CHAPTER

23

Preclinical Development of Monoclonal Antibodies

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OUTLINE

INTRODUCTION

The use of mAbs as therapeutics has provided patients with targeted and effective medicines to treat numerous diseases. Currently 31 mAbs are approved by the US FDA, and five of these mAbs, infliximab (Remicade), rituximab (Rituxan), trastuzumab (Herceptin), bevacizumab (Avastin), and adalimumab (Humira), generated sales of over $4 billion in 2008, and global sales for the entire sector surpassed $30 billion in sales that year [2].

As knowledge and experience in the field of mAbs has grown, the biopharmaceutical industry has greatly accelerated its pursuit of therapeutic mAb products. Presently over 250 mAbs are in various stages of clinical development [3]. Of the product candidates in the pipelines of 27 biotechnology companies, about 88%

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(114 out of 130) are in early Phases I and II clinical development. More than half (55%) are intended for the treatment of cancer, while 32% target immunological and inflammatory disorders, and 13% are in development for treating infectious diseases, cardiovascular indications, and other illnesses. There has been a relatively high success rate (23%) for mAb development, with notable success in immunological and inflammatory indication pursuits (24%) and cancer indications (14%) [3]. In contrast, the overall success rates for new chemical entities (NCEs) as a whole and for NCEs for oncology are reported to be 11% and 5%, respectively [4]. The current FDA-approved mAbs are summarized in Table 23.1.

As this field has evolved and matured, so has the practice of nonclinical safety evaluation supporting their clinical development. The nonclinical development of mAbs is distinct from that of small-molecule therapeutics, with a greater focus on selecting the appropriate species for testing, species that show pharmacological activity with the therapeutic mAb. Guidance documents from international and national regulatory authorities have set the stage for the philosophy and principles behind nonclinical safety evaluation, emphasizing that a case-by-case approach is necessary for mAb development. This case-by-case approach takes into account the disciplines of pharmacology, pharmacokinetics, and toxicology. Following a brief introduction to antibody history, structure, and function, this chapter will summarize the general principles and practice of nonclinical safety evaluation of therapeutic mAbs.

HISTORY OF ANTIBODY THERAPEUTICS: THE DISCOVERY OF SERUM THERAPY

The concept of using antibodies to treat disease originated in the late 1800s, during the great expansion of knowledge in the field of bacteriology, when the utility of serum antitoxins to target infectious agents was beginning to be elucidated. In 1882, Robert Koch assembled a team of researchers in Berlin, including Paul Ehrlich, Emil von Behring, Erich Wernicke, and Shibasaburo Kitasato. All would make significant contributions to the early stages of antibody-based therapy (reviewed in Refs. [2,5]). Berlin was the center of bacteriology in Germany, similar to France under the guidance of Louis Pasteur.

In 1901, Behring was awarded the first Nobel Prize for his work on serum therapy and its uses to treat diphtheria. This work, conducted with Kitasato, was based on observations of immunity to diphtheria and tetanus infection in rats. They discovered that substances in the blood, referred to as antitoxins, were able to neutralize toxins produced by these infectious organisms. Antitoxin produced in one animal was capable of protecting another animal from infection, and could cure an animal actually showing symptoms of diphtheria [6,7]. Similar observations were made with anthrax infection in rats, demonstrating the breadth of the principle.

In 1892, prior to the availability of antidiphtheria serum therapy, approximately 50,000 children died of diphtheria in Germany, and lethality exceeded 50% [2,6]. Behring and Ehrlich worked together to conduct experiments that generated a high-quality, standardized antidiphtheria serum in dairy cattle. Their collaboration with the pharmaceutical manufacturer Farbwerke Hoechst provided seven pediatric wards in Berlin hospitals with a vast amount (75,225 vials) of standardized serum [6]. Roux followed Behring's methods to produce antidiphtheria serum in Paris, and this decreased mortality in Paris from 52% to 25% [8]. Behring used the funds from his Nobel Prize to seed a new company in 1904, which still exists today as Novartis (Chiron) Behring, a vaccine manufacturer, still located on Emil von Behring Strasse in Marburg, Germany [6].

In 1908, Ehrlich was awarded the Nobel Prize in Physiology and Medicine for his work that set the foundation for the concept of a magic bullet for treating disease. Ehrlich demonstrated that the toxin-antitoxin reaction was a chemical reaction, accelerated by heat and diminished by cold. He extended this concept to chemical substances, hypothesizing that chemicals, like antitoxins, must have certain affinities for pathogenic organisms. Chemicals thus could serve as magic bullets, heading straight to the organisms at which they were aimed [9]. His work with arsenical compounds targeting treating syphilis supported this concept. Today, with the advent of mAbs, the specificity of the magic bullet concept has been realized.

