

Chapter 2

Discovery Process for Antibody-Based Therapeutics

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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 ($A\beta$) 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

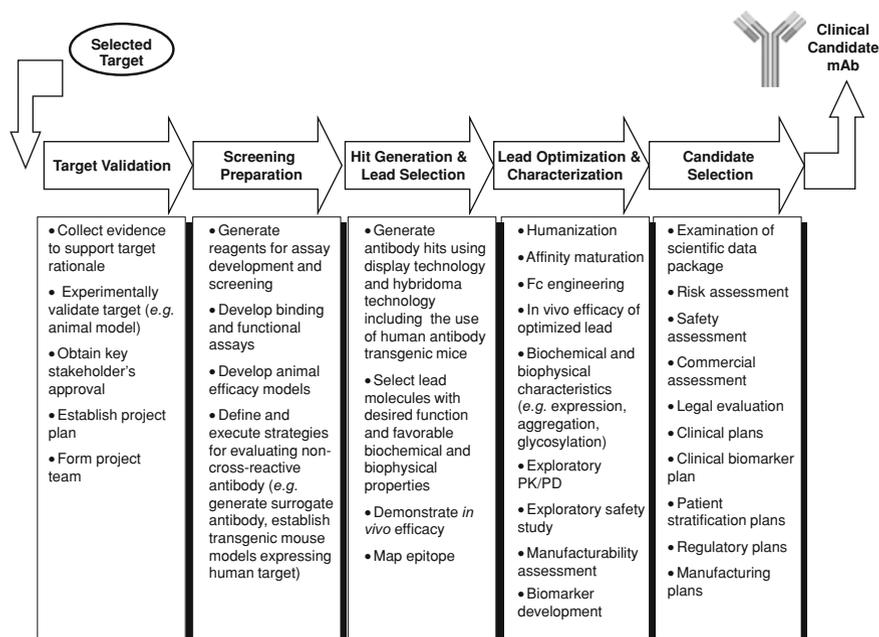


Fig. 2.1 Overview of discovery process for therapeutic monoclonal antibodies leading to the selection of a clinical candidate. The overall process can be divided into five stages: target validation, screening preparation, hit generation and lead selection, lead optimization and characterization, and 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 μ M 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 Joussem 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.

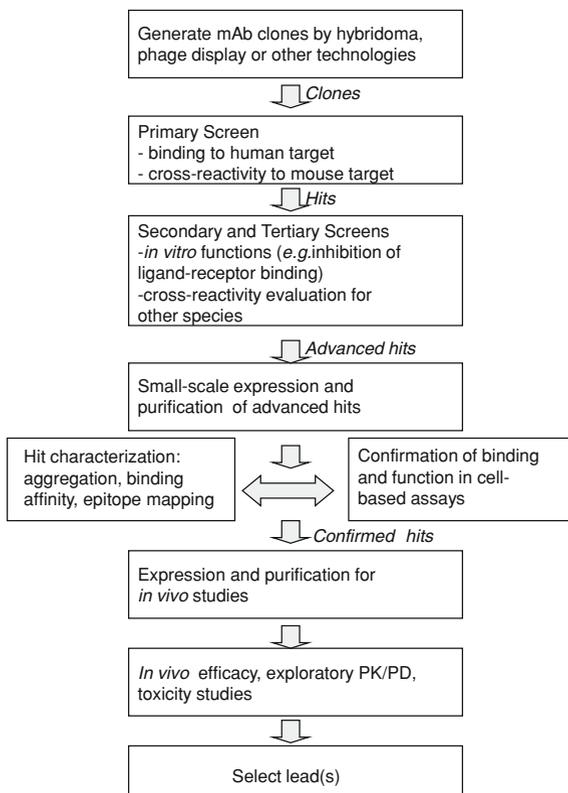
Once a target is selected, the therapeutic targeting strategy should be defined. For example, a common molecular mechanism for an antibody-based therapeutic is the blockade of a ligand-receptor interaction, for which there are three conceivable targeting strategies: an anti-ligand antibody, an anti-receptor 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

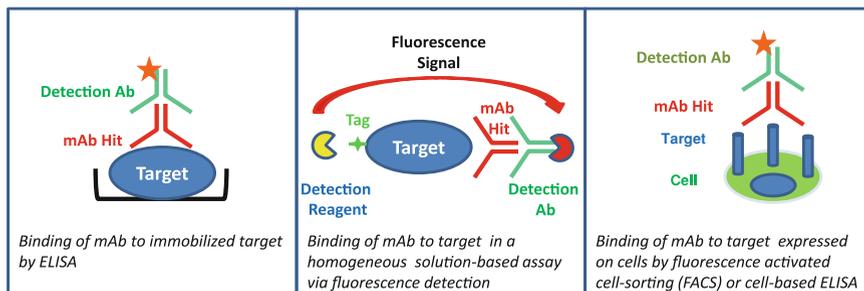
The screening preparation phase, rather than the screening phase, is often the bottleneck of the early discovery process. High quality reagents and optimized functional assays are the key steps for a successful screening phase. A common mistake is to rush into the antibody generation process before reagents 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

