our understanding of biologics and mAbs both in preclinical and clinical settings. In addition, there appears to be some discord across regulatory regions as a result of differences in implementation and interpretation of the ICH S6 guidance. Therefore, it was agreed that an Addendum to ICH S6 should be formulated to facilitate its understanding and harmonize its application. The main topics that are addressed in the Addendum include species selection, study design, reproductive/developmental toxicity, and carcinogenicity. At present the ICH S6 Addendum has reached the Step 2 version of the four-step ICH process. As the draft guideline—if approved in its current form—could have profound implications on the conduct of preclinical safety studies with mAbs, the key proposed changes and/or clarifications are discussed here. It should be noted however, that this is a draft guideline, and that significant changes could still be made. At present therefore, it is advised to adhere to the ICH S6 guidance and only adopt recommendations made in the ICH S6 Addendum if there is a strong scientific basis to do so.

Species Selection

The S6 Addendum continues to emphasize the use of relevant species and a case-by-case approach for preclinical safety assessment as outlined above. If there are relevant rodent and non-rodent species for preclinical safety assessment of the mAb, both species should be used in short-term toxicity studies. However, if the toxicological findings in both species are similar, the Addendum indicates that longer term studies in one single species are usually considered sufficient. One would presume that in this case, only the rodent species would be used for chronic toxicity and reproductive toxicity assessments. With regard to TXR, the general consensus is that the text in ICH S6 is no longer appropriate, and TXR should not be used for selection of relevant species for safety evaluation. Moreover, TXR studies with tissues from nonclinical species are considered to have limited value and therefore are not generally recommended. In the case of bi-specific antibodies, evaluating each binding site separately is not considered useful. Specific reference is also made to mAbs directed against foreign antigens (e.g. bacterial or viral antigens). For these mAbs, it is suggested that safety is evaluated in an animal model of disease. If this is not feasible, a short-term safety study in a single species—with the choice of species being justified by the sponsor—can be considered to support clinical dosing. For ADCs, the requirement for two species safety testing is dependent on the conjugated drug. If a novel toxin or toxicant is incorporated, two species safety testing is recommended, as the conjugated drug is likely to be associated with off-target effects. These species would not necessarily

have to be pharmacologically relevant. At the same time, for toxins or toxicants for which there is a sufficient body of scientific information available, safety evaluation of an ADC in a single relevant species should suffice.

Study Design

The study design section of the ICH S6 Addendum emphasizes dose selection, study duration, and recovery. With regard to dose selection, specific attention is given to setting the high dose for preclinical safety studies. It is recommended that the high dose should be the highest of either: (1) the dose which gives the maximum intended pharmacological effect in the preclinical species or (2) the dose which gives an up to 10-fold exposure multiple over the maximum anticipated exposure in the clinical setting. In these assessments, corrections should be made for differences in target binding and in vitro pharmacological activity between the preclinical species and humans as outlined above. This guidance could lead to significant changes in the design of preclinical studies, as many of these have used very high doses to avoid questions from regulators around maximum feasible dose when no toxicity is observed. However, based on experience, if toxicity cannot be demonstrated by the suggested approach for high dose selection, then additional toxicity studies at higher multiples of human dosing are unlikely to provide further useful information. This could also impact the number of dose groups for preclinical safety studies, as there would appear to be less scope for three dose groups based on a dose range of up to 10-fold the maximum anticipated human exposure.

The ICH S6 Addendum also enforces the view that studies of 6-month duration should be sufficient to support chronic dosing in the clinic, in line with the published literature (Clarke et al. 2008). Finally, with regard to recovery, the ICHS6 Addendum states that in case of any adverse effects, recovery should be assessed in at least one study. The purpose of the recovery period is to examine reversibility of these adverse effects only—not to assess delayed toxicity or evaluate immunogenicity, nor a demonstration of complete recovery. If there is evidence of sustained PD activity (target suppression), without evidence of concomitant abnormal PK that can be ascribed to ADAs and/or no evidence of immune-mediated reactions during the dosing phase of a study, measurement of ADAs in nonclinical studies is not routinely required. As stated above, preclinical immunogenicity is not predictive of clinical immunogenicity, and therefore, the main purpose of the ADA assessment is to ensure study validity and to provide proof of exposure. This emphasizes that recovery animals should be included for recovery from drug-induced lesions or adverse effects, and not for assessment of immunogenicity.

Reproductive and Developmental Toxicity

The guidance for DART studies does not generally deviate from ICH S6 or ICH S5 as outlined above. When the mAb is pharmacologically active in rodents and rabbits, the ICH S6 Addendum indicates these species should be used unless there is a scientific reason to use an NHP. When the clinical candidate is pharmacologically active only in NHP, then assessment of reproductive toxicity in NHPs is generally preferred over alternative approaches, with the potential for effects on male and female fertility being assessed by standard histopathological evaluation and assessment of menstrual cycles in repeat dose toxicity studies of at least 3 months duration using sexually mature NHPs. Only if there is a specific cause for concern, should specialized assessments such as sperm count, sperm morphology/motility, testicular volume, and male or female reproductive hormone levels be evaluated. This also means that the use of alternatives (surrogate mAb or transgenics) just to assess reproductive toxicity is not recommended when the clinical candidate is cross-reactive to NHP. The ICH S6 Addendum recognizes that if the mAb is pharmacologically active only in NHPs, one well-designed and powered ePPND study in NHPs could suffice for registration. The duration of the postnatal phase of this ePPND should be justified and is dependent on the endpoints that are considered relevant for the pharmacological activity of the mAb. Bearing in mind that many mAbs target the immune system, this implies that postnatal periods upwards from 6 months' duration may be desirable, as demarcation of splenic architecture is not complete until 6 months postpartum, and germinal centers do not appear until approximately 9 months postpartum (Buse 2005).

Carcinogenicity

The ICH S6 Addendum reinforces statements in the ICH S6 main text indicating that a product-specific assessment of the carcinogenic potential of a mAb is required. This could be based on a weight of evidence approach based on literature data, known class effects, in vitro data, and data from chronic toxicology and clinical studies. The end result of this product-specific assessment of carcinogenic potential should be the communication of risk that may serve as a basis for the clinical risk management plan in conjunction with labeling proposals, clinical monitoring, and post-marketing surveillance. This weight of evidence approach may in itself be sufficient to address carcinogenic potential and inform clinical risk without conducting additional nonclinical studies, as outlined above. Rodent bioassays or short-term carcinogenicity studies with homologous products are generally of limited value to assess carcinogenic potential of the clinical candidate. Consideration should be given to the inclusion of additional endpoints in toxicity studies.

Selection of Starting Doses for First-Time-In-Man Studies Based on Preclinical Data

The severe and life threatening adverse events that occurred in the first-time-in-man (FTIM) trial with TGN1412, an anti-CD28 super-agonistic mAb, have significantly changed the emphasis of the approach to starting dose calculation for mAbs (Suntharalingam et al. 2006). While this chapter is not aimed at providing an in-depth review of FTIM dose setting with mAbs, and excellent reviews on best practices and experience since the TGN1412 incident can be found elsewhere (Nada and Somberg 2007; Agoram 2009; Lowe et al. 2009; Milton and Horvath 2009; Muller and Brennan 2009; Muller et al. 2009), some general principles for dose setting based on preclinical data are outlined below, using the TGN1412 example to highlight potential pitfalls.

The key regulatory guidance for starting dose selection for FTIM trials are the FDA's 'Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers' and the EMA's 'Strategies to Identify and Mitigate Risks for First-in-Human Clinical Trials with Investigational Medicinal Products', issued by the Committee for Medicinal Products for Human Use (EMEA/CHMP/SWP/28367/07). The FDA guideline describes a four-step process for selection of the maximum recommended starting dose (MRSD) that involves: (1) determination of the NOAEL in toxicity studies and providing justification for extrapolating animal NOAELs to a human equivalent dose (HED) based on mg/kg or mg/m^2 (the latter is often used for mAbs), (2) converting each animal NOAEL into a HED, (3) selecting the HED from the most appropriate species; by default the most sensitive species should be used (i.e. the lowest HED) unless there is a scientific justification not to do so (e.g. species-specific effects), and (4) dividing the HED by an appropriate safety factor (usually at least 10). This algorithm was used for calculation of the starting dose for TGN1412, and based on a NOAEL of 50 mg/kg in the NHP toxicity study, following a scaling factor of 3.1 from NHP to man, a 10-fold and an additional 16-fold safety factor, a 0.1 mg/kg starting dose was determined (Lowe et al. 2009).

However, the FDA guidance also recognizes that consideration needs to be given to the pharmacologically active dose (PAD), and that once an MRSD has been calculated as described above, that it may be of value to compare it to the PAD derived from appropriate pharmacodynamic models. If the pharmacologically determined HED is lower than the MRSD, it would be appropriate to lower the starting dose. This is especially relevant to mAbs whose toxicity is driven mainly by on-target effects or exaggerated pharmacology. The FDA guidance does not, however, describe in detail how the PAD can be determined.

The CHMP guidance for setting the starting dose for FTIM trials was issued in response to the TGN1412 incident, and the subsequent report by the Expert Scientific Group on Phase I clinical trials led by Sir Gordon Duff and issued in November 2006. The CHMP guidance not only emphasizes the use of the PAD for setting starting doses for FTIM trials, but also that the full range of the

pharmacological dose-response should be explored. In addition, the CHMP guideline introduces and defines the concept of the MABEL, as the anticipated dose level leading to a minimal biological effect in humans. The MABEL approach is an all-encompassing strategy that takes into account all the in vitro and in vivo information available including: data from in vivo toxicology, PK/PD and pharmacology studies, PK/PD simulations, data on target binding and affinity, receptor occupancy (or effects on total antigen, or free plus antibody–antigen complexes in the case of soluble targets), dose response data from in vitro cell-based assays, and known or anticipated target expression in humans versus the preclinical species. The most sensitive measure of pharmacological activity in man should be used to determine the MABEL. It is important to note however, that the MABEL approach should not be applied generically as the principal means to determine starting doses for FTIM trials. Determining the MABEL and the MRSD for each mAb that undergoes FTIM testing based on the principles outlined above would be good practice. However, in deciding which approach to follow for dose setting for human trials, consideration should be given to the potential risks associated with the pharmacology of the mAb. This risk assessment should take into account the mode of action of the mAb, the nature of the target, and the relevance of the animal species and models used. In particular, for molecules with a novel mode of action that activate multiple signaling cascades and are highly species specific and/or with a steep dose/response, a MABEL approach should be considered.

The key to FTIM dose setting is to consider all the available data and the relevance of the models applied, and then using the most sensitive measure of biological activity. For example, with regard to TGN1412, based on PK/PD simulations and experimentally determined CD28 receptor expression on human T cells, at a starting dose of 0.1 mg/kg, it is predicted that up to 90% of CD28 receptors would be occupied, and therefore almost maximal pharmacology would be achieved (Waibler et al. 2008; Lowe et al. 2009). Given the relative risks associated with the pharmacology of TGN1412, a starting dose at a much lower level of receptor occupancy would be desirable. Furthermore, in retrospect, the lack of a proliferative response of NHP-derived T cells to TGN1412 relative to human T cells should have been a concern and points toward the NHP not being a relevant species (Stebbings et al. 2007). Indeed, Eastwood et al. demonstrated that differences in CD28 expression on $CD4^+$ effector memory T cells—key to driving the cytokine storm in man—explain the marked differences in tolerability of TGN1412 in the NHP versus man (Eastwood et al. 2010; Pallardy and Hunig 2010). At the same time, data obtained with the murine or rat anti-CD28 super-agonistic antibodies (D665 and JJ316 respectively) also did not predict a cytokine storm, due to much more rapid suppression of cytokine release by T regulatory cells (Gogishvili et al. 2009). In hindsight, it would appear that the human response to TGN1412 would have been very difficult to predict, regardless of the design of the preclinical program. Nevertheless, by using the PAD or the MABEL approach, it seems likely that lower starting dose levels would have been selected, potentially reducing the severity of the clinical signs in patients.

Concluding Remarks

Since the introduction of the ICH S6 guideline in 1997, there have been significant advances in our understanding of biologics and mAbs in both the preclinical and clinical settings. The ICH S6 guideline and Addendum (once approved) provide a good framework for safety testing of mAbs. From the above it is clear that the preclinical safety assessment of biologics is a holistic approach that first and foremost takes into account species relevance and requires an in-depth scientific understanding of the in vitro and in vivo properties of the mAb. Only this in-depth understanding will allow for the prediction of safe starting doses for FTIM trials, as well as for continued patient safety during subsequent clinical development. It should be recognized however, that a number of key clinical safety risks encountered with mAbs in the clinic—e.g., infusion reactions, increases in susceptibility to bacterial or viral infections, reduced tumor surveillance, and PML—cannot be assessed preclinically. In order to assess these risks, good clinical risk management strategies and monitoring as well as identification of safety biomarkers and diagnostics will be essential.

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Chapter 11
Application of Pharmacokinetic/ Pharmacodynamic Modeling in the Development of Antibody-Based Therapeutics

Donald E. Mager

Abstract Mathematical modeling of the time-course of drug exposure (pharmacokinetics, PK) and associated pharmacological effects (pharmacodynamics, PD) has evolved from a simple descriptive endeavor to an essential component of model-based drug development. The role of PK/PD modeling can be more critical for antibody-based therapeutics owing to their complex pharmacological properties. Nonlinear behavior can manifest in most of the physiological processes controlling antibody PK, and mechanisms of drug response are inherently nonlinear and often involve turnover of endogenous ligands and biomarkers. In this chapter, model concepts and features that are common to antibody-based therapeutics are highlighted, along with applications of such models for understanding inter-species differences in concentration-effect relationships, selecting first-in-human doses, designing regimens for clinical studies, and linking PK/PD relationships to clinical outcomes. To improve prospective model predictions of drug efficacy and safety, new approaches will be needed to link antibody disposition with robust systems pharmacology models of drug and disease mechanisms.

Introduction

The efficacy and safety of new chemical entities and their role in the therapeutic management of diseases, particularly at early stages of drug discovery, are associated with large uncertainty and risk. Pharmacometric techniques seek to

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streamline the development process, providing a quantitative, model-based framework for integrating information from multiple platform technologies, across scales of organization, to inform critical decisions (Powell and Gobburu 2007). The application of pharmacokinetic/pharmacodynamic (PK/PD) modeling in drug development is well established (Sheiner and Steimer 2000) and represents a critical element of model-based drug development (Lalonde et al. 2007). Such models describe the temporal relationships between drug exposure and pharmacological and toxicological responses following acute and chronic administration. Useful models associated with well-designed experimental and clinical studies can provide a strategic advantage over empirical analysis strategies and are used to identify and understand determinants of variability among compounds, animal species, and patients. The impact of environmental, pharmacological, and pathophysiological factors on responses to drugs can be assessed in a quantitative manner to improve confidence in drug properties and for gauging the risk of moving compounds forward in development.

The case for integrating PK/PD modeling into the development strategy for protein-based therapeutics and monoclonal antibodies is reinforced by their distinct pharmacological properties (Galluppi et al. 2001; Mould and Sweeney 2007). The PK/PD relationships for monoclonal antibodies tend to be more complex than for small molecules due to a combination of pharmacological and (patho-) physiological factors that have been well reviewed (Lobo et al. 2004; Roskos et al. 2004). Nonlinear dose-dependent effects on drug absorption, disposition, and response are commonplace. Two major processes that can complicate the assessment of antibody pharmacokinetics are the FcRn salvage pathway and target-mediated disposition (Lobo et al. 2004; Tabrizi et al. 2006). Brambell was the first to suggest that a receptor-mediated pathway was responsible for protecting IgG molecules from degradation, resulting in their relatively long half-life (Brambell et al. 1964). This hypothesis was confirmed and the ubiquitous FcRn pathway was identified in independent studies (Ghetie et al. 1996; Israel et al. 1996; Junghans and Anderson 1996). Although this high-capacity mechanism is rarely saturated to produce an increase in antibody clearance, this pathway may be responsible for an inverse relationship between dose and bioavailability following subcutaneous (SC) administration (Wang et al. 2008). Antibody binding to its pharmacological target not only initiates drug response, but it can also influence the overall disposition of the compound. So-called target-mediated drug disposition (TMDD) (Levy 1994; Mager 2006) may result in dose-dependent distribution, and receptor-mediated transport might also represent a major elimination mechanism for many monoclonal antibodies (Lobo et al. 2004; Tabrizi et al. 2006). Other recognized factors controlling antibody disposition include target location (i.e., soluble vs. tissue bound), immunogenicity, concomitant medications, and disease and patient-specific variables (Tabrizi et al. 2006). Such complexities form unique challenges for the characterization of pharmacological properties, dose selection, and development of new antibody-based drugs.

Simple descriptive or so-called non-compartmental methods are a useful starting point for PK/PD data analysis to assess the degree of system linearity,

identify a base structural model, and rationalize initial parameter estimates (Jusko 2005). However, the frequent dose-dependent nature of antibody PK/PD and the slow equilibration between drug in plasma and the sites of elimination and action violate the fundamental assumptions of linearity and time-invariance in statistical moment theory. Error-prone point estimates will fail to fully characterize drug and system-specific properties, and the construction of mechanism-based mathematical models becomes essential. A structural model (commonly using either explicit or ordinary differential equations) is specified and parameters are estimated from fitting the model to PK/PD data via nonlinear regression analysis or population mixed effects modeling. The methodological issues involved in the development, application, and interpretation of PK/PD models are discussed in detail elsewhere (Bellissant et al. 1998; Gabrielsson and Weiner 2000). Mathematical modeling and computer simulations using mechanism-based PK/PD models are well suited for assessing complex concentration-effect relationships and may be used to guide the development of therapeutic monoclonal antibodies throughout the development lifecycle.

Data-Driven Pharmacokinetic Models

The open two-compartment model is one of the most popular structural models applied to antibody PK data:

$$\begin{aligned} \frac{dC_p}{dt} &= \frac{I(t)}{V_c} - \left(\frac{CL}{V_c} + k_{12}\right) \times C_p + k_{21} \times A_p / V_c \\ \frac{dA_p}{dt} &= k_{12} \times C_p \times V_c - k_{21} \times A_p \end{aligned} \tag{11.1}$$

where C_p is plasma drug concentration, A_p represents the amount of drug in the peripheral compartment, $I(t)$ represents a function describing the appearance of drug based on the route of drug administration, k_{12} and k_{21} are first-order distribution rate constants between the central and peripheral compartments, and V_c is the central volume of distribution. For rapid intravenous (IV) injection, the input function is set equal to zero and the dose (D) is reflected in the initial condition [$C_p(0) = D/V_c$]. A constant rate IV infusion is easily handled by setting $I(t)$ equal to the zero-order infusion rate for time (t) less than or equal to the infusion time, otherwise $I(t) = 0$. Although the absorption of macromolecules from subcutaneous (SC) or intramuscular injection can be complicated by molecular weight-dependent convection through lymphatic vessels and injection site catabolism (Charman et al. 2000; Kagan et al. 2007; Supersaxo et al. 1990), the absorption of antibodies after extravascular administration (e.g., efalizumab and omalizumab) has been described using simple first-order uptake kinetics (Meno-Tetang and Lowe 2005; Ng et al. 2005). The clearance (CL) of some antibodies may be characterized with linear first-order elimination ($CL = V_c \times k_{10}$); however, the

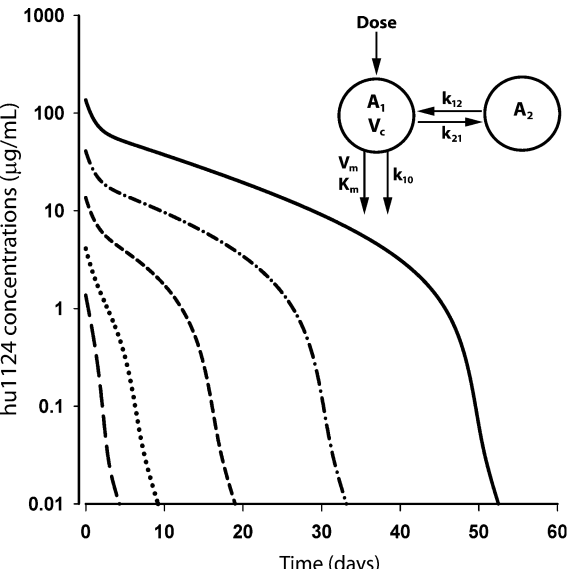

Fig. 11.1 Standard two-compartment model with parallel Michaelis–Menten and linear first-order elimination of monoclonal antibodies. Lines are simulated pharmacokinetic profiles of a human anti-CD11a monoclonal antibody using the model (*inset*) and parameters from Bauer and et al. (1999). Symbols are defined in text

majority of antibody-based drugs demonstrate saturable or capacity-limited clearance:

$$\mathrm{CL} = \frac{V_{\mathrm{max}}}{K_{\mathrm{m}} + C_{\mathrm{p}}} + \mathrm{CL}_{\mathrm{ns}} \tag{11.2}$$

where V_{max} and K_{m} are the traditional Michaelis–Menten parameters and $\mathrm{CL}_{\mathrm{ns}}$ represents non-saturable clearance pathways (e.g., intracellular catabolism following pinocytosis). The nonlinear Michaelis–Menten function (quotient in Eq. 11.2) may be specified alone (Mould et al. 1999) or in parallel with $\mathrm{CL}_{\mathrm{ns}}$ (Bauer et al. 1999). The pharmacokinetic model developed by Bauer et al. for efalizumab, an anti-CD11a monoclonal antibody, following IV administration to monkeys and human psoriasis patients, is a classic example of this common model structure (Bauer et al. 1999). Simulated human pharmacokinetic profiles for this drug are shown in Fig. 11.1. For high plasma drug concentrations (i.e., $C_{\mathrm{p}} \gg K_{\mathrm{m}}$), the saturable clearance function approaches a limiting value (V_{max}), and at relatively low concentrations will represent a first-order elimination rate ($V_{\mathrm{max}}/K_{\mathrm{m}}$).

A major source of the nonlinear elimination (and sometimes distribution) of antibodies is receptor-mediated clearance or target-mediated drug disposition (TMDD). Meijer et al. showed how initial exposure to anti-human CD3 antibody resulted in removal of target T cells, coinciding with a decrease in drug clearance for subsequent doses (Meijer et al. 2002). Whereas the Michaelis–Menten function can be used to describe this behavior, basic assumptions, such as greater drug concentration relative to the target and negligible turnover processes, does not hold

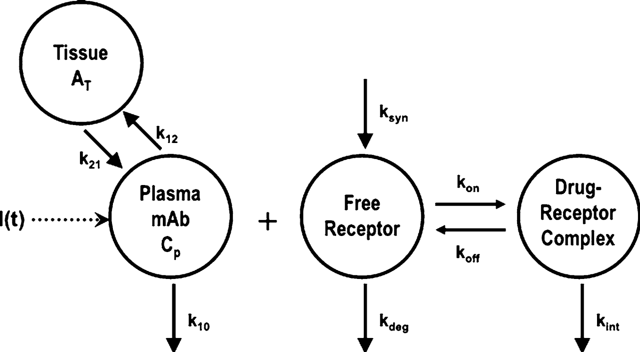

Fig. 11.2 Model diagram of pharmacological target-mediated drug disposition. Adapted from Mager and Jusko (2001)

for all systems. A general pharmacokinetic model of TMDD has been described (Mager and Jusko 2001), and the operative equations extending Eq. 11.1 include:

$$\begin{aligned}\frac{dC_p}{dt} &= -k_{on} \times R_f \times C_p + k_{off} \times RC \\ \frac{dRC}{dt} &= k_{on} \times R_f \times C_p - (k_{off} + k_{int}) \times RC\end{aligned} \tag{11.3}$$

where k_{on} and k_{off} are second- and first-order rates of association and dissociation, R_f and RC represent free and bound receptor (or pharmacological target) concentrations, and k_{int} is a first-order internalization rate constant. Under certain conditions, the total receptor concentration (R_{tot}) may be assumed to be time-invariant (Mager and Jusko 2001; Sugiyama and Hanano 1989), and thus $R_f = R_{tot} - RC$. Therefore, at high drug concentrations, $RC \rightarrow R_{tot}$ and can effectively limit both the distribution and elimination of drug from the central compartment. Alternatively, an additional equation may be introduced that directly describes the rate of change of R_f. Ng et al. co-modeled the pharmacokinetics of the anti-CD4 antibody TRX1 with the time-course of free and total CD4 concentrations using the model shown in Fig. 11.2. The model well captured the data in healthy volunteers and provided a method to assess the overall contribution of the saturable elimination pathway and guide dose selection in subsequent clinical trials.

Several approximations to the general TMDD model have also been developed to address the challenge of identifying the drug-binding micro-constants (k_{on} and k_{off}) from routine pharmacokinetic data (Gibiansky et al. 2008; Mager and Krzyzanski 2005). The equilibrium solution assumes that drug-target binding is relatively fast, and the binding micro-constants are replaced with the equilibrium dissociation constant ($K_D = k_{off}/k_{on}$). Hayashi et al. used this technique to co-model omalizumab with free and total target (i.e., IgE) concentrations (Hayashi et al. 2007). Guidelines for developing and selecting appropriate models have been proposed (Gibiansky et al. 2008; Yan et al. 2010), and models should be fit-for-purpose

(consistent with study objectives) and strike a balance between mechanisms of disposition and the ability to reliably estimate parameters from experimental data.

Pharmacodynamic Models

The primary use of antibody-based drugs include: (1) immunotoxicotherapy or antibodies targeting soluble drugs, cytokines, and other ligands, (2) elimination of target cells, (3) alternation of cellular function, and (4) targeted drug delivery (Lobo et al. 2004; Wang et al. 2008). General approaches to modeling antibody pharmacokinetics are feasible; however, the array of mechanisms of drug action limits generalizable pharmacodynamic models to specific categories or types of effects. Diverse models are available that seek to characterize the intensity and time-course of pharmacological effects and facilitate the estimation of drug- and system-specific parameters that control drug pharmacology and biological rate-limiting steps (Mager et al. 2003b). In terms of measurements, efforts should be made to understand the kinetics of the drug target when feasible (e.g., soluble or circulating ligands or receptors). In addition, mechanism-based biomarkers, or substances in the causal pathway connecting drug-target interactions and the ultimate clinical response, can provide insights into concentration-effect relationships, opportunities for streamlining the development process, and improved understanding of the pharmacological treatment of diseases. Validated assays for these measurements are essential for PK/PD systems analysis.

One of the simplest pharmacodynamic models is the direct effect or $E_{\max}$ model, which represents a linear transduction of the Hill equation for receptor occupancy (Wagner 1968):

$$E = \frac{E_{\max} \times C_{\mathrm{p}}}{\mathrm{EC}_{50} + C_{\mathrm{p}}} \tag{11.4}$$

where E is the drug effect and $E_{\max}$ and EC_{50} are the efficacy and potency parameters. For this relationship, the time to peak effect corresponds with the time of peak drug concentration, and there is no temporal disconnect between exposure and response. A PK/PD model for abciximab, a Fab fragment that binds to the GPIIb/IIIa receptor on platelets, combined a TMDD model with an inhibitory $E_{\max}$ model (Eq. 11.4 subtracted from a baseline) to describe the time-course of drug concentrations and the inhibition of ex vivo platelet aggregation (altered cell function) in patients undergoing percutaneous coronary angioplasty (Mager et al. 2003a). Simulations suggested that changes in receptor concentrations could contribute to inter-subject variability in abciximab dynamics, which is indirectly supported by clinical observations (Kereiakes et al. 2000).

Direct effect models are pharmacologically based on target occupancy, and the influence of efficacy and potency parameters is intuitive; however, few antibody-based drugs exhibit pharmacodynamic responses that can be described

using such simple models. For most antibody interactions with endogenous targets and the time-course of proximal mechanism-based biomarkers, turnover processes characteristic of so-called indirect response models are required, where drug influences the production or loss of the receptor or response variable (Dayneka et al. 1993). The four basic models in this class include a zero-order production rate constant and a first-order elimination or removal rate constant to describe the turnover of the pharmacodynamic variable, and drug concentrations inhibit or stimulate either process, through use of the Hill function, depending on the mechanism of action. The pharmacodynamic model developed for efalizumab essentially utilizes drug concentrations to drive the stimulation of removal of CD11a+ cells (Bauer et al. 1999), which is mathematically consistent with indirect response model IV (Dayneka et al. 1993; Gibiansky and Gibiansky 2009):

$$\frac{dR}{dt} = K_{in} - K_{out} \times \left(1 + \frac{S_{max} \times C_p}{SC_{50} + C_p}\right) \times R \qquad (11.5)$$

with R as the biomarker (CD11a%), K_{in} and K_{out} represent zero-order and first-order production and elimination rate constants, S_{max} is the maximal fold increase in K_{out}, and the SC_{50} is the drug concentration producing 50% of S_{max}. Simulated response profiles of Eq. 11.5 using parameters from the original study are shown in Fig. 11.3, revealing the characteristic prolonged nadir of effect with greater dose levels owing to the time drug concentrations remain above the SC_{50}. Mould et al. first modeled the time-course of drug-receptor binding of clenoliximab, and subsequently integrated fixed profiles of the drug-receptor complex as forcing functions for characterizing the inhibition of CD4 receptor density in patients with moderate to severe rheumatoid arthritis (Mould et al. 1999). The inhibition of production of eosinophils from an anti-IL-5 monoclonal antibody, which prevents IL-5 mediated signaling of eosinophil proliferation and differentiation, is a good example of using an inhibitory indirect response model for a proximal biomarker of antibody drug action (Zia-Amirhosseini et al. 1999). Thus, whereas TMDD models can leverage drug kinetics and target dynamics to simultaneously characterize PK/PD properties, indirect response models can accurately recapitulate drug and system properties controlling the pharmacodynamics of many antibodies under conditions where drug pharmacokinetics are linear or the experimental data do not fully support the identifiability of TMDD model parameters (Gibiansky and Gibiansky 2009).

More detailed pharmacodynamic models can be constructed for cases where additional mechanisms of drug action are measured or have been previously modeled. The immunotoxicotherapy model for denosumab, an IgG2 antibody directed against the receptor activator of nuclear factor-**κ**B (RANK) ligand (RANKL), is an example of effectively linking PK processes and a systems model with a clinically meaningful biomarker—serum N-telopeptide (NTX), reflecting bone turnover (Marathe et al. 2008). Original data were digitized from a clinical study in multiple myeloma patients (Body et al. 2006), and the pharmacokinetics

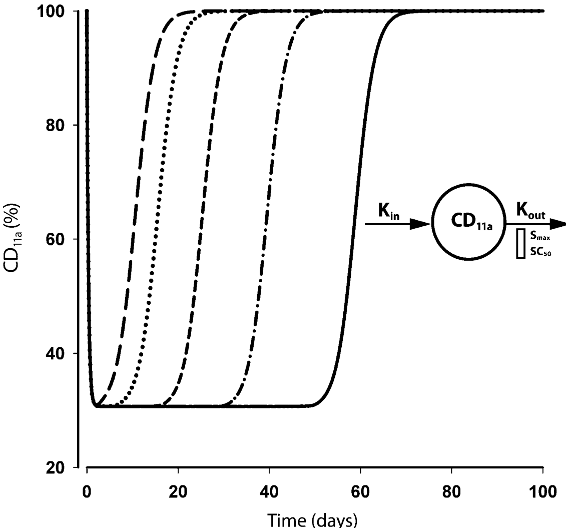

Fig. 11.3 Simulated CD11a suppression using an indirect response model (*inset*). Pharmacokinetic profiles shown in Fig. 11.1 were fixed as driving functions and the lines are simulations using Eq. 11.5 and the pharmacodynamic parameters in Bauer and et al. (1999)

of denosumab was well described using a TMDD model. Although the time-course of NTX could be captured with a simple indirect response model, denosumab PK was integrated into a mechanistic model by Lemaire et al. that includes the tight coupling between osteoblasts and osteoclasts as well as several key regulatory factors in the essential RANK–RANKL–osteoprotegrin pathway (Lemaire et al. 2004). The final model recapitulates the processes that are thought to regulate the turnover of NTX and has the advantage of providing a platform for evaluating the role of physiologically important substances (e.g., RANKL) on drug response and new testable hypotheses for designing effective therapeutic strategies.

Predicting Human PK/PD Properties

One of the major goals of preclinical studies is to obtain relevant information on the in vivo disposition and dynamics of drugs early in the drug development process and to anticipate probable PK/PD properties in humans for initial dose selection in clinical trials. Allometric scaling and physiologically based pharmacokinetic (PBPK) models are the most popular techniques for predicting pharmacokinetic properties of drugs in humans from data collected in other species. Although these methods have been applied to small molecular weight compounds for decades with variable and often limiting success, it is hypothesized that these techniques are more reliable for macromolecules owing to the relative species conservation of mechanisms that control the disposition of such compounds (Ferraiolo et al. 1992; Mordenti et al. 1991b).

Allometric Scaling

Organ sizes and many physiological processes, such as renal clearance, scale across species according to a well known power-law relationship (Adolph 1949):

$$Y = a \times W^b \tag{11.6}$$

where Y is a physiological parameter of interest, W represents body weight, and a and b are the allometric coefficient and exponent. The units and absolute value of a will depend on the specific property of interest, whereas b is unit-less and describes how Y will change with body weight. For clearance processes, b tends to be around 0.75, whereas organ sizes or physiological volumes tend to be directly proportional ($b = 1$) (Adolph 1949). Physiological times or the duration of physiological events (e.g., heartbeat and breath duration, lifespan or turnover times of endogenous substances or processes) typically scale across species with b values around 0.25 (Adolph 1949; Boxenbaum 1982). These inter-species relationships appear to manifest from the fractal nature of biological systems (West et al. 1997; West and Brown 2005).

Mordenti et al. (Mordenti et al. 1991a) were the first to apply allometric scaling to therapeutic proteins, where Eq. 11.6 was applied to five compounds, one of which was an anti-CD4 IgG1 antibody. Exponents for total systemic clearance and the volumes of distribution (central compartment and steady-state value) were in agreement with the expected theoretical values. Mahmood confirmed that simple allometry may be used for most proteins in a review of predicted total clearance values of 15 therapeutic proteins from interspecies scaling (Mahmood 2004). Despite such promising signals, the prospective use of allometric scaling for predicting human pharmacokinetics of monoclonal antibodies must be done cautiously. Retrospective analyses suggest that human antibody pharmacokinetics can be reasonably predicted from simple allometric scaling of monkey data alone when drug disposition is linear (Dong et al. 2011; Ling et al. 2009). Although PK parameters should also scale from rodents under these conditions, human antibodies may not cross-react to the same degree as in primates, making it difficult to anticipate whether nonlinear clearance is expected in humans. Evidence of nonlinear pharmacokinetics in monkey studies should raise concerns for prospective human projections from allometry. Even when antibody exposure in monkeys can be captured with traditional Michaelis–Menten models (Eqs. 11.1 and 11.2), scaling-up these terms can fail to predict human pharmacokinetic characteristics, especially for relatively low doses levels (Dong et al. 2011). It remains to be determined if more complex TMDD models can be used to better translate monkey data (Kagan et al. 2010) and whether a combination of in vivo preclinical data, compartmental modeling, and in vitro uptake studies might reliably anticipate macromolecule pharmacokinetics in humans under such nonlinear conditions (Proost et al. 2006). Understanding inter-species differences in target affinity, expression, and turnover processes is critical for such extrapolations.