Serum therapy was not without its challenges, and patients developed fevers, chills, anaphylaxis, and serum sickness, characterized by malaise, rash, fever, and arthralgias (reviewed in Ref. [10]). Techniques to purify the serum improved, but foreign polyclonal Abs continued to induce significant immune responses in humans. With the discovery and development of antibiotics in the early 1900s, the focus on serum therapy diminished.

ANTIBODY STRUCTURE AND FUNCTION

Antibodies are heavy (~150 kDa) globular plasma proteins that belong to the immunoglobulin superfamily and function to identify and neutralize foreign

TABLE 23.1 Summary of FDA-Approved Therapeutic Monoclonal Antibodies

Product Trade Name (Generic Name)	Target Description	mAb Isotype	Indication	AntiDrug Antibodies[a]	Date Approved
THERAPEUTIC AGENTS					
Cyramza (ramucirumab)	Human vascular endothelial growth factor receptor 2 antagonist	IgG1	Gastric cancer, as a single agent, or in combination with paclitaxel, is indicated for the treatment of patients with advanced or metastatic, gastric, or gastroesophageal junction adenocarcinoma, with disease progression on or after prior fluoropyrimidine or platinum-containing chemotherapy Nonsmall cell lung cancer, in combination with docetaxel, is indicated for the treatment of patients with metastatic nonsmall cell lung cancer with disease progression on or after platinum-based chemotherapy; patients with EGFR or ALK genomic tumor aberrations should have disease progression on FDA-approved therapy prior to receiving Cyramza Colorectal cancer, in combination with FOLFIRI, is indicated for treatment of patients with metastatic colorectal cancer with disease progression on or after prior therapy with bevicizumab, oxaliplatin and a fluoropyrimidine	Yes	2014
Sylvant (siltuximab)	Human IL-6	Human-mouse chimeric IgG1	Treatment of patients with multicentric Castleman's disease who are human HIV negative and human Herpes Virus-8 negative	Yes	2014
Entyvio (vedolizumab)	α4β7 integrin	IgG1	Adult patients with moderately to severe ulcerative colitis or Crohn's disease who have had an inadequate response with, lost response to or were intolerant to a TNF blocker or immunomodulator, or had an inadequate response to or demonstrated dependence on corticosteroids	Yes	2014
Keytruda (pembrolizumab)	Human programmed cell death receptor (PD-1)	IgG4	Treatment of patients with unresectable or metastatic melanoma and disease progression following ipilimumab and, if BRAF V600 mutation positive, a BRAF inhibitor	No	2014
Blincyto (blinatumomab)	Bispecific CD-19-directed CD3 T-cell engager	2 scFvs	Treatment of Philadelphia chromosome-negative relapsed or refractory B-cell precursor acute lymphoblastic leukemia (ALL)	Yes	2014
Opdivo (nivolumab)	Human programmed cell death receptor (PD-1)	IgG4	Treatment of patients with unresectable or metastatic melanoma and disease progression following ipilimumab and, if BRAF V600 mutation positive, a BRAF inhibitor	Yes	2014
Kadcyla (ado-transtuzumab emtansine)	HER-2 receptor and microtubule inhibitor conjugate	IgG1	Treatment of patients with HER-2 positive metastatic breast cancer who previously received treatment of trastuzumab and a taxane, separately or in combination	Yes	2013
Simponi Aria (golimumab)	Human TNFα	IgG1	Treatment of adult patients with moderately to severely active rheumatoid arthritis in combination with methotrexate	Yes	2013
Gazyva (obinutuzumab)	Human CD20	IgG1	Treatment of patients with previously untreated chronic lymphocytic leukemia, in combination with chlorambucil	Yes	2013
Perjeta (pertuzumab)	HER2/neu receptor	IgG1	In combination with trastuzumab and docetaxel, treatment of patients with HER2-positive metastatic breast cancer who have not received prior antiHER2 therapy or chemotherapy for metastatic disease, or as neoadjuvant treatment with HER2-positive, locally advanced, inflammatory or early state breast cancer	Yes	2012