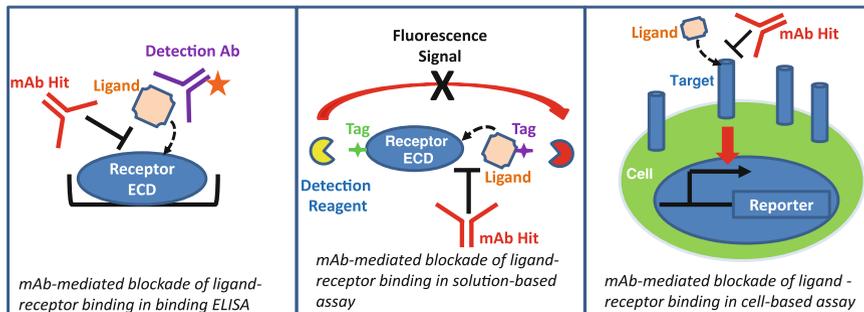


Fig. 2.3 Depiction of typical binding assays and functional assays used for screening antibodies. ECD stands for extracellular domain

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 Fc γ receptors (Fc γ R) 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 Fc γ R 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 (Stebbins 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 indispensable 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 (EMA) (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 syncytial 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 debilitating 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 ($A\beta$) for the treatment of AD. The molecular target for bapineuzumab, amyloid β ($A\beta$), 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). $A\beta$ 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 $A\beta$. Consistently, transgenic mouse models recapitulating these human genetic mutations have demonstrated that increased accumulation of $A\beta$ 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 $A\beta$ 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 $A\beta$ 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 semegastat (Eli Lilly), a small molecule inhibitor of γ -secretase that blocks $A\beta$ production. Despite the controversy around the amyloid hypothesis, there is irrefutable scientific evidence supporting $A\beta$ as a validated therapeutic target.

$A\beta$ 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- $A\beta$ 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- $A\beta$ antibody is based on the initial landmark observation that active immunization of transgenic mice overexpressing $A\beta$ (PDAPP mice) with $A\beta$ peptides led to a decrease in brain $A\beta$ 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- $A\beta$ antibodies including 3D6, the parental murine antibody for bapineuzumab in PDAPP mice, led to brain $A\beta$ plaque clearance (Bard et al. 2000). In the above study, anti- $A\beta$ antibodies were shown to enter the brain and directly bind $A\beta$ amyloid plaques. Multiple therapeutic mechanisms for anti- $A\beta$ antibodies have since been proposed (Brody and Holtzman 2008), including the “peripheral sink” hypothesis that postulates an anti- $A\beta$ antibody can exhibit biological activity outside the brain by sequestering peripheral $A\beta$ in an immune complex, thus altering $A\beta$ equilibrium, resulting in a net efflux of soluble $A\beta$ 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 $A\beta$ 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 $A\beta$ conjugated to a carrier protein (Bard et al. 2003; Schenk et al. 1999). *In vitro*, 3D6 has been shown to bind soluble $A\beta$ by ELISA and $A\beta$ 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 $A\beta$ 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 $A\beta$ as well as brain $A\beta$ plaques, but not APP (Bard et al. 2000). It is important to note that the epitope for a specific anti- $A\beta$ antibody may influence its therapeutic efficacy. *In vitro*, antibodies targeting different epitopes on $A\beta$ demonstrate different binding profiles for free $A\beta$ versus plaques (Bard et al. 2003). Interestingly, a large panel of antibodies targeting distinct $A\beta$ epitopes is under evaluation in clinical trials. Compared with bapineuzumab that binds to the *N*-terminus of $A\beta$, 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- $A\beta$ antibodies in clinical trials are reported to target theoretically toxic $A\beta$ oligomers (Morgan 2011).

In vivo and ex vivo evaluation of murine anti- $A\beta$ 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- $A\beta$ antibody, solanezumab, presently with unknown clinical implications (Siemers et al. 2010).

The clinical trial studies of bapineuzumab utilize three biomarkers: the levels of $A\beta$ 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- $A\beta$ antibody can lead to the clearance of $A\beta$ 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- $A\beta$ 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- $A\beta$ 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 A β 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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