Physiologically Based PK Models

In contrast to classical pharmacokinetics, physiologically based PK (PBPK) modeling represents a systems-driven approach and seeks to mimic physiological pathways and processes controlling the time-course of plasma and tissue drug concentrations. As Dedrick noted, "Physiologic modeling enables us to examine the joint effect of a number of complex inter-related processes and assess the relative significance of each" (Dedrick 1973). Most PBPK models contain principal components, such as arterial and venous blood pools, liver, and kidney, along with additional tissues depending on their role in specific disposition processes, whether it represents a potential site of action (biophase), or is likely to account for a significant proportion of the administered dose (Gerlowski and Jain 1983; Nestorov 2003). A series of mass-balance equations are specified that define the time-course of drug concentrations within each tissue/organ compartment, which are connected according to anatomical and physiological relationships. Physiological parameters, such as tissue/organ volumes and blood flows, are frequently fixed to experimentally measured values or the literature reported estimates (Brown et al. 1997; Davies and Morries 1993); however, these terms have also been estimated during model fitting (Xu et al. 2003).

Reports of PBPK modeling of antibody pharmacokinetics are relatively sparse. The experimental and computational requirements are often seen as formidable; however, advances in analytical techniques and computer hardware and software are bringing this methodology within common reach. Baxter et al. developed and evaluated a bi-functional antibody PBPK model in mice and scaled the model to predict its pharmacokinetics in humans (Baxter et al. 1995). A membrane-limited model was utilized, featuring equations for describing up to nine molecular species in each tissue (resulting from specific and non-specific binding), and mass transport across the membrane included convective and diffusive components (so-called two-pore system). Friedrich et al. further incorporated lymphatic circulation and identified critical properties requiring optimization for this therapeutic agent to be successful (Friedrich et al. 2002). PBPK models that include FcRn-mediated antibody binding in endosomal spaces are a major advance and enable the systematic evaluation of the influence of FcRn binding and trafficking on the half-lives of antibody drugs (Ferl et al. 2005; Garg and Balthasar 2007). The model from the Balthasar lab reasonably predicts typical properties of common linear IgG antibodies and also has been extended to include TMDD properties (Urva et al. 2010). PBPK models are well suited for scaling preclinical data to anticipate human pharmacokinetics in various relevant tissues (including target sites) and may represent a strategic advantage in drug development. Furthermore, this bottom-up or systems-driven approach provides a means for understanding the pharmacological implications of many complexities associated with the disposition of monoclonal antibodies.

First-in-Human Dose Selection

An integrated preclinical PK/PD model that is likely relevant in humans and establishes a relationship between drug exposure and a meaningful mechanism-based biomarker may be used to facilitate the selection of first dose(s) in humans. For antibodies that exhibit complex nonlinear properties, model-based dose selection can avoid the potential limitations of simple methods that are associated with inappropriate assumptions. The development of a TMDD model for an anti-Dickkopf-1 (Dkk-1) antibody in rats and monkeys and its translation for predicting the minimal anticipated biological effect level (MABEL) in humans is an excellent example (Betts et al. 2010). A large range of dose levels was tested (0.1–100 mg/kg) in rats and monkeys, and no significant adverse events were observed. Thus, the no observable adverse event level (NOAEL, FDA Guidance, 2005) resulted in doses predicted to show high target occupancy, even after scaling to account for body surface area and a 100-fold safety factor (0.16 and 0.32 mg/kg from rats and monkeys). On the other hand, applying the simple receptor occupancy (RO) equation (Duff): $\mathrm{RO}(\%) = C_\mathrm{p}(0) \times 100/\left[K_\mathrm{D} + C_\mathrm{p}(0)\right]$, and selecting the dose associated with 10% occupancy yielded an extremely low value of 1×10^{-6} mg/kg (Fig. 11.4). This substantial under-prediction is likely associated with failing to consider turnover processes and inappropriate assumptions, such as rapid binding conditions and drug concentrations in far excess of the receptor. The final preclinical PK/PD model resembled Fig. 11.2 (with a time-dependent production of free receptor) and successfully described free serum drug and Dkk-1 concentrations. In order to scale the model, human parameters were taken from the literature, experimentally measured, or scaled from rat and monkey values via allometric scaling (Eq. 11.6). This mechanistic approach predicted a starting dose of approximately 0.008 mg/kg (Fig. 11.4), which is much lower than NOAEL, but more reasonable than simple target occupancy calculations. An efficacious dose level was also predicted using the scaled model, and this study highlights how complex processes such as TMDD may limit the use of simple dose selection techniques and the need for more appropriate model-based algorithms to ensure clinical safety and efficacy.

The PK/PD model for omalizumab, an anti-IgE antibody, is another clear example where modeling can be used to inform antibody-based drug development. A rapid binding or equilibrium approximation to the TMDD model (Fig. 11.2) was developed using data from healthy volunteers and asthmatic patients (Meno-Tetang and Lowe 2005). There is a clear correlation between asthma symptoms and the suppression of free IgE concentrations (Slavin et al. 2009). Interestingly, the PK/PD model can reasonably predict free IgE concentrations from only fitting free drug and total IgE concentrations (Meno-Tetang and Lowe 2005). Monte Carlo simulations of model outcomes supported empirical regression-based dose selection. In addition, a sensitivity analysis revealed that investing in the development of higher affinity antibodies would not likely result in any advantage in efficacy over omalizumab owing to an offset in the turnover of the drug-IgE complex (Agoram et al. 2007).

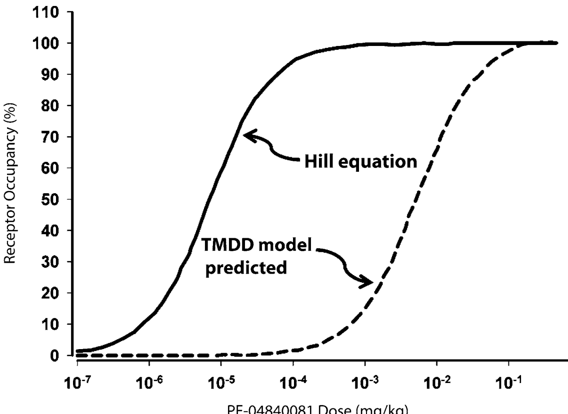

Fig. 11.4 Simulated Dkk-1 target occupancy using a simple Hill-type equation (*solid line*) or the TMDD model and parameters reported by Betts and et al. (2010)

Regimen Design for Clinical Trials

Once the decision is made to move a compound forward in clinical development, study designs and regimens can be rationally derived and evaluated using PK/PD modeling. The nonlinear mixed effects TMDD model for TRX1 (Fig. 11.2), which was based on data from a dose escalating study in healthy volunteers, was used to simulate the percentage of total drug elimination due to receptor-mediated clearance and an appropriate dosing regimen for subsequent clinical trials (Ng et al. 2006). As expected, the percent contribution of receptor-mediated elimination was dose-dependent and decreased from almost 100% to approximately 60% for simulated responses to dose levels ranging from 1 to 10 mg/kg. Dosing regimens of 1, 2, 3, 4, and 5 mg/kg, administered as a 2-hour infusion on days 0, 4, 9, and 13, were simulated, and the time-course of free CD4 profiles were assessed. The 1 mg/kg dosing regimen clearly showed CD4 levels breaking through the targeted suppression of 20%, recovering to almost 50%, prior to the next dosing event.

Returning to the omalizumab PK/PD model, Lowe et al. applied the model using nonlinear mixed effects modeling to a relatively large data set of 1,928 asthmatic patients and healthy volunteers from over four phase III trials (Lowe et al. 2009). Individual model predicted free IgE concentrations correlated with total clinical symptom scores, morning peak expiratory flow, and use of rescue medication. Model simulations were also performed to calculate doses and regimens for 1,000 patients for each subset of bodyweight and baseline IgE concentration. Short increments of bodyweight and baseline IgE were used, and the final model-derived dosing table is in good agreement with currently licensed tables (albeit with some slight discrepancies). The mechanism-based nature of the model, coupled with its ability to accurately describe PK/PD profiles for a wide-range of patients, suggests that it may be useful for the individualization of therapy for patients currently outside the licensed table as well. The omalizumab TMDD model was also integrated into the development of HAE1, a high-affinity anti-IgE

antibody (Putnam et al. 2008). In this case, published experience with the innovator compound (e.g., model and parameter values) could be leveraged with in vitro and in vivo data to predict PK/PD properties of the second-generation antibody and guide phase I and II study designs. At each stage, trial data were used to refine the model, address uncertainties associated with parameter values, and to assist in defining the risk of moving the compound forward.

Extending Models to Clinical Outcomes

Understanding the dynamics of antibody targets and proximal biomarkers can provide key insights into the exposure-response relationships for antibody-based drugs. However, a major frontier in PK/PD modeling is effectively linking such biomarkers to clinical outcome variables. For example, Ng and et al. expanded the PK/PD model for efalizumab (Eq. 11.5) to include a clinical efficacy model of the psoriasis area and severity index (PASI) score (Ng et al. 2005). The turnover model for the PASI score included a rate of psoriasis skin production that was directly proportional to the amount of free surface CD11a on T cells and offset by a first-order skin-healing rate constant. A population-based approach was utilized to estimate mean parameters and between subject variability in 240 patients from phase I and II clinical trials. The final model well captured the data and simulations suggested that administering greater dose levels less frequently might result in similar efficacy and improved patient convenience.

The binary or categorical nature of many clinical outcome variables represents a challenge to establishing efficacy models from PK/PD relationships. Rheumatoid arthritis patients are frequently assessed in clinical studies as to whether or not they achieve the American College of Rheumatology response criterion of 20% (ACR20); a dichotomous response variable. For such outcomes, logistic regression modeling is frequently used to model the probability (p) of achieving a positive event. The logit transform of this probability can be written as:

$$\text{logit}(p) = \ln \frac{p}{1-p} = f(x,t) + \eta \tag{11.7}$$

where x represents a measurement of drug exposure (and placebo response if applicable), t is time, and η represents inter-subject variability. Lee and et al. utilized Eq. 11.7 to correlate the cumulative area under the concentration–time curve (AUC; exposure) of etanercept (a dimeric fusion protein), and an empirical placebo function, with the probability of rheumatoid arthritis patients achieving the ACR20 (Lee et al. 2003). The model adequately described the time-course of clinical response and suggested that less frequent dosing might be possible. The ability of the model to capture the relatively long delay in the onset of effect (about 3–6 months) is probably related to the use of cumulative AUC as the exposure variable. In contrast, indirect response models are best suited for characterizing

exposure-response hysteresis when a drug serves to inhibit (or stimulate) turnover parameters of physiological systems (Dayneka et al. 1993). Hutmacher et al. described the application of an indirect response model and an unobservable continuous latent variable to link drug exposure to the probability of achieving the ACR20 (Hutmacher et al. 2008). Here, the probability tends to equal 1 as the continuous latent variable falls below a threshold. This approach has been further extended to simultaneously model ACR20, ACR50, and ACR70 as categorical variables in response to golimumab treatment, an IgG1κ anti-TNFα monoclonal antibody (Hu et al. 2010). Again, the data were well characterized, and this study nicely demonstrates a mechanistic (yet parsimonious) approach to categorical clinical outcomes for antibody therapeutics.

Conclusions

Antibody-based therapeutics typically exhibit complex nonlinear drug disposition and dynamics. Understanding the mechanisms controlling these in vivo processes is critical for efficient drug discovery, development, and applied therapeutics and can provide insights into designing novel drug candidates and delivery systems to optimize exposure-response relationships or therapeutic efficacy. Mathematical modeling of preclinical and human PK/PD data is necessary for characterizing such properties, and techniques like allometric scaling and PBPK modeling may be used to anticipate human PK/PD properties under certain conditions. Modeling of novel mechanism-based biomarkers and potential surrogates of drug effects (both therapeutic and adverse) can further enhance PK/PD models to include meaningful clinical outcome variables.

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Chapter 12
Application of Population Pharmacokinetic-Pharmacodynamic Approaches in the Design of Translational Strategies for Development of Antibody-Based Therapeutics

Feng Jin

Abstract Population-based pharmacokinetic-pharmacodynamic (PK-PD) approaches have been successfully applied in various stages of drug development over the last few decades. The development of antibody-based therapeutics has benefited substantially from the utilization of population approaches. Moreover, almost all FDA-approved monoclonal antibody therapeutics have been evaluated using population approaches. In this chapter, application of population PK-PD methods will be reviewed in the context of translational strategies employed during the development of antibody-based therapeutics.

Introduction

Much like traditional small-molecule drugs, a major challenge during the development of antibody-based therapeutics is maintaining an effective information flow and translation of accumulated knowledge throughout the various development phases. A science-based approach for translation of the PK-PD data is vital for effective advancement of antibody-based therapeutics. To this end, population-based PK-PD approaches have been applied successfully in various stages of development of antibody-based therapeutics (Betts et al. 2010). This chapter will provide an overview of some of the most prevalent population approaches employed for development of antibody-based therapeutics (Aarons 1999; Bauer et al. 2007; Ette and Williams 2004; Pillai et al. 2005). Additionally, relevant

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examples will be presented to showcase the utility and value of population PK-PD in aiding development of antibody-based therapeutics.

Population PK-PD Methodologies

Methodologies employed in the application of population PK-PD have evolved to a highly sophisticated level since the first publication of population PK by Sheiner et al. in 1972. Over the course of four decades, numerous population PK-PD methods have been developed to evaluate the potential sources of variability in PK and PD throughout various stages of drug development. In this section, major population methodologies, such as the Two-Stage method, the Non Linear Mixed Effect method, and the Bayesian method are reviewed (Aarons 1999; Bauer et al. 2007; Ette and Williams 2004; Pillai et al. 2005).

Two-Stage Method

In the standard two-stage (STS) approach, data from individual subject are fitted to a model and individual parameter estimates are obtained. During the second stage, the individual parameters are used to generate population parameter summary statistics (Sheiner and Beal 1983). In this approach, individual parameter estimates are combined to calculate empirical mean and variance as if the set of parameters are obtained from a true N-sample multivariate distribution. This method is simple and can generate reasonable mean estimates of the population parameters. However, a limitation of this approach is that it has a tendency to overestimate the random effects (Sheiner and Beal 1980, 1981, 1983). Hence, additional alternatives have been proposed to overcome the limitation associated with this approach. In the global two-stage (GTS) approach, it is assumed that the individual parameter estimates follow normal distribution centered on true expectation with variance. The GTS method provides a maximum likelihood estimate of the true population expectation and the true population variance–covariance. In general, the GTS methodology leads to a less biased estimation, particularly for population variance–covariance estimates (Steimer et al. 1984).

An alternate two-stage approach, the iterative two-stage method (IT2S), is an extension of the GTS methodology (Steimer et al. 1984). In the IT2S method, individual data are fitted (E step) repeatedly using Bayesian estimates obtained from the previous fitting, until the convergence is reached (M step) where the difference of prior and new parameter distribution is minimized. In the E step, individual parameter estimates are obtained through minimization of individual objective function by the linearized approximation. In the M step, population parameters are updated using the similar approximation method (Aarons 1999; Bauer et al. 2007). Although the IT2S method is computationally more demanding

due to its iterative nature, this approach is more robust and can provide relatively more accurate results for a rich dataset; however, analysis of the sparse dataset using IT2S may lead to biased results (Bauer and Guzy 2004; Ette et al. 1995). This limitation could be partially attributed to the linearization method used in this approach and the validity of the normality assumption of fixed effect parameter θ (Bauer et al. 2007). The iterative two-stage method currently has been implemented in various software packages such as IT2S routine (Forrest et al. 1993) and P-Pharm/Kinetica (Mentre and Gomeni 1995).

Nonlinear Mixed Effect Method

The nonlinear mixed effect method (NLME) was first proposed by Sheiner and Beal in their seminal papers (Sheiner and Beal 1980, 1981, 1983; Sheiner et al. 1977, 1972). As inferred by the name, NLME is a statistical method that integrates the fixed effects (known or explainable effects) and random effects (unknown or unexplainable effects) and evaluates both simultaneously. The NLME method involves a nonlinear hierarchical model with two levels of random effects (or errors) to describe the parameter variability (Davidian and Giltinan 1995, 2003). The first level of error comes from the individual level and the model describing the system at the individual level can be written as:

$$Y_{ij} = f\left(x_{ij}, \phi_i\right) + \varepsilon_{ij} \tag{12.1}$$

"Y_{ij}" is the *j*th observation in the *i*th subject, and "*f*" denotes the function of the structural model with respect to certain independent variables (i.e., time and dose, etc.). "ϕ_i" is the parameter(s) for the *i*th subject and "ε_{ij}" is the residual error (intra-subject variability) associated with the *i*th subject. The first level of the random effect is depicted by "ε_{ij}" which typically represents measurement error that follows a distribution with a mean of 0 and variance of σ^2. The second level of random effect happens at the population level due to variation of parameters among different subjects (inter-subject variability). The inter-subject variability can be generally described as:

$$\phi_i = g(v_i, \theta) + \eta_i \tag{12.2}$$

"ϕ_i" represents the parameters for the *i*th subject, "g" denotes the function that defines the covariate model of the parameters, and "v_i" represents a vector of the covariates for the *i*th subject; "θ" is the typical value for the parameters representing the fixed effects while "η_i" denotes the random error (inter-subject variability) of the parameters for the *i*th subject. "η_i" is assumed to follow a distribution with mean of 0 and covariance matrix of Ω.

The common estimation method used for fitting the population data using NLME is based on the statistical principle of maximum likelihood (ML). Because of the nonlinear nature of the models used in the area of PK-PD, estimation methods used in NLME usually employ a linearization approximation to compute the likelihood function. The simplistic approximation method is a first order (FO) approximation method (Beal and Sheiner 1982; Sheiner and Beal 1982). In this method, both random effect models characterizing η_i and ε_{ij} are linearized using first-order Taylor series expansion. The inter- and intra-subject variability are combined and the objective function is minimized toward the sum of both random errors. This methodology is established based on the assumptions that the random effects are independent and normally distributed. Under this condition, the population parameters can be estimated using an extended least squares criterion and is equivalent to maximum likelihood estimation. The FO method was the original estimation method implemented in the initial release of the NONMEM program. In general, the FO method requires much less computational power as compared to more sophisticated methods such as FOCE (first-order conditional estimation) due to its simplicity and generally yields relatively accurate estimates when the entire inter- and intra-subject variability is small. However, when the residual and inter-subject variances are large, the parameter estimation can be highly inaccurate (Ette et al. 1995, 1998). Hence, this approach can serve as a tool for model exploration rather than the final analysis. Compared to the FO method, FOCE methodology only uses linearized approximation at the individual level. This method was first published by Lindstrom and Bates (1990). First, with θ, Ω, and σ set at their initial values, the individual parameter is estimated by fitting the individual data to maximize the posterior distribution of ϕ_i (individual likelihood). Then, a quasi-Newton search routine is implemented to maximize the total likelihood to update the population parameters. A similar approach, called Laplace method, was also developed where a second order expansion is used in assessing the variance–covariance of individual parameters (Pinheiro and Bates 1995). Both the FOCE and Laplace methods were implemented in the NONMEM software package. The process of FOCE is highly computationally intensive, but the accuracy of the parameter estimation is generally good regardless of the magnitude of the variability and is relatively efficient in handling PK-PD model equations with close forms, compared to the complex PK-PD modeling with numerous differential equations. Newer methods (i.e., Gaussian-Hermite and Adaptive Gaussian-Quardrature methods (Pinheiro and Bates 1995)) have been developed to compute more exact integration of the likelihood function to overcome the inaccuracy arisen from linearized approximations. Both methods are implemented in the SAS PROC NLMIXED module (SAS/STAT 9.2 User's Guide: The NLMXED procedure 2008). More recently, the Expectation–Maximization (EM) algorithm combined with various Monte-Carlo integration methods (i.e., Monte Carlo important sampling-IMP; Bauer and Guzy 2004) and the Markov Chain Monte Carlo stochastic approximation-SAEM (Monolix Users Manual 2008) were developed. These methods present a more exact integration approach and have been

implemented in different software packages (IMP is implemented in NONMEM 7 and S-ADAPT and SAEM is implemented in NONMEM 7 and Monolix).

Full Bayesian Stochastic Method

Compared to the methods discussed which are two-stage in nature, the full Bayesian method involves a third level of random effect (Lunn et al. 2002). In the full Bayesian method, the uncertainty of population parameters θ, Ω, and σ are also considered and taken into account for the total likelihood:

$$(\theta, \Omega, \sigma) \sim p(\theta, \Omega, \sigma) \tag{12.3}$$

Instead of maximizing the likelihood, the full Bayesian method performs a series of simulations according to possible population parameter values based on their priors. In this sense, an entire distribution profile of the population parameters is generated and one can evaluate the mode of these distributions through approximation. The full Bayesian method is implemented in the WinBugs and markov chain monte carlo (MCMC) technique is used to facilitate the integration and simulation. Recently, the full Bayesian method has also been incorporated into NONMEM 7 as an estimation algorithm option. This method is particularly useful when there is prior knowledge regarding the population parameters preceding the actual analysis.

Learn-and-Confirm Through the Development Cycle

The concept of "learn and confirm" cycles was coined by Sheiner (1997). In his landmark paper, Sheiner described the process of clinical development as a repetitive process that involves two major cycles: "learn and confirm". What has been learned from previous clinical trials can be utilized and confirmed in future clinical trials; the outcome of those trials will again serve as a learning tool for the next ones. The whole process is iterative and dynamic in nature, and the learning evolves through the development cycles. The concept of learning and confirming should not only apply to clinical development, but also in the preclinical development stage. By implementing the "learn and confirm" process in both the nonclinical and clinical setting, the development gap between these steps can be filled to form a seamless translation of information from discovery into the clinic, and vice versa (Fig. 12.1). For instance, learnings from clinical studies can be used to aid preclinical research in terms of target and pathway identification. Translating the learnings from the previous stage to the next is an essential step for successful application of the "learning and confirming" philosophy in drug development. Modeling and simulation have been demonstrated to be powerful tools to facilitate translational research throughout

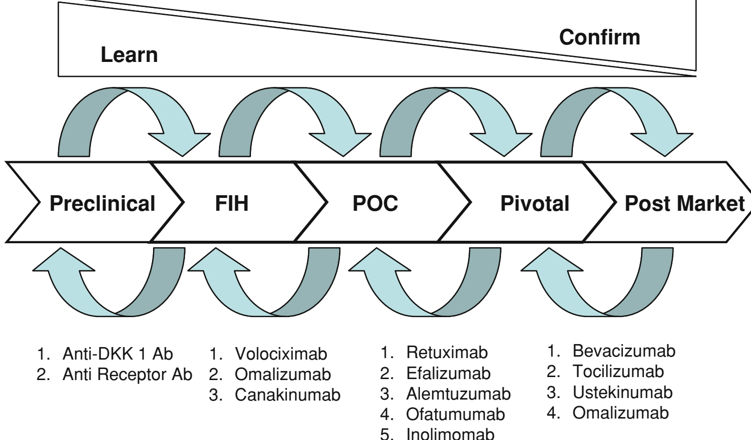

Fig. 12.1 Learn and confirm cycles within drug development from preclinical to post-marketing. Learn and confirm cycles are a continuum throughout the whole drug development process. The knowledge learned in the previous stage will be confirmed in the next stage. Alternatively, new hypotheses can be formed to feedback to previous stages for additional testing and learning. A number of examples were given in the following section to illustrate the utility and importance of population PK-PD in driving the Learn and confirm process

the various development stages. For the successful translation of information across various development stages, it is critical to understand the source of variability. Thus, population PK-PD approaches have proven to be invaluable in achieving this objective. In the following sections, a number of examples (as listed in Fig. 12.1) will be presented to showcase the utility and value of population PK-PD approaches in aiding monoclonal antibody development via the "learn and confirm" approach.

Preclinical to First-In-Human

During the preclinical development stage, critical goals are to identify potential leads and to select compounds with desirable safety, efficacy and PK-PD properties. Successful translational strategies for the development of antibody-based therapeutics across species should enable understanding of the dose–effect relationship with respect to both beneficial and deleterious effects from early stages of development (Tabrizi et al. 2009). Based on the information collected from the in vitro and in vivo studies early on, a quantitative framework can be established to characterize the PK-PD and toxicity relationships for the lead candidate. Integration of knowledge with respect to target antigen properties, target pharmacology, antigen isoforms and pharmacological redundancy in health and disease, PK, PD, and safety information is crucial for effective and safe design of the First-In-Human (FIH) studies. In addition, translation of the stimulus–response mechanisms across

species that convert receptor occupancy into pharmacological response can be greatly facilitated by application of population PK-PD approaches.

Due to unique specificity for its target antigen, the magnitude of the interaction between the antibody and its target can be generally linked to the target antigen occupancy, as well as PD and safety profiles (Wang et al. 2008). One of the major objectives for preclinical translational PK-PD work is to delineate and understand such relationships across various species in order to ensure that reasonable doses are selected for FIH studies. In general, the PK-PD models used for evaluation of the exposure–response relationships during the preclinical development of monoclonal antibodies are typically mechanism-based. For antibody-based therapeutics that target soluble antigens, relationships between antibody exposure and the effect on free antigen reduction or antigen binding to the antibody (bound antigen) can be evaluated. Evaluation of the relationships between the free antigen and antibody concentrations in vivo can provide valuable information regarding the antibody potency, EC_{50} (i.e., antibody concentration resulting in 50% suppression of the antigen) and the maximum system efficiency, or E_{max}. Additionally, for many of the currently marketed antibodies that recognize membrane-associated internalizing antigens, interaction of antibody-based therapeutics with the target antigens can greatly influence their PK properties. When antigen binding alters the clearance of an antibody, the effect is usually manifested in terms of a dose-dependent clearance rate and half-life (Mager and Jusko 2001). In these instances, a generalized PK model, referred to as the target mediated disposition model (TMDD) (Mager 2006), can be utilized to evaluate antibody pharmacokinetics as a proof-of-principle biomarker, following antibody treatment (Lammerts van Bueren et al. 2006; Vugmeyster et al. 2009; Xiao et al. 2010). Using such mechanistic models, the key physiologically relevant PK-PD parameters can be accurately estimated and readily extrapolated to project the human dose after accounting for differences in the antigen density across species.

In the past, PK-PD modeling at the preclinical stage generally focused on a naive pool or the mean data. Little emphasis was placed on estimating parameter variability from animal data. However, population PK-PD modeling approaches can be useful in separating the potential inter-animal variability from the total variability observed in the experimental data. Application of population approaches at early stages has improved prediction of parameters with added accuracy and precision for FIH dose projections. For example, population methods were utilized in analyzing the PK-PD results of an antibody against Dickkopf-1 (DKK-1) obtained from the nonclinical species (Betts et al. 2010). The modeling outcome was further used to project the human efficacious dose and to aid FIH trial design. PF-04840082 is a humanized anti-DKK-1 antibody for the treatment of osteoporosis. In both rat and monkey, the pharmacokinetics of PF-04840082 exhibited nonlinear properties. The full TMDD model was implemented in the analysis of PF-04840082 PK data in rats and monkeys. Population analysis was conducted using NONMEM V. Since information regarding the changes in the time-course of the target antigen was also collected, total DKK-1 (both free and bound DKK-1) data were modeled simultaneously with the PK data. The model-predicted

parameters were further scaled to patients based on allometry and were utilized to project the efficacious clinical dose.

In another example, a population PK-PD approach was utilized in the translational analysis for an antibody directed against a membrane-associated antigen (Lowe et al. 2010). The non-linear mixed effect model was applied to analyze the antibody pharmacokinetics using NONMEM V. Body weight was included as a covariate in the population model. Based on the historical data, the allometric exponents were set to 0.75 for clearance and 1 for volume of distribution. The model was first fitted to cynomolgus monkey data. Model-estimated parameters in nonhuman primates, together with patient-specific information (i.e., body weight, dose schedule, etc.), were further employed to generate random simulations for each patient. The simulated profiles, in a majority of cases, predicted human profiles well by agreeing with the observed human data. Subsequently, both monkey and human data were combined to update the model. The only covariate required to improve the goodness of fit was the antigen turnover parameter that was species and disease specific. The model successfully identified the differences in the target density between the toxicology species and the disease population (Lowe et al. 2010). By using the population approach, it was possible to evaluate the impact of antigen expression and variability on the antibody PK-PD profiles across species. These examples highlight that when information regarding target expression and density are available during early studies, the predictions of efficacious human doses can be more precisely predicted by recognizing and adjusting for the antigen expression differences across species using population PK-PD approaches.

FIH to Proof-of-Concept (POC)

Phase I studies are designed to explore a wide range of dose levels and provide information regarding safety, tolerability, and PK of the lead candidate for the first time in humans. Because most of the safety concerns for monoclonal antibodies are stemming from exaggerated pharmacology due to excessive interaction with the target, the FIH studies are typically conducted in the intended patient populations with relevant expression of the target antigen. In turn, the PD data are typically collected in these studies to better understand the pharmacodynamic and toxic effects. Availability of rich data sets for both PK and PD provides a great opportunity to assess the PK-PD relationships for the lead candidate in a relevant population. Robust PK-PD models can be established to elucidate not only the pharmacokinetic properties of the lead, but to further explore the time-course for the changes in the pharmacodynamic profile. Generally, the structural PK-PD models developed during the early preclinical stages is used for evaluation of Phase I clinical data in line with the concept of a learn-and-confirm development strategy. This approach allows for confirmation of the learning from preclinical studies and guides preclinical assessment for second generation compound

targeting the same pathway. Although PK-PD samples are collected over a wide range of doses, the sample size for Phase I study is typically small. Nevertheless, population methodologies are highly useful in evaluating Phase I data. During Phase I clinical studies, addition of a population modeling component can provide the opportunity to better understand the potential sources for inter-subject variability. As a result, more precise estimation of the key system parameters, such as drug potency and the maximum system efficiency, can be evaluated. Thus, by evaluating modeling data generated from such integrated PK-PD models, one can further explore various simulation scenarios to aid selection of the optimal dose(s) for Phase II studies.

Volociximab is a chimeric IgG4 antibody that specifically binds $\alpha5\beta1$ integrin and prevents it from binding to fibronectin. $\alpha5\beta1$ has been shown to be a key mediator of vascular development; therefore, volociximab was developed as an anti-angiogenic agent for the treatment of solid tumors. During the Phase I single arm dose-escalation study, volociximab was administered as an IV infusion to adult subjects with solid tumors who were unresponsive to standard therapies (Ng et al. 2010). A total of 21 subjects were enrolled into six dose cohorts at 0.5, 1, 2.5, 5, 10, and 15 mg/kg. Each subject received five IV doses at weeks 1, 3, 4, 5, and 6. Samples for both PK and PD analysis (saturation of $\alpha5\beta1$ integrin on circulating monocytes) were collected. A population PK-PD model was developed to evaluate the PK and PD data simultaneously. For the system model, a modified target-mediated PK-PD model was used to characterize both volociximab PK and the free fraction of monocytes in circulation. For the variance model, inter-subject variability was evaluated using an exponential model. Residual models for PK and PD data were characterized by proportional and Poisson models, respectively. The PK and PD model was fitted to the data simultaneously using the monte-carlo expectation–maximization (MCPEM) algorithm implemented in the S-ADAPT program. The modeling output was evaluated using diagnostic plots, visual predictive check, and simplified numerical predictive check. The final model was reported to generate unbiased predictions and reasonable parameter estimations. The intra- and inter- subject variability parameters were also well estimated with percent standard error of all parameters below 44%. The final model parameters were used to simulate PK-PD profiles based on preset criteria on the $\alpha5\beta1$ free fraction ($\leq$5%) on circulating monocytes to aid Phase II study design. Predictions generated via model simulations suggested that a volociximab dose of $\geq$10 mg/kg IV every other week should be the biologically active dose and dosing frequency.

In another example, omalizumab was developed for the treatment of asthma by interrupting the allergic cascade mediated by IgE. Omalizumab is a humanized IgG1 antibody directed against human IgE. Administration of omalizumab significantly decreases the circulating levels of free IgE. The initial population PK-PD model for omalizumab was published using a small Phase I dataset (Meno-Tetang and Lowe 2005). In this report, a full target-mediated disposition model was used to describe omalizumab PK and free and total IgE concentrations. The population model was developed using NONMEM V. The goodness-of-fit was evaluated based on parameter estimation precision and diagnostic plots. The model provided

good fits to all subjects for all three variables and the PK-PD parameters were precisely estimated with low standard error. The population PK-PD model was further refined using two additional Phase I studies conducted in Japan (Hayashi et al. 2007). A quasi-equilibrium target-mediated disposition model was used to simplify the model and enhance model stability. Because binding of omalizumab to IgE can generate different forms of oligomers depending on their relative concentrations, the binding affinity K_D was defined as an empirical function:

$$K_D = K_{D0} \times \left(\frac{X_{TX}}{X_{TE}}\right)^{\alpha} \quad (12.4)$$

To account for the concentration dependency for formation of complexes, K_{D0} was experimentally determined in vitro. X_{TX} and X_{TE} represented total omalizumab and total IgE in serum, respectively. The clearance of omalizumab-IgG complexes showed higher clearance compared to omalizumab alone. This observation was attributed to a decrease in binding affinity of omalizumab from the complex to FcRn due to steric hindrance, or complex elimination via the IgE clearance pathway. Therefore, an additional clearance parameter for the complex was incorporated. Inter-subject variability was described by an exponential error model. Residual error was described by a proportional error model. Body weight, baseline IgE, age, and sex were evaluated as covariates. Both body weight and baseline IgE were included as covariates in the final model helping the model to converge. Final fitting results showed that most of the individual observed omalizumab, total IgE, and free IgE concentrations were within the 80% (single-dose study 1101) or 95% (multiple-dose study 1305) prediction interval that was generated for 1,000 simulated subjects for each cohort (Hayashi et al. 2007). Inter-subject variability was reported to range from 13 to 40%. Intra-subject variability varied from 17 to 22%. Diagnostic plots revealed good fits of the model to the experimental data with no significant bias. The incorporation of concentration-dependent K_D function significantly decreased the objective function value by 107. The model predicted values for omalizumab and IgE half-lives are close to what have been reported before. The estimated omalizumab-IgE complex clearance was between those of omalizumab and IgE alone, consistent with the hypotheses proposed. The model also accurately identified the PD response, namely free IgE levels, as a function of body weight, baseline IgE, and the administered dose. Additionally, the external model validation successfully bridged the results between the Japanese and Caucasian populations. These findings provided key criteria for future development of the compound from the trial and dosing perspective.