Continued

TABLE 23.1 Summary of FDA-Approved Therapeutic Monoclonal Antibodies—cont'd

Product Trade Name (Generic Name)	Target Description	mAb Isotype	Indication	AntiDrug Antibodies[a]	Date Approved
Raxibacumab	PA component of *Bacillus anthracis*	IgG1	Treatment of adult and pediatric patients with inhalational anthrax in combination with appropriate antibacterial drugs, and for prophylaxis or inhalational anthrax when alternative therapies are not available or not appropriate	No	2012
Adcetris (brentuximab)	CD30-directed antibody – monomethyl auristatin E (MMAE; drug (microtubule disrupting) conjugate	IgG1	Treatment of patients with Hodgkin's lymphoma after failure of autologous stem cell transplant or after failure of at least two prior multiagent chemotherapy regimens in patients who are not ASCT candidates, and patients with systemic anaplastic large cell lymphoma after failure of at least one prior multiagent chemotherapy regimen	Yes	2012
Benlysta (belimumab)	Human mAb to B-lymphocyte stimulator (BLyS)- specific inhibitor	IgG1	Treatment of adult patients with active, autoantibody-positive, systemic lupus erythematous who are receiving standard therapy	Yes	2011
Yervoy (ipilimumab)	Human mAb to cytotoxic T-lymphocyte antigen 4 (CTLA-4) blocking antibody	IgG1	Treatment of unresectable or metastatic melanoma	Yes	2011
Actemra (tocilizumab)	Human mAb to IL-6 receptor	IgG1 k	Treatment of adult patients with moderate to severe active RA who have inadequate response to one or more TNF antagonist therapies; patients 2 years of age or older with active sJIA	Yes	2010
Prolia (denosumab)	Human mAb to RANKL (receptor activator of nuclear factor kappa-B ligand)	IgG2	Treatment of postmenopausal women with osteoporosis at high risk for fracture	Yes	2010
Simponi (golimumab)	Human mAb to TNF alpha	IgG1k	Treatment of severely active rheumatoid arthritis; active psoriatic arthritis; active ankylosing spondylitis; +/−MTX	Yes	2009
Ilaris (canakinumab)	Human mAb to IL-1β	IgG1 k	Treatment of cryopyrin-associated periodic syndromes (CAPS), in adults and children 4 years and older including familial cold auto inflammatory syndrome (FCAS) and Muckle–Wells syndrome (MWS)	Yes	2009
Stelara (ustekinumab)	Human mAb to p40 protein subunit used by both IL-12 and IL-23	IgG1k	Treatment of adult patients with moderate to severe plaque psoriasis who are candidates for phototherapy or systemic therapy	Yes	2009
Arzerra (ofatumumab)	Human mAb to CD20	IgG1 k	Treatment of patients with chronic lymphocytic leukemia (CLL) refractory to fludarabind and alemtuzumab	None detected	2009
Arcalyst (rilonacept)	Fusion protein of human Fc and ligand binding domains of IL-1 receptor component and IL-1 receptor accessory protein	IgG1 Fc	Treatment of Cryopyrin-associated periodic syndromes (CAPS), including familial cold autoinflammatory syndrome (FCAS) and Muckle–Wells syndrome (MWS)	Yes	2008
Cimzia (certolizumab pegol)	Humanized Fab' fragment to human TNF-alpha conjugated to PEG	Fab' fragment	Treatment of moderate to severely active Crohn's and rheumatoid arthritis	Yes	2008
Soliris (eculizumab)	Humanized mAb to complement protein C5	IgG2/4	Treatment of paroxysmal nocturnal hemoglobinuria to reduce hemolysis	Yes	2007

Lucentis (ranibizumab)	Humanized mAb fragment to human VEGF-A	IgG1 Fab′	Treatment of wet age-related macular degeneration and macular edema following retinal vein occlusion (intravitreal injections)	Yes	2006
Vectibix (panitumumab)	Human mAb to epidermal growth factor receptor (EGFR)	IgG2	Treatment of EGFR-expressing metastatic colorectal carcinoma with disease progression on or following chemotherapy. Approval based on progression-free survival; no data demonstrate improvement in disease-related symptoms or increased survival	Yes	2006
Orencia (abatacept)	Fusion protein of human IgG1 Fc and human T-lymphocyte associated antigen 4 (CTLA-4)	IgG1 Fc fused to CTLA-4	Treatment of moderate to severe active RA; juvenile idiopathic arthritis	Yes	2005
Avastin (bevacizumab)	Humanized mAb to human VEGF	IgG1 k	Treatment of metastatic cancer of the colon or rectum; nonsquamous small cell lung cancer; metastatic breast cancer; glioblastoma; renal cell carcinoma	Yes	2004
Erbitux (cetuximab)	Chimeric human/murine mAb to epidermal growth factor receptor	IgG1 k	Treatment of EGFR expressing metastatic colorectal cancer; advanced squamous cell carcinoma of the head and neck	Yes	2004
Tysabri (natalizumab)	Humanized mAb against α4 family of integrins (on all leukocytes except neutrophils)	IgG4	Treatment of relapsing forms of multiple sclerosis; treatment of Crohn's disease	Yes	2004
Amevive (alefacept)	Human fusion protein of and CD2 binding portion of human leukocyte function antigen 3 (LFA-3)	IgG1 Fc fused to CD2	Treatment of moderate to severe plaque psoriasis	Yes	2003
Bexxar (tositumumab)	mAb to CD20 covalently linked to I-131	Murine IgG2a	Radioimmuno-therapeutic agent for patients with CD20 positive follicular non-Hodgkin's lymphoma	Yes	2003
Xolair (omalizumab)	Humanized mAb to IgE	IgG1	For patients with moderate to severe persistent asthma who have a positive skin test or reactivity to a perennial aeroallergen	Yes	2003
Humira (adalimumab)	Human mAb to TNFα	IgG1	Treatment of moderate to severe active RA; juvenile idiopathic arthritis; psoriatic arthritis; ankylosing spondylitis; Crohn's disease; plaque psoriasis	Yes	2002
Zevalin (ibritumomab tiuxetan)	Chimeric murine mAb to CD20, covalently bound to linker-chelator tiuxetan to chelate Indium-111 and Yttrium-90; administered with rituxamab	IgG1	For treatment of relapsed or refractory B-cell non-Hodgkin's lymphoma; previously untreated follicular NHL	Yes	2002
Campath (alemtuzumab)	Humanized mAb to CD52	IgG1	Treatment of B-cell CLL patients (treatment with alkylating agents and failed fludaribine)	Yes	2001
Mylotarg (gemtuzumab ozogamicin)	Humanized mAb targeting CD33 (adhesion protein on cell surface of leukemic blasts and immature myelomono-cytic cells) and conjugated with cytotoxin antibiotic calicheamicin	IgG4	Treatment of CD33 positive acute myeloid leukemia in relapse (60 years or older)	Yes	2000