In another example, efficacy of canakinumab, a human anti-IL-1β antibody developed for treating patients with cryopyrin-associated-periodic syndromes (CAPS), was evaluated in a Phase I/IIa study. CAPS is an orphan condition resulting from mutation of NLRP3, the gene encoding cryopyrin, that leads to excessive production of the pro-inflammatory cytokine IL-1β. While CAPS patients present with a variety of autoimmune conditions, established IL-1

therapies such as anakinara have shown efficacy in treating this disease. The Phase I/IIa study for canakinumab was designed to assess safety, pharmacokinetics, phamacodynamics, and clinical efficacy of this compound in treating CAPS patients (Lachmann et al. 2009). An integrated population PK-PD model was developed to describe the PK, PD, and clinical efficacy data collected. For the system PK-PD model, a mechanism-based model was developed to describe the binding between canakinumab and IL-1β. The binding process was defined to occur in both plasma and interstitial tissue compartments. The model assumed exchange of both canakinumab and antibody-IL-1β complexes between plasma and tissue. Tissue IL-1β was linked to acute phase reactant responses by a Hill equation. A population PK-probability model was developed to characterize the relationship between canakinumab PK and clinical response. The pharmacokinetics of canakinumab was described by a simple two-compartmental model. The final PK-efficacy model was simplified to a Hill equation linking total canakinumab concentration and relapse probability. In this model, the inhibition constant, K_i, was defined as the canakinumab concentration at which there is a 50% probability that a flare may occur. The Hill coefficient approximated the inverse of the variance of a Gaussian distribution of canakinumab concentration over which a patient is transitioning from remission to flare. The population nonlinear mixed effect model was implemented in NONMEM V. The model fitted the time of flare and the need for drug administration as well. Despite the very small sample size ($N = 7$), the inter-subject variability was reasonable, ranging from 3 to 60% and the residual errors were acceptable at 18 and 49%. The model-estimated parameters were further used in simulations to help the design of future clinical trials. Based on the Monte Carlo simulations, it was determined that the desirable treatment option was 150 mg SC every 8 weeks in order to keep patients free of flares.

POC to Pivotal Trials

In Phase II studies, the focus is geared more towards expanding the learnings from Phase I and conducting trials in the disease population intended for registration. These studies typically recruit hundreds of patients to ensure appropriate statistical power and may be conducted in multiple geographic locations. One of the most important objectives for Phase II studies involves testing drug effects at multiple dose levels to enable exposure–response analysis in order to guide Phase III study design. Population PK-PD modeling is a powerful tool at this stage and has greater utility including: (a) Assessing the population PK and identifying the potential covariate(s) effect and sub population; (b) evaluating exposure–response relationships in order to facilitate inference of optimal efficacious dose and dosing frequency; (c) Confirming the PK-PD relationship previously defined in Phase I studies and (d) Generating simulations based on modeling outcome to support Phase III study development.

For example, the population pharmacokinetics of rituximab, a chimeric antibody targeting CD20, a B-lymphocyte antigen, was evaluated in a single Phase II study. Rituximab is a B cell-depleting antibody developed for RA, NHL, and CLL (Ng et al. 2005a). In a Phase II study in RA patients, efficacy of rituximab alone or in combination with DMARD (Disease-Modifying Anti-Rheumatic Drugs) was evaluated. Patients received a 1,000 mg IV infusion of rituximab on days 1 and 15. NONMEM V was used for the population nonlinear mix-effect modeling. Both one- and two- compartment linear PK models were evaluated as potential structure models. FOCE with interaction was used throughout the model building process. Inter-subject variability was described by an exponential error model. Covariate effects were assessed using a multiplicative covariate regression model. Intra-subject variability was described by both proportional and additive error models. The covariate model for biological covariates (age, sex, body surface area (BSA), height, baseline B cells, and weight) was established using the forward selection method followed by the backward elimination method. The covariate effect of co-medication with DMARD was assessed subsequently to obtain the final model. Structural and covariate model comparison and evaluation were performed using diagnostic plots, likelihood ratio test, and objective function value comparison for alternative hierarchical models. The final model was further evaluated with the bootstrap re-sampling technique. After comparison, a two-compartment PK model was selected as the base structural model with inter-subject variability added on CL and V_c. Inclusion of additional nonlinear clearance did not improve model performance, presumably due to the low B cell baseline in RA patients. This observation was different from what has been observed for NHL patients, who typically have high B cell counts. Consistently, baseline B cell counts did not show a significant covariate effect. In the final model, BSA and sex were the significant covariates for CL and V_c. The covariate effects addressed about 32% of the inter-subject variability for CL and 42% of the inter-subject variability for V_c. No difference in CL and V_c was detected between groups with rituximab alone or rituximab with DMARD co-administration. Model parameter estimation precision was good in general with percent CV lower than 29%. Bootstrap runs showed around a 84% success rate in minimization steps, suggesting the model was relatively stable. Although BSA was determined as one of the major covariates, the improvement of AUC variability after dose adjustment based on BSA was minimal. As a result, BSA-adjusted dosing was not recommended. This work proved critical for dose selection not only in RA patients, but in oncology-related indications such as NHL (non-Hodgkin's Lymphoma) and CLL (Chronic Lymphocytic Leukemia).

Efalizumab is a humanized anti-CD11a monoclonal antibody that binds to the integrin alpha L chain expressed on T lymphocytes and was developed for the treatment of psoriasis. This antibody inhibits the movement of T lymphocytes from the circulation to the dermal compartment. The PK-PD and efficacy data for efalizumab was collected from five Phase I and Phase II studies (Ng et al. 2005b). The PK of efalizumab was characterized by a two-compartment model with first-order absorption and dual linear and Michaelis–Menten clearance. The PD result,

measured as percent free CD11a, was modeled using an indirect response model to correlate efalizumab PK with the observed effect. Efalizumab clinical efficacy was measured by the disease severity score of psoriasis, or PASI (psoriasis area and severity index score). The PASI score was assumed to be directly related to the turnover of psoriatic skin and the effect of efalizumab was described by partial inhibition of psoriatic skin production that was proportional to the percent-free CD11a. Inter-individual variability was assumed to be log-normally distributed. Intra-individual variability for PK, CD11a concentrations, and PASI score were modeled with Poisson and constant additive error respectively. These data were analyzed by a nonlinear mixed-effect model using the Monte Carlo parametric expectation maximization algorithm (MCPEM) implemented in S-ADAPT, and all data were modeled simultaneously (Ng et al. 2005b). The model outcome was evaluated by diagnostic plot, posterior predictive check, and external validation. The predicted and observed efalizumab PK data were in good agreement and diagnostic plot suggested no systematic bias. Both PK parameters and inter-subject variability were well estimated with good precision (SE below 22%). The model also provided good estimation of CD11a data with good parameter estimation precision and no systematic bias. Inter-subject variability for PD was generally higher than that in PK. The efficacy model described the observed PASI data well and both efficacy parameters and inter-subject variability were estimated with good precision (<47%). The posterior predictive check based on simulations also demonstrated that the model was adequate to predict the distribution of the data. As a way of external validation, the model predicted parameters were further used to run Monte Carlo simulations to generate PK-PD and efficacy results based on a completed Phase III study and demonstrated good agreement with the study observations. Overall, the model satisfactorily described the complex PK-PD/ efficacy data with good precision and directly tied PK-PD information with clinical outcome in a temporal fashion. The modeling results allowed further trial simulations for different regimens with different dosing intervals shown in Fig. 12.2 (adopted from ref. Ng et al. 2005b).

The overall PK-PD and efficacy simulations suggested that less frequent antibody administration of higher doses would have similar effects on total CD11a expression and PASI relative to more frequent administration with lower doses. The projected results from these simulations were employed for the design of future Phase III trials.

Application of population PK-PD methodology also proved critical for clinical development of Alemtuzumab. Alemtuzumab is a humanized IgG1 antibody directed against CD52, a glycosylphosphatidylinositol (GPI)-anchored antigen expressed on various cell types including lymphocytes and monocytes. Alemtuzumab is currently approved for the treatment of B-cell lymphocytic leukemia (B-CLL). The population PK-PD and efficacy analysis reported previously utilized the data collected from four Phase II lymphoma and leukemia studies (Mould et al. 2007). The PK-PD data were analyzed using Nonlinear Mixed effect models by NONMEM V. Different structural PK models were tested. Inter-subject variability was assumed to be log-normally distributed and the intra-subject variability was

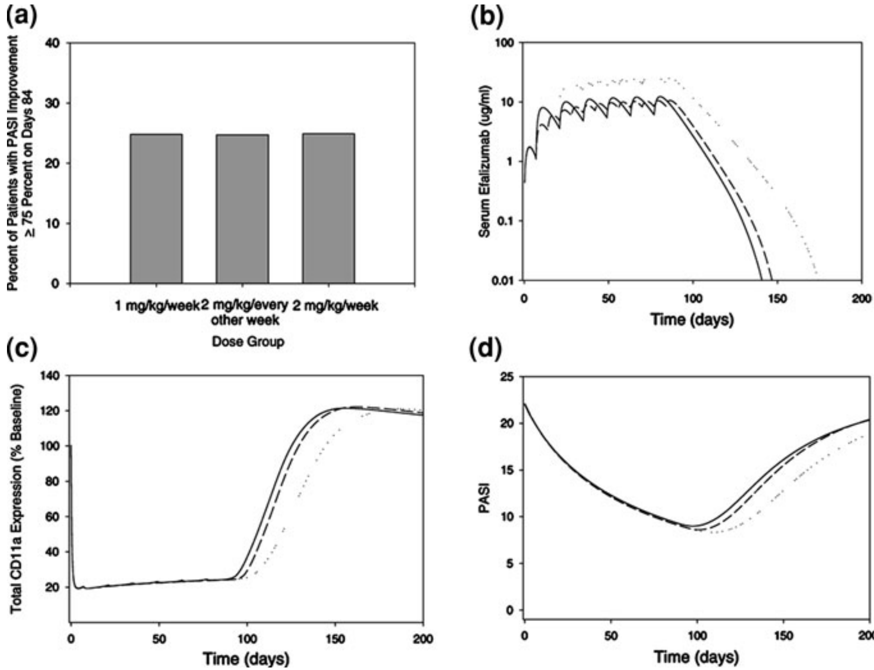

Fig. 12.2 Simulated profiles of efalizumab PK-PD/PASI responses following 3 different dosing regimens at: 0.7 mg/kg for the first week, 1 mg/kg/week subsequently (*dashed line*), 2 mg/kg every other week (*solid line*), or 2 mg/kg/week (*dotted line*). **a** Model predicted percent of subjects with improvement in PASI at least 75%, and simulated **b** plasma efalizumab time profile, **c** Total CD11a expression-time profile, and **d** PASI score-time profiles (adopted from reference (Ng et al. 2005b))

modeled as a combination of constant coefficient of variation and additive error. Various covariates (age, sex, height, BSA, BMI, race, study, WBC count and lymphocyte count) were modeled using power function centered or scaled to a hypothetical reference value. The model was fitted using the FOCE method with interaction. Covariate analysis was carried out using forward selection which was followed by backward deletion methodology. The final model that optimally fitted the data was a two-compartment model with Michaelis–Menten elimination kinetics. WBC count was identified as the major covariate for V_{max} (maximal rate of elimination). Since WBC varied over time, V_{max} also changed over time accordingly. All parameters were estimated with good precision and diagnostic plots showed no obvious bias. WBC, indicative of tumor burden, was measured as a relevant PD marker and was modeled using an indirect response model with stimulation acting on k_{out}. Random error was described by an exponential error model, and residual error was described by an additive error model. The model was fitted using the FO method. The estimated inter-subject variability was high, which could be reflective of the differences between patients for CD52 positivity and disease status. The model-

estimated a high WBC baseline and was consistent with observed values. Diagnostic plots suggested that the model performed reasonably well and was adequate to describe the complex PK-PD relationships observed in the study. Exposure–response modeling was conducted using SAS and only data from trails for B-CLL were included in the analysis. Logistic regression models were developed to characterize the relationship between various exposure measurements and treatment response outcome. The analysis revealed that both maximal trough concentration and $AUC_{0\text{-tau}}$ were significant predictors of clinical outcome. In summary, the PK-PD model accurately described the dose and time-dependent pharmacokinetics of alemtuzumab and its relationship with WBC dynamics. Exposure–response modeling identified the good predictors for clinical responses and linked PK-PD information with outcome measures.

Ofatumumab is a human IgG1 monoclonal antibody targeting a unique epitope on CD20, a B-lymphocyte antigen. This antibody ablates B-cells via Fc-dependent effector mechanisms including CDC (complement-dependent cytotoxicity) and ADCC (antibody-dependent cell-mediated cytotoxicity). The data used for the PK and efficacy analysis outcome were generated in a Phase I/II study that assessed ofatumumab safety and efficacy in patients with CLL (Coiffier et al. 2010). The antibody was administered as 4 weekly IV infusions at three different dose levels. The population PK was evaluated only in a cohort where patients received a first infusion of 500 mg and three subsequent infusions of 2,000 mg. The PK data were fitted with a nonlinear mixed effect model using SAS 9.2. A two-compartment linear model was used and random effects were assumed to be log-normally distributed, where residual errors were assumed to be normally distributed and covariate effects were assessed using ANOVA. For the exposure–response analysis, both objective response and time-to-event endpoints were evaluated and logistic regression methodology was used to correlate PK measures to the clinical responses. As ofatumumab showed a trend of nonlinearity in the preliminary PK analysis, a more complex model was developed. Covariate assessments showed a gender effect on clearance. In addition, high SPD (sum of the product of diameters of the lymph node size), a surrogate marker for tumor burden, was associated with high clearance. Gender, body weight, and BMI were associated with high volume of distribution. In the exposure–response analysis, greater exposure (measured as $AUC_{0\text{-infinity}}$ or CL adjusted by SPD) was associated with higher probability of objective response and time-to-event endpoints. This population analysis satisfactorily characterized the relationship between PK, tumor burden, and clinical endpoint.

Inolimomab is a murine monoclonal antibody that specifically targets the α chain of the IL-2 receptor. It was developed as an immunosuppressive agent for the treatment of corticosteroid-resistant graft-versus-host disease (GVHD). A Phase I/II study was conducted to evaluate inolimomab efficacy in combination with steroids in treating patients who developed acute GVHD following hematopoietic stem cell transplantation (HSCT) (Dartois et al. 2007). Twenty-one subjects were assigned to four different dose groups (0.1, 0.2, 0.3, and 0.4 mg/kg) and received inolimomab as IV infusions. Patients with complete response after seven daily infusions of the inolimomab induction regimen were assigned to

receive a maintenance regimen. Patients without a complete response remained on induction treatment for one more week before they were assigned to the maintenance regimen. Acute GVHD grade and performance status (combination of organ scores and clinical performance) were evaluated daily as clinical response endpoints. Population PK-PD analysis was conducted with a nonlinear mixed effect model using NONMEM V. For a structural model, various PK models were tested. Inter-subject variability was assumed to be log-normally distributed, while intra-subject variability was assumed to be multiplicative. FOCE methodology with interaction was used in the model fitting. Model outcome was evaluated using diagnostic plots and visual predictive check. Nested models were compared based on likelihood ratio testing. A two-compartment model provided the best results and was chosen as the final base model. Model predictions were in strong agreement with the observed data and visual predictive check confirmed the model was adequate. To evaluate the exposure–response relationship, a proportional odds model was used. Exposure variables (i.e., C_{max}, cumulative AUC, $AUC_{intensity}$, and AUC_t) were correlated with the cumulative probability of the ordinal categorical clinical response variables after logit transformation. Various correlation models were tested such as E_{max}, linear, and log-linear models. Inter-subject variability was assumed to follow either normal or log-normal distribution. The Laplacian method in NONMEM was used for model estimation and modeling results were evaluated by diagnostic plots and visual predictive check. After exploratory graphical analysis, cumulative AUC was shown to be correlative to three-organ (skin, intestinal tract, and liver) score cumulative probabilities. Consequently, exposure–response models were developed to correlate cumulative AUC with organ score cumulative probabilities. As to skin, an E_{max} model was used for correlation. Additional mixture probability distribution was assumed to account for two subpopulations with respect to their potency parameter EA_{50}. Linear models were used for intestinal tract and liver score and visual predictive check revealed overall good agreement of the 80% quintile and the observed data. The PK and exposure–response models were then combined to simulate global acute GVHD scores for further model qualification. The simulations demonstrated good agreement of the 90% confidence interval for the global score versus observed value and confirmed the global therapeutic effect on decreasing GVHD scores. The population modeling from this work helped identification of the most sensitive exposure variable to be correlated to response. In addition, the authors successfully developed exposure–response models for different organs which allowed prediction of different organ damage under the same exposure. Moreover, sub-populations with respect to skin response sensitivity were identified. This analysis aided determination of the dosing scheme for a specific patient population. Additionally, the models can be used to predict global disease responses by combining the simulated scores for the three organs. Based on these results, future trials with efficacy criteria set on global treatment activity, instead of individual organ score, can be designed.

Phase III and Beyond

The primary goal of Phase III clinical studies is to confirm, in a larger patient population, efficacy and safety information previously learned during the earlier stages of clinical development. These studies generate key results that are used for regulatory filing. During Phase III studies, population PK-PD modeling approaches have been utilized extensively to confirm exposure–response relationships in the target population and support the dosing regimens that will be critical during the registration phase. Additionally, population modeling has been employed to confirm the pharmacokinetic properties including covariate effect learned from previous studies. Therefore, application of population PK-PD during this stage of development provides the opportunity for the final population PK model to be established; moreover, application of population PK-PD in this phase enables further refinement of the exposure and efficacy target relevant for the design of effective dosing strategies in special patient populations, as well as in post-marketing evaluation. Although confirmatory population analysis has been performed using one or multiple Phase III studies, it is also a common practice to conduct a meta-analysis in order to integrate data from all phases of clinical development to enhance statistical power and robustness.

A case in point is bevacizumab. Bevacizumab is a humanized IgG1 monoclonal antibody directed against vascular endothelial growth factor (VEGF) and was developed as an anti-angiogenic agent for the treatment of cancer. Bevacizumab is the first biologic agent approved as first-line treatment for metastatic colorectal cancer in combination with intravenous 5-Fu chemotherapy. The population pharmacokinetics of bevacizumab was conducted using a meta-analysis approach by combining data generated from eight Phase I to Phase III clinical trials (Lu et al. 2008). In these trials, bevacizumab was given as either a single agent or in combination with chemotherapeutics. The Phase I trials enrolled patients with various types of solid tumors. Phase II and III trials were conducted in cancer patients with colorectal carcinoma, breast carcinoma, and non-small cell lung cancer (NSCLC). Bevacizumab, given as an IV infusion, was tested at different dose levels and frequencies ranging from 0.1 to 20 mg/kg every week to every 3 weeks. The PK data were analyzed with a nonlinear mixed effect model using NONMEM V. The FO estimation method was used for model building and the FOCE method was used to generate the final model with different structural models evaluated. For covariate analysis, different types of covariate effects, such as demographic factors, disease-related covariates and disease severity, as well as concomitant chemotherapies were assessed. The initial covariate analysis was limited to CL and V_c. The covariate models were developed using forward addition followed by the backward elimination method. In the end, a multiplicative covariate regression model was built to relate PK parameters with the most significant covariates. Inter-subject variability was modeled by an exponential model and the intra-subject variability was modeled by a combination of proportional error and additive error models. A final model was evaluated using diagnostic plots

and bootstrap analysis. During the development of the full population PK model, a two-compartment model was found to better describe the data and was therefore selected as the basis for the structural PK model. A total of 16 disease-related covariates were evaluated and body weight, gender, serum albumin, SGOT, and alkaline phosphates were found to have a significant effect on CL, while the V_c parameter correlated significantly with body weight, gender, and serum. Chemotherapies were found to have a significant effect on CL. In the final model, the total covariate effects explained about 40% of the inter-subject variance for V_c, and 60% of the inter-subject variance for CL. Among these covariates, body weight and gender were the two most significant. Diagnostic plots showed good agreement between model predictions and observed concentrations. Estimated final model parameters were consistent with the 95% confidence interval generated from bootstrap analysis. In summary, the above meta-analysis successfully established a two-compartment linear model to describe bevacizumab PK. The linear model enabled simulation of different dosing regimens to achieve target serum bevacizumab concentrations. Since body weight has been identified as the major covariate, this finding supported the weight-based dosing strategy for this antibody.

IL-6 is a pro-inflammatory cytokine that plays a central role in the pathogenesis of rheumatoid arthritis (RA). Tocilizumab is a humanized anti-IL-6 receptor (IL-6R) monoclonal antibody that competitively inhibits the binding of IL-6 to both soluble and membrane-bound IL-6R, and suppresses pro-inflammatory activities mediated by IL-6. Various studies have shown that tocilizumab, currently approved for the treatment of moderate to severe RA, inhibited joint damage and reduced disease activity in RA patients. The data used for the population PK analysis were obtained from four Phase III studies. In these studies, tocilizumab was administered as an IV infusion at 4 or 8 mg/kg every 4 weeks. In addition to sparse sampling in all Phase III studies, intense samples were collected in 20% of subjects enrolled. Nonlinear mixed effect modeling implemented in NONMEM VI was used to analyze the PK data (Frey et al. 2010). The PK data were log-transformed and the FO method was used for the estimation process. Parallel linear and Michaelis–Menten elimination pathways were employed to describe tocilizumab elimination. Inter-subject variability was described by an exponential model and residual error was described by a combination of proportional and additive error models. A list of continuous and categorical covariates including demographic, laboratory, and biomarker data were included in the covariate analysis. The covariate analysis was conducted using stepwise generalized additive modeling (GAM) and bootstrap of the GAM analyses in Xpose 3.102 using S-PLUS 7. A covariate effect was identified as relevant when the covariate resulted in a statistically significant change to the Akaike information criterion, and if the total inclusion frequency from the bootstrap of the GAM was >0.8. All the identified covariates were further evaluated in NONMEM using a stepwise forward addition followed by a backward elimination approach. A randomization test was done subsequently to confirm the selection of the covariate. After a baseline-covariate model was built, three additional time-varying covariates including IL-6, soluble IL-6R, and the anti-tocilizumab antibody were assessed by plotting population and individual weighted residuals versus the covariates.

The continuous covariate was modeled by a power function with the covariate normalized to its respective median value. The categorical covariate was modeled using equations as follows:

$$\mathrm{TVP} = \theta_p \text{ for males} \tag{12.5}$$

$$\mathrm{TVP} = \theta_p \times (1 + \theta_{\mathrm{sex}}) \text{ for females} \tag{12.6}$$

Combined continuous and categorical covariate effects were defined as multiplicative. The modeling results were evaluated using diagnostic plots and visual predictive check. Once the final model was established, simulations were performed to generate the tocilizumab concentration–time course profile to evaluate the impact of covariates on key secondary PK parameters such as steady state AUC, C_{max}, and C_{min}. Tocilizumab PK was best described by a 2-compartment model with combined linear and Michaelis–Menten elimination kinetics. Model predictions were in good agreement with the observed data, suggesting this model is sufficient to be the base model. The inter-subject variability was applied to CL, central compartment volume V_1, peripheral compartment volume V_2, $\mathrm{V}_{\mathrm{max}}$, and covariate effects were tested on these parameters. In the final model, sex, BSA, HDL-C, and the logarithm of rheumatoid factor on CL, total protein and albumin on V_1, Creatinine CL, and smoking on V_{max}, were identified to be significant covariates. Although BSA was the only body size parameter retained in the final model, others like BW and BMI revealed a similar relationship to CL. Time varying parameters, including anti-tocilizumab antibody, did not affect the concentration–time course. Concomitant medication (mainly DMARDs) showed no effect on tocilizumab PK; therefore, there was no need for dose adjustment when tocilizumab was given together with other traditional DMARD therapies. Visual predictive check results showed that approximately 90% of the observed concentration data fell within the 90% quintile of the simulated profile, suggesting that the model was adequate to describe the population data. Simulations of tocilizumab concentration–time profiles demonstrated that non-linear target-mediated clearance was dominant at low concentrations, while linear clearance was dominant at higher concentrations above the K_{m}. Although body size parameter had a positive correlation with linear clearance, it was determined that body weight-based dosing may not be required if the target-mediated pathway can be saturated. However, from the simulations, neither 4 nor 8 mg/kg at monthly dosing intervals were shown to fully saturate the nonlinear pathway (Frey et al. 2010). Therefore, a weight-based adjustment for dosing was needed and the dosing regimens currently approved are 4 and 8 mg/kg administered monthly.

Another case in point is Ustekinumab, a human IgG1 monoclonal antibody directed against IL-12 and IL-23 cytokines. IL-12 and IL-23 have been associated with the pathogenesis of psoriasis; ustekinumab has been shown to be efficacious in treating patients with moderate to severe psoriasis in Phase I through Phase III studies by neutralizing IL-12 and IL-23 activities. A population-based exposure–response model was developed to correlate the ustekinumab exposure

with therapeutic response and PASI score for optimal dose determination (Zhou et al. 2009). The PK and PASI data used in the analysis were obtained from two Phase III studies: PHOENIX I and PHOENIX II. The nonlinear mixed effect modeling implemented in NONMEM VI was used for model development. FOCE with interaction between Ω and σ was used as the minimization method. The exposure–response (PK and PASI) relationships were modeled in a sequential manner, where PK was fitted first, followed by PASI-time data estimation using post hoc empirical base estimates of the PK parameters in the forcing functions. A one-compartmental linear model with first-order absorption was used to characterize the pharmacokinetics of ustekinumab (Zhu et al. 2009). To describe the relationships between ustekinumab exposure and PASI score, a semi-mechanistic model (modified indirect response model (Dayneka et al. 1993)) was used to relate the ustekinumab concentration–time profile with PASI scores following repeated-dose treatment with the antibody. In this model, ustekinumab inhibited the formation of psoriatic skin lesions which were assumed to be directly correlated with the PASI scores. The inhibition was described with an $E_{\max}$ model. The placebo effect on PASI, which eventually plateaus to $\mathrm{plb}_{\max}$ (maximum placebo effect) post treatment, was described by an empirical equation:

$$\mathrm{plb} = \mathrm{plb}_{\max} \times \left(1 - \exp^{-k_{\mathrm{plb}} \times t}\right) \tag{12.7}$$

Inter-subject variability was assumed to be log-normally distributed and residual errors were described by a combination of proportional and additive error models. Different covariates were evaluated in the graphical covariate search process. Factors identified in the graphical analysis were included in the covariate model building step using standard forward addition and backward elimination methods. For highly correlated covariates, only one was used in the model. The final model was assessed by diagnostic plots, bootstrap re-sampling analysis, and visual predictive check. Although the placebo effect was small, incorporation of the placebo model did improve the overall fit and was therefore included in the final model. No apparent bias was detected in the individual model prediction plots. The inter-subject variability for IC_{50} was large (283%) and had a trend of bimodal distribution. The $E_{\max}$ estimate was close to 1; however, fixing $E_{\max}$ to 1 actually led to worsening of the final fit. As a result, the $E_{\max}$ parameter was estimated in the final model. None of the covariates added were able to decrease inter-subject variability; consequently, no covariate effect was included in the final model. Overall, the means and percent relative standard errors generated from bootstrap analysis were in good agreement with the parameter estimates generated using the final model. A visual predictive check was performed to simulate the percent responders (75% improvement of PASI score achieved at the end of 28 week treatment) and the results were compared to the percent responders observed from the two Phase III trials. There was strong agreement between the observed PASI 75 response rates and the simulated results. Additional investigation of the results revealed a trend of correlation between low exposure and partial/non-responders. Following subcutaneous administration, the analysis

demonstrated a trend of lower median exposure to the antibody in non-responders (CL/F 0.844 L/day) and partial responders (CL/F 0.609 L/day) relative to patients responding to ustekinumab treatment (CL/F 0.462 L/day). In line with these observations, a 30-fold higher potency (lower IC_{50}) was reported in the patients responding to the treatment relative to the partial responders, consistent with the bimodal distribution of individual IC_{50} estimates. These findings suggested that this population may require higher doses of ustekinumab, or alternatively, more frequent dosing, to achieve comparable efficacy. The model was proposed to support future alternative dosing strategies during development of ustekinumab (Zhou et al. 2009).

Furthermore, the development of omalizumab exemplifies the impact of population PK-PD on translational research and presents probably the best case study in support of personalized medicine (Lowe et al. 2009; Slavin et al. 2009). As aforementioned, in the case of a monoclonal antibody directed against IgE, its effect is tightly related to the body load of IgE in each individual patient. Based on the population PK-PD model developed in early phase clinical trials (Hayashi et al. 2007; Meno-Tetang and Lowe 2005), a more elaborate model was built to not only relate omalizumab PK with free IgE as the PD endpoint, but to also connect the PK-PD relationships with clinical outcome (Lowe et al. 2009). Five clinical studies (four of the studies were Phase III trials) were used in the model development phase. The model was developed using nonlinear mixed effect modeling implemented in NONMEM VI. The base PK-PD model was an expansion of the model that was established previously (Hayashi et al. 2007). In the base model, instead of fixing the K_D parameter as it was designed in the original simulation, the revised model allowed the K_D to be fitted in order to account for potential inter-individual differences in Fcε-expressing factors that may compete with omalizumab binding to IgE. Additionally, body weight and baseline IgE concentrations were used as covariates for structural parameters. More specifically, body weight was determined as a covariate for all clearance and volume parameters as well as IgE production rate. Baseline IgE was modeled as a covariate for IgE production rate, IgE clearance, and K_D. Covariance matrices were incorporated for estimation of free omalizumab clearance and volume, as well as omalizumab-IgE complex clearance and IgE production rate. Despite producing similar results as FO, the FOCE method led to termination as the maximum number of iterations was exceeded. Therefore, the PK-PD dataset was modeled using the FO method. The model was evaluated using diagnostic plots and predictive check; model diagnostic plots showed no obvious deviation between observed data and the predictions. Parameter estimations were fairly precise with percent SEE ranging from 1.6 to 16% for structural parameters, 2.6–24% for covariates, and 12–47% for intra-subject variance. The precision of unexplained inter-subject variance was in general reasonable, ranging from 22 to 40%, with the exception of inter-subject variance for absorption rate at 141%. Although a wealth of PK-PD data from a bioequivalence study were included in the modeling, most of the Phase III data were sparse and contained limited information regarding the absorption phase. Importantly, this approach may have resulted in the high inter-subject variability

for estimation of the absorption rate (K_a). A predictive check was performed to simulate the steady state level of free IgE according to Phase III study designs for different subsets of patient populations based on their body weight and baseline IgE. Both the median steady-state IgE levels and the shape of the distribution were well predicted. As a result, a robust PK-PD model to describe omalizumab and IgE profiles simultaneously was developed. The model was further extended to correlate free IgE levels with clinical outcome (total asthma symptom score, morning peak expiratory flow, and rescue medication use). Following this analysis, a temporal discordance was observed between the IgE concentration–time profile and the time-course for clinical responses, reflecting a time delay between IgE changes and the clinical response following omalizumab treatment. However, when the clinical response data were grouped into blocks at every 4 weeks to remove the time variable, a strong correlation was identified between the mean clinical response and the model-inferred free IgE levels at the midpoint of each block. This correlation analysis was successful in identifying a target-free IgE level (14 ng/mL) for selection of the ideal dosing frequency. Subsequently, a Monte-Carlo simulation was performed to deduce the dose level and the administration frequency required for achieving the free IgE suppression target based on different body weight and baseline IgE concentrations. The simulations allowed generation of a model-driven dosing table for effective administration of omalizumab in the patients with severe asthma (Lowe et al. 2009).

Other Related Topics

Immunogenicity

Similar to other biologic products, monoclonal antibodies have the ability to induce an immune response. Although antibody engineering technology has evolved tremendously over the last few decades, and the potential of immunogenicity for antibody products has been greatly alleviated, even fully human antibodies can still induce an immune response (Wang et al. 2008). Since an immune response may affect the PK and PD of antibody-based therapeutics, it has been evaluated as a potential covariate in population analysis. For example, the status of the immune response developed against infliximab was evaluated as a covariate for clearance in the population PK analysis for patients with Ankylosing Spondylitis (Xu et al. 2008). Immune response status was evaluated as a binary variable with positive patients coded as 1 and non-positive patients coded as 0. Inclusion of immune response status adequately described the impact of immunogenicity on altering clearance and the overall PK characteristics. A similar model was also used in the population PK modeling for infliximab in patients with ulcerative colitis (Fasanmade et al. 2009). These analyses suggested that a positive immune response may cause an over 40% increase in the systemic clearance of infliximab. Immune response status was also evaluated as a covariate for the ustekinumab clearance parameter

(Zhu et al. 2009). Positive immune responses led to a 35% increase in ustekinumab clearance. Although it is generally expected that development of immune responses against therapeutic antibodies should accelerate their pharmacokinetic elimination, population PK analysis has not always been successful in identifying the impact of anti-drug-antibody (ADA) responses as a significant covariate. For example, in the population analyses for golimumab (Xu et al. 2009) and panitumumab (Ma et al. 2009), the presence of response only affected the systemic exposure of the therapeutic antibodies by approximately 10%.

Fixed Dosing Versus Body Weight Dosing

It is tempting to speculate that body size parameters (body weight, body surface area, etc.) are the most important covariates for antibody-based therapeutics since in numerous population PK analyses these parameters are identified as significant covariates with the highest impact on antibody dosing. For many monoclonal antibodies, their volumes of distribution are typically restricted within the systemic and lymphatic system. Hence, volume parameters are highly correlated to body size parameters; therefore, body-weight or BSA-adjusted dosing has been employed. However, the benefit of adjusting antibody dose based on body size parameters has not been systematically demonstrated. A recent study utilized a population approach to investigate the level of impact on antibody pharmacokinetics between body size-based dosing versus fixed dosing (Wang et al. 2009). Mixed-effect modeling using NONMEM VI was performed to simulate PK profiles for 12 selected antibodies. Monte-Carlo simulations were performed based on the final population PK model published for each compound to generate concentration–time profiles following fixed-dose or body weight-adjusted dosing. For these simulations, BSA was assumed to be normally distributed with a median at 1.82 m^2 and an artificially assigned standard deviation to generate a range from 1.2 to 2.4 m^2. The distribution of body weight was described by a power function with a power coefficient equal to -0.5, generating a body weight distribution profile with a median at 75.7 kg and a range of 38.8–187.2 kg. The fixed dose was set to the dose that would be given to a subject with median body weight or BSA. The performance of both approaches was evaluated by comparing: (a) the 95th percentile intervals of the simulated 1,000 concentration–time profiles; (b) the distribution of AUC and $C_{\max}$; and (c) the inter-subject variability of AUC and $C_{\max}$. From the twelve monoclonal antibodies selected, there was no marked difference in the 95% PK intervals between the two dosing strategies. The distribution of AUC and $C_{\max}$ were also similar for fixed dosing and body size adjusted dosing. In addition, neither approach showed a clear advantage in reducing PK variability. Overall, the simulations suggested that there is no obvious PK advantage by body size-adjusted dosing compared to the fixed dosing approach. When the body size parameters are strongly correlated to PK parameters, i.e., power coefficient >0.68, there appears to be greater benefit for body size based dosing. However, such

benefit should also be considered against other factors, such as relative contribution of other covariates, PK variability relative to PD variability, and the therapeutic window. Considering benefits from multiple perspectives such as cost, and convenience of delivery, fixed dosing could be preferred over body size-adjusted dosing because of their comparable performance on PK.

Drug: Drug Interactions

With an increase in the number of approved antibody-based therapeutics, information regarding interactions between monoclonal antibodies and other concomitant medications including small molecule drugs and therapeutic proteins is emerging (Keizer et al. 2010). Unlike small molecule drugs in which drug–drug interactions have been extensively studied and their clinical implications are well recognized, the mechanism and impact of interactions induced by antibody-based therapeutics are not well defined. The predominant mechanisms of drug interactions for small molecule drugs are metabolism- and transporter-based interactions. Since monoclonal antibodies are primarily cleared through high-capacity and nonspecific clearance pathways or through interaction with their specific targets, the likelihood of direct interaction with small molecules in a metabolic or transport pathway is minimal. In contrast, drug–drug interactions induced by monoclonal antibodies relate more toward their interactions with a specific target and immunogenicity (Huang et al. 2010; Lee et al. 2010).