Continued

TABLE 23.1 Summary of FDA-Approved Therapeutic Monoclonal Antibodies—cont'd

Product Trade Name (Generic Name)	Target Description	mAb Isotype	Indication	AntiDrug Antibodies[a]	Date Approved
Enbrel (etanercept)	Fusion protein of human and human ligand-binding domain of TNF receptor	IgG1 Fc fused to TNF R	Treatment of moderate to severe active RA, JIA, psoriatic A, anklyosing spondylitis	Yes	1998
Herceptin (trastuzumab)	Humanized mAb to human epidermal growth factor receptor 2 (HER2)	IgG1	Metastatic breast cancer and metastatic gastric or gastroesophageal junction adenocarcinoma overexpressing HER2	Yes	1998
Remicade (infliximab)	Chimeric (murine variable and human constant regions) mAb to human TNFα	IgG1k	Treatment of moderate to severe active or fistulizing Crohn's disease; ulcerative colitis; RA; ankylosing spondylitis, psoriatic arthritis, plaque psoriasis	Yes	1998
Simulect (basaliximab)	Chimeric (human/-murine) mAb to IL-2Rα (CD25)	IgG1k	For prophylaxis of acute organ rejection in renal transplant recipients	Yes	1998
Synagis (palivizumab)	Humanized mAb to respiratory syncytial virus (RSV)	IgG1	Prevention of serious lower respiratory tract disease caused by RSV in pediatric patients at high risk for RSV disease (infants)	Yes	1998
Rituxan (rituximab)	Chimeric human/murine mAb to CD20 antigen on human B cells	IgG1 k	For treatment of relapsed or refractory CD20 positive B-cell non-Hodgkin's lymphoma	Yes	1997
Zenapax (daclizumab)	Humanized mAb to alpha subunit of IL-2 receptor on T cells	IgG1	For prophylaxis of acute organ rejection in renal transplant recipients (adults and pediatrics)	Yes	1997
Reopro (abciximab)	Fab fragment of chimeric human/murine mAb 7E3 inhibiting platelet aggregation	Murine IgG2a	As adjunct to percutaneous transluminal coronary angioplasty intervention for the prevention of cardiac ischemic complications	Yes	1993

[a]*As noted in labeling or regulatory documents.*

substances such as viruses and bacteria. There are five classes, or isotypes, of antibodies in humans and other placental mammals. These differ in their heavy chain sequences including alpha (α), gamma (γ), delta (δ), epsiolon (ε), and mu (μ) found in IgA, IgG, IgD, IgE, and IgM antibodies, respectively (for a review see Ref. [11]). The most prevalent isotype is IgG, which constitutes about 85% of serum immunoglobulins, with an average concentration of 11–14 g/L in normal adults. Because of its role in humoral protection, the IgG isotype and its derivatives are the primary focus of therapeutic development [12].

An antibody is shaped like the letter Y, and is composed of two identical heavy and two identical light chains connected by disulfide bonds (Fig. 23.1). Each heavy chain can pair with one of two light chains, either kappa (κ) or lambda (λ). The majority of antibodies are monomers, composed of two heavy and two light chains, but IgA can exist as a dimer and IgM as a pentamer. Antibody size, evaluated by biophysical analysis, is estimated to be about 12 nm long, with three rod-shaped arms each about 3.5 nm in diameter. They are intermediate in size between albumin and mammalian hemoglobin [13].