In general, antibody-based therapeutics that modulate cytokine activities could indirectly alter CYP enzymatic systems or transporter expression, resulting in PK changes for small molecule drugs whose disposition is highly dependent on the affected metabolic or transporter pathway(s). For example, tocilizumab is shown to modulate CYP expression and further affect the pharmacokinetics of drugs that are CYP substrates (i.e., omeprazole is a CYP219 substrate and simvastatin is a CYP3A4 substrate) (Zhang et al. 2009). On the other hand, the PK of monoclonal antibody therapeutics can be altered by drugs affecting the immunogenicity potential or regulating expression of the target that the antibody interacts with. Overall, the interactions between monoclonal antibodies and other medications due to these mechanisms are typically mild to moderate. In the recent guidance document issued by the FDA regarding drug–drug interactions (Guidance for industry: drug interaction studies- study design, data analysis, and implications for dosing and labeling 2006), population approaches have been described as valuable tools for evaluating and characterizing the clinical impact of known or newly identified interactions. Traditionally, the DDI potential within small molecule drugs is investigated extensively and dedicated DDI studies are often conducted for known or suspected interactions. Because of the long half-life of monoclonal antibodies and the indirect nature of their PD interactions, dedicated DDI studies are not routinely conducted for monoclonal antibodies. As dedicated DDI studies for monoclonal antibodies are still rare, meta-analysis using data from multiple studies

to evaluate DDI potential has been adopted. For example, the effect of immunosuppressants such as azathioprine and mycophenolate mofetil (MMF) on the pharmacokinetics of basiliximab following combination therapy in renal transplant patients was assessed by combining data from different studies (Kovarik et al. 2001). A previously established population PK model (Mentre et al. 1999) was used to fit data from these studies. Model-estimated basiliximab clearance values, co-administered with or without azathioprine or MMF, were compared. Basiliximab clearance was 29 mL/h when co-administered with azathioprine and 18 mL/h when co-administered with MMF. Both were significantly lower compared to a clearance of 37 mL/h from a previous study of basiliximab without concomitant administration of either drug. With respect to DDI evaluation for specific combination therapies used in certain disease areas (i.e., organ transplantation, solid tumors, and chronic autoimmune conditions), the population approach is more commonly used in the covariate analysis, as part of the population PK assessment effort, to examine the DDI potential for co-administered medications. In the population analysis conducted for ustekinumab (Zhu et al. 2009), 28 concomitant medications that were most frequently used in the analysis were evaluated for potential drug–drug interactions. The effect of each concomitant medication on CL/F was evaluated using the maximum likelihood null hypothesis testing methodology against the final covariate model. None of the concomitant medications showed a significant effect on the PK parameter estimates. With greater recognition of the DDI potential for monoclonal antibodies, it is anticipated that more dedicated DDI studies will be conducted and more sophisticated population methods can be applied to these assessments (Duan 2007; Zhou 2006).

Concluding Remarks

Population PK-PD analysis has now become a routine methodology employed in all stages of drug development. Population PK-PD modeling has tremendously improved our understanding about drug targets, disease conditions, and the properties of the drug of interest. With ever enhancing computing power and emerging new technologies such as parallel computing, it is anticipated that population PK-PD methodologies will be applied in an even broader spectrum during the development of antibody-based therapeutics.

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Chapter 13
Translational Biomarkers: Essential Tools in Development of Antibody-Based Therapeutics

Mohammad A. Tabrizi and Cherryl B. Funelas

Abstract A science-based approach for translation of exposure–response data is vital for effective advancement of antibody-based therapeutics and application of relevant biomarkers can streamline this process significantly. Selecting and evaluating relevant biomarkers early on not only lessens the time and cost associated with clinical evaluation, but also fosters implementation of rational decision-making processes throughout various antibody development phases.

Introduction

A critical consideration during the development of antibody-based therapeutics (ABTs) is selection and evaluation of relevant biomarkers during the early preclinical stage. In general, the use of biomarkers during clinical evaluation helps facilitate the process by determining if a drug is: (a) reaching/affecting the molecular target in humans, i.e., Proof-of-Mechanism, POM, (b) resulting in any measurable down-stream activity/signaling, i.e., Proof-of-Principle, POP, and/or (c) providing measurable endpoints that allows prediction of desirable or deleterious outcomes, i.e., Proof-of-Concept, POC (Young 2009; Lee et al. 2007; Chau et al. 2008). Evaluation of comparable biomarkers in early preclinical development in "relevant" animal models should allow for the design of

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successful translational strategies from discovery to the clinic while identifying potential risks to humans and establishing safe First-In-Human (FIH) dosing strategies (Tabrizi et al. 2009; Tabrizi and Roskos 2007). This review will address application of translational biomarkers in development of ABTs.

Translational Biomarkers

Evaluation of relevant biomarkers in appropriate animal models can greatly enhance translation of exposure–response relationships across species (Tabrizi et al. 2009; Tabrizi and Roskos 2007; Buckley et al. 2008). When appropriate immunoassay methodologies are available, relationships between antibody pharmacokinetics (PK) and the ensuing effects on POM or POP biomarkers can be effectively examined (Young 2009; Lee et al. 2007; Chau et al. 2008; Tabrizi and Roskos 2006; Tabrizi et al. 2006). Evaluation of PK–PD relationships in vivo can provide invaluable information with respect to antibody potency (EC_{50}), and the maximum response efficiency (E_{max}), hence, facilitating determination of the FIH dose and the clinical dose escalation plans (Tabrizi et al. 2006, 2009; Tabrizi and Roskos 2006; US Department of Health and Human Services 2005).

Application of biomarkers should guide selection of safe and effective first (1st)-generation leads for advancing through various development stages. Additionally, relevant biomarkers can further provide a clear opportunity for evaluation of differentiating characteristics relevant to development of second (2nd)-generation bio-superior drug candidates and lead evaluation during preclinical phases. In many instances, improvements in affinity, antibody PK, and/or antibody effector functions, namely CDC (complement-dependent cytotoxicity) and/or ADCC (antibody-dependent cell-mediated cytotoxicity), are generally the desirable differentiating characteristics vital in selection of next-generation antibody-based therapeutics (Chaps. 4 and 16). Effective and intelligent design of preclinical studies using translational biomarkers along with careful consideration with respect to clinical performance of the 1st-generation candidate should facilitate advancing the most effective 2nd-generation leads into the clinic.

A major challenge in the development of a novel monoclonal antibody therapeutic is identification of the most pharmacologically relevant species (Tabrizi et al. 2009; Tabrizi and Roskos 2007; Buckley et al. 2008). This can be facilitated by analyzing sequence and structural properties of the antigen, critical residues in the binding region (epitope mapping), understanding of sequence conservation and cross-species homology, and characterization of affinity and functional potency (Chap. 10). Accurate biophysical determination is a key step in development of successful translational strategies as data obtained from biophysical studies will allow for adjustment of affinity differences for the lead candidate across species (Chap. 5). Information regarding antigen concentrations can be obtained experimentally by direct measurements of target antigen(s) in the

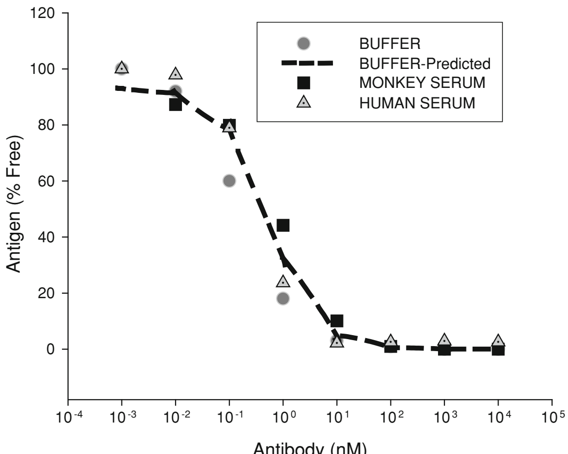

Fig. 13.1 Evaluation of antigen suppression sensitivity in serum from monkeys and humans relative to suppression detected in buffer following incubation of human antigen in the presence of various antibody concentrations

intended patient population that can be compared to antigen concentrations in the preclinical animal models (Fig. 13.1).

Immunogenicity of therapeutic antibodies can be a significant problem in the therapeutic use of antibodies containing xenogeneic protein sequences (Chap. 2). As immunogenic responses against administered antibody drugs can alter PK, PD, and biodistribution, evaluation of antibody immunogenicity in preclinical studies is a critical consideration for appropriate evaluation and interpretation of exposure–response relationships obtained from animal studies (Tabrizi et al. 2006, 2009; Tabrizi and Roskos 2006, 2007; Buckley et al. 2008).

Application of PK–PD Modeling

Implementation of successful translational strategies during development of ABTs necessitates integration of knowledge with respect to antigen distribution, antigen expression, kinetic properties, target pharmacology, PD system efficiency and redundancies, antibody isotypes as well as evaluation of composite factors that regulate or impact (i.e., immunogenicity) antibody PK and PD properties (Tabrizi et al. 2009). Interaction of ABTs with soluble or cell-associated targets provides a unique opportunity for selection and evaluation of relevant biomarkers during the early preclinical stage. Proof-of-Mechanism (POM) biomarkers allow for evaluation of ABTs interaction with the molecular target while POP biomarkers further address if the target modulation results in measurable down-stream activity/ signaling. As safety concerns associated with antibody-based therapeutics are often an extension of their intended pharmacological activity, evaluation of desirable or deleterious outcomes should be accomplished by POC biomarkers.

Proof-of-Mechanism Biomarkers

Application of translational biomarkers has proven highly effective for the design of translational strategies for many of the 1st-generation antibodies marketed against soluble antigens such as omalizumab (Ruffin and Busch 2004; Milgrom et al. 1999; Saban et al. 1994). Due to the central role of IgE in asthma, omalizumab has proven efficacious in adults and adolescents with moderate to severe chronic asthma. Omalizumab is a humanized IgG1 antibody that inhibits binding of IgE to its high affinity receptor, FcεRI, in both man and monkey (Saban et al. 1994). Omalizumab was shown to lower free IgE levels in a dose- and baseline IgE-dependent manner. Reduction in free IgE level, a POM biomarker, was shown to correlate with concomitant decrease in mast cell degranulation and significant reduction in inflammatory cells and mediators, the clinically relevant POP/POC markers (Ruffin and Busch 2004; Milgrom et al. 1999; Saban et al. 1994). The magnitude of reduction in IgE was directly related to the efficacy of omalizumab in asthma, and when the concentration–response relationships in man were corrected for the affinity differences across species, results were in good agreement with the data obtained from preclinical studies in monkeys. A major limitation in the clinical application of omalizumab has been the narrow weight range and limited baseline IgE levels, between 30 and 700 IU/ml, where the antibody can be applied (Putnam et al. 2008). Hence, it was determined that a higher affinity 2nd-generation anti-IgE antibody (HAE1) could offer the potential advantage of expanding the baseline IgE window to include subjects with >700 IU while reducing the dose levels or dosing frequency relative to omalizumab therapy (Putnam et al. 2008). Owing to a higher affinity, the 2nd-generation molecule was expected to require a lower molar ratio of drug-to-IgE to achieve the target free IgE level necessary for reported therapeutic efficacy ($\leq$10 IU). Evaluation of exposure–response relationships using a clinically validated mechanism-based PK–PD model (Putnam et al. 2008; Hayashi et al. 2007) along with therapeutically relevant translational biomarkers (i.e. free IgE) allowed for confirmation of clinical projections from preclinical data and the effective design of translational strategies for HAE1 from the preclinical stage to clinical development phases (Putnam et al. 2008).

Recently, a similar approach was implemented for determination of exposure–response (i.e., POM biomarkers: suppression profile of free antigen) relationships and FIH clinical dose selection for a fully human IgG2 antibody in humans from data obtained in monkeys (Fig. 13.2). Preliminary data obtained from in vitro and in vivo studies allowed construction of a mechanistic PK–PD model shown in Fig. 13.2a. The model accounted for antibody PK, antibody affinity for interaction with the target antigen, free ligand turnover rate, and elimination of antibody–ligand complexes. Preliminary projections using the PK–PD model and the single dose data in monkeys guided the design of a long-term safety study in non-human primates (Figs. 13.2a, b). Following administration of the multiple weekly doses of the lead antibody in monkeys over a 60-fold dose range, concentration-dependent suppression of the free antigen was observed (Fig. 13.2b).

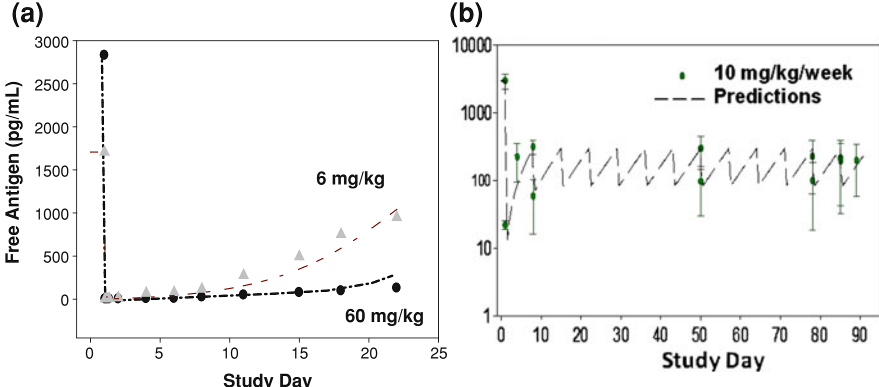

Fig. 13.2 In vivo suppression of antigen in monkeys following administration of a single dose of a fully human antibody (**a**). In vivo suppression of the antigen following administration of multiple antibody doses in monkeys (**b**). The symbols represent the actual data (mean ± SD). The *lines* in **b** represent the predictions made using the data shown in **a**

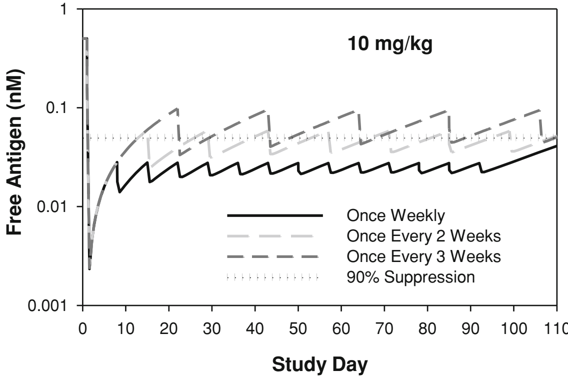

Fig. 13.3 Predicted antigen suppression in man (based on the information summarized in Fig. 13.2) and translation of antibody PK across species. The impact of the changes in dosing frequency for the antibody administration in man is predicted

Biophysical experiments previously revealed a similar antibody affinity for the orthologous antigen in monkey as compared to that in man (also see Fig. 13.1), hence, no correction for the affinity differences across species was necessary. This information allowed for determination of the pharmacological system efficiency (EC_{20}, EC_{50} and E_{max}) in patients facilitating the clinical dose selection (Fig. 13.3).

Biomarker Assays

Development of robust bioanalytical methodologies is the first step in implementing effective translational strategies during ABT development (Chap. 7). Appropriate immunoassay methodologies should examine effectively the relationships between

antibody pharmacokinetics (PK) and the ensuing effects on POM biomarkers. Assays for detection of free antigen post-ABT dosing in vivo can employ appropriate configurations where ABT can be used as a capture reagent. Detection of the free antigen can be accomplished using a tagged anti-antigen antibody that recognizes non-competing epitopes on the antigen recognized by the therapeutic antibody. Application of these assays for determination of POM biomarkers have been described previously (Lee et al. 2007; Tabrizi et al. 2009). Similarly, appropriate assay configurations should allow quantification of bound antigen to the therapeutic antibody in samples collected from in vivo studies post-ABT treatment.

Many of the ABTs raised against soluble antigens such as circulating TNF, VEGF, IgE, and various cytokines (e.g., IL-8, IL-5) have undergone extensive research both in animal models and clinical studies. For this class of ABTs, application of mechanistic PK–PD models using POM biomarkers can prove highly effective for the design of successful translational strategies throughout various development stages. Following administration of therapeutic doses of an ABT, it is anticipated that the free concentrations of the antigen are suppressed. However, in general the elimination rate of small antigens is reduced upon binding to the ABT causing a simultaneous increase in the ABT-antigen complex concentration. The magnitude of the in vivo increase in ABT-antigen complex concentration will be dependent on the turnover rate of the antigen (i.e., antigen synthesis and clearance rates) relative to that observed for the antibody and the elimination rate of the complex (Chap. 6). In many instances, due to the lack of the detection limit of the bioanalytical methodologies used for quantification of free antigen concentrations, experimental evaluation of the free antigen concentration post-ABT dosing is not feasible. However, as a decrease in free antigen concentration is correlated with an increase in bound ABT-antigen complex, application of PK–PD modeling approach should allow for extrapolation of the free antigen concentration when the bound complex is experimentally measured. This approach was employed for evaluation of free IL-8 concentration post anti-IL8 antibody dosing in patients with inflammatory diseases. A mechanistic PK–PD model (Fig. 13.4a) predicted the time-course of the antibody concentration–time profile in relation to the changes in free IL-8 (Fig. 13.4b) and total/bound antigen concentrations (Fig. 13.4c) in serum following administration of multiple antibody doses ranging from 0.1 to 3.0 mg/kg. The theoretical relationship between dose-dependent suppression of free antigen and the reciprocal increases in total antigen concentration at steady-state immediately following administration of the 4th dose are shown in Fig. 13.4d and e. These simulations underline the inverse relationships between the changes in free and bound antigen concentrations at steady-state and further highlight the translational application of POM such as total/bound antigen concentrations where experimental evaluation of free antigen is not achievable.

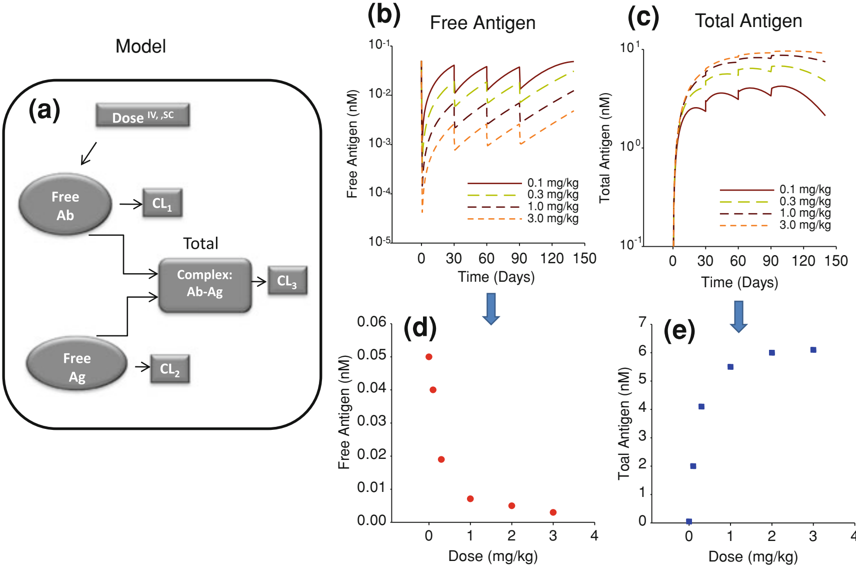

Fig. 13.4 A mechanistic PK–PD model **a** predicted the time-course of the antibody concentration–time profile in relation to the changes in free antigen **b** and total/bound antigen concentrations **c** in serum following administration of multiple antibody doses ranging from 0.1 to 3.0 mg/kg. The theoretical relationship between dose-dependent suppression of the free antigen and the reciprocal increase in the total antigen concentration at steady-state immediately following administration of the 4th dose is shown in **d** and **e**, respectively

Proof-of-Principle/Proof-of-Concept Biomarkers

Many of the currently marketed antibodies recognize membrane-associated internalizing antigens. Interaction of ABTs with this class of target antigen can greatly impact their PK (Tabrizi et al. 2006). When the antigen alters the clearance of an antibody, the effect is usually manifested as a dose-dependent clearance rate and half-life (Chaps. 11, 12). At low antibody doses that do not saturate the antigen, the half-life is shorter; however, as the antibody dose is increased, the antigen is progressively saturated, and an increase in the half-life (or a decrease in the clearance rate) is observed. Dose-dependent saturation of this nonlinear clearance pathway can be used as an effective translational POM/POP biomarker across species. For example, implementation of a science-based translational strategy using POP for efalizumab, a humanized IgG1 antibody against CD11a, proved effective during development of this antibody (Coffey et al. 2004, 2005; Bauer et al. 1999; Sun et al. 2005). As the pharmacokinetics of efalizumab was highly influenced by target expression, a predictive translational strategy was obtained from preclinical studies with respect to antibody internalization, antigen binding, receptor occupancy, and correlation to the in vivo potency in chimpanzee. From this information, therapeutic effective doses were determined during early

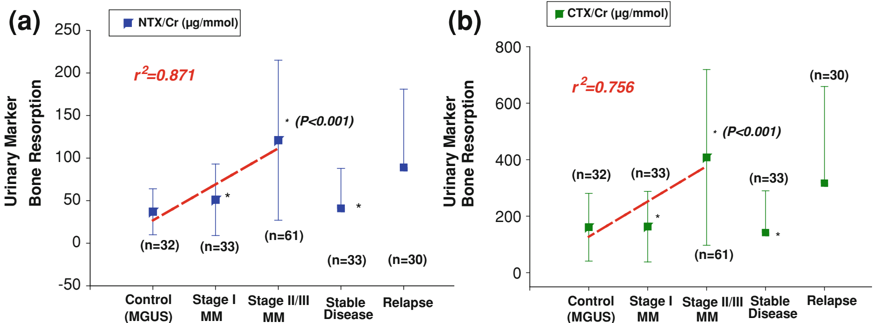

Fig. 13.5 Changes in urinary bone resorption markers (CTX, in **a** and NTX in **b**) in patients with monoclonal gammopathy of undetermined significance (MGUS) and Multiple Myeloma (MM) versus disease progression. Retrieved from Facon et al.

development (Coffey et al. 2004, 2005; Bauer et al. 1999; Sun et al. 2005). Initially, using purified mouse and human T-cells, internalization of anti-CD11a antibodies was evaluated following interaction with the antigen in vitro. In line with these observations, target-mediated clearance of efalizumab following administration of a single intravenous dose in chimpanzee was evaluated. In vitro half-maximal binding of efalizumab to lymphocytes was achieved at an EC_{50} of 0.1 µg/ml (similar to the observed K_m for saturation of the antigen sink in chimps), with saturation requiring concentrations around 10 µg/mL. When corrected for differences in the PK across species, similar steady-state effective serum trough concentrations were achieved in man at relevant therapeutic doses.

In many instances, a POP biomarker, i.e., target-mediated clearance can be effectively correlated to additional POP/POC markers of activity across species. Denosumab (AMG162) is a fully human Xenomouse®-derived IgG_2 antibody directed against human RANKL and is currently undergoing clinical evaluation in cancer and osteoporosis (Kearns et al. 2007; McClung et al. 2006; Lipton et al. 2007; Body et al. 2006; Marathe et al. 2008; Peterson et al. 2004; Facon et al.). Pharmacokinetics of denosumab is highly influenced by target (RANKL) expression and density. A strong correlation has been observed between the target saturation and other mechanistic bone resorption markers such as N- and C-telopeptides, NTX, and CTX (POP biomarkers), in humans and monkeys. These biomarkers have shown a strong correlation with other POC markers such as bone mineral density (BMD), a critical endpoint for evaluation of denosumab therapeutic efficacy in patients with osteoporosis, and with disease progression in cancer patients (Fig. 13.5). Application of translational biomarkers like target-mediated clearance, N- and CTX, along with strong correlation with clinical outcome and disease progression are critical for further clinical development of denosumab and development of bio-superior 2nd-generation anti-RANKL antibodies.

Concluding Remarks

As more advanced antibody-based modalities are emerging, implementation of effective translational strategies using relevant mechanistic biomarkers are becoming essential. Translation of the stimulus–response mechanisms across species that convert receptor occupancy into pharmacological response can be greatly facilitated using relevant POM and POP biomarkers. Application of translational biomarkers during ABT development can provide a predictive framework for generation of vital indices that can guide development of safer and more efficacious 1st- and 2nd-generation leads.

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Chapter 14
Translational Research in Alzheimer's Disease for Development of Antibody-Based Therapeutics

Eric C. Yuen, Enchi Liu and Gene G. Kinney

Abstract Clinical and non-clinical biomarkers play a critical role in translational research in Alzheimer's Disease (AD). Validation of such biomarkers provides the necessary tools to predict target engagement and efficacy in non-clinical and clinical studies. For therapies that target amyloid-beta (Aβ), it is desirable for a compound that decreases brain Aβ in non-clinical studies to correlate with a clinical decrease in brain amyloid load as measured by PET scan of an amyloid binding ligand. Such results provide a set of non-clinical and clinical biomarkers to test potential compounds before moving into large Phase 3 clinical trials. CSF phospho-tau and total tau are also promising biomarkers for therapies that target either Aβ or tau. Volumetric MRI and FDG–PET require further studies before they can be considered biomarkers indicative of response to AD disease modifiers.

Introduction

Alzheimer's disease (AD) is a fatal disorder characterized by a progressive decline in cognition. Approximately 5.3 million people in the US have AD, and it is the seventh leading cause of death in the US. With the rapid growth of the elderly population, the total prevalence of AD in the US is expected to skyrocket to 11–16 million by 2050 (Alzheimer's Association 2010.) In addition to the human toll, AD causes an enormous economic burden. Total health care and long-term service costs total about $172 billion in the US, with an additional $144 billion in unpaid caregiver costs borne predominantly by family members.

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Current treatments of AD are limited to symptomatic therapies that do not alter the underlying progression of neurodegeneration. There is a significant unmet medical need for disease modifying treatments to slow the progression of cognitive decline. A worldwide effort within academia and industry is underway to develop disease modifying treatments but efforts thus far have not been successful. Improvements in translational research are necessary to increase the probability of success of developing such treatments.

Much progress toward an understanding of the pathophysiology of AD has come from autopsy examination of AD brains, which show abnormal deposition of amyloid plaques and neurofibrillary tangles (Braak and Braak 1991). Amyloid plaques consist primarily of beta amyloid (Aβ) deposition, whereas neurofibrillary tangles are made up of abnormal, hyperphosphorylated tau (Crowther et al. 1989; Hardy and Selkoe 2002; Goedert and Spillantini 2006). Research regarding the impact of various forms of Aβ has been underway for many years. Scientific evidence supports the concept that the accumulation of Aβ may play an important role in the pathophysiology of AD. This is captured as the amyloid cascade hypothesis, which proposes that both soluble and insoluble forms of Aβ are toxic and trigger a cascade that includes tau hyperphosphorylation and neuronal death (Hardy and Selkoe 2002).

Two of the main difficulties of clinical research in AD include the variability inherent in cognitive endpoints, as well as the insensitivity of many endpoints (e.g., functional endpoints) required by regulatory authorities in clinical trials compared with non-clinical measures (Vellas et al. 2007, 2008). Such clinical endpoint variability and insensitivity has resulted in the need for large, expensive, and long Phase 3 clinical trials in an attempt to detect efficacy with cognitive endpoints for AD disease modifiers (Green et al. 2009; Gauthier et al. 2009). Translational research with non-clinical and clinical studies is critical to establish that potential therapeutics are demonstrating the desired biological effect in humans and is therefore a critical step toward generating data to justify embarking on large, extensive Phase 3 trials.

Translational Research

Translational research connects bench research to clinical development. Successful use of translational research requires an iterative approach, such that results from bench research can be used to design clinical studies, the results of which can be used to inform bench research to further improve clinical research. Through this feedback process, more predictive animal models and more appropriate biomarker and clinical endpoints across animal models and patients can be chosen in order to improve the chance for successful development of novel treatments.

For AD, reliance on clinical endpoints in animal models has not been predictive of success in clinical trials of disease modifiers to date. This failure may in part be due to the poor correlation between cognitive endpoints in animal models and patients

in clinical trials, possibly due to cognitive testing in animals being focused on specific domains with narrowly defined endpoints compared with clinical endpoints tending to assess a number of cognitive domains (Zahs and Ashe 2010). Empirically, there have been a number of compounds tested in large human Phase 3 studies in which success in multiple endpoints in AD animal models failed to translate into successful clinical trials (Zahs and Ashe 2010). This lack of correlation could be explained by the fact that AD animal models only partially recapitulate all of the clinical and pathological findings in AD patients (Spires and Hyman 2005; Zahs and Ashe 2010) as well as the lack of sufficient evidence of target engagement and/or changes in disease state biomarkers at the doses tested in the clinic.

Thus, in light of the difficulties in utilizing clinical endpoints for AD translational research, biomarkers are increasingly seen as playing a key role. Correlation in biomarker response between animals and humans may result in animal model findings that are more predictive of clinical trial outcomes, which in turn may provide more confidence in entering the clinic, as well as moving into large clinical trials based on positive results from these biomarkers in the clinic. Another advantage of biomarkers is the ability to detect positive results in much smaller clinical trials (e.g., $N < 100$) than those required to detect efficacy in cognition (e.g., $N \sim 1{,}000$) (Beckett et al. 2010; Green et al. 2009; Gauthier et al. 2009).

Biomarkers

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001; FDA 2010). Significant research has been conducted on various biomarkers of AD progression and predictors of AD progression (Wallin et al. 2010; Beckett et al. 2010; Shaw et al. 2009; Okello et al. 2009; Chetelat et al. 2003; Drzezga et al. 2003; Killiany et al. 2000; Vemuri et al. 2009a, b; Henneman et al. 2009; Forsberg et al. 2008; Wolk et al. 2009; Hansson et al. 2006; Mattsson et al. 2009). The most promising biomarkers correlating with or predicting Alzheimer's disease progression include:

1. Positron emission tomography (PET) imaging of brain amyloid load using a radioisotope labeled ligand that binds to fibrillar amyloid
2. Cerebrospinal fluid (CSF) Aβ levels
3. Aβ synthesis and clearance rates measured by stable isotope labeling kinetic (SILK) technique
4. CSF measurements of phospho-tau (p-tau) and total tau (t-tau)
5. Volumetric magnetic resonance imaging (MRI)
6. PET imaging of fluoro-deoxy-glucose (FDG) as a measure of metabolic rate of neurons

7. Other biomarkers such as MRI arterial spin labeling, MRI diffusion tensor imaging, MRI magnetic resonance spectroscopy, CSF isoprostanes, and other biomarkers, all requiring further data to determine whether they would be appropriate response biomarkers.

Correlations between these clinical biomarkers with identical or analogous biomarkers in animal models may provide clues as to the most predictive biomarkers for measuring response to AD disease modifying treatments. Such correlations may also enable the design of non-clinical studies that lead to a better understanding of the pathophysiology that accounts for such biomarker changes in the human disease.

PET Imaging of Brain Amyloid Load

The majority of AD disease modifying treatments in advanced clinical development target Aβ production, clearance, or aggregation (Mangialasche et al. 2010). Most or all of these compounds have been tested in AD animal models and have been shown to decrease brain Aβ (Zahs and Ashe 2010). Such animal studies have been used to determine the optimal doses to study in clinical trials. Successful translational research would suggest that it would be advantageous to develop a method of quantifying amyoid load in the brains of patients during a clinical trial.

Radiolabeled Pittsburgh B compound (PiB) has been used as a PET imaging agent to measure fibrillar amyloid load in the brains of patients, as well as in AD animal models (Klunk et al. 2003; Bacskai et al. 2003). PET–PiB signal correlates well with the clinical diagnosis of AD (Klunk et al. 2004), as well as the quantity of amyloid plaque load in autopsy examination of AD brains (Bacskai et al. 2007; Leinonen et al. 2008; Ikonomovic et al. 2008). Longitudinal studies of patients utilizing PET–PiB suggest that amyloid deposition likely occurs many years before the onset of cognitive decline (Villemagne et al. 2008; Jack et al. 2009; Morris et al. 2009; Rabinovici and Jagust 2009; Wolk et al. 2009; Resnick et al. 2010).

In non-clinical studies, treatments that decrease brain amyloid load as measured by Western blot or immunohistochemistry have also been shown to decrease amyloid load as measured by PET (Weng et al. 2010).

These aforementioned non-clinical and clinical studies of PET amyloid load suggest that this biomarker could also be used to measure response to pharmacologic treatments in clinical trials, especially those that target Aβ production, clearance, or aggregation. One such study was with a monoclonal antibody, bapineuzumab, which binds to the N-terminus of Aβ (Rinne et al. 2010). Patients with mild-to-moderate Alzheimer's disease were randomly assigned to receive bapineuzumab IV ($N = 19$) or placebo ($N = 7$) treatment for 18 months. PET amyloid load was measured at baseline and weeks 20, 45, and 78 utilizing ^{11}C-PiB. Treatment with bapineuzumab reduced cortical ^{11}C-PiB retention over 18 months compared with both baseline and placebo (Fig. 14.1). Importantly, this

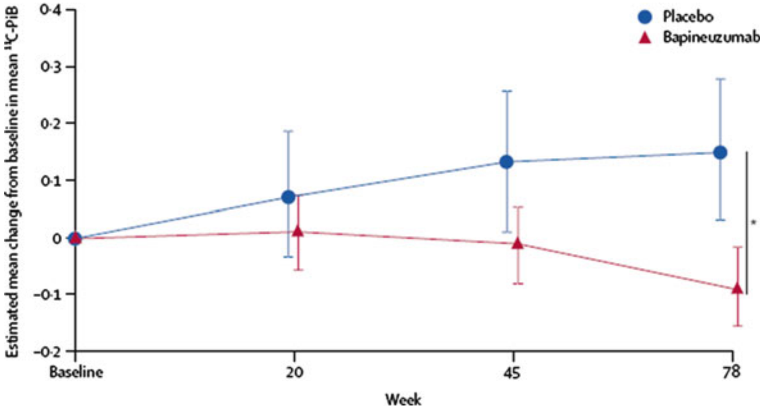

Fig. 14.1 Estimated change from baseline over time in mean uptake of ^{11}C-PiB PET. Data are least squares means and 95% CIs. *Difference between patients in the placebo group and those in the bapineuzumab group at week 78 = −0.24 ($p = 0.003$). PiB = Pittsburgh compound B. Rinne et al. (2010). Disclosure Janssen Alzheimer Immunotherapy is partnered with Pfizer, Inc. to co-develop bapineuzumab

study suggests that ^{11}C-PiB PET is useful biomarker in assessing the effects of potential AD disease modifiers on cortical fibrillar amyloid-β load in vivo. However, since the study was not powered to detect clinical efficacy, determination of the predictability of ^{11}C-PiB change on clinical efficacy requires results from ongoing Phase 3 studies.

A number of other PET amyloid ligands are being studied to determine whether they would also be useful as a response biomarker (Clark et al. 2011; Koole et al. 2009; Kung et al. 2010; Rowe et al. 2008; Small et al. 2006). These ligands have the advantage of being radiolabeled with F18, which has a much longer half-life than ^{11}C.