Antibody structure, elucidated using X-ray crystallography, has been shown to comprise folds of repeated ~110 amino-acid segments (Fig. 23.2), or domains, that form compact functional units (see Ref. [11]). The domains can be further grouped into Fab and Fc regions. Fab stands for fragment-antibody binding; the tips of the Fab bind to the target antigen, and this region is composed of heavy and light chain, variable, and constant regions. This region includes the CH1 heavy-chain domain and the C light-chain domain.

The antigen-binding site is comprised of the Fab variable regions of light and heavy chains. Six linear polypeptide segments called complementarity determining regions (CDR) loops form the antigen recognition site. Three of these CDR loops come from the light chain and three from the heavy chain. The enormous diversity of antigen binding is derived from these six CDR loops, which are hypervariable in sequence [14,15].

The Fc region (standing for fragment-crystallizable) is the most easily crystallized. It is composed of two heavy-chain regions in most antibody isotypes, and three regions in IgM and IgE. The Fc region is responsible for effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). This region binds to a specific class of Fc cellular receptors and other immune molecules, such as complement proteins, which leads to the generation of the appropriate immune response to a specific antigen.

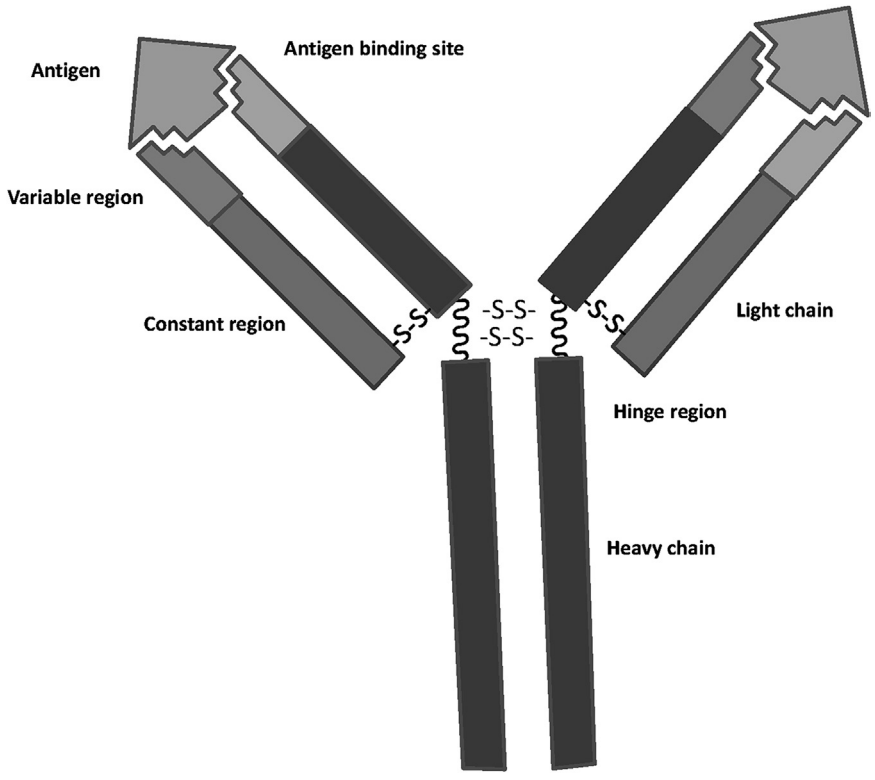

FIGURE 23.1 **The basic structure of an antibody is composed of two heavy chains and two light chains held together by disulfide bonds.** The antigen-binding section consists of the variable regions from both heavy and light chains.