CSF Aβ Levels

CSF Aβ levels have been studied as a possible biomarker in clinical trials. The rationale is that treatments that lower brain Aβ levels may also decrease CSF Aβ levels. However, there is a complex equilibrium among brain, CSF, and plasma Aβ. In patients with AD, CSF Aβ levels are lower than normal. Indeed, there is an inverse relation between PET–PiB and CSF Aβ1-42 (Fagan et al. 2006). Thus, it is unclear as to whether a treatment that decreases brain Aβ would be expected to further decrease the already low levels of CSF Aβ, or normalize (and increase) the level. Furthermore, some treatments, such as Aβ immunotherapy (vaccination or antibody directed against Aβ) may result in penetration of antibody into the CSF, resulting in binding to the antigen, Aβ, thereby changing Aβ clearance kinetics and ultimately being reflected as an increase in Aβ in

the CSF. Thus, the utility of CSF Aβ levels as a biomarker of treatment response may depend on the mechanism of action.

An additional level of complexity involves the likelihood that different forms of Aβ may change differentially (e.g., Aβ40 vs. 42; N-terminally truncated; oligomers vs. monomers, etc.) Thus, the specific nature of the interaction of the therapeutic treatment with Aβ forms and the assays by which these Aβ levels are measured can result in difficulties predicting the direction of change of CSF Aβ.

Aβ immunotherapy has been shown to decrease brain and CSF Aβ and increase plasma Aβ in Vervet primates following Aβ vaccination (Lemere et al. 2004). In contrast to the changes seen with Aβ vaccination, Aβ immunotherapy with a monoclonal antibody had the opposite effect on CSF Aβ (increased) with similar effects as seen with Aβ vaccination in brain Aβ (decreased) and plasma Aβ (increased) in transgenic mice (DeMattos et al. 2001; Seubert et al. 2008). Clinical trials of bapineuzumab and AN1792 showed no change in CSF Aβ despite decrease in PET amyloid load by bapineuzumab and a change in tau-related CSF markers for both treatments (Gilman et al. 2005; Salloway et al. 2009; Rinne et al. 2010). In contrast, solanezumab, a monoclonal antibody that binds Aβ, increased CSF Aβ (Siemers et al. 2010). These contradictory data suggest that CSF Aβ is not yet a valid biomarker for demonstrating response to Aβ immunotherapy treatment, but instead may require further studies of the time course of change and other experimental conditions.

For a treatment that decreases Aβ production, such as a gamma-secretase inhibitor (GSI), non-clinical studies have generally shown decreases in Aβ levels in brain and CSF, but with decreases or increases in plasma Aβ depending on the experimental conditions (Lanz et al. 2004; Oddo et al. 2004; Barten et al. 2005; Best et al. 2005; Lanz et al. 2010). The GSI LY450139 showed no change in CSF Aβ in Phase 2 studies (Siemers et al. 2006, 2007; Fleisher et al. 2008), and also showed no benefit in clinical efficacy in Phase 3 (Lilly news release 2010). It is likely that the dose of LY450139 in clinical trials was too low due to safety concerns at higher doses owing to relatively poor selectivity for this compound's ability to inhibit Aβ cleavage from the amyloid precursor protein (APP) over inhibition of notch cleavage, and thus the studies of LY450139 are likely non-informative with respect to CSF Aβ as a biomarker. A notch-sparing GSI, BMS-708163, decreased CSF Aβ with lowering in a dose-dependent manner (Albright et al. 2008). Higher gamma-secretase inhibition was possible with BMS-708163 compared with LY450139 because of less safety concerns around notch inhibition. CSF Aβ is a promising treatment response biomarker for compounds that inhibit Aβ production.

Aβ Synthesis and Clearance Rates Measured By Stable Isotope Labeling Kinetic Technique

The SILK technique has been studied as a biomarker of newly synthesized Aβ. Its advantage is that it is a sensitive measure of the decrease in Aβ synthesis of 47, 52, and 84% by a GSI, LY450139 at doses of 100, 140, and 280 mg, respectively

(Bateman et al. 2009). This technique was able to detect a drug effect even though there was no change in CSF Aβ at equivalent doses (Fleisher et al. 2008). However, LY450139 showed no clinical benefit at the 100 and 140 mg doses in the discontinued Phase 3 studies (Lilly news release 2010). These results suggest a lack of predictive concordance between the SILK technique and clinical outcome measures, at least at levels of 47–52% for a GSI. Further clinical studies are required to determine if rates of inhibition of newly synthesized Aβ greater than 52% will predict clinical efficacy.

CSF phospho-tau and total tau

Tau is an intracellular protein in neurons that binds to and regulates the assembly and stability of neuronal microtubules. When tau is found in an abnormal, hyperphosphorylated form, it has the propensity to aggregate, and it is this abnormal form of tau that is the major component of neurofibrillary tangles. The presence of hyperphosphorylated tau and tau in general can be monitored by measuring their levels in CSF in the form of p-tau and t-tau, since CSF levels may largely reflect levels from dead neurons due to the intracellular location of these proteins. Indeed, CSF p-tau and t-tau levels are increased in AD patients (Blennow 2004, 2005; Blennow et al. 2010a, b; Marksteiner, Hinterhuber and Humpel 2007; Itoh et al. 2001).

Non-clinical data suggest that hyperphosphorylation of tau is a downstream effect of Aβ toxicity. For example, in vitro studies have shown that Aβ can phosphorylate tau (Takashima et al. 1998; Zheng et al. 2002; De Felice et al. 2008). In tau transgenic mice, local injection of Aβ produces neurofibrillary tangles (Götz et al. 2001). Furthermore, neurofibrillary tangles seen in tau transgenic mice were enhanced when crossed with Aβ transgenic mice (Lewis et al. 2001).

The effects of Aβ on tau have been shown to be reversed by Aβ immunotherapy or GSI treatment (Oddo et al. 2004). In this study, local intracerebral injection of anti-Aβ monoclonal antibodies in 3 $\times$ Tg mice initially reduced Aβ deposits, followed by a reduction in tau pathology in neurons. Treatment with a GSI replicated these findings. In a different study in 3 $\times$ Tg mice, reduction of both soluble Aβ and tau levels via active or passive immunization against Aβ were required to rescue the cognitive impairments. Notably, decreasing soluble Aβ without affecting soluble tau levels did not improve cognition (Oddo et al. 2006). Wilcock et al. similarly found that, in two different transgenic mouse models, vaccination against Aβ reduced brain Aβ levels, decreased brain hyperphosphorylated tau levels, ameliorated neuronal loss, and reversed cognitive deficits (Wilcock et al. 2009). The mechanism by which Aβ may induce tau pathology could involve decreases in the levels of C terminus of heat shock protein70-interacting protein by Aβ (Oddo et al. 2008).

These non-clinical findings support the notion that decreasing tau may be a necessary step in the successful treatment of AD and that CSF p-tau and t-tau may be important response biomarkers in patients.

Clinically, the quantity of neurofibrillary tangles appears to be more closely correlated with cognitive decline than amyloid plaques (Arriagada et al. 1992; Wilcock and Esiri 1982; Giannakopoulos et al. 2003; Nelson et al. 2007). CSF p-tau and t-tau are increased in patients with AD versus healthy elderly people (Itoh et al. 2001). Both of these findings support the notion that hyperphosphorylated tau leads to active neurodegeneration in AD patients.

Vaccination against Aβ with AN1792 showed a significant decrease in CSF t-tau (Gilman et al. 2005). CSF p-tau was not evaluated due to the lack of a robust assay at the time [Liu (2009) Personal communication, 15 Dec 2009].

In an exploratory, post hoc analysis of pooled data from two Phase 2 studies, bapineuzumab treatment over 12 months decreased CSF p-tau versus placebo ($p = 0.027$), while there was a trend toward decreased CSF t-tau versus placebo ($p = 0.086$; Blennow et al. 2010a, b). These clinical data, in addition to the non-clinical findings, also support the utilization of CSF p-tau and t-tau as response biomarkers. A reduction of CSF p-tau or t-tau could indicate a downstream effect of a drug targeting a reduction of brain Aβ.

Volumetric MRI

Progressive brain atrophy is a well-studied characteristic of AD (Fotenos et al. 2005; Jack et al. 2005; Karas et al. 2004). Atrophy occurs in specific regions of the cortex initially and more rapidly than other regions (Vemuri et al. 2009a; Henneman et al. 2009; Duara et al. 2008; Jack et al. 2005). Brain atrophy can be monitored using volumetric MRI (vMRI). Brain atrophy has not been a biomarker typically used in non-clinical studies, but has been studied extensively in clinical studies. It has been well established as a possible diagnostic biomarker and as a marker of natural disease progression.

Brain atrophy has been less studied as a biomarker for response to an AD disease modifier. In a Phase 2 study of an Aβ vaccine, AN1792, there was a paradoxical acceleration of brain atrophy in AN1792 antibody responders versus placebo as measured by whole brain atrophy, ventricular volume, or hippocampal volume over 12 months (Fox et al. 2005). More rapid loss of brain volume did not correlate with worsening cognition over 12 months, since cognition as measured by the neuropsychological test battery favored AN1792 antibody responders over placebo ($p = 0.008$). In a long-term follow-up of this Phase 2 study, no difference was seen on whole brain atrophy or hippocampal atrophy between antibody responders and placebo-treated patients, while the paradoxical acceleration of ventricular volume enlargement seen over the first 12 months was maintained over 4.6 years ($p = 0.021$). These long-term vMRI changes occurred in the setting of possible clinical benefit as observed on the disability assessment for dementia

scale among antibody responders compared with placebo-treated patients ($p = 0.015$) after 4.6 years (Vellas et al. 2009).

Volumetric MRI was further studied as a treatment response biomarker with bapineuzumab. A Phase 2 study showed no difference in the rates of brain atrophy between bapineuzumab (all doses pooled) and placebo in the modified intent-to-treat population. However, in a sub-group analysis of the cohort of patients who were ApoE4 non-carriers, the rate of whole brain atrophy was reduced in the bapineuzumab-treated group compared to the placebo group ($p = 0.004$). Interestingly, it is this same subgroup of ApoE4 non-carriers that also showed clinical benefit in a post hoc analysis of bapineuzumab over placebo as measured by ADAS-cog ($p = 0.026$), suggesting the possibility of a correlation between slowing of brain atrophy and clinical benefit (Salloway et al. 2009).

The contradictory findings (albeit post hoc, exploratory findings) on correlation between clinical benefit and slowing of brain atrophy for bapineuzumab versus clinical benefit and more rapid brain atrophy for AN1792 are difficult to explain. The authors for the AN1792 results hypothesize that more rapid brain atrophy with AN1792 treatment could be explained by reduction in amyloid plaque that is more impactful on brain volume than any deceleration of neuronal loss, since amyloid plaque may account for up to 9% of the volume of some cerebral regions (Gilman et al. 2005). However, this explanation would not explain the opposite effect that bapineuzumab may have on the rate of brain atrophy. These discrepant results suggest that more studies of vMRI as a response biomarker are required, and its utility may be limited to one compound and not generalizable to other compounds.

PET Imaging of fluoro-deoxy-glucose (FDG)

The metabolic rate of neurons can be measured utilizing PET imaging after administration of radiolabeled fluoro-deoxy-glucose (FDG). A number of studies utilizing FDG–PET have shown that patients with AD have lower levels of FDG–PET signal compared with cognitively normal, age-matched elderly people (Silverman et al. 2001; Jagust et al. 2007; Foster et al. 2007), suggesting its utility as a diagnostic biomarker. FDG–PET signal correlates with cognitive decline, suggesting that it is a marker of normal disease progression.

FDG–PET was studied as a possible biomarker of response to bapineuzumab treatment (Rinne et al. 2010). In an 18 month, randomized, placebo-controlled trial, there was no difference between FDG and PET signal in the bapineuzumab-treated group versus placebo. In contrast, this same study showed a difference in PET–PiB signal. These results suggest that FDG–PET is not a sensitive response biomarker for amyloid clearance, at least with the small sample size ($N = 25$) in this study.

Concluding Remarks

Translational research in development of therapies for treatment of AD requires validation of biomarkers that can predict target engagement and efficacy in non-clinical and clinical studies. For compounds that target Aβ, it is desirable for a treatment that decreases brain Aβ in non-clinical studies to correlate with a clinical decrease in PET amyloid load and possibly a change in CSF Aβ or SILK. Such results provide a set of biomarkers to test potential compounds before moving into large Phase 3 clinical trials. CSF p-tau and t-tau are also promising biomarkers for treatments that target either Aβ or tau. vMRI and FDG–PET require further studies before they can be considered biomarkers for AD disease modifiers.

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Chapter 15
Considerations in Manufacturing Process Development for Antibody-Based Therapeutics

Paula C. Miller and Peiling Xu

Abstract Antibody-based therapeutics are a rapidly growing sector of new pharmaceutical drug candidates. As the therapeutic utility of monoclonal antibodies continues to grow, development of robust manufacturing processes has been a significant focus within the pharmaceutical industry. This chapter describes the work-processes, challenges, and opportunities for platform production of antibodies with a focus on production for use in human clinical trials and marketing applications.

Introduction

The use of monoclonal antibodies in research is widespread. Antibodies are used as therapeutic (Sekhon 2010) and diagnostic agents (Hagemeyer et al. 2009), as well as tools to identify targets for treatment of disease (Sioud 2007) and to research disease pathways. As the utility of monoclonal antibodies continues to grow, development of high-yielding, robust manufacturing processes to produce them has been a significant focus within the pharmaceutical industry.

Antibodies share a common structural framework over large regions of the protein sequence and therefore exhibit similar physiochemical properties (Wang et al. 2007). The similarities in structure and physiochemical properties render

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antibodies ideal candidates for a platform approach for production and purification. Currently, most monoclonal antibodies are produced in mammalian cell culture since mammalian cells possess the requisite machinery capable of performing complex post-translational modifications (PTMs) that are often required for drug efficacy and stability (Jenkins et al. 2008). Comparative studies also indicate that expression titers and specific productivity are higher for antibodies produced in mammalian expression systems (Geisse et al. 1996).

First generation antibody therapeutics were murine derived, followed later by chimeric, and more recently, fully human antibodies are being developed as therapeutics. It has been suggested that relative to murine and chimeric antibodies, fully human antibodies minimize the potential to elicit an immunogenic response in humans (Lonberg 2008). Although antibodies as a class are well suited for developing platforms for production and purification, each candidate must be evaluated individually to assess whether the operations and conditions defined by a given platform will be suitable for production. The following sections describe the work-processes, challenges, and opportunities for platform production of antibodies, with a focus on production for use in human clinical trials.

Cell Line Development

Once a development candidate has been identified (Chap. 2), cell line development begins with transfection of a suitable expression vector into a host cell line. Mammalian expression systems predominate for the large-scale production of antibodies since they can perform complex PTMs that are important for correct protein folding, stability, multimer formation, and secretion into the medium (Jenkins et al. 2008). Chinese Hamster Ovary (Owyang et al. 2011) cell lines are some of the more commonly used host cell lines for antibody production and employ selectable markers based on dihydroxy folate reductase (DHFR) and glutamine synthetase that are available in suitable stable expression vectors for these cell lines (Bebbington et al. 1992). While strong promoters are used to drive product expression, selection markers are often driven by weaker promoters to increase selection stringency (Shukla and Thommes 2010). Cell line stability is another key consideration during development. Typically a good production cell line will be stable for 70–100 generations. The primary goal is to create a stable cell line with the appropriate growth properties and a high specific productivity (Q_p). The selection process is typically facilitated by automation technology such as Fluorescence Activated Cell Sorting since large numbers of clones are typically screened to identify a clone that exhibits the best profile with regard to stability, productivity, and protein quality.

The selection process typically begins in microtiter plates, for example 256- or 96-well plates, progressing to higher volumes and lower well numbers as the selection process proceeds. The selection process also includes evaluation of a subset of promising clones in shake flasks and small bioreactors to assess cell

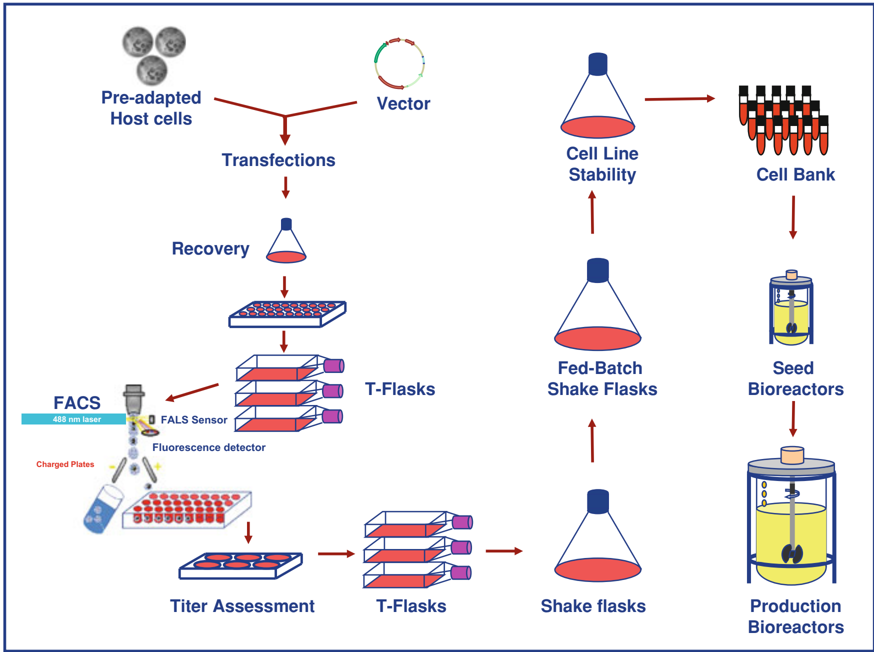

Fig. 15.1 Schematic of a generic cell line selection and production platform

viability and expression. During the selection process, the material that is produced is used to develop or confirm a suitable formulation, downstream processing methods, and to evaluate analytical platform methods. This material and material produced during subsequent cell culture process development are critical for an efficient and integrated strategy for production of clinical trial material. Once a clone is selected, a master cell bank (MCB) is prepared. The MCB, or a working cell bank (WCB) prepared from the MCB, is used in the scale-up and production of material for enabling regulatory toxicology studies and clinical trials (Fig. 15.1). It is worth noting that the MCB is prepared under GMP conditions and is used to generate all the antibody supplies for the lifetime of the product should the candidate be successful. Thus, the preparation of a MCB represents the earliest definition of the commercial process.

Cell Culture Process Development

Platform cell culture process development presents a number of challenges, including the need to adequately control for protein misfolding, aggregation, oxidation, deamidation, proteolysis, and glycosylation variants. Each of these

product-related impurities should be monitored and controlled under the platform conditions. In some cases the platform may need to be modified to address candidate-specific issues.

Scaling the process involves thawing vials from a MCB or WCB. This initial inoculum is expanded in shake flasks and small bioreactors and is then transferred to progressively larger seed reactors prior to transfer of the cell mass to the production bioreactor. There are two phases during process scaling. During the growth phase, the primary objective is to increase the viable cell mass, while during the protein production phase, cell growth slows and antibody expression and secretion ensues. A typical cycle-time in the bioreactor is 10–12 days. In traditional batch production, the media containing the required nutrients and additives is not replaced and production of cellular waste products limits the productivity of the cells. The more commonly employed fed-batch production involves replacing limiting components of the media during the growth and protein production phases (Shukla and Thommes 2010). Titers between 2 and 4 g/l have been attained using this technique and use of optimized media can increase titers further (Glynn et al. 2009). Perfusion technology employs various strategies to retain the cells in the bioreactor while continuously feeding new media to the trapped or immobilized cells. In these systems, high productivity can be maintained for months. The technology is as not widely employed in the pharmaceutical industry due to challenges in maintaining sterility over long periods of time and difficulties in maintaining a consistent product profile (Shukla and Thommes 2010; Kim 2007). Delivery of oxygen, pH, and temperature can impact cell productivity as well as protein quality and are, therefore, controlled during the cell culture process. The mass transfer of oxygen and carbon dioxide at the gas–liquid interface is directly affected by scale and is an important factor to control during scale-up of cell culture processes (Shukla and Thommes 2010).

Harvesting involves separating the cell culture from the growth medium or broth containing the desired antibody, and is accomplished using centrifugation, depth filtration, microfiltration, and various membrane filtrations. Centrifugation takes advantage of the density differences between the cells and the surrounding liquid and accelerates settling in the growth media. The cells are relatively fragile, so care must be taken to avoid excessive shear stresses during centrifugation that can result in cell lysis and the release of host proteins, DNA, and other impurities into the broth. Further removal of cellular debris is carried out using filtration based on size and charge. Depth filtration refers to the use of a porous medium that is capable of retaining debris from the broth throughout its matrix, rather than just on its surface. This can prevent fouling in subsequent membrane filtrations, and it has also been shown that depth filters are able to remove host cell protein contaminants from a recombinant monoclonal antibody process stream (Yigzaw 2006). Size-based membrane filtration is often the last step in the harvest process after which, the clarified broth is further processed through a number of chromatographic and virus removal steps in downstream purification.

Downstream Process Development

The common structural features of antibodies make them well suited to platform purification. Most if not all large-scale purification schemes incorporate Protein-A or MabSelect™ chromatography as the first downstream purification step. The Protein-A immobilized ligand binds to the Fc region of antibodies while host cell proteins (HCP), DNA, and other process related impurities flow through in the mobile phase. Typically Protein-A chromatography affords product in greater than 90% purity after elution from the column at low pH (Darcy et al. 2011).

Ion exchange chromatography is commonly used to further purify the antibody product from HCP, DNA, and other process and product-related impurities. Additional chromatography steps can be employed to address antibody-specific issues; for example, hydrophobic interaction chromatography (HIC) is often selected to remove high levels of aggregate (Shukla 2007). For mAbs, the trend has been to select either a 2- or 3-column downstream platform that efficiently purifies the majority of antibody candidates. In addition to Protein-A, three column platforms have employed anion (AEX) and cation (CEX) exchange steps. Protein-A followed by AEX chromatography using a weak partition mode, or Protein-A followed by CEX where the AEX column is replaced by a membrane are examples of 2-column platforms (Glynn et al. 2009; Shukla and Thommes 2010). Recent technological advances have made membranes an attractive addition, or a potential alternative, to traditional resin column chromatography. For example, the commercially available Sartobind Q membrane outperformed traditional AEX resins for DNA removal when compared side by side and could be reused up to 10 times without affecting its ability to remove DNA. The same membrane, however, is less effective than traditional resins when removing HCP (Yigzaw 2006).

Since antibodies are produced in mammalian cell culture that can harbor viruses harmful to humans, two orthogonal methods to remove viral particles are typically incorporated, in addition to the chromatography steps in the downstream process. These generally include a low pH viral inactivation step followed by viral filtration. Filter pore sizes of approximately 50 nm, capable of removing retroviral contaminates, have been used widely in the past; recently, however, filters designed for the smaller viruses ($\cong$20 nm) such as the Parvovirus, are being incorporated as a result of European Medicines Agency (EMA) requirements introduced in 2008 (EMA 2008). Often, the viral filters can be some of the more costly components in the purification process and smaller pore sizes are more susceptible to fouling, so they are often incorporated late in the platform when most other impurities have been removed. For clinical trials, it is necessary to demonstrate that the process is capable of clearing known and unknown viruses to appropriate levels. Typically this is accomplished using a panel of representative model viruses. The work is often outsourced to dedicated contract organizations due to the special precautions and culture methodology required to carry out this work in-house. In addition, demonstrating viral clearance may involve in-house development of a scaled-down model that mimics the downstream clearance steps

and uses viral mimetics to demonstrate viral clearance prior to outsourcing the clearance studies using live viruses. For First-In-Human (FIH) clinical trials it is generally acceptable to demonstrate clearance of two representative viruses, assayed once, using new resin; however, since subsequent trials in Europe would fall under the 2008 EMEA guidelines, and because viral clearance studies are expensive and time consuming, more and more companies are choosing to comply with the more recent EMEA guidelines in US trials even at early stages of development. Viral clearance validation for Phase 3 and Biologic License Application (BLA) filing is much more comprehensive including, but not limited to, demonstrating clearance for a minimum panel of four representative viruses, assayed in duplicate using new and recycled resins (Zhou and Tressel 2008).

The last downstream step is commonly ultrafiltration/diafiltration to reduce the volume, increase the protein concentration to an appropriate level for formulation, and to incorporate selected stabilizing excipients to facilitate storage and preparation of the fully formulated drug product. The Food and Drug Administration (FDA) defines a drug in part as "A substance intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease"(FDA 2012). This definition, along with others provided, serves to differentiate a drug substance from a drug product. The same drug substance can be found in multiple drug products (e.g. multiple strengths, formulations, or routes of delivery that all contain the same drug substance). For an antibody biotherapeutic, the drug substance refers to the antibody preparation after purification and addition of appropriate stabilizing agents. The drug product is the finished dosage form containing a specific strength of a drug substance, generally, but not necessarily, in association with additional active or inactive ingredients. The final drug product may also be incorporated into various delivery devices including pre-filled syringes and single or multiple use auto-injectors.

Production of clinical-grade material involves execution of multi-step processes and often multiple facilities to support manufacture of the drug substance, drug product, and the required analytical testing. In order to simplify logistics, it is often desirable to develop and define hold conditions for downstream process intermediates and storage conditions for the drug substance. Typical storage conditions are 2–8 °C and require supporting stability studies to define the use period for the stored drug substance. If an antibody is particularly unstable, even after including stabilizing excipients, the drug substance can be frozen to minimize degradation. In this case, however, it is additionally necessary to define and demonstrate drug substance freeze–thaw cycle conditions that do not negatively impact protein quality.

Formulation Development

Like other proteins, antibodies are subject to various degradation pathways. Degradation can occur during the process to prepare the drug substance or drug product and upon storage in the liquid, frozen, or solid state of either the drug

substance or drug product. The downstream process for production of the drug substance is designed to eliminate or reduce impurities and degradation products to within acceptable limits and to minimize formation during subsequent processing to confer a drug product with the appropriate quality. An understanding of the various degradation pathways is critical not only to minimize and remove impurities during production, but also to maintain the appropriate quality attributes of the final drug product post production, and for the duration of shipping and storage.

Degradation of antibodies can be generally categorized as either physical or chemical. Physical degradation includes surface adsorption, denaturation, and aggregation, while examples of chemical degradation include deamidation, oxidation, isomerization, fragmentation, and cross-linking (Chap. 4).

Proteins, including antibodies, can be readily adsorbed onto a variety of surfaces including storage containers, I.V. bags, tubing, etc. Surface adsorption, particularly at very low drug concentrations, can measurably reduce the concentration of antibody in solution and hence the drug available to the patient. In some cases, surface adsorption can be minimized by adding detergents or other surfactants (Doran 2006). Denaturation is another form of physical degradation and can occur as a result of shear, temperature changes, or lyophilization (Wang et al. 2007).

By far, the most significant form of physical degradation of antibodies is aggregation. As the demand for sub-cutaneous and intramuscular injection routes of administration to support patient focused delivery approaches increases, the ability to formulate antibodies at higher and higher concentrations has become a major focus in the pharmaceutical industry. These high concentrations present unique challenges, including the possibility for aggregation and high viscosity solutions that can be difficult to administer to patients. As concentration increases, hydrophilic and hydrophobic surfaces, transiently exposed during normal molecular motion, have an increased probability of interacting in an energetically favorable manner with exposed surfaces in other antibodies and forming stable aggregates (Daugherty 2010). Aggregation can be induced by sheer stress, shaking, temperature changes, freeze–thaw operations, long-term storage, and lyophilization. A variety of excipients including various sugars and surfactants are commonly employed to reduce or prevent aggregation. High concentration solutions can also present solubility and viscosity challenges. Typically high concentration solutions are necessary to deliver the required antibody dose as a single subcutaneous or intramuscular injection of 1–2 ml volume via a syringe. Precipitation and high viscosity can interfere with delivery via a syringe or other device. Screening for optimal pH as well as additives like sodium chloride or histidine have been used to decrease viscosity of antibody solutions (Wang et al. 2007).

Deamidation is one of the more prevalent forms of chemical degradation of antibodies and is a primary contributor to charge heterogeneity. Most deamidation proceeds via a succinimide intermediate and can be influenced by pH, steric effects, and primary sequence (Wang et al. 2007; Daugherty 2010). Typical strategies to control deamidation during processing and storage include screening

for optimal pH and the use of stabilizing buffering agents. Isomerization to afford isoaspartic acid species, often observed for antibodies, also occurs mostly via a succinimide intermediate and can be difficult to control. Since it does not require water to proceed, it can also occur in the solid state. Like other isomerization reactions and deamidation via a succinimide intermediate, steric factors and primary sequence can affect the rate of isomerization degradation pathways (Wang et al. 2007). Oxidation is another degradation pathway, although not as prevalent as deamidation. Methionine and cysteine are some of the more commonly oxidized residues, but oxidation of tyrosine, tryptophan, and histidine has also been observed (Daugherty 2010). Like deamidation, adjustment of the pH to minimize oxidation pathways is a common control strategy. Cross-linking via disulfide bond formation of unpaired cysteine residues or free thiol groups is a chemical degradation pathway that can lead to aggregation. Since antibodies contain several points of flexure within their framework, fragmentation is a common concern in addressing antibody stability. The hinge region between the Fc and Fv domains is one potential site of fragmentation that can affect the efficacy of the drug. The resulting fragments may be more susceptible to subsequent degradation or have a different bio-distribution and clearance profile than the intact monoclonal antibody. Typically optimization and control of pH, temperature, and process and handling conditions are sufficient to control fragmentation.

IgG antibodies produced in mammalian cells contain a biantennary complex attached to each of the two heavy chains within the Fc domain. When produced in CHO host cells these oligosaccharides are often fucosylated and microheterogeneous in nature. Although heterogeneity in glycosylation patterns is not unusual, proper glycosylation and distribution of variants may be critical for proper antibody function (Wang et al. 2007).

Although antibodies share a common framework, many of their degradation pathways are associated with specific primary sequences or the unique antigen binding regions (i.e. CDRs). Additionally, the rates of various degradation pathways for a single antibody differ for a specific set of conditions including pH, temperature, concentration, as well as processing and handling conditions. Therefore, determining the optimal formulation and storage conditions can be very compound specific. Most platform strategies strive to define a formulation or set of formulations that are suitable for the majority of candidates, but are not optimized for any specific candidate. This approach has been quite successful for antibodies, but does not eliminate the need to understand the degradation pathways for a specific compound and to document the selected formulation is suitable for a given antibody. This is particularly important for antibody drug candidates that ultimately become successful commercial products since the dose and dosage form generally evolves as development progresses from FIH studies to regulatory approval and launch.

Analytical Development

In the previous sections, a common theme is the importance of understanding how the production process, formulation, handling, and storage conditions affect the critical quality attributes of antibody drug candidates. Key to this understanding is the ability to monitor and measure the process and product-related impurities observed during development and production, and relate them to the efficacy and stability of a given candidate. A variety of methods can be used to identify, monitor, and quantify the various process and product-related impurities.

Of the various process related impurities, HCP, DNA, and endotoxins can have negative side effects on patients and are therefore of primary concern. Endotoxins, or pyrogenic lipopolysaccharides (LPS) are derived from Gram-negative bacteria such as *Escherichia coli*. In mammalian production hosts, endotoxins can be introduced into a process via raw materials. Endotoxin levels have been examined by the limulus amoebocyte lysate assay for the presence of LPS (GE 2007). HCPs are typically analyzed by Western blotting or 2D gel electrophoresis (GE 2007, Brass, 1996). DNA can be extracted and quantified by standard techniques, including qPCR. Additionally, a number of host cell specific ELISA-based assays to quantify HCP and DNA are also available (Glynn et al. 2009).

Product-related impurities are equally important to monitor and a variety of analytical methods have been employed to quantify the physical and chemical degradation pathways of antibodies. Physical degradation such as denaturation can be measured by various biophysical methods such as nuclear magnetic resonance spectroscopy, circular dichroism, or intrinsic fluorescence. Detecting aggregation can be challenging because aggregates can vary in size from simple dimers to complex multimers and can be soluble or precipitate out of solution. Size exclusion chromatography (SEC-HPLC) has become a standard method for detecting and quantitating aggregates but this method can underestimate high molecular mass species. Therefore, regulatory agencies often request an orthogonal method to confirm SEC-HPLC (Jenkins et al. 2008). Examples of other methods to detect aggregates include aggregate sensitive dyes or analytical ultracentrifugation. The latter method relies on the differences in sedimentation coefficients to separate species based on size. Since cross-linking via disulfide bonds can lead to formation of aggregates, measuring the total free thiol content by diagonal electrophoresis can provide an assessment of the propensity for cross-linking and intra- and intermolecular disulfide bond formation (Jenkins et al. 2008).

Chemical degradation, like fragmentation, can also be detected using methods that separate based on size. Deamidation is the primary source of charge heterogeneity and initial identification may involve detection of differences in charge distribution by isoelectric focusing or high performance cation exchange chromatography (Andya et al. 2001). Similar methods can be used to assess oxidative degradation. Further analyses to identify specific amino acid residues subject to deamidation, oxidative degradation, or other PTMs can be accomplished using

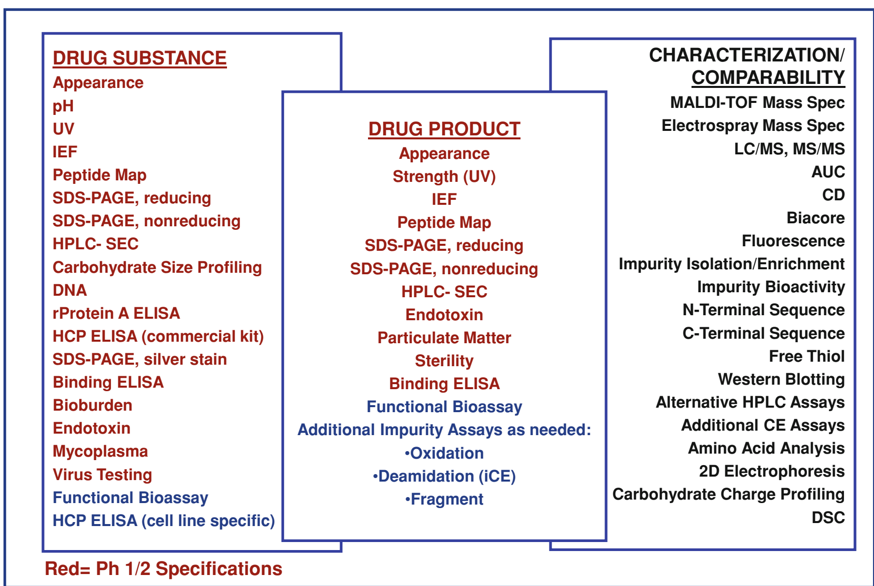

Fig. 15.2 Typical mAb analytical methods for release and characterization

amino acid sequencing, peptide mapping, tryptic digestion, HPLC, and mass spectrometry in various combinations.

Many of the methods described above are amenable to platform development for antibodies. Platform analytical methods are designed to detect and quantify the expected degradation pathways for antibodies, though the impurity profile for any specific antibody tends to be unique. Ultimately, however, for each candidate it is necessary to develop candidate-specific identity and bioassays. N-terminal sequencing and peptide mapping can serve as identity assays. Often simple binding ELISA-based (or Biacore) bioassays are initially developed for early clinical development. It is necessary, however, to develop a cell-based or functional bioassay for later stage clinical trials.