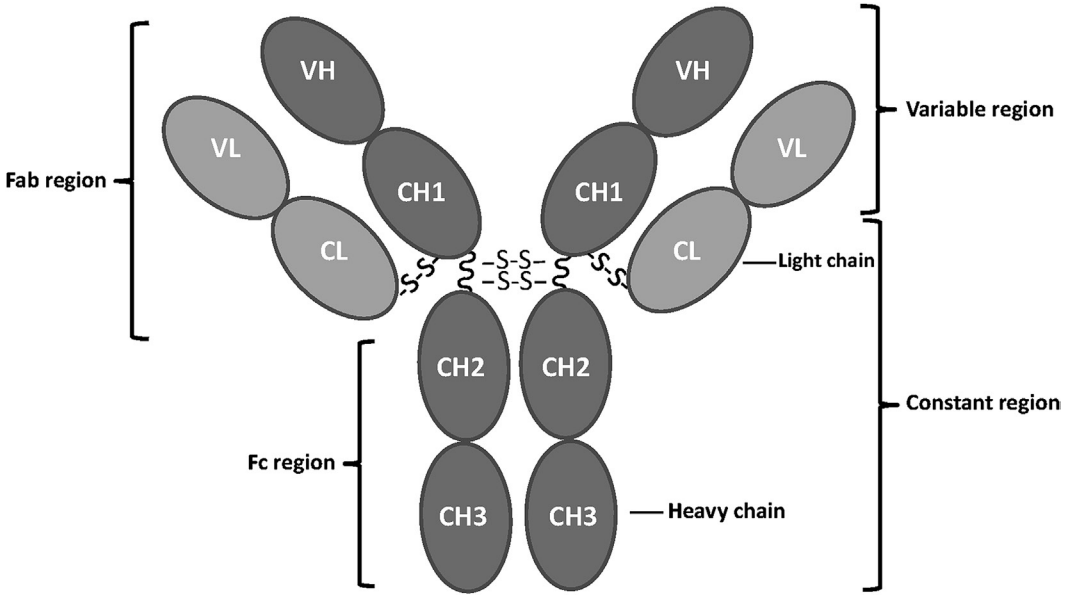

FIGURE 23.2 Antibody structure has been shown to fold into ~100 amino domains that form compact functional units referred to the Fab and Fc regions. For the IgG molecule, the heavy chain is made up of one variable and three constant domains, and the light chain is composed of one variable and one constant domain.

This interaction with the Fc receptor results in different physiological effects including recognition of opsonized particles, lysis of cells, degranulation of mast cells, basophils, and eosinophils [16,17].

The Evolution of Therapeutic Monoclonal Antibodies

Over 70 years passed between the early use of serum therapy for treating diphtheria and the availability of the first licensed monoclonal antibody therapeutic. In this time, a greater understanding of immunology coupled with key discoveries in cell and molecular biology paved the way for generating monospecific mAbs. The first mAbs were of mouse origin, and their repeated administration to patients resulted in robust immune responses generating antidrug antibodies (ADA) and subsequent loss of efficacy. With advances in DNA technology, animal sequences in the mAb were gradually replaced with human sequences, gradually producing fully human mAbs, for use as therapeutics as well as laboratory reagents (Fig. 23.3). This evolution in mAb structure generally reduced the immune response to the foreign mAb, although even fully human mAbs can elicit immune responses (however, these are often milder than their mouse or chimeric mAb cousins). This next section will briefly describe the evolution of mouse mAbs to fully human mAbs.

Mouse mAbs

A great paradigm change in the field of antibody technology came when mAbs could be produced to bind to a desired target. In 1975, Köhler and Milstein published their seminal paper describing the hybridoma methodology and the capability of producing unlimited quantities of mAbs of predefined specificity [18]. They fused mouse myeloma cells with B lymphocytes from a mouse immunized with a specific antigen (sheep red blood cells), thus generating hybrid immortal mouse cells that produced a specific mAb directed against the immunizing antigen. Hybridoma technology was not patented, thus allowing many scientists to utilize the technology and to produce mAbs. Köhler, Milstein, and Jerne (whose plaque assay was used in the hybridoma process) were awarded the 1984 Nobel Prize in Physiology and Medicine for this discovery [5]. However, a major limitation of this technology was the inability to produce human mAbs.

Eleven years later, the first approved mAb derived from this technology was muromonab-CD3 (Orthoclone OKT-3), indicated for the inhibition of transplanted organ rejection. This mouse IgG_2 mAb targets the membrane surface protein CD3 on human T lymphocytes, essential for T-lymphocyte function. Muromonab-CD3 binds to CD3, initially leading to T-lymphocyte activation and subsequently inducing inhibition and apoptosis of the T lymphocyte. Normal T-lymphocyte function is restored within 1 week of administration. Use of this mAb has been associated with anaphylactoid and anaphylactic reactions as well as cytokine release syndrome. Subjects treated with muromonab developed neutralizing antidrug antibodies to the murine mAb during or following the 2nd week of therapy [19]. Newer mAbs targeting human CD3 such as otelixizumab (chimeric