In addition, antibody preparations are evaluated or assayed for appearance, particulate matter, adventitious virus contamination and protein concentration; the latter usually by ultraviolet spectroscopy. Figure 15.2 illustrates a typical strategy for analytical release testing and characterization where methods (shown in red) are used for early Phase 1/2 analysis and additional methods (shown in blue) are added as development proceeds to Phase 3 and filing.

Project Management and Critical Path to Clinical Material

Production of clinical supplies requires close collaboration between multiple scientific disciplines. Often various teams, each with a different focus, are working in an integrated and iterative way during research, process development, and

manufacture with multiple hand-offs between these teams. More often than not, research, process development, and manufacture occur over multiple locations and long periods of time, where personnel move in and out of various roles on the teams. Furthermore, outsourcing some or all of the process development or manufacturing to contract manufacturing organizations (CMOs) is becoming increasingly common which brings with it additional complexity. The outsourced work typically requires a technology transfer and some level of oversight personnel to facilitate and act as technical consultants. The lead times to identify a CMO, conduct a quality audit, negotiate a contract, and schedule a slot for the work can cause delays if appropriate planning is not in place. Moreover, managing the contract and the payment schedule can require additional non-technical oversight. While the pharmaceutical industry's focus on speed to the clinic in recent years has fueled the development of platform process development for antibodies, the technical and business complexities have necessitated careful planning as well as project management to facilitate and streamline platform antibody production across scientific disciplines to ensure an appropriate balance between speed, cost, and quality.

Usually, the transfer of DNA or the appropriate expression vector for a specific candidate from the discovery team to the process development team is the first critical hand-off that initiates process development and manufacture of clinical supplies. For streamlined candidate development, it is also important to have representative protein material to enable early formulation screening and initial assessment of analytical methods. This material can come from the discovery team or can be prepared by the process development team after the hand-off from the discovery team. Within the process development team, cell line development, formulation development, and analytical method development all proceed in parallel. The cell culture development group continues to supply protein to the downstream purification group, and the downstream group supplies protein to the formulation scientists and analysts as process development proceeds. The analysts use this material to iteratively evaluate the capabilities of platform analytical methods to quantify impurities as the process and impurity profile changes during development. In addition to early screening of candidates to identify potential challenges, formulation scientists evaluate the impact of the platform formulation(s) on the selected candidate with regard to stability, storage, and handling. For these studies, it is preferable to use protein produced from the final or near final process to ensure the changes to the impurity profile during process development do not negatively affect the stability of the antibody in a given formulation. The selection of a formulation for the final drug product occurs before the drug substance manufacture begins. This is because some excipients defined in the final formulation are typically added during the last step of the drug substance manufacture to help stabilize the material for storage and for shipping to another facility where the drug product manufacture is completed. It is necessary, therefore, to define the final formulation before manufacture of the drug substance begins. This is different from small molecule development, where the manufacture of the drug substance can usually be decoupled from selection of a formulation that affords the

final drug product. Another difference between development of small molecules and biologics is that real-time stability studies to support clinical shelf-life are required for biologics, while for small molecules predictive stability is often sufficient. This is because proteins, with their associated tertiary structure, do not necessarily conform to Arrhenius-predicted degradation rates like their small molecule counterparts (Weiss et al. 2009). From a timing perspective, this means that after producing the clinical GMP material, a portion needs to be evaluated in a study to demonstrate that it is stable over time before it can be administered to patients. The need to perform real-time stability studies on the GMP clinical supplies can become a critical path to the clinic. Typically GLP material is prepared first to enable the regulatory toxicology studies and while the safety study is underway, the GMP clinical supplies are manufactured and evaluated for stability. The clinical supplies can be packaged and labeled for use while the stability studies proceed in parallel. The process development work and manufacture is documented in the CMC section of the Investigational New Drug or Investigational Medicinal Product Dossier submission and should demonstrate a robust process that consistently produces stable antibody preparations within the defined specifications.

In an accelerated development strategy, availability of representative protein generally defines the critical path to the clinic; first the material from cell culture development is used to enable the parallel development of the downstream purification process, analytical methods, and a suitable formulation; the GLP material is then used for a regulatory toxicology study, and finally, packaged GMP supplies with supporting stability studies are employed to enable clinical dosing. Since GMP supplies can be prepared while the regulatory toxicology is underway, from a production perspective, it is the process development and manufacture of GLP material for regulatory toxicology that is on the critical path to the clinic. Figure 15.3 illustrates the decrease in mAb cycle times for preparation of regulatory toxicology material over time. This graph demonstrates that platforms for antibody production along with careful planning, communication, and project management can afford increasingly fast cycle times.

Considerations for Biosimilar Development and Production

Biosimilars are generic versions of an “innovator” drug with the same amino acid sequence. Additionally, biosimilars are expected to demonstrate a high level of similarity to the innovator drug in a given indication even though they are produced from a different clone and manufacturing process. While generic small molecule drugs have been available for decades, biosimilars have only recently become available, and the first biosimilar antibodies are still in the development pipeline (Beck 2011; McCamish and Woollett 2011). The regulatory pathway for generic small molecules in the US was enabled by the Hatch-Waxman Amendments to the Drug Price Competition and Patent Term Restoration Act of 1984,

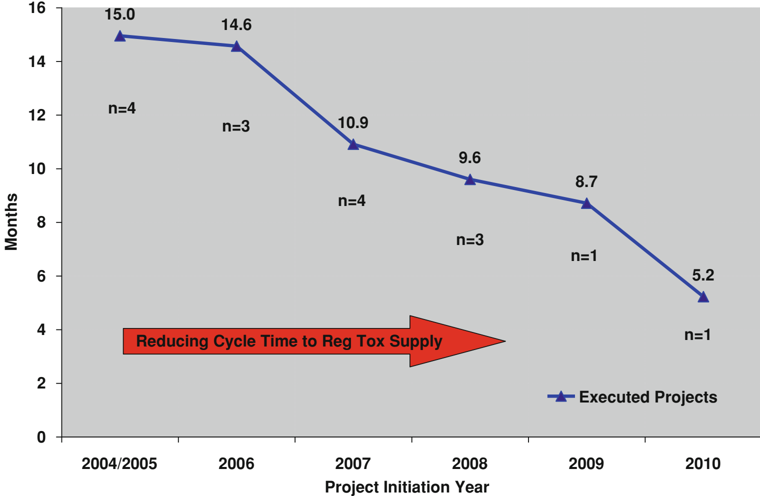

Fig. 15.3 Cycle times for fully executed platform monoclonal antibody development and manufacture of regulatory toxicology material over time (Pfizer Biotherapeutics)

which allowed an abbreviated New Drug Application as long as the generic sponsor could demonstrate bioequivalent safety and efficacy with the innovator drug. Additional clinical trials were not required by the generic sponsor as long as bioequivalence was demonstrated (FDA 1984). In comparison to small molecules, biotherapeutics are large, complex molecules that fold to incorporate specific tertiary structure and are subject to a variety of PTMs. As such, a biotherapeutic drug is a complex mixture of species and is nearly impossible to replicate in every detail.

Nevertheless, batch-to-batch variations are typically small during routine manufacture using the same clone and manufacturing process. Innovator manufacturing process changes, however, are frequently required after approval and during the product life cycle to meet changing demand and/or to decrease cost. Changes to increase productivity, scale of manufacturing, or in order to transfer the process to different manufacturing facilities are some common examples, and as previously discussed, such changes can result in a significant shift in quality attributes that can impact the safety and efficacy of the final drug. To address these concerns, regulatory agencies, and the pharmaceutical industry as a whole, have adopted a data-driven comparability approach where there is a direct comparison of the product before and after the manufacturing changes. Although it is necessary to demonstrate comparable safety and efficacy, these types of changes by the innovator rarely require additional clinical studies (McCamish and Woollett 2011).

Europe led the way in creating a pathway for the approval of biosimilars. In 2004, the European Medicines Agency (EMA) enacted legislation that granted their authority to approve biosimilar products that explicitly refer to a previously approved biotherapeutic for a given indication, based on a comparability approach

(EMEA 2004). For biosimilars, this typically means an abbreviated clinical strategy relative to a new drug, but it is not the same comparability exercise mentioned above- used to monitor product quality before and after manufacturing changes by the innovator- for which additional clinical trials are generally not required. Until recently, the FDA had lacked the authority to approve biosimilars, but in March 2010, as a part of the larger healthcare reform legislation, the Biologics Price Competition and Innovation Act (BPCIA) provided a path for the FDA to consider biosimilars, and furthermore, permits the FDA to designate interchangeability, an issue not addressed by the current EMA guidelines (FDA 2009). If a biosimilar is designated as interchangeable, then the biosimilar may be substituted for the innovator product without requiring prior consultation with the prescribing physician, and may, in turn, increase patient access to this important class of drugs. Currently there is no path to designate interchangeability in EU markets.

In highly regulated markets like the EU and US, a biosimilar is a compound that is "highly similar" to the innovator drug. In practice, determining what "highly similar" means for a given innovator drug can be challenging. The biosimilar sponsor may not rely on the innovator data or the published literature, but must determine the variability in the quality attributes of the innovator drug, preferably over a period of time, by obtaining and analyzing samples of commercial innovator reference product (McCamish and Woollett 2011). Furthermore, and in contrast to small molecule generics, the sponsor must carry out head-to-head clinical trials using both the innovator drug and the biosimilar. Obtaining innovator drug from multiple lots during the product life cycle can be difficult and expensive, but is necessary to define the so-called "goal posts" for the range of innovator quality attributes that define a "highly similar" compound. After defining the "goal posts", a sponsor can carry out iterative process development and analytical characterization to define the process parameters that afford a "highly similar" product. As the complexity of the innovator drug increases, so does the probability that multiple iterations will be required to be successful. The biosimilar sponsor may choose to narrow the quality target range in order to provide additional confidence that the quality attributes remain within the "goal posts" defined by the innovator product during the clinical development and approval process for a biosimilar. Having defined a "highly similar" candidate, the subsequent preclinical and clinical studies may be streamlined relative to a new biological entity. To complicate matters however, regulatory agencies generally require that the innovator reference product is approved and labeled in the local region. This makes a combined strategy for approval in the US and EU markets difficult and expensive. First-generation biosimilars introduced in the EU were "highly similar" to smaller, less complex recombinant innovator drugs like erythropoietin, somatropin, and insulin. The first mAb examples are currently in development (Beck 2011).

Selected Examples from the Literature

Immunogenicity: Hypersensitivity

Cetuximab (Erbitux), with annual sales over one billion US dollars, is a chimeric mouse-human IgG1 monoclonal antibody directed against the epidermal growth factor receptor, and is approved for use in colorectal cancer and squamous-cell carcinoma of the head and neck. A high prevalence of hypersensitivity reactions to cetuximab has been reported in some areas of the US (Chung et al. 2008).

Among 76 cetuximab-treated subjects, 25 exhibited a hypersensitivity reaction to the drug. IgE antibodies against cetuximab were found in *pretreatment* samples from 17 of these subjects, and only one of the 51 subjects who did not have a hypersensitivity reaction had such antibodies. The IgE antibodies were shown to be specific for an oligosaccharide, galactose-α-1,3-galactose, which is present within the Fab portion of the cetuximab heavy chain. Cetuximab is produced in the mouse cell line SP2/0, which expresses the gene for α-1,3-galactosyltransferase. A control study using cetuximab produced in CHO cells did not demonstrate the presence of the aforementioned glycosylation in the Fab region, and was thus not recognized by the preexisting IgE antibodies.

Monoclonal antibody glycosylation is important to the function and safety profile of the molecule and a detailed analysis of atypical glycosylation of monoclonal antibodies is important. An increasingly preferred approach is the use of CHO cells, which after extensive evaluation and experience in the pharmaceutical industry, have been shown to produce glycosylation patterns similar to those observed in humans. Another important consideration is post-market monitoring. In this case, the authors were able to identify preexisting antibodies from the control group, where no cetuximab was administered, and develop a post-market screen to identify the potential for a hypersensitivity reaction in certain patient populations.

Viral Contamination

Viral contamination may have a negative impact on the production process, and more importantly on patient safety. For these reasons, viral clearance continues to be a significant focus within the industry. This example describes Genentech's experience in dealing with two separate viral contaminations and a general approach for viral clearance and control. Additionally, polymerase chain reaction technology was first introduced here for the routine monitoring of viral contamination (Garnick 1996). In both cases of viral contamination, Minute Virus of Mice (MVM) was positively identified. MVM is a mouse-specific parvovirus. This virus is particularly resilient and can therefore be difficult to eradicate from colonies of laboratory animals. Although MVM has not been shown to infect humans,

parvoviruses from related families can cause disease in children and in immune-suppressed individuals (Heegaard and Brown 2002). In this case, the definitive source of the two contamination events was not conclusively identified, but the investigation suggested the contamination may have been introduced via raw materials used during the cell culture process.

In a second example, a DHFR mutant CHO K1 cell was used to produce an unnamed biotherapeutic—the culture was found to become rapidly acidic and widespread cell death was observed (Rabenau et al. 1993). Various methods were used to eliminate the possibility of bacterial, fungal or mycoplasma contamination. Using serum neutralization, electron microscopy, and RNA analysis, the authors identified the contamination as Epizootic Hemorrhagic Disease Virus, isolate 318 (EHDV-318). EHDV-318 was first isolated from a sentinel calf herd at the Khartoum University farm in Sudan, but at least 10 serotypes have been identified globally. The virus causes an often fatal hemorrhagic disease in North American white-tailed deer, but very little information is available about the impact of EHDV in domestic cattle and other ruminants. Thus far, transmission to humans has not been reported (Mohammed et al. 1996). A definitive source of the viral contamination in this case, however, was not determined due in large part to the multiple potential sources of contamination including the cell line, media supplements, inoculated/transfected materials, or laboratory staff.

Although mammalian expression systems like CHO offer advantages over bacterial expression systems, they may also harbor viruses that may be harmful to patients. In addition, viral contamination can have a significant negative impact on production and identifying the source of contamination poses a significant challenge due to the complex and multi-step nature of production processes. Because of the potential disruption of the production process and more importantly for patient safety, it is important to implement a clear quality assurance and control strategy to reduce the potential for viral contamination.

Concluding Remarks

The growth of the biotherapeutic pipeline within the pharmaceutical industry over the past 10 years, and specifically the growth of the monoclonal antibody sector, has fueled the development of platforms to more efficiently develop and manufacture these drugs. Moreover, antibodies are particularly well suited to platform process development and manufacture because they share a common structural framework over large portions of the protein sequence, and therefore exhibit similar physiochemical properties. This approach has been successful within the industry and typically relies on defining a set of development and production parameters that are designed to be suitable for the majority of antibody drug candidates, albeit not optimized for any specific candidate.

Further candidate-specific optimization, if required, can be deferred until the probability of commercial success merits the additional investment. Platforms for

process development and manufacture have also enabled progressively faster cycle times from candidate selection to the clinic. With increased speed, however, there is increased complexity as work proceeds across multiple teams and locations over long periods of time. The importance of project management to facilitate efficient and integrated development throughout the candidate and product life cycle cannot be understated.

While platforms for antibody production have been used to decrease cost and increase speed, it is still necessary to evaluate each candidate individually to assess whether the operations and conditions defined by a given platform will be suitable for production. This is particularly important for antibody drug candidates that ultimately become successful commercial products, since the production process generally evolves as development progresses from FIH studies to regulatory approval and launch. For biotherapeutics, product quality is inherently linked to the process by which the candidates are produced. This link is established early in the choice of a host cell line and clone selection, and continues in defining process parameters for cell culture fermentation and downstream purification. Formulation and drug storage conditions are equally important in maintaining the product quality attributes after manufacture.

Since manufacturing changes will likely be required during the life cycle of a successful product, a robust understanding of the process and the critical parameters that affect product quality is critical for the integration of these changes without disruption of product supply. Finally, as the patents on early biotherapeutics expire and some of the most profitable antibody drugs approach patent expiry, biosimilars are appearing in the marketplace. With regulatory paths for approval of biosimilars defined in both the EU and US markets, this is sure to be a growing segment of research and development. A fundamental understanding of the process parameters that impact product quality is an absolute requirement to develop a biosimilar that meets the "highly similar" standard when compared to the innovator product.

Lastly, the use of platforms to decrease cost and increase speed to the clinic, coupled with a robust understanding of the factors that affect product quality as the production process evolves over time, will ultimately decrease the cost of this important class of drugs, and more importantly, will greatly expand patient access to these life-saving medicines.

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Chapter 16
Strategies for Development of Next Generation Antibody-Based Therapeutics

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Abstract With the anticipated emergence of bio-generics, next generation antibody-based therapeutics have garnered much attention as future contributors to the growth of the biologics market. As next generation modalities confront their first-generation rivals, it is critical that next generation products present a clear differentiating advantage over the existing competition and hence offer the potential to displace their first-generation rivals based on improved therapeutic activity, safety, and increased dosing convenience. Improvements in antibody affinity, specificity (i.e. toward homologous and orthologous cognate antigens), binding epitopes, pharmacokinetics, and potency offer critical differentiating characteristics for next generation antibody-based therapeutics. Herein we discuss recent approaches employed for development of next generation antibody-based therapeutics.

Introduction

With the increasing number of patent expirations of innovative biologics and improved clarity in regulatory requirements for development of biologics, much attention has been directed to the development of next generation antibody-based therapeutics (Beck et al. 2010; Oflazoglu and Audoly 2010; Weiner et al. 2010). Continued innovation in the antibody field has been fueled by improved

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understanding of the disease biology and advances in the technologies available for antibody generation. As the most prevalent therapeutic indications have become increasingly crowded, it will become important for new antibodies to demonstrate improved utility over existing therapeutic options. Products that differentiate in a meaningful manner against existing therapies are more likely to garner market penetration and continue expanding the antibody market. Therefore, the key challenge for the biotechnology industry will be to continue generating antibody-based therapeutics to counteract the market erosion caused by increasing pricing pressures, reimbursement issues, and biosimilar legislation. This chapter will provide an evaluation of the recent approaches employed for development of next generation antibody-based therapeutics.

Antibody Drug Conjugates

Antibody drug conjugates consist of three basic components: the monoclonal antibody, the cytotoxic drug, and the linker coupled to the antibody. The basic premise of an antibody drug conjugate is to confer higher tumor selectivity to a cytotoxic drug that is too toxic to be used on its own, or alternatively, to bestow improved cell killing activity to a monoclonal antibody that is tumor-selective but inadequately cytotoxic (Chari 2008). Tumor selectivity is essential and the antigen target should exhibit elevated and uniform expression on the tumor cell surface, with minimal expression on vital tissues in order to minimize collateral damage and thus toxicity. It is also important to note that antibody drug conjugates are prodrugs that require drug release for activation; activation occurs following internalization of the antibody drug conjugate into the target cell.

Previous failures with this class of anticancer agents have been due to shortcomings with the antibody, the cytotoxic drug, and/or the linkers coupled to the antibody. Monoclonal antibodies used in the early conjugates were either of murine origin or chimeric, and thus immunogenic (Chari 2008; Teicher 2009); the immunogenicity observed in patients prevented repeated cycles of therapy. To increase efficacy and decrease immunogenicity, advances in recombinant DNA technology enabled the generation of humanized and fully human antibodies, including transgenic animals, whereby endogenous antibody genes were replaced by the equivalent human sequences (Khazeli et al. 1994; Green et al. 1994; Lonberg et al. 1994; Bruggemann et al. 1991).

The first generation of antibody drug conjugates demonstrated only modest potency and weaker activity than the parent drug (Chari 2008). These conjugates employed clinically used chemotherapeutic drugs, such as methotrexate, Vinca alkaloids, and doxorubicin, by coupling them to monoclonal antibodies (Chari 1998).

The stability of the linker in circulation is a critical parameter, as spontaneous release of the small molecule drug is likely to compromise potency and exacerbate toxicity of the antibody drug conjugate (Polakis 2005). The three general types of

linkers employed for conjugating small molecule cytotoxics to antibodies can be classified by their mode of cleavage. The hydrazone linkers are susceptible to acid pH and thus release drug under acidic conditions within the lysosomes of target cells. The disulfide linkers undergo intracellular reduction—their mode of action is based on the observation that the intracellular concentration of thiols, such as glutathione and cysteine, is much higher as compared to plasma (Ducry and Stump 2010). The peptide linkers on the other hand, are hydrolyzed by lysosomal proteases (Toki et al. 2002; Dubowchik et al. 2002; Willner et al. 1993; Kaneko et al. 1991; Greenfield et al. 1990; Liu et al. 1996; Miller et al. 2004; Senter 2009). Early antibody drug conjugates employed acid hydrazone linkers; however, the major pitfall of this class of linkers is their tendency to undergo cleavage at non-target sites (Gerber et al. 2009). More recent linkers employ disulfide and peptidic moieties, as described above, which exhibit improved stability in circulation (Polakis 2005; Gerber et al. 2009). The key strength of peptide-based linkers is that their hydrolysis is enzymatic; enzymes can be selected for preferential expression within tumor cells, thereby minimizing the likelihood of drug release outside the cells and into the circulation (Ducry and Stump 2010). Hence, because peptide linkers exhibit improved serum stability, they are also associated with improved antitumor activity (Wu and Senter 2005; Kovtun and Goldmacher 2007). Furthermore, it is worth noting that conjugates coupled via a reducible disulfide bond linker demonstrate bystander cytotoxicity (Kovtun et al. 2006; Doronina et al. 2006). With this class of disulfide-linked conjugates, the cytotoxic drug undergoes disulfide reduction followed by methylation (Chari 2008). This modification of the drug, when released, is able to diffuse out of the cell and kill neighboring cells. Such a mechanism may prove particularly effective in eradicating tumor cells that do not express the target antigen within a tumor mass.

Recently, newer cytotoxic molecules have been developed with improved potencies as compared to first-generation conjugates that employed conventional chemotherapeutic agents (Chari 1998, 2008). For example, auristatins and maytansinoids (Chari 2008; Doronina et al. 2003; Aboukameel et al. 2007) embody new classes of cytotoxics that are under clinical evaluation. These drugs act by binding to tubulin, thereby inhibiting tubulin polymerization. Maytansinoids are derived from a natural product, while auristatins are synthetic compounds. Several maytansinoid antibody drug conjugates have been characterized with demonstrated preclinical activity, and more recently, clinical activity, targeting cell surface tumor antigens including CD19, PSMA, CD33, CD138, and CD56 (Henry et al. 2004; Legrand O et al. 2007; Polson et al. 2009; Tassone et al. 2004a, b; Lewis Phillips et al. 2008). Trastuzumab-DM1 (Beeram et al. 2007) is an antibody drug conjugate that targets $HER2^{+}$ breast tumors and carries an antimitotic maytansine derivative. Recently, Trastuzumab-DM1 was reported to induce 44% Objective Response Rates (ORRs) in breast carcinoma patients when administered at 3.6 mg/kg in phase I and II clinical studies, with dose limiting toxicities of thrombocytopenia (Krop et al. 2007; Vogel et al. 2009). All patients who participated in these clinical trials experienced prior progression on trastuzumab therapy. Importantly, not only did Trastuzumab-DM1 exhibit improved efficacy in this

resistant patient population, patients receiving Trastuzumab-DM1 exhibited a lower toxicity profile as compared to patients receiving trastuzumab therapy in combination with docetaxel. Moreover, Trastuzumab-DM1 clearly demonstrated high clinical activity as a single agent, without the need for additional concurrent chemotherapy. Thus, these clinical studies represent a paradigm shift in the antibody drug conjugate field and highlight the potential of these agents in treating solid tumor malignancies. However, whether Trastuzumab-DM1 will represent a significantly superior agent as compared to other approved or investigational agents remains to be seen.

Calicheamicin has been under investigation due to its ability to induce DNA double-strand breaks and mediate cell killing at significantly lower concentrations compared to most drugs used in cancer chemotherapy (Kovtun and Goldmacher 2007; Hamann et al. 2002a; DiJoseph et al. 2004a). Current antibody drug conjugates employing this toxin are directed against liquid and solid tumor antigens, including CD22 and 5T4, respectively (DiJoseph et al. 2004b, 2005; Boghaert et al. 2008). Moreover, clinical demonstration of this class of warhead is the antibody drug conjugate gemtuzumab ozogamicin (Mylotarg; Pfizer), a humanized anti-CD33 IgG4 conjugated to calicheamicin (Bross et al. 2001; Hamann et al. 2002b; Larson et al. 2005). Mylotarg was approved in 2000 by the FDA for the treatment of acute myeloid leukemia but has been recently withdrawn due to minimal clinical activity and safety concerns owing to the rate of fatal toxicity observed in treated patients. Nonetheless, at least one additional targeted agent is under evaluation in the clinic that currently employs a calicheamicin warhead (Advani et al. 2010).

It is also important to note that one of the major limitations of gemtuzumab ozogamicin was that patients developed resistance to the drug when their tumors overexpressed P-glycoprotein (Linenberger et al. 2001, 2005; Matsui et al. 2002). In fact, the majority of cytotoxics employed in antibody drug conjugates (Walter et al. 2007, 2003; Tang et al. 2009; Kovtun et al. 2010) are substrates for the P-glycoprotein transporter. When an antibody drug conjugate is internalized into the cell, the conjugate is processed and cytotoxic metabolites are generated; these metabolites may be substrates for P-glycoprotein and can therefore be susceptible to P-glycoprotein-mediated resistance. A new approach to circumvent this resistance has been recently developed through modifications in the linker conjugating the antibody to the drug (McDonagh et al. 2006). Kovtun and colleagues describe a maleimidyl based hydrophilic linker coupled to antibody maytansinoid conjugates that appear to be a poor substrate for P-glycoprotein, thereby enabling the cytotoxic drug to remain inside the cell. Moreover, they report that these conjugates bypass P-glycoprotein-mediated resistance in vitro and in vivo, with an improved therapeutic index (McDonagh et al. 2006). The potential utility of this improved linker design is compelling, although it remains to be seen whether such modifications will result in improved clinical responses in cancer patients. Lastly, IgGs have been recently engineered to contain predetermined sites for drug conjugation to yield uniform and more homogeneous drug conjugates with defined stoichiometries. McDonagh and colleagues describe the substitution of cysteine residues

within the constant domains (that form the interchain disulfide bonds) with serine (Junutula et al. 2008). A similar approach involves the incorporation of cysteines at defined sites available for drug conjugation; these mAbs, referred to as THIOMABs, contain two free cysteines in the antibody constant region (Hamblett et al. 2004). The key driver behind this strategy is based on studies that reveal that increased drug loading of an antibody results in reduced efficacy (Sanderson et al. 2005; Cartron et al. 2002). Data demonstrated that antibody drug conjugates with high drug to antibody ratios were cleared more quickly from the circulation, compromising efficacy and tolerability (Sanderson et al. 2005; Cartron et al. 2002). Thus, this novel approach is anticipated to generate highly cytotoxic drugs with increased tolerability, efficacy, and ultimately, more durable clinical responses.

Improvement in Antibody Properties

Selection of an appropriate antibody is the initial step in development of antibody candidates. Manipulation of binding affinity, interaction with effector functions, pharmacokinetic (PK) properties (i.e. half-life and biodistribution properties) and other characteristics, such as improved cross-reactivity and specificity profiles, manufacturability and low immunogenicity, are among the considerations critical in development of next generation antibody candidates.

Improvements in Antibody Effector Functions

Multiple technologies have recently emerged to enhance Fc-dependent antibody effector function (Chap. 4). Despite the success of rituximab, a chimeric IgG1 anti-CD20 monoclonal antibody with demonstrated efficacy in the treatment of non-Hodgkin's lymphomas, there is a recognized need to develop new agents with improved therapeutic activity. While the primary mode of action of rituximab is antibody effector function, new technologies have been developed with the aim of improving antibody drug efficacy and patient survival while circumventing the observed reduced patient responses caused by genetic polymorphisms (Weng et al. 2003; Kubota et al. 2009). Fc-dependent antibody effector functions, namely ADCC and CDC, are mediated by the interaction of the antibody Fc domain with its cognate receptor. In the case of ADCC, the primary receptor is FcγRIIIa, and for CDC, C1q. Given the fact that the antibody Fc domain mediates effector mechanisms, engineering of this region to enhance cytotoxic activity has been extensively studied (Chap. 4).

Several strategies have been implemented to elicit enhanced ADCC activity, including glycoengineering and mutagenesis (Stavenhagen et al. 2008; Lazar et al. 2006; Shinkawa et al. 2003; Yamane-Ohnuki et al. 2004). The basic principle of these approaches is to improve Fc binding to the activating FcγRIIIa receptor, or to minimize binding to the inhibitory FcγRIIb receptor. As discussed in Chap. 4, a

number of technology platforms have emerged that alter the glycosylation of the Fc domain. For example, BioWa has developed a Fut8 knockout CHO cell line that produces afucosylated antibodies (Yamane-Ohnuki et al. 2004; Niwa et al. 2004). Elimination of fucose dramatically improves Fcγ RIIIa binding (Yamane-Ohnuki et al. 2004; Niwa et al. 2004) and afucosylated antibodies have been demonstrated to elicit up to 100-fold higher ADCC activity in vitro when compared to fucosylated counterparts, with concomitant enhanced in vivo antitumor activity (Ferrara et al. 2005). Another case in point is GlycArt; GlycArt employs cell lines that overexpress recombinant 1,4-N-acetylglucosaminyltransferase III to generate antibodies with enriched bisected oligosaccharide (Umana et al. 1999). Recently, an antineuroblastoma IgG1 and an anti-CD20 IgG1 antibody with enriched bisected oligosaccharides demonstrated more than tenfold improvement of ADCC activity as compared to their non-bisected counterparts (Davies et al. 2001; Shields et al. 2001). Another approach to enhance ADCC activity is via mutagenesis of the human IgG1 Fc domain (Lazar et al. 2006; Shinkawa et al. 2003; Shields et al. 2001). For instance, Shields and colleagues have reported Fc domain variants (S298A, E333A, K334A) with enhanced binding to Fcγ RIIIa, resulting in improved ADCC activity (Shields et al. 2001). Other Fc variants (F243L, R292P, Y300L, V305I, P396L) (Lazar et al. 2006) reported up to 10-fold improvement in binding affinity to Fcγ RIIIa with a concomitant improvement in ADCC activity. Lastly, computational design algorithms (Shinkawa et al. 2003) have also been exploited to design novel IgG1 Fc variants with up to 100-fold improvement in binding affinity to FcgammaRIIIa, yielding enhanced ADCC activity in vitro and in cynomolgus monkeys.

Approaches to enhance CDC activity to improve lysis of target cells have also been the subject of meticulous study. For human IgG1, several residues within the CH_2 domain have been implicated as key to the interaction between the Fc domain and C1q (Idusogie et al. 2000; Thommesen et al. 2000) and efforts have focused on increasing the affinity of this interaction. As a case in point, Xencor, Inc. has reported a series of Fc engineered variants of the humanized anti-CD20 monoclonal IgG1 antibody ocrelizumab with improved ability to mediate complement (Moore at al. 2010). More specifically, Xencor, Inc. described three single substitutions (S267E, H268F, S324T) that yielded potency increases of 1.9- to 3-fold relative to native IgG1 ocrelizumab (Moore at al. 2010). Combination of the single substitutions within the same antibody further increased potency, ranging from 3.3- to 5.4-fold for double substitution variants and 6.9-fold for triple substitution variants (Moore at al. 2010).

Improvements in Antibody Specificity

Optimal cross-reactivity to orthologous antigens is critical for selection of relevant species intended for the conduct of preclinical safety and pharmacology studies (Bornstein et al. 2009; Tabrizi and Suria 2009). Limited antibody cross-reactivity

across species exacerbates the complexities and challenges encountered during the course of antibody development as alternate approaches become necessary during antibody development (Bornstein et al. 2009; Tabrizi and Suria 2009). Since favorable cross-reactivity can greatly facilitate preclinical evaluation early on, it is no surprise that novel technologies that allow selection of the most potent antibody leads with improved orthologous cross-reactivity profiles will be of critical importance for development of bio-improved antibody therapeutics. For example, the introduction of tumor necrosis factor-α (TNFα) inhibitors represents a significant advancement in the management of chronic inflammatory diseases (Palladino et al. 2003). Infliximab was the first anti-TNFα antibody introduced into the US market. Due to the limited cross-reactivity of infliximab to non-human orthologs, preclinical safety studies were conducted in chimpanzee (a highly protected species that is no longer accepted as a species of choice for conducting preclinical toxicology studies); additionally, development of a surrogate molecule—a rat-murine chimeric IgG2 antibody (cvlq)—as well as surrogate animal models (i.e. transgenic mouse models) were required for further evaluation of infliximab safety and pharmacology studies (Black and Green 1998). In contrast, development of golimumab, a bio-improved IgG1 anti-TNFα monoclonal antibody, was greatly facilitated by its cross-reactivity to the cynomolgus monkey antigen. Assessment of golimumab pharmacokinetics, pharmacodynamics, and safety in preclinical studies in this cross-reactive species allowed evaluation of the optimized attributes of the bio-improved antibody during preclinical development [Summary Basis of Decision (SBD) 2009].

In many instances, limited cross-reactivity to proteins with close identity and homology within a given family, such as neurotrophins, angiopoietins, or various chemokines might be a preferred design strategy necessary for achieving the optimal clinical safety profile. For instance, the angiopoietin (Ang) family comprises Ang1, 2, 3, and 4 along with their cognate receptor Tie2 and the homologous receptor Tie1 (Brown et al. 2009). Whereas Ang1 and Ang2 share close to 60% identity in their primary amino acid sequence, they bind Tie2 with similar affinity [KD: 3 nM (Maisonpierre et al. 1997)]. Binding of Ang1 is reported to induce tyrosine phosphorylation of Tie2 and activation of its signaling pathway; in contrast, Ang2 is reported to antagonize Ang1 signaling (Maisonpierre et al. 1997; Asahara et al. 1998; Shim et al. 2007; Thurston et al. 1999). Whereas Ang1 mediates vascular protective effects, such as suppressing plasma leakage, inhibiting vascular inflammation, preventing endothelial death, and blood vessel stabilization, Ang2–Tie2 interactions are believed to increase vascular permeability, sprouting, proliferation, and tumor remodeling (Maisonpierre et al. 1997; Thurston et al. 1999). Therefore, it is not surprising that "selective" inhibition of Ang2–Tie2 receptor interaction has been utilized as potential antiangiogenic therapy for treatment of solid tumors (Brown et al. 2009). Similarly, neurotrophins are well known for their role in neuronal survival and growth. Anti-NGF (Nerve Growth Factor) antibodies are currently under development and have proven promising in the treatment of pain in various pathological conditions (Cattaneo and Tanezumab 2010). While NGF and its receptor TrkA are involved in pain transduction

mechanisms in many chronic and inflammatory pain states (Patapoutian et al. 2009), signaling of neurotropin-3 (NT-3) through trkC is believed to play a critical role in proprioception— the sense of position and movement of the limbs (Maisonpierre et al. 1990; Fan et al. 2000; Ramer et al. 2002). Therefore, isolation of anti-NGF antibodies as potential pain therapeutics requires a high degree of specificity for NGF over related molecules such as NT-3.

As discussed in Chap. 6, application of quantitative pharmacology can greatly facilitate establishing the affinity design goals aimed at optimizing the differential reactivity to homologous proteins in vivo (Tabrizi et al 2010). Figure 16.1a illustrates a simple kinetic model of an antibody interaction with two antigens simultaneously within plasma pools. Using differential equations, the model accounts for changes in the unbound antibody, unbound antigens, and the antibody–antigen complexes (Fig. 16.1a). The optimal affinity design goal for antigen 1 and the differential binding to antigen 2 can then be calculated in parallel using the predicted clinical dose, administration frequency, estimates of the unbound antigen clearance rate, the clearance of the antibody–antigen complex, and the percent suppression of the pre-dose antigen(s) concentrations (Fig. 16.1b). As shown, under the simulation conditions employed in Fig. 16.1b, greater than 100-fold affinity differences are necessary to achieve differential reactivity to homologous antigens 1 and 2 at a maximum therapeutic clinical dose of 10 mg/kg administered every 3 weeks. Important considerations for establishing the affinity design goal estimates are the antigen concentrations, antigen turnover rate, and the anticipated antibody–antigen complex clearance that can be directly influenced by the complex size. This topic has been discussed in detail previously (Chap. 6 and Tabrizi et al. 2009).

Epitope Recognition and Target Modulation

Diversity in target modulation is a critical consideration for generation of bio-improved monoclonal antibodies. Antibodies can mediate target modulation by binding to a receptor or its cognate ligand. Modulation of signaling events by manipulating different epitopic domains within the same target can directly influence the antibody potency profile in vivo. For instance, based on their engagement of the target antigen, two classes of anti-CD20 antibodies have been described (Oflazoglu and Audoly 2010; Hammadi et al. 2010). Type-I antibodies, such as rituximab, redistribute CD20 into lipid rafts and elicit complement activation; in contrast, type-II antibodies, such as tositumomab, are capable of inducing non-apoptotic cell death (Beers et al. 2010; Lim et al. 2010). Beers et al. (2010) recently demonstrated that type-II anti-CD20 antibodies were 5-fold more potent and elicited more pronounced elimination of circulating B-cells in a transgenic CD20 mouse model when compared with their type-I counterparts. The enhanced ability of type-II antibodies to deplete B-cells was attributed to a lack of or limited internalization of the type-II antibody following interaction with

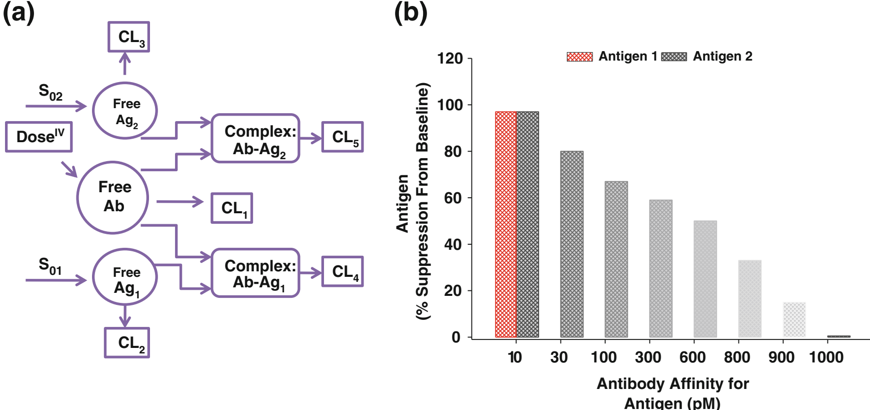

Fig. 16.1 **a** A simple tri-molecular kinetic model of an antibody interaction in vivo with two antigens simultaneously within the plasma pools. The model accounts for antibody elimination and distribution, the affinities for interaction of the antibody with antigens 1 and 2, free antigen turnover rates and elimination of the antibody–antigen complexes (*Ag* Antigen, *Ab* Antibody, *Ab–Ag* Antibody–Antigen Complex, *CL* Clearance, S_0 Antigen synthesis rate). **b** The simulations, conducted as a function of affinities for antigen 1 (set at 10 pM) and antigen 2 (changed from 10 to 1,000 pM), predict that greater than 100-fold affinity differences will be necessary to achieve differential reactivity between two homologous proteins (antigens 1 and 2) at a relevant clinical dose of 10 mg/kg administered every 3 weeks. The following parameters were used as constants: antigen concentrations (Ag1 = Ag2 = 0.3 nM), antigen clearance ($CL_2 = CL_3 = 2.5$ L/day/kg), and the antibody and antibody–antigen complex clearance ($CL_1 = CL_4 = CL_5 = 0.025$ L/day/kg)

the CD20 antigen. In contrast, type-I anti-CD20 antibodies induced significant internalization of the antibody–antigen complex; the more pronounced internalization of the type-I antibodies following interaction with the CD20 antigen resulted in reduced effector cell depletion of B-cells (desired therapeutic effect) due to shorter residence time on the cell surface and enhanced antibody elimination via the target-mediated clearance pathway (Beers et al. 2010; Lim et al. 2010).

Antibodies can also prevent a receptor from assuming a requisite conformation for signal transduction; interference with signal transduction pathways can thus mediate apoptosis and/or inhibit cellular proliferation. This approach has been explored in the design of a second-generation anti-human epidermal growth factor receptor 2 (HER2) antibody (Agus et al. 2005; Baselga et al. 2010). Trastuzumab and pertuzumab are both recombinant humanized monoclonal antibodies that target different extracelluar regions of the HER2 tyrosine kinase receptor. To inhibit signaling, trastuzumab binds to the juxtamembrane portion of the extracellular domain of HER2, while pertuzumab targets the dimerization epitope of the HER2 receptor (Hudis 2007; Nahta et al. 2004). Interestingly, results obtained from clinical studies indicate that pertuzumab can be effective in the treatment of HER2-positive breast cancer in patients that progressed during prior trastuzumab

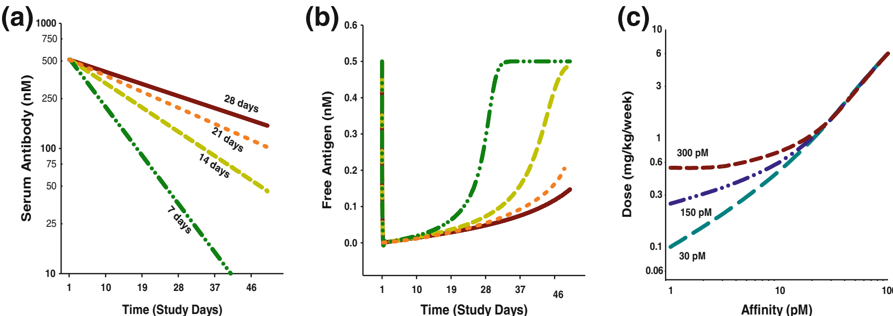

Fig. 16.2 The theoretical impact of improvements in antibody half-life and affinity on the antigen suppression profile. Improvements in antibody half-life from 7 to 28 days results in more prolonged serum exposure (**a**) and antigen suppression profiles (**b**). Relationships between the clinical dose and antibody affinity at three different baseline antigen concentrations (**c**). Decreases in antibody affinity improve the required clinical dose and antibody potency until affinity is reduced to about 1/5th–1/10th of the baseline antigen concentrations

therapy (Agus et al. 2005; Baselga et al. 2010; Langdon et al. 2010). Additionally, preclinical data highlighted that pertuzumab blocks heregulin-dependent association of HER3 with HER2 substantially more effectively than trastuzumab, and further reflected that pertuzumab may exhibit efficacy against tumors expressing normal or only moderately elevated levels of HER2 where trastuzumab showed poor efficacy (Agus et al. 2005; Baselga et al. 2010; Nahta et al. 2004; Langdon et al. 2010).

Improvements in Antibody PK and Affinity

Improvements in antibody affinity and PK half-life are among the critical factors that can reduce clinical dose, dosing frequency, and the extent and duration of antigen suppression profiles. The theoretical impact of improvements in antibody half-life and affinity on the suppression profile of a circulating antigen can be evaluated using relevant antibody–antigen interaction models as described previously (see Fig. 16.1, and Chap. 6). As shown, improvements in antibody half-life from 7 to 28 days results in more prolonged serum exposure and pronounced antigen suppression profiles (Fig. 16.2a, b). Figure 16.2c represents the relationships between the clinical dose and antibody affinity at three different antigen concentrations. As shown, improvements in antibody affinity can impact the required clinical dose; however, the potency ceiling for affinity (a point where further improvements in affinity do not yield additional improvements in potency and clinical dose requirement) occurs when affinity is reduced to about 1/10th of the antigen concentration(s) (Roskos et al. 2007). This is a critical consideration for antibody design in order to maximize binding potency of a therapeutic antibody in vivo.

Improvements in antibody affinity and PK half-life have also been employed in generation of bio-improved monoclonal antibodies. For example, the most recent anti-TNF antibody marketed in the US, golimumab, has the highest potency and longest PK half-life relative to other anti-TNF antibodies with the lowest required effective clinical dose and dosing frequency. Similarly, motavizumab (MEDI-524), an IgG1 anti-RSV antibody currently in development, offers approximately 20-fold higher in vitro potency for viral neutralization as compared to palivizumab. Prophylaxis studies in cotton rats revealed an approximate 5-fold reduction in required serum concentrations ($\sim$8 vs. 40 μg/ml) as compared to palivizumab for a greater than $2\log_{10}$ decrease in lung viral load, the desired clinical endpoint in RSV immune-prophylaxis in infants. Additionally, improvement in the PK half-life via enhancement of FcRn binding (by tenfold) for MEDI-524-YTE resulted in approximately a 4-fold increase in the MEDI-524-YTE circulating half-life relative to that observed for MEDI-524.

Concluding Remarks

Following the success of first-generation antibody therapeutics, next generation antibody-based therapeutics are currently in development against a variety of targets. Knowledge of the structure–activity relationships for antibody molecules has provided the opportunity to generate highly tailored therapeutics by fine-tuning their pharmacological properties, such as target binding, in vivo half-life, effector recruitment, and antigen reactivity. However, many challenges still remain in order to bring more efficient and affordable antibody therapeutics to the market. In addition to antibody-related considerations, the potential impact of bio-improved properties on clinical safety and the risk-to-therapeutic benefit profile in the target patient population will further shape the future development path for next generation antibodies. The recent unfavorable recommendation by the FDA Antiviral Drug Advisory Committee not to license motavizumab due to a higher incidence of allergic reactions in infants as compared to its first-generation counterpart, palivizumab, further underlines the significance of these considerations for development of next generation bio-improved monoclonal antibodies (Motavizumab should not be licensed for marketing: FDA Antiviral Drugs Advisory Committee 2010).

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Chapter 17
Immune Complex Therapies for Treatment of Immune Thrombocytopenia

Rong Deng and Joseph P. Balthasar

Abstract Approximately 30% of patients with chronic immune thrombocytopenia (ITP), a common autoimmune disease, are refractory to standard therapies. In the last several years, several published reports have suggested that gamma globulin immune complexes may inhibit pathways of platelet destruction in ITP, attenuating thrombocytopenia in human patients and in animal models of ITP. This chapter reviews the literature associated with the use of immune complexes as a treatment of ITP, including a discussion of immune complex therapies that are in current clinical use (e.g., IVIG, anti-D), mechanisms proposed for the effects of immune complexes in ITP, possible adverse effects associated with immune complexes, and translational considerations for the development of novel immune complex therapies (e.g., antibody-coated liposomes).

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disease that is characterized by thrombocytopenia, increased rates of platelet (PLT) destruction, and normal or enhanced rates of platelet production (George et al. 1996; Cines and Blanchette 2002; British Committee for Standards in Haematology General Haematology Task Force 2003). The disease was first described by Werlhof as

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Morbus Maculosis Haemorrhagicus in 1735 (Jones and Tocantins 1933); however, the pathogenesis of ITP was not established until the 1950s. In 1951, Harrington et al. demonstrated that the administration of plasma from ITP patients led to the development of thrombocytopenia in normal volunteers (Harrington 1951), and the causative factors in plasma were later identified as antiplatelet antibodies (van Leeuwen et al. 1982; Hou et al. 1997; McMillan 2000a). It is believed that antibody-sensitized platelets in ITP patients are destroyed rapidly by phagocytes in the reticuloendothelial system (RES) through Fcγ receptor-mediated or complement-mediated processes (McMillan 2000b; Cines and Blanchette 2002). Platelet counts in ITP patients are typically <30,000/μL (~ 3–20% of platelet counts found in the general population) (George et al. 1996; British Committee for Standards in Haematology General Haematology Task Force 2003). ITP patients are at risk for the development of bleeding events, and it is reported that about 5–10% of chronic ITP patients will experience a fatal hemorrhage (George et al. 1996; Cines and Blanchette 2002; British Committee for Standards in Haematology General Haematology Task Force 2003).

ITP is typically treated with immunosuppressants (corticosteroids or chemotherapeutics) and/or splenectomy; however, stemming from the pioneering work of Imbach and coworkers (Imbach et al. 1981), there is increased use of high dose intravenous immunoglobulin (IVIG) and anti-D immunoglobulin to treat ITP (Bussel et al. 1991; Scaradavou et al. 1997; Cines and Blanchette 2002). Although the mechanism(s) of IVIG and anti-D action remain controversial, several groups have provided data that suggests that these therapies directly provide, or lead to the in vivo formation of, gamma globulin immune complexes that inhibit pathways of PLT elimination in ITP, thereby attenuating thrombocytopenia in this disease (Tankersley 1994; Teeling et al. 2001b). Based on this hypothesis, several groups have recently introduced new "immune complex" strategies to treat ITP, and promising results have been reported (Bazin et al. 2004; Clynes 2005; Siragam et al. 2005). This review will discuss the use of IVIG and anti-D as therapies for ITP, present a review of new immune complex strategies that are under development, discuss possible mechanisms associated with the effects of immune complexes in ITP, discuss toxicities that may result following the use of such therapies in human patients, and discuss translational considerations for the development of novel immune complex therapies (e.g., antibody-coated liposomes).

Are Immune Complexes Responsible for IVIG Activity?

IVIG therapy calls for the administration of immunoglobulin that is prepared from the plasma of at least 1,000 healthy donors via the Cohen alcohol fractionation method (Good and Lorenz 1991; Lemieux et al. 2005). The pooled immunoglobulin preparation is primarily comprised of IgG (>98%) with trace quantities of IgM, IgA, and other plasma proteins (Lemieux et al. 2005). Imbach et al. first reported in 1981 that the administration of high-doses (2 g/kg) of IVIG led to a

rapid increase in platelet counts in ITP patients (Imbach et al. 1981). Following this report, IVIG has been widely used to treat ITP and other autoimmune diseases (Kazatchkine and Kaveri 2001), such as Kawasaki syndrome (Newburger et al. 1986), systemic lupus erythematosus (Jordan 1989), dermatomyositis (Jolles et al. 1998), rheumatoid arthritis (Maeda et al. 2001), and Guillain-Barré syndrome (Kleyweg et al. 1988).

Proposed mechanisms of IVIG action in ITP include (Kazatchkine and Kaveri 2001; Hansen and Balthasar 2004): (1) neutralization of anti-platelet antibodies by anti-idiotypic antibodies contained within the IVIG preparation (Berchtold et al. 1989); (2) suppression of anti-platelet antibody production (Tsubakio et al. 1983); (3) inhibition of complement-mediated platelet destruction (Basta et al. 1989; Hed 1998); (4) inhibition of Fcγ receptor-mediated platelet destruction (Fehr, Hofmann and Kappeler 1982; Bussel 2000); (5) increasing platelet production (Grossi et al. 1986); (6) acceleration of the clearance of anti-platelet antibodies (Hansen and Balthasar 2002a, b); and (7) increasing the expression of inhibitory receptors that block platelet phagocytosis (Samuelsson et al. 2001). Although many hypotheses have been proposed, most discussions of IVIG action have favored the hypothesis that IVIG increases PLT counts by inhibiting Fcγ receptor-mediated platelet phagocytosis (Crow and Lazarus 2003). This hypothesis has been supported by research that has demonstrated the importance of Fcγ receptor-mediated PLT elimination in ITP, and by data demonstrating that the Fc-portion of IVIG is required for IVIG efficacy in ITP. For example, knockout mice lacking expression of Fcγ receptors were shown to be resistant to the development of experimental immune thrombocytopenia (Clynes and Ravetch 1995), and infusion of monoclonal antibodies against FcγRI (Wallace et al. 1997) and FcγRIII (Clarkson et al. 1986) has led to increases in PLT counts in patients with ITP. Additionally, administration of Fc fragments to ITP patients, at doses similar to those used in IVIG therapy, has been shown to increase PLT counts (Debre et al. 1993), whereas administration of Fab fragments (i.e., lacking the Fc domain) was found to be much less effective than intact IVIG (Tovo et al. 1984).

The Fcγ receptor family includes: FcγRI (CD64), which demonstrates high affinity for monomeric IgG ($k_d \approx 10^{-9}$ M), FcγRII (CD32) and FcγRIII (CD16), which bind to monomeric IgG with a dissociation constant of $\sim 10^{-6}$ M (Ravetch and Bolland 2001). Interestingly, the typical IgG concentration in human plasma is $\sim 7 \times 10^{-5}$ M, which is far greater than the K_D values for all of the Fcγ receptors; consequently, it is anticipated that Fcγ receptors will be essentially saturated prior to IVIG administration. High dose IVIG administration is not expected to lead to a significant change in the fraction of Fcγ receptors bound to monomeric IgG and, therefore, it is unlikely that monomeric IgG contained within IVIG leads to substantial competitive inhibition of Fcγ-receptor-mediated PLT phagocytosis.

However, IVIG contains significant quantities of polymeric IgG, and several recent reports have suggested that IgG dimers and IgG aggregates may bind to Fcγ receptors with high avidity, thereby allowing efficient inhibition of Fcγ receptor-mediated PLT elimination. For example, Tankersley speculated in 1994 that IVIG dimers and aggregates bound to low affinity Fcγ receptors and blocked platelet

clearance in ITP (Tankersley 1994). Consistent with this hypothesis, Telling and coworkers found that the aged IVIG preparations, with increased quantities of dimers and IgG aggregates, were more effective than fresh IVIG when studied using acute and chronic, passive-immune, murine models of ITP (Teeling et al. 2001b). Augener et al. reported that the efficacy of IVIG treatment in ITP was not associated with the monomeric IgG levels, but with the presence of IgG aggregates in the preparation (Augener et al. 1985).

It appears that the quantity of IgG dimers in IVIG is mainly dependent on the size of the donor pool (with dimer quantity increasing with the number of donors) and on the conditions used for IVIG storage (Farrugia and Poulis 2001). The formation of IgG aggregates may occur through anti-idiotypic interactions or via hydrophobic interactions, which may be influenced by temperature, ionic strength, pH, excipients (e.g., sucrose, glucose), and the duration of the manufacturing process (Farrugia and Poulis 2001). Bleeker and his colleagues compared 16 different IVIG preparations from 11 different manufacturers, and they found that commercially available IVIG preparations contained variable amounts of IgG dimers (range: 5–15%) (Bleeker et al. 2000).

Additionally, IgG multimers may form in vivo, following IVIG administration. That is, IVIG is known to contain a wide spectrum of antibodies, including the natural auto-reactive antibodies (auto-IgG). Auto-IgGs are often polyreactive, demonstrating affinity for a variety of self and non-self structures (Avrameas and Ternynck 1993). Natural auto-IgGs have been shown to be much more prone to form immune complexes than non-specific antibodies (Berneman et al. 1993). Based in part on these considerations, Lamoureux et al. hypothesized that injection of large amounts of IVIG could oversaturate the normal mechanisms for control of auto-IgGs in plasma (e.g., anti-idiotype IgM-dependent inhibition of auto-IgGs (Avrameas and Ternynck 1993)) resulting in the formation of soluble autoimmune complexes (Lamoureux et al. 2003). Indeed, incubation of IVIG with human serum in vitro has been shown to lead to the formation of immune complexes (Lamoureux et al. 2004), and purified polyreactive auto-IgG has been shown to provide protection against inflammation in several autoimmune diseases models (collagen-induced arthritis, experimental autoimmune encephalomyelitis in rats, and spontaneous diabetes mellitus in non-obese diabetic mice) (Bruley-Rosset et al. 2003). Recently, it has been shown that administration of complexes formed by incubation of human IgG (from IVIG) and a mouse monoclonal anti-human IgG prevented and reversed thrombocytopenia in mice more efficiently than IVIG (Bazin et al. 2006).

In summary, it is now clear that IVIG contains significant quantities of IgG multimers, and the available data suggest that additional IgG immune complexes form in vivo upon IVIG administration. IgG complexes have been shown to inhibit PLT elimination in vitro, and IVIG effects in vivo have been shown to correlate with the quantity of multimers in the IVIG formulation. As such, there is substantial support for the hypothesis that gamma globulin immune complexes are responsible, at least in part, for the efficacy of IVIG therapy.

Anti-D: An Immunotherapy of ITP Designed for In Vivo Formation of Immune Complexes

In 1983, Salama et al. proposed that IVIG contained anti-red blood cell (RBC) antibodies, which led to the opsonization of RBC in vivo following IVIG administration (Salama et al. 1983). Additionally, Salama et al. hypothesized that antibody-opsonized RBC competed for binding to Fcγ receptors, effectively inhibiting the Fcγ-receptor-mediated elimination of PLT in ITP patients (Salama et al. 1983). Based on this hypothesis, anti-D, a human polyclonal antibody preparation with high titer for the Rho(D) antigen of RBC, was evaluated for treatment of ITP (Bussel et al. 1991; Scaradavou et al. 1997; Ware and Zimmerman 1998). Consistent with the antibody-coated RBCs hypothesis, measurable hemolysis in IVIG-treated and anti-D-treated ITP patients was observed (Salama et al. 1983), and anti-D was found to increase platelet counts at much lower doses of total immunoglobulin than those required for IVIG effects (50 μg anti-D/kg vs. 1–2 g IVIG/kg) (Scaradavou et al. 1997; Ware and Zimmerman 1998). Bussel et al. demonstrated that anti-D was ineffective in ITP patients that did not express the Rho(D) antigen (Bussel et al. 1991), supporting the hypothesis that anti-D achieves effect by the formation of antibody-RBC immune complexes. Additionally, antibody preparations directed against the C antigen of RBC were found to increase platelet counts in three ITP patients who were Rho(D) antigen negative, but C antigen positive (Oksenhendler et al. 1988). Moreover, Song et al. demonstrated that several anti-RBC monoclonal antibodies effectively increased platelet counts in a murine ITP model (Song et al. 2003), and the efficacies of these monoclonal antibodies were correlated with their ability to inhibit Fcγ-receptor-mediated phagocytosis (as measured by the clearance of antibody-opsonized RBC) (Song et al. 2003). In summary, the available data strongly suggest that anti-D leads to the efficient formation of antibody-RBC immune complexes, where the immune complexes lead to efficient blockade of Fcγ-receptors, thereby slowing PLT elimination in ITP. Anti-D is an effective therapy for ITP; however, administration of anti-D leads to several troubling toxicities, including hemolysis, bone pain, and anemia (Hong et al. 1998). Consequently, there is substantial interest in the development of new immune complex strategies that may show the high dose potency of anti-D, yet avoid the main toxicities of anti-D immunotherapy (e.g., anemia).

Preclinical Considerations for Translational Investigations of Novel Immune Complex Treatments of ITP

Discussion of novel immune complex treatments of ITP requires consideration of the preclinical experimental models that are available for evaluating new therapies, and consideration of the potential for the quantitative translation of preclinical

results to predict effects in human ITP. Several animal models of thrombocytopenia have been reported, including models where thrombocytopenia is induced by chemotherapy (Kuter and Rosenberg 1995), platelet activating factor (Meade et al. 1991), immunization against MPL ligand (i.e., thrombopoietin) (Dale et al. 1997), and infection with the dengus-2 virus (Huang et al. 2000). These animal models of thrombocytopenia may serve as poor models of human ITP, as the mechanisms associated with thrombocytopenia are not consistent with ITP (i.e., where thrombocytopenia is mediated by antiplatelet antibodies).

Immune-mediated models of ITP may be categorized as passive-immune models or as active immune models. Several passive models of ITP have been reported, where exogenous antiplatelet antibodies are administered to induce thrombocytopenia (Teeling et al. 2001b; Hansen and Balthasar 2002b; Song et al. 2003; Deng and Balthasar 2007a). These models have been shown to allow induction of controlled, reproducible states of antiplatelet antibody-mediated thrombocytopenia. Passive-immune models have been used extensively to evaluate new treatments of ITP and to evaluate mechanisms associated with drug action in ITP, including investigations of anti-D, IVIG, antibody-coated liposomes, and anti-CD44 antibodies (Teeling et al. 2001b; Hansen and Balthasar 2002b; Song et al. 2003; Deng and Balthasar 2007a; Crow et al. 2011). However, given the nature of passive-immune models, where exogenous pathogenic antibody is used to induce the symptoms of the clinical disease, such models have little or no translational utility when applied to investigate treatments targeting mechanisms associated with the production of pathogenic antibodies.

In active immune models, the pathogenic antibodies are generated by the host immune system. With respect to ITP, the most frequently utilized active immune model employs male W/B F1 mice, which develop a spontaneous autoimmune condition that, at early stages, closely approximates human ITP (Oyaizu et al. 1988; Mizutani et al. 1993). However, the induced thrombocytopenia is of variable severity, and the model is complicated by a high risk rate for fatal myocardial infarctions. Due to their high reproducibility and due to the similar mechanisms of elimination of opsonized platelets in pre-clinical species and in man, the vast majority of translational investigations of new ITP treatments have employed passive-immune models.

To date, little work has been performed to employ model-based methodologies to translate findings from ITP animal models to man, or to quantify the importance of competing mechanisms in ITP animal models (Hansen and Balthasar 2003; Deng and Balthasar 2007b). Pharmacokinetic/pharmacodynamic (PKPD) modeling has the potential to facilitate such quantitative investigations. For example, modeling work conducted by Hansen and Balthasar (2003) and Deng and Balthasar (2007b) allowed demonstration that IVIG effects on anti-platelet antibody clearance (i.e., via competitive inhibition of FcRn) accounted for $\sim$50% of the overall effect of IVIG in passive rat and mouse models of ITP. Future efforts in PKPD modeling of preclinical data may assist in mechanistic evaluations of ITP therapies, while also facilitating the translation of findings from animals to man.

Novel Immune Complex Therapies for Treatment of ITP

Bazin et al. demonstrated that pre-formed tetramolecular immune complexes, containing human Fc fragments and mouse anti-human IgG, were at least six times more efficient than IVIG in preventing the phagocytosis of opsonized red blood cells in vitro, and in attenuating thrombocytopenia in a murine ITP model (Bazin et al. 2004). Siragam et al. reported that antibodies for soluble antigens or insoluble antigens can palliate thrombocytopenia in a murine ITP model (Siragam et al. 2005). They found that mice experimentally treated with soluble ovalbumin and anti-ovalbumin antibodies were protected from immune thrombocytopenia. Additionally, these investigators found that mice treated with ovalbumin-conjugated RBC and anti-ovalbumin antibodies were also protected from ITP (Siragam et al. 2005). Interestingly, antibodies reactive with endogenous soluble albumin or transferrin also ameliorated ITP (Siragam et al. 2005), suggesting that antibodies specific for endogenous proteins may have potential for use in the treatment of ITP. In the above studies, the immune complex therapies were found to be much more potent than IVIG, achieving similar effects at a protein dose that was about 100-fold lower than that required for IVIG.

In parallel with the work of Bazin et al. and Siragam et al. we have proposed that antibody-coated liposomes (ACL), designed to mimic anti-D therapy, may be used as a new strategy for the treatment of ITP (Deng and Balthasar 2005, 2007a). We hypothesize that ACL may compete with antibody-coated platelets for occupation of Fcγ receptors, in a manner that is analogous to that proposed for anti-D opsonized RBC. Additionally, antibody-coated liposomes may allow the inhibition of complement-mediated platelet elimination, which does not appear to be possible with anti-D therapy. The advantages of ACL over IVIG and anti-D are summarized as follows: (1) antibody-coated liposomes are synthetic particles, thus minimizing risk for the transfer of human pathogens; (2) similar to results found for anti-D, in vivo investigations have demonstrated that antibody-coated liposomes achieve effects at much lower doses of immunoglobulin than those required for IVIG; (3) ACL may be engineered to allow sufficient antibody surface density to activate the complement system and inhibit complement-mediated elimination of antibody-coated platelets (which does not appear to be possible with anti-D therapy); (4) antibody-coated liposomes would not opsonize red blood cells and, therefore, it is expected that ACL will not precipitate hemolysis, anemia, or bone pain, which are major side effects associated with anti-D treatment.

Two types of antibody-coated liposomes have been prepared, anti-methotrexate antibody (AMI)-coated liposomes and IVIG-coated liposomes (Deng and Balthasar 2005, 2007a). AMI-coated liposomes were designed to allow reversible binding of murine, monoclonal AMI to the liposome, based on our intent to mimic antibody-coated platelets, which demonstrate reversible antibody-platelet binding. With respect to the future development of clinical formulations of antibody-coated liposomes, the use of human immunoglobulin is desirable as such formulations would be expected to be less likely to induce the development of anti-drug

immune responses in ITP patients. Additionally, covalent attachment of antibody may be more stable in storage and in vivo. Accordingly, the IVIG-coated liposomes preparation that we have tested was developed by covalent amide bond conjugation of phospholipid carboxyl groups and free amine groups of IVIG, using a carbodiimide catalyzed reaction.

AMI-coated liposomes were found to inhibit complement deposition in vitro and to inhibit macrophage phagocytosis of antibody-coated red blood cells in vitro (Deng and Balthasar 2005). In vivo studies demonstrated that AMI-coated liposomes attenuated 7E3 (an anti-platelet antibody)-induced acute, passive thrombocytopenia in rats (Deng and Balthasar 2005). The effects were dependent on liposome dose, size, antibody content within the liposome formulation, and on the dosing schedule employed (Deng and Balthasar 2005).

In a follow-up study, AMI-coated liposomes, IVIG-coated liposomes, IVIG, and an anti-RBC monoclonal antibody (TER119) were found to attenuate experimentally induced thrombocytopenia in a dose-dependent manner (Deng and Balthasar 2007a). However, the effects of TER119 were associated with severe hemolysis, as TER119 decreased RBC counts by $\sim$50%. The antibody-coated liposome preparations were found to achieve increases in platelet counts at a much lower immunoglobulin dose than that required for IVIG, and without the side effects associated with anti-RBC therapy.

Mechanisms of Immune Complex Action in ITP

As introduced above, immune complexes may block platelet destruction in ITP via the engagement and competitive inhibition of FcγR (e.g., FcγRI, FcγRIII) (Dijstelbloem et al. 2001; Clynes 2005); however, immune complexes may also achieve effects indirectly via engagement of the inhibitory Fcγ receptor, FcγRIIB. FcγRIIB signals via immuno-receptor tyrosine-based inhibition motifs (ITIMs), which trigger pathways leading to the inhibition antibody-dependent cellular cytotoxicity, phagocytosis, and cytokine release (Ravetch and Lanier 2000). Inhibitory effects of IgG immune complexes were first recognized over 30 years ago, with the observation that B cell activation could be attenuated by immune complexes (Chan and Sinclair 1971). More recently, Samuelsson et al. reported that the protective effects of IVIG in ITP were dependent on the upregulation of FcγRIIB on splenic macrophages, and these investigators found that IVIG effects were abrogated in mice lacking FcγRIIB (Samuelsson et al. 2001). Song et al. have confirmed that IVIG effects in murine ITP were dependent on FcγRIIB expression; however, they reported that anti-RBC monoclonal antibodies (e.g., TER119 and M1/69) did not require FcγRIIB for activity in the murine model of ITP (Song et al. 2005). The role of FcγRIIB in the effects of immune complexes has been clarified by Siragam et al. These investigators found that FcγRIIB expression was required for efficacy of soluble immune complexes and that FcγRIIB was not required for efficacy of insoluble immune complexes (e.g., antibody-opsonized

RBC) (Siragam et al. 2005). Given the similarities between antibody-coated liposomes and antibody-coated RBCs, antibody-coated liposomes may be expected to inhibit FcγR-mediated phagocytosis through FcγRIIB-independent pathways. However, the mechanisms of action of antibody-coated liposomes in ITP have not yet been thoroughly investigated.

Immune complexes may also elevate platelet counts in ITP patients by inhibiting complement-mediated destruction of antibody-coated platelets (Hed 1998; Ravetch 2002). Immune complexes may modulate complement by the following mechanisms (Mollnes et al. 1995; Basta 1996; Mollnes et al. 1997): (a) binding activated complement components, particularly C3b and C4b, thus functioning as a "scavenger"; (b) binding C1q, and consuming C1q in plasma (i.e., "depletion"); (c) enhanced inactivation of C3b bound to immune complexes; and (d) blockade of the C1R receptors. All of these mechanisms may lead to inhibition of complement-mediated clearance of PLT in ITP; however, at present, there is a paucity of published reports investigating the relationship of these mechanisms to the effects of immune complexes in ITP.

Immune Complexes: Possible Untoward Effects

Large amounts of immune complexes may lead to type III hypersensitive reactions and tissue damage (Goldsby et al. 2000). The extent of damage has been shown to depend on the quantity of immune complexes as well as their distribution within the body (Goldsby et al. 2000). Following formation of immune complexes in blood, it appears that primary sites of complex deposition include blood vessels, synovial membranes, the glomerular basement membrane of the kidney, and the choroid plexus of the brain (Fernandez et al. 2004). Additionally, immune complexes may bind to cells via FcγR, leading to the release of vasoactive substances that increase vascular permeability and decease blood pressure (Bleeker et al. 1987; Bleeker et al. 2000). Bleeker and coworkers found that IVIG treatment led to hypotension in a rat model with vasoactive effects dependent on the quantity of IgG polymers present in the dosing solution (Bleeker et al. 1987; Teeling et al. 2001a). Infusion of IgG aggregates has been shown to induce the "acute lethal toxicity syndrome", which is associated with vascular leakage and hemodynamic shock (Jancar and Sanchez Crespo 2005).

It is hypothesized that IgG immune complexes stimulate these effects through the activation of macrophages and neutrophils (Teeling et al. 2001a). IVIG has been shown to stimulate neutrophils in vivo and in vitro through the binding of IgG dimers and polymers to Fcγ receptors, potentially leading to cytokine release and neutrophil trafficking to extravascular sites (Bleeker et al. 1989). Interestingly, the clinical side effects of IVIG in humans have been associated with a transient decrease in neturophil and monocyte numbers in peripheral blood and an increase in the serum concentration of TNF (Andresen et al. 2000). Correlations have been demonstrated between IgG dimer content in IVIG preparations and the occurrence

of clinical adverse reactions in humans (Spycher et al. 1999). As such, there is concern that the administration of immune complexes therapies (e.g., IVIG, anti-D, antibody-coated liposomes) may lead to a variety of adverse events, and this risk must be balanced by the potential for patient benefit.