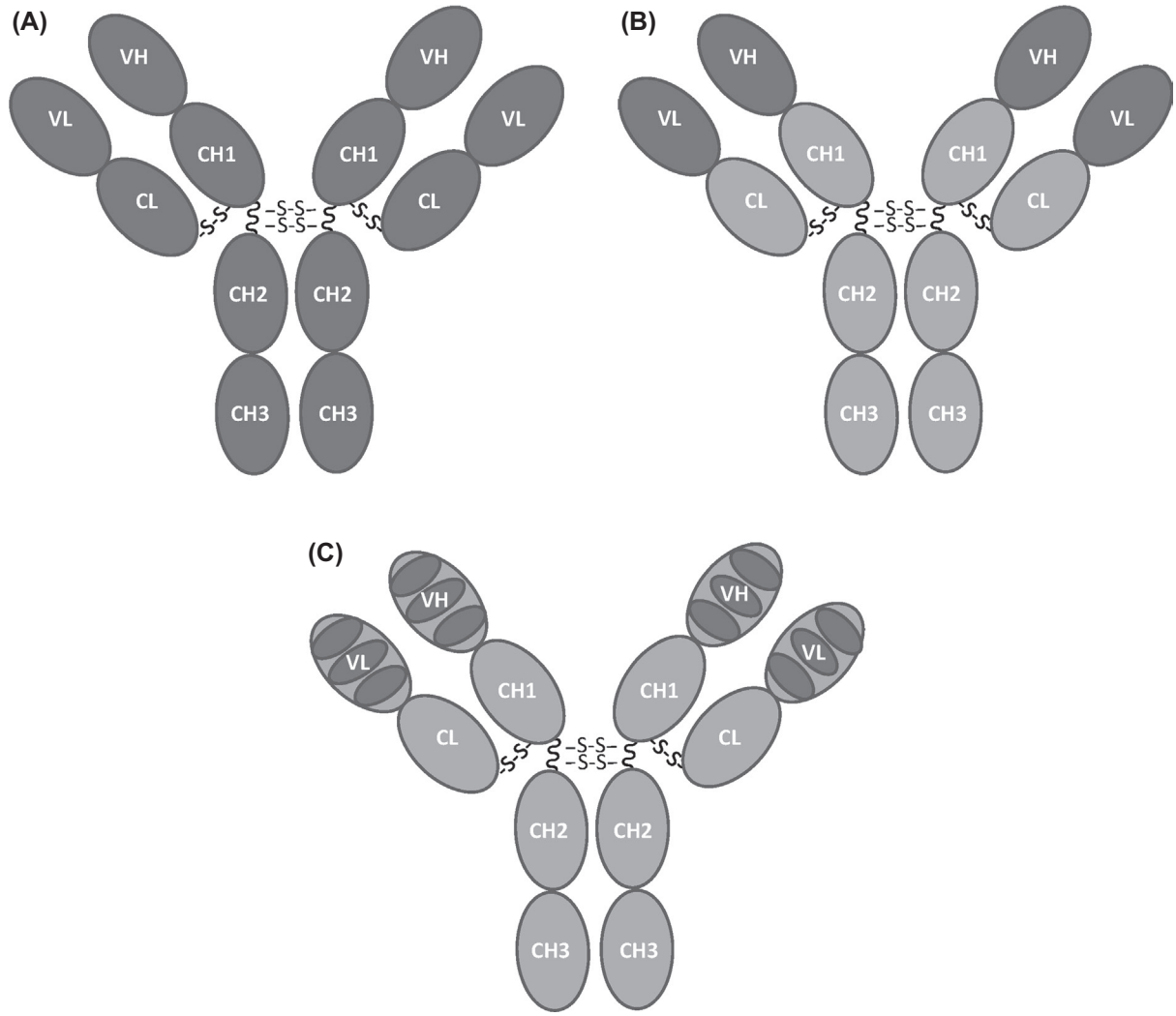

FIGURE 23.3 The different forms of monoclonal antibodies (mAb), including (A) a mouse mAb; (B) a chimeric mAb with human amino acid regions (light gray) replacing the mouse regions (dark gray); (C) a humanized mAb (light gray) with mouse regions (dark gray) remaining only in the complementarity determining region responsible for binding.

mAb) and visilizumab (humanized mAb) are currently in development [20].

The main drawback of administering therapeutic mouse mAbs to humans was that the mouse-protein sequences were recognized as foreign, often resulting in a robust immune response, including the generation of human antimouse antibodies, increased clearance of the mAb, and injection site and hypersensitivity reactions.

Chimeric and Humanized mAbs

Experience with recombinant DNA technology, along with an increased understanding of mAb structure and function, led the way to the production of less immunogenic alternatives. Murine immunoglobulin VH and VL chains were fused with human immunoglobulin constant regions [21,22], generating chimeric mAbs. These proteins contain about one-third murine sequences (2VH and 2VL subunits) and two-thirds human sequences, including the human Fc region, and are produced using cell-culture technology.

The first chimeric mAb to be produced was abciximab (ReoPro), which was made in immunoglobulin form, then cleaved to a Fab fragment, and marketed as a mAb fragment. The first chimeric IgG to be produced was rituximab (Rituxan), still a strongly selling product, with >$3 billion in sales annually [5]. Antidrug antibodies have been reported following treatment with rituximab. However, since the drug targets and depletes B lymphocytes, the potential producers of ADA, it is not a good representation of the ADA response found with chimeric mAbs. A better example is infliximab, which is also an immunosuppressant. Data for this drug show that up to 51% of psoriasis patients in the Phase III study developed ADA, following IV administration at 3 mg/kg every 8 weeks for 1 year [23]. The development

of ADA with infliximab has resulted in shorter dosing intervals and increases in dose level to maintain efficacy [24].