Concluding Remarks

It is now well established that the efficacy of two accepted therapies for ITP, anti-D, and IVIG, is mediated, in part, by immune complexes that are either contained in the dosing formulation or generated in vivo. Based on the success of these therapies, several recent reports have introduced new immune complex strategies for treatment of ITP, including soluble IgG/protein complexes and antibody-coated liposomes. These new immune complex strategies have shown promise in animal models of ITP, palliating thrombocytopenia at much lower doses of immunoglobulin than those required for efficacious IVIG therapy, and providing benefit without inducing the main toxicities associated with anti-D (e.g., intravascular hemolysis, anemia). Mechanistic investigations suggest that immune complexes increase PLT counts in ITP by inhibiting Fcγ receptor-mediated phagocytosis of PLT, either via FcγRIIB-dependent (for soluble immune complexes) or by FcγRIIB-independent pathways (for insoluble immune complexes). The possible effect of immune complexes on complement-mediated PLT elimination has not been evaluated thoroughly. Although the new immune complex therapies have shown excellent efficacy in preclinical ITP models, there is some concern for toxicities, including "acute lethal toxicity syndrome" and hemodynamic shock. Further research will be required to weigh the potential benefits and toxicities provided by immune complexes in the treatment of human immune thrombocytopenia.

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Chapter 18
Application of Bioinformatics Principles for Target Evaluation

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Abstract In order to reduce time spent during the lead optimization phase of a drug discovery project, many researchers have attempted to incorporate computerized modeling approaches as part of their program. Various software tools have been developed in support of this pursuit. Molecular modeling, which attempts to produce an electronic representation for the structure of a molecule of interest, and docking, which attempts to discover which molecules are likely to bind to one another, are two approaches that have been used successfully. Although these approaches have worked best with static molecules, recently new tools have been developed to aid in working with more challenging targets, such as antibodies designed to interfere with the function of many cell surface receptors such as G protein-coupled receptors (GPCRs).

Introduction

As drug discovery as a whole has moved toward rational drug design, computational and bioinformatics methods have been adopted widely. In order to utilize computational methods such as docking (a computational process that attempts to determine which molecules are likely to interact) in the discovery process, computerized representations of the structures in question are required. If the structures have already been described via experimental methods such as X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy, these representations

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can be used. The first 3D-structure of an antibody was solved early in the 1970s (Edmundson et al. 1970). However, although the number of experimentally available structures continues to increase (the SACS online database contains an automatically updated summary http://www.bioinf.org.uk/abs/sacs/), there are not nearly enough structures available to completely sustain computer-aided examination. In fact, of the over 70,000 structures in the protein data bank (PDB, http://www.pdb.org), only several hundred are of antibodies. Given the high likelihood that the antibody of interest does not have a structure available, a model representation is often needed.

Molecular Modeling

Models can be produced in one of two ways: Given that the amino acid sequence of proteins (referred to as the "primary structure") determines the three-dimensional (the "tertiary") structure, one can infer that conserved amino acid sequences in molecules will produce the same structure. Thus, for molecules with homologous sequences, a "homology" model of their structure can be produced. However, as is more often the case, molecules of interest do not share sequence homology with molecules in which structural information has already been found experimentally. Therefore, "ab initio" methods, which attempt to predict the tertiary structure of a molecule via the sequence itself, are needed.

Web-Based Modeling

Nonspecific web-based modeling methods such as SwissModel (Arnold et al. 2006) are frequently employed for protein-based homology modeling. However, applications such as SwissModel are not ideal when working with antibodies, as they often do not produce reliable results for the hyper-variable regions, due the high degree of sequence variation. Antibodies essentially have three areas that have proven challenging for classic homology modeling: The conformation of the complementarity determining region (CDR) loops; the hyper-variability of the CDR-H3 loop; and, the relative orientation of the light and heavy chains.

Web-based modeling methods such as WAM (Whitelegg and Rees 2000), PIGS (Marcatili et al. 2008), and RosettaAntibody (Sircar et al. 2009) all allow one to produce models of the variable regions quickly, cheaply, and with fairly little expertise. Using the web antibody modeling (WAM, http://antibody.bath.ac.uk) service is straightforward. Previously, users needed to perform their own alignments via a relatively tedious process. This process is still available to users, and is referred to as the "traditional" method by the program's authors. In this method, the user may enter the antibody sequence for both the heavy and light chains into a web form. The known antibody sequences are provided for each chain so that the user may insert deletions in the CDRs to produce an alignment. Once this is done,

the user must select a screening method before submitting the job. An algorithm called "WAMpredict" is available for those who would like to use this manual alignment method. A new automated method, named "autoalign," is now available. This method automatically adds deletions to the CDRs based on the positions of certain residues. If the user′s sequence meets the specified set of conditions, users may simply paste the sequence into the web site and submit the sequence to produce a model for their antibody of interest.

The WAM method was the first automated and broadly available web service for modeling antibodies. However, it has several limitations. For example, the user is not given insight into what structural templates were used to generate the model. This obfuscation is detrimental, as structural biologists often want to overlay and compare the template and model structures to analyze the differences. Additionally, the resulting structures often need a great deal of further preprocessing due to steric clashes of atoms before being incorporated into a docking workflow. The Prediction of Immunoglobulin Structure method (PIGS) addresses some of the shortcomings that exist with the WAM service. The PIGS method is another web-based service for the modeling of antibodies based on the traditional structure method. A key differentiator is that PIGS allows the user to choose templates (for the frameworks and the loops) and modeling strategies in an automatic or manual fashion.

Using the PIGS service is intuitive, even for those with limited knowledge of modeling. Similar to WAM, PIGS allows pasting in the standard FASTA (a widely recognized text-based format which uses single letters to represent either nucleic acid or amino acid sequences) formatted sequence of the variable regions of the light and heavy chains of the antibody of interest. This service will then display a list of putative templates for both loops. The user can select which templates to use. For users with limited knowledge of modeling, the method of template production is limited enough that one from each type can be selected. Although the PIGS method may have some value, it is notoriously inaccurate in the absence of information about the CDR H3 loop, which often occurs in novel antibody sequences. The most recently developed web service for modeling antibodies, RosettaAntibody, addresses some of these shortcomings.

The RosettaAntibody service (http://antibody.graylab.jhu.edu) is similar in utility to the previously described methods. RosettaAntibody generates ten antibody homology models for each input sequence, and this set of models can be used simultaneously with EnsembleDock, a docking method and tool produced and maintained by the same lab. However, errors in the CDRs of RosettaAntibody homology models (particularly H2 and H3) can still frustrate docking, and only the ten pre-generated backbone conformations are sampled during ensemble docking. The user can incorporate the sequences of the light and the heavy chain of the antibody of interest. Then two options can be selected: the "quick" method and the "long" method. The long method can take significantly more time, due to the fact that it will attempt to model the CDR H3 loop in an ab initio fashion—that is, it will have to generate the loop portions without using any pre-existing structures for comparison. The RosettaAntibody service is the most broadly used web-based

antibody service currently available, and the included refinement of VL-VH relative orientations along with CDR H3 loop modeling and minimization of all the CDR loops are the key strengths of this method.

All of the above methods can be used to generate a model for the structure of an antibody. This alone can be useful for structure-based studies. In a review by (Schwede et al. 2009) homology modeling was stated to be integral to the development of 11 of the first 21 marketed antibodies including Zenapax, Herceptin, and Avastin. However, it is often even more desirable to use the resultant structure in a docking study.

Molecular Docking

Regardless of which method is used to produce the computerized representation of the structures, once they are available, docking methods can be utilized. Computerized docking methods are often used to identify novel structures or scaffolds that bind to a receptor in the context of a "virtual" screen, or evaluate the likelihood of different "poses", or protein–ligand pairs, to support the "lead optimization" phase of drug-discovery projections. Docking methods, though varying in the algorithmic foundations that separate them, all work by attempting to identify the macromolecular complex with the position that has the lowest free binding energy. Essentially, all docking methods can be broadly described as having to go through several phases.

In the searching phase, the goal is to produce a number of protein–ligand pairs, or "poses", to serve as inputs for a scoring function. Due to the extremely high number of potential poses, docking approaches typically limit the search space to a reasonable subset of the total number of conformations. Once the poses are generated, the scoring phase applies a mathematical method, known as a scoring function, to quantify the predicted interaction strength. Finally, based on their scores, the poses are evaluated and ranked.

Various docking methods have been developed and used, mainly in the study of protein–protein and protein–ligand interactions. Docking methods are useful in the study of antibody-antigen and antibody-hapten interactions, as the antigen binding site of antibodies is well characterized, and the general structure of most antibodies (except for the hypervariable region, which consists of six loops of variable sequence and structure located on Beta sheets on the antibody's surface) is highly similar.

There are three phases that are common to all docking applications. First, docking programs must find useful ways for representing molecules. Second, docking programs must algorithmically determine all potential poses of the receptor and ligand of interest. Finally, docking applications need a method to calculate and express the complementarity of those poses.

There are two approaches to electronically represent structures: abstract descriptions and atomic representations. Most approaches use abstract descriptions of the protein to reduce the macromolecular structural information to a level that is

manageable. Additionally, there are two common approaches that are employed to represent molecules: Geometric shape descriptors and grids. Geometric shape descriptors generally use common shapes to represent chemical elements and/or properties, while grid approaches use grids based on energies and forces. Antibodies have been shown to undergo significant changes upon binding, such as adjustments of single amino acid side chains, loop rearrangements, and entire domain movements. Therefore, successful docking protocols must account for these changes.

Applications of Docking Methods

Given that there is known to be structural flexibility in the various segments of an antibody fragment variable (FV) region, docking methods that can address flexible or non-rigid molecules will inevitability be more predictive. A recent report (Agostino et al. 2009) highlights that of the four programs evaluated (e.g. Glide, AutoDock, GOLD, and FlexX), the Glide method was the most predictive for docking carbohydrate ligands to antibodies. These algorithms, although highly useful, are only available as applications that must be downloaded and installed. Additionally, some of them (i.e. Glide) are now commercial products.

ClusPro (Comeau et al. 2004) was one of the first docking services that was web based. Moreover, ClusPro has features specifically designed to improve docking results with antibody-antigen complexes. Presently, the inventors of the program have not disclosed how antibodies are treated compared with other receptors. The ClusPro service (http://cluspro.bu.edu/login.php) is extremely simple to use. To utilize the service, the user needs to enter the PDB ID (i.e. the 4-character unique identifier of every protein in the protein data bank) for both the receptor (the antibody) and ligand (the antigen) of choice, or the user may upload a PDB file directly. To enable the antibody-specific functionality, the user must select “antibody mode”. If desired, the user also has the option of automatically masking the non-CDR regions. ClusPro is just one of the available online docking services that use fast-Fourier transform methods for grid matching. Others include GRAMM-X (Tovchigrechko and Vakser 2006) and ZDOCK (Chen et al. 2003).

PatchDock (http://bioinfo3d.cs.tau.ac.il/PatchDock/) and SymmDock (http://bioinfo3d.cs.tau.ac.il/SymmDock) are docking algorithms available as web services, but both use shape complementarily principles instead of brute-force methods that search the entire conformational space. The advantage of this approach is that it is much quicker in environments without significant computing power available. They are also of interest because they include a set of parameters, known as a “complex type,” formulated especially for antibody docking runs. If the user selects the antibody–antigen complex type, the program automatically detects the CDRs of the antibody and limits the search to these regions.

RosettaDock (http://rosettadock.graylab.jhu.edu) by Lyskov and Gray (2008) has emerged to become one of the most widely used and accurate algorithms

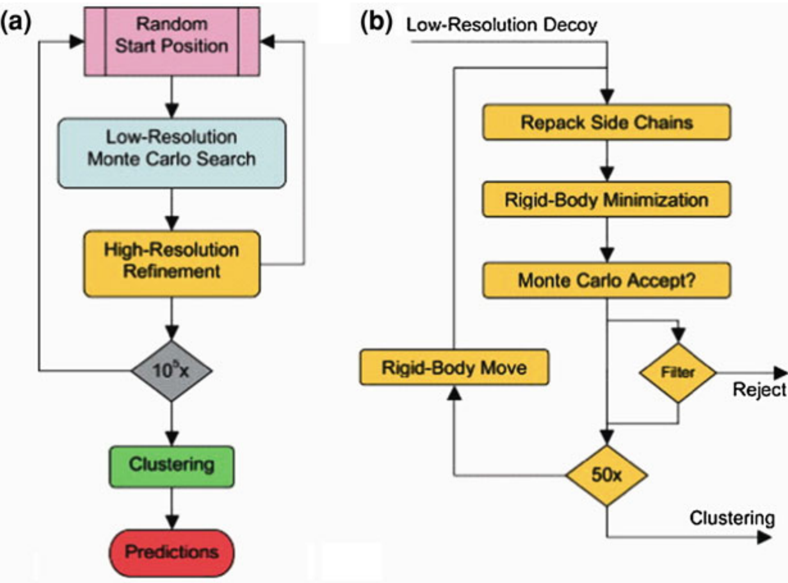

Fig. 18.1 The RosettaDock protocol. **a** The docking process flowchart. $10^5\times$ refers to the number of decoys used in this step. **b** The process flowchart of the refinement step. 50× refers to the number of times this step is repeated. Figure originally from Gray et al. (2003)

available (Lensink et al. 2007). This algorithm was developed by the Gray lab, the same group that created the RosettaAntibody approach (Fig. 18.1). Application of the RosettaDock server is similar to other web-based docking tools. To utilize the service, the user must enter or upload the PDB files of the proteins to be docked. The user can use either a combined method, or to separate the two files. The site currently only allows local docking jobs (the jobs only explore conformation within 10–20 angstroms RMSD-Root Mean Square Deviation-from the upload starting conformation) to be run, in order to prevent excessive CPU times and ensure that all users are provided reasonable access to the service.

SnugDock (Sircar and Gray 2010) is the first docking algorithm with targeted antibody flexibility. SnugDock is built upon RosettaDock using the sampling components of RosettaAntibody. This new protocol performs multibody docking by allowing simultaneous structural optimization of the relative orientations of antibody-antigen and VL-VH. SnugDock simulates induced fit by simultaneous optimization of the binding interface by allowing flexibility of the CDR loops and interfacial side chains. Even better predictions can be made by combining SnugDock with EnsembleDock, resulting in a protocol that accounts for conformer selection, multibody docking, and a flexible paratope.

No matter which docking method is used to study antibody-antigen complexes, one must consider that an antibody docking protocol can take months to complete when working with large complexes, even when using computer clusters.

Researchers who do not have access to a computer cluster or powerful workstation may find that the time to run even a single docking job may make this approach unfeasible. In these situations, there is a strong impetus to utilize "cloud" computing services.

Cloud computing refers to the practice of utilizing another organization's resources to process or analyze data. The user transfers the data to another company's physical servers over the Internet, and visualizes the results remotely, or re-transfers the data back to his or her computer for further review. The user has no idea where or how the actual calculations take place. Although many types of workloads have not been widely utilized in the cloud, antibody docking workflows and services are readily available by utilizing RosettaDock in the Amazon "EC2" cloud. A recent article described Pfizer's success in running part of their antibody docking pipeline via this service (http://www.bio-itworld.com/issues/2009/may-jun/antibody-docking-EC2.html).

Case Studies

Influenza Virus Hemagglutinin

In a recent publication (Pedotti et al. 2011; Worn et al. 2000), models of two antibodies, CR6261 and F10, were developed using both the PIGS and Rosetta Antibody programs. The structure of both of these antibodies in complex with influenza virus hemagglutinin (HA) has already been solved, so it is possible to assess the accuracy of the models.

Both applications generated accurate results. Using the PIGS method, the authors were able to obtain the best results using the "same antibody" option. When using this option, the model was able to achieve an RMSD of 1.3 Å for F10 and 1 Å for C6261 in comparison to the solved structures. As previously discussed, Rosetta Antibody does not allow a choice of methods, but returns ten different models. Rosetta also achieved good results, producing a structure with an RMSD of 1.7 Å for antibody F10, and 1.1 Å for antibody C6261. Figure 18.2 illustrates the differences in these models. The notoriously difficult to model H3 loop is colored green for the crystal structure (the actual structure), pink for the PIGS representation, and yellow for the Rosetta models. This depiction illustrates the idea that computer-based models are highly accurate for certain regions, but display some variability for others. If the area of interest is in a region of the model that is uncertain, the model is obviously of lesser value.

This publication also explored the use of three different applications, Roseta-Dock, ZDock, and HADDOCK. Of the three, RosettaDock returned the best results, even though HADDOCK incorporated a flexible backbone approach.

Interest in using computational approaches such as modeling and docking in studying antibodies directly is rapidly increasing. However, computational methods have reached even greater levels of maturity in studying a class of structures

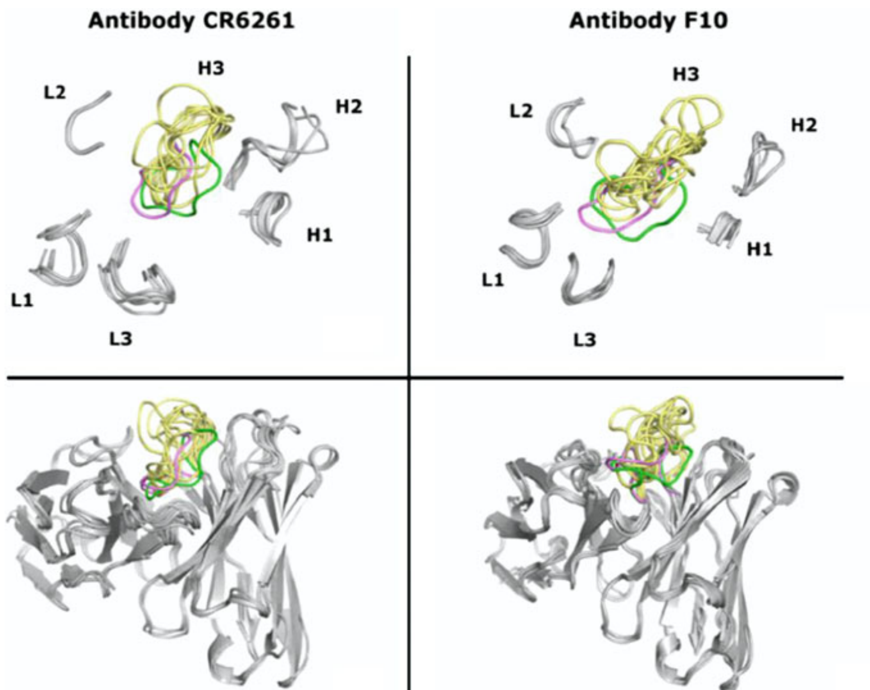

Fig. 18.2 Cartoon representation of antibodies CR6261 (*left*) and F10 (*right*). Only the CDR loops are shown at the *top*. *Green* represents the original structure, *yellow* the model produced by Rosetta, and violet the model produced by PIGS. Figure originally from Pedotti et al. (2011)

that are often linked with antibodies for therapeutic considerations—G Protein-Coupled Receptors (GPCRs).

Therapeutic Antibodies and GPCRs

Classically, interactions between receptors and their corresponding extracellular ligands have been used as an essential point of interference for many therapeutic agents. The G protein-coupled family of receptors (GPCRs) are among the most commonly targeted receptors for a variety of therapeutic agents, including therapeutic antibodies. GPCRs are seven-transmembrane domain receptors that are known to mediate many key normal physiological processes as well as many disease states, including cancer, cardiovascular disease, and neurological disorders (Conn et al. 2009; Dorsam and Gutkind 2007; Escolar et al. 2005; Rozec and Gauthier 2006; Smith and Luttrell 2006; Volante et al. 2004). Members of the GPCR family share a common structure consisting of an extracellular N-terminal domain, an intracellular C-terminal domain, and seven-transmembrane domains,

linked by three extracellular and three intracellular loops. The GPCR family of receptors is divided into 4 subfamilies: A, B, C and the Frizzled family. Family A receptors include rhodopsin-like receptors that typically interact with small molecules such as amino acids, nucleotides, lipids, and peptides. Members of this family are the largest group of targets for drugs currently available. Ligands to this family of receptors can either interact with the transmembrane domain or their intracellular loops. Family B receptors include secretin-like and adhesin-like receptors. Secretin-like receptors are activated by ligands such as large peptides and hormones. Adhesin-like receptors comprise a very large N-terminus, but their ligands have not been identified. Currently no small molecule drugs are available for these two subfamilies. Family C receptors comprise metabotropic glutamate receptors (mGluR), γ-aminobutiric acid (GABA) receptors, and the Ca^{2+} sensing receptors (Eglen and Reisine 2009; Heilker et al. 2009; Jacoby et al. 2006). These receptors can function both as homo- and hetero-dimers, and ligand binding leads to dimerization between the extracellular domains and the transmembrane domains (Pin et al. 2004). Lastly, the Frizzled family of receptors has been shown to couple to G proteins and have structural similarities to Family A and B receptors.

Attempts to develop high affinity antibodies to GPCRs have been limited by an inability to generate highly purified GPCR preparations due to high levels of receptor glycosylation and sulphation, low levels of surface expression, and a high degree of cross-reactivity to closely related receptors. To overcome these limitations, antibodies to the receptor regions with higher sequence diversity are generated. These regions include short peptide sequences in the N- or C-terminal regions, as well as the third intracellular loops of most GPCRs (Gupta et al. 2008). Antibodies have also been successfully raised against extracellular domains (ECDs) of Family B and C receptors (Hutchings et al.). However, these antibodies are limited in use as therapeutic agents, since they do not affect receptor function. To increase antigenicity, these peptides are conjugated to either carrier proteins or to an inert Poly-L lysine backbone. In fact, Gupta and Devi (2006) have generated several monoclonal antibodies against the N-terminal region of a variety of family A GPCRs including μ, δ, and κ opioid receptors to study antibody-induced conformational changes of these receptors in their native environment (Gupta et al. 2008). Moreover, these antibodies can be used to identify receptor selective ligands (partial agonists had lower levels of recognition by these antibodies, whereas full agonists and antagonists did not affect the recognition). This allowed Gupta et al. (2008) to develop high throughput screening (HTS) assays to search for agonists, partial agonists, and antagonists for GPCRs of interest. These antibodies did not affect the functional properties of the receptors, limiting their use as therapeutic agents.

Antibodies to GPCRs can also function as receptor agonists or antagonists. For example, a monoclonal antibody raised against δ opioid receptor has been shown to function as an agonist and increase the intracellular cAMP levels after long-term treatment (Gomes et al. 1999). Furthermore, anti-thyrotropin receptor antibodies have shown to function as an inverse agonist (Chen et al. 2007).

Moreover, agonist-like monoclonal antibodies (mAbs) have been raised against human β_2 adrenoreceptor (Lebesgue et al. 1998).

One method utilized to generate monoclonal antibodies to GPCRs is the phage-display technology. This technology has been used to generate human antibodies against the N-terminal region of C5aR and the second extracellular loop of C3aR. These antibodies did not interfere with the ligand binding to the receptors, suggesting that the immunodominant domains for these receptors do not interfere with receptor-ligand binding (Hawlisch et al. 1998; Huang et al. 2005).

As previously mentioned, computational methods can play an integral role in generating better mAbs. There has been success in using homology modeling to create representations of GPCRs of interest (Li et al. 2010). In turn, this has allowed more precise peptide designs that mimic GPCR epitopes in their native environment (Li et al. 2010). This method has been used to generate mAbs against extracellular loop-2 of CCR5. Cells exposed to these mAbs were resistant to R5-HIV infection (Misumi et al. 2006).

Antigens can also be conjugated and assembled into virus-like particles (VLPs) (Sommerfelt 2009). Cell surface display of antigens onto assembled VLPs can help trigger a high-titer response to the antigen without any requirements for adjuvant. This method has also been used to inhibit HIV binding to this receptor (Hunter et al. 2009). An alternative method to capture antigens in their native environment is to inject cell lines expressing the antigens or the whole receptor into laboratory animals (Graeler et al. 2002; Ohno et al. 2008). Using this method, functional mAbs against S1P1 have been generated that can bind to the native receptor but not the denatured protein. Similar techniques have been used to generate functional mAbs against CXCR4 homo-dimers and CXCR4/CXCR2 hetero-dimers, as well as GLP-1 receptor (Hutchings et al. 2010). GPCR antigens or whole functional receptors can also be presented on dendritic cell exosomes or baculovirus insect systems (Breckpot and Escors 2009; Loisel et al. 1997).

One of the most recent approaches used to generate structurally stable GPCRs is to generate StaR® GPCRs. StaR® GPCRs carry a number of point mutations in their structure which renders them thermostable (Robertson et al. 2011). This method has been used to generate thermostable GPCRs including NTSR1, Adenosine A_{2a}, and B1-adrenergic receptors (Magnani et al. 2008; Serrano-Vega et al. 2008; Shibata et al. 2009). This method can stabilize the receptor in an either agonist or antagonist conformation, allowing generation of conformation-specific mAbs that could serve as antagonists or agonists.

The partial vesicle solubilization method can be used to reconstitute GPCRs into phospholipid bilayers after their purification. NTSR1 homo-dimers have been generated using this method (Harding et al. 2009). Antibodies against interacting hetero-dimeric GPCRs can be generated using this approach as described for CXCR4/CXCR2 hetero-dimers (Hutchings et al. 2010).

Immunization of animals with the DNA sequence for the GPCR of interest has also been described as an alternative mode for antibody production. In this approach, the DNA is injected either intramuscularly (Fujimoto et al. 2009; Kaptein et al. 2008), or DNA-coated microparticles are bombarded into the

animal's skin (Elagoz et al. 2004). Antibodies against CXCR4, CCR3, CCR5, and FPRL-1 have been generated using these approaches (Hutchings et al. 2010).

Despite significant developments in generating mAbs against GPCRs in recent years, no approved therapeutic antibodies have been introduced to the market to date. It is likely that developments in GPCR stabilizing techniques will allow major progress in generating effective therapeutic antibodies. Recently Hutchings et al. (2010) reported a list of therapeutic antibodies that are currently under development by multiple pharmaceutical companies. The list includes antibodies against C5aR, C3aR, CXCR4, CCR8, CCR2, CCR8, CCR9, GCG-R GLP1R, VPAC-1, CRTH2, LGR5, and CXCR3. Due to the limited availability of peer-reviewed articlces on GPCR therapeutic antibodies, it is likely that many other anti-GPCR antibodies that are currently under development have been overlooked.

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Chapter 19
Concluding Remarks

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Abstract Following the success of first-generation antibody therapeutics, next generation antibody-based therapeutics are currently in development against a variety of antigen targets. Knowledge of the structure–activity relationships for antibody-based modalities has provided the opportunity to generate highly tailored therapeutics by fine-tuning their pharmacological properties. As more advanced antibody-based modalities are emerging, implementation of effective translational strategies is becoming increasingly essential.

> "When you have eliminated the impossible, whatever remains, however improbable, must be the truth."
>
> *Sherlock Holmes*

Antibody-based therapeutics (ABTs) are emerging as one of the major classes of drugs effective in the treatment of many human diseases. With advances in science, the field is now benefiting from both an increased understanding of the mechanistic basis of ABTs and development of sophisticated technologies to generate safe and highly tailored modalities. In addition, ABTs are now evolving as a significant component of financial deals within the biotechnology and pharmaceutical sectors of the economy. In 2008, antibody-based therapeutics grossed

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approximately US $35 billion worldwide, representing ~43% of the total global sales of biologics. Growth in this segment is estimated to continue, with 10.8% annual cumulative growth in sales through 2013 as existing ABTs expand their approved usage and new entrants launch into the marketplace. It is estimated that ~200 new antibody-based molecules are currently at various stages of development and several are predicted to reach the marketplace in the next few years.

Continued innovations in the field have been fueled by expansion of the knowledge of target biology and an increase in the understanding of the structure–activity relationships for ABTs. This trend has provided the opportunity for generation of highly targeted therapeutics by fine-tuning their pharmacological properties. The evolution of therapeutic antibodies has encompassed multiple engineering efforts in the hope of improving the efficacy, safety, and duration of effects for antibody-based drugs. Improvements in protein engineering technologies have afforded investigators the ability to overcome problems associated with introducing foreign antibodies into humans. In addition, with current improvements in antibody engineering technologies, many classes of novel antibody-derived molecules are now emerging as promising next generation therapeutics. These new antibody-based formats are carefully designed and engineered to possess special features such as improved selectivity as well as enhanced efficacy, and may potentially revolutionize treatments of various disabling diseases.

Although the field continues to advance, many challenges remain in order to bring more efficacious and affordable antibody-based candidates to the market. Similar to small molecule drugs, translation of accumulated knowledge across various development stages poses a major challenge hindering effective development of ABTs. However, unlike small molecule drugs, antibody-based therapeutics generally exhibit exclusive specificity for the target antigen. The advancement of therapeutic monoclonal antibodies to various stages of the drug development process can be effectively streamlined when appropriate translational strategies are applied. In general, the concentration of drug at the biological receptor determines the magnitude of the pharmacological or toxicological response. As shown in Fig. 19.1a, for drugs with a wide therapeutic index, the separation of the concentration–effect curves for toxic and beneficial effects should generally allow efficacy while avoiding the toxic effects at clinically relevant doses. In contrast, for narrow therapeutic index drugs, a clear delineation between the beneficial and toxic effect curves may not be possible (i.e., in instances where the target antigen is also expressed on normal tissues, Fig. 19.1b). It is evident that successful translation of safety and efficacy information into the clinic requires a clear understanding of the many factors that can critically influence and define the relationships between the biophase drug concentrations and the anticipated beneficial or toxic effects across species. The importance of translational challenges encountered during development of antibody-based therapeutics is highlighted by severe adverse events, not predicted from preclinical studies, as demonstrated in the First-In-Human (FIH) clinical trial in healthy subjects receiving the starting dose of TGN1412. This example highlights the importance of a science-based

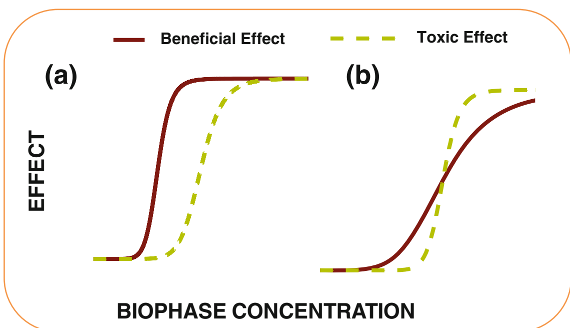

Fig. 19.1 A theoretical relationship between drug dose or concentrations and the pharmacological and toxicological effects. **a** A drug with wide or **b** narrow therapeutic index

decision-making approach for translation of the exposure-response relationships during the various stages of antibody drug development.

As discussed throughout this book, implementation of successful translational strategies during development of antibody-based therapeutics necessitates integration of knowledge with respect to the following: antigen distribution, antigen expression, kinetic and equilibrium or steady-state properties of the Antigen/ABT complex, target pharmacology, PD system efficiency and redundancies, comparative investigations of target antigen properties, species-dependent pharmacology, and antibody design criteria. Critical evaluation of the many factors that regulate antibody exposure-response relationships in relevant animal models is necessary for the design of successful translational strategies from discovery to the clinic and establishing the FIH dose and dosing strategies. Evaluation of the pharmacodynamic system efficiency and stimulus-response mechanisms that convert receptor occupancy into pharmacological response(s) along with effective application of quantitative pharmacology are among the key translational considerations throughout the development process. Quantitative pharmacology is a critical translational tool facilitating the information flow throughout the drug development process. Inclusion of appropriate mechanistic PK–PD models that account for both the antibody-based modalities and target antigen properties early on in the drug development process serves as an informative tool for predicting FIH dose and dosing strategies.

In this book, we have provided a comprehensive discussion of various topics critical for establishing successful translational strategies for the development of antibody-based therapeutics. Like an engrossing detective novel, we hope that the topics covered here guide the interested reader in developing a keen sense of "observation, evaluation, and deduction" while approaching the critical issues relevant to development of antibody-based therapeutics.

Editors Biography

Gadi G. Bornstein, Ph.D. has over 10 years of experience in R&D with an emphasis in preclinical antibody discovery and development. Dr. Bornstein is currently a Research Fellow at Pfizer CTI (Centers for Therapeutic Innovation) in New York. He received his B.S. in Biochemistry at the University of California, Davis and his doctoral degree in biochemistry at the University of Southern California Keck School of Medicine. Dr. Bornstein completed his postdoctoral training at Stanford University in the Division of Immunology and Rheumatology.

Following his postdoctoral training, Dr. Bornstein joined Amgen Fremont, Inc. (formerly Abgenix, Inc.) as a Staff Scientist in the Preclinical Oncology Department. During his tenure at Abgenix, he was a project team leader and lead biologist for multiple antibody programs. Prior to joining Pfizer CTI, Dr. Bornstein was a Principal Scientist at AstraZeneca, where he led several antibody programs within the Preclinical oncology portfolio.

Scott Klakamp Ph.D. is a Senior Research Fellow at Takeda California. He leads the Biophysical Chemistry Group and has extensive experience in research and development involving the biophysical and analytical characterization of biologics and monoclonal antibodies. Dr. Klakamp is one of the leading scientists in utilizing Biacore®, KinExA®, and FACS techniques to measure the binding kinetics and equilibrium constants of human monoclonal antibody/antigen complexes. Prior to joining Takeda California, Dr. Klakamp served as a Principal Scientist at AstraZeneca R&D Hayward (merged with MedImmune, Inc), where he led the Biophysical Characterization Group. Prior to AstraZeneca, he headed the Biophysical Group at Abgenix, Inc. (acquired by Amgen, Inc.) from 2001 to 2006, and previously held scientific positions in Chiron, Inc., GeneMedicine, Inc, and the Pennsylvania State Police. Dr. Klakamp has been an author on over 30 research and review papers, book chapters, and patents, and has been an invited speaker at numerous international and national meetings. He

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Mohammad Tabrizi Ph.D. is a leader in translational sciences as related to development of antibody-based therapeutics. He has extensive experience in research and development of monoclonal antibodies and biologics. His product development experience spans many therapeutic areas including oncology and inflammatory diseases, and his technical expertise includes preclinical pharmacology and safety, preclinical and clinical pharmacokinetics, pharmacodynamics (PD), GLP-compliant bioanalytics, and clinical pharmacology relevant to the development of therapeutic monoclonal antibodies. He has been an author or co-inventor of more than 40 original papers, reviews, book chapters, and patents and has been an invited speaker to numerous national and international conferences.

Dr. Tabrizi is currently a Senior Fellow at Merck Inc. in Palo Alto, California. Prior to Merck, he held various positions as Vice President, Preclinical Development at AnaptysBio, Director of Translational Sciences at MedImmune, a wholly owned subsidiary of AstraZeneca, where he was involved in the design and implementation of effective translational strategies for development of therapeutic monoclonal antibodies from discovery to the clinic. In addition, he was one of the key contributors in successful establishment of the AstraZeneca R&D, Hayward (currently MedImmune) and played a vital role in the integration of the Hayward team into the global company. He served in numerous managerial and scientific positions at AstraZeneca, Abgenix Inc. (later acquired by Amgen Inc.), Coulter Pharmaceutical Inc. (later acquired by Corixa), and TAP holdings Inc. Dr. Tabrizi received his bachelor's degree in Pharmacy from University of Houston (Summa Cum Laude) and his PhD from University at Buffalo, State University of New York (SUNY) in the area of Pharmacokinetics and Pharmaceutical Sciences. He completed postdoctoral training at SUNY with a focus on therapeutics.

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