Due to the immune response in patients, efforts were made to replace the mouse-variable-region DNA sequences in the CDR with human sequences. This process is called humanization, and was first described by Winter and colleagues in 1986. They grafted the CDR from a murine antibody into its most closely related human framework, then made the amino-acid changes required to stabilize the engineered constructs [25]. Queen and colleagues developed a detailed process for CDR grafting, which formed the basis for humanization of many mAbs currently on the market and in clinical trials [26–28].

In 1997, the FDA approved the first humanized mAb, which was daclizumab, an anti-CD25 (IL-2 alpha subunit indicated for the treatment of transplant rejection). Antidrug antibodies (14%) were detected in adult patients, but a reduction in clinical efficacy was not observed [23,29].

Fully Human mAbs Using Phage Display or Transgenic Mice

The ultimate goal was to make fully human mAbs, which were anticipated to elicit a minimal immune response when administered to humans. This was made possible with the further evolution of recombinant DNA techniques leading to the generation of phage-display techniques and transgenic animals.

In phage-display technology, filamentous bacteriophages naturally display minor and major coat proteins on their surface. Using molecular biology techniques these proteins can be engineered to display repertoires of antibody fragments fused to the minor coat protein [30–33]. To obtain the genes that code for the antibody fragments displayed by the phage, the light- and heavy-chain variable (V) gene repertoires are harvested from a population of lymphocytes, or assembled in vitro, and cloned, yielding surface expression on the phage. These phage-displayed antibodies can be selected by binding to a target protein/antigen and then used to infect bacteria that will then secrete the target-specific single-chain antibody fragment. Human antibody fragments have thus been isolated with specificities against both foreign and self-antigens, including haptens, carbohydrates, secreted and cell surface proteins, viral coat proteins, and intracellular antigens from the lumen of the endoplasmic reticulum. The DNA from the selected phage is then reformatted into a full-length mAb and expressed in mammalian cells. The first phage-display-derived, fully human mAb on the market was adalimumab (Humira, 2002), an IgG1 mAb that targets human TNF alpha, indicated for the treatment of moderate-to-severe rheumatoid arthritis. Approximately 5% of rheumatoid arthritis patients in the Phase 3 study developed ADA that were neutralizing when tested in vitro at least once during treatment. Patients concomitantly treated with methotrexate had a lower ADA response than patients on monotherapy (1% vs. 12%), and there was no apparent correlation of ADA with adverse effects.

At the same time, the generation of transgenic mice capable of producing fully human mAbs was taking place. The ability to introduce genes, hundreds of kb in size, into mouse embryonic stem cells provided the opportunity to replace the mouse immunoglobulin loci with human antibody genes [34]. These mice can be immunized with specific antigens and their B cells can then be selected for generation of hybridoma, which will yield fully human mAbs of predefined specificity. The first fully human mAb to be derived using this technology was panitumumab (Vectabix), a human IgG2 antibody discovered using the Abgenix XenoMouse technology (2006) and targeting the human epidermal growth factor receptor. Antidrug antibodies (~4.6%) were detected in the Phase III study, with about 1.6% of patients testing positive for neutralizing antibodies. Antidrug antibodies were not correlated with altered pharmacokinetics or toxicity.

The main advantage of humanized and fully human therapeutic mAbs compared with the earlier murine and chimeric mAbs is that there is a lower risk of patients developing a robust immune response, which would result in injection-site reactions, hypersensitivity, and increased clearance of the drug, with subsequent loss of efficacy. However, it is not unexpected that ADA will be generated by human/humanized mAbs, especially with chronic administration. In clinical trials, ADA are routinely measured to understand their overall incidence and the magnitude of a response. The incidences cannot truly be compared between mAbs as the bioanalytical assays measuring the drug differ between products, with different limits of sensitivity among the assays.

As assessed by evaluation of the immunology sections in the package inserts of the FDA-approved human/humanized mAbs (Table 23.1), there are ADA responses seen in the trials supporting registration of all approved mAbs except of atumumab, which targets CD20 on B lymphocytes, and pembrolizumab, which targets the human programmed cell-death receptor (PD-1). The absence of detectable ADA for these mAbs may be related to the true lack of ADA, low sensitivity of the assay, and/or assay interference in the presence of high circulating levels of mAb.

The half-life of mAbs generally increases with the degree of humanization. The half-life of a murine

mAbs is about 1.5 days, of chimeric mAbs, about 10 days, of humanized mAbs, about 12–20 days, and of fully human mAbs, about 15–20 days [35]. The shorter half-life of murine mAbs is attributed to the lack of binding to Fc neonatal receptor (FcRn), which has been demonstrated to be responsible for protection of IgG against systemic elimination by recycling the receptor-bound mAb back into the systemic circulation [36–38].

Next-Generation Antibody Therapeutics

Once humanized and fully human mAbs, with reduced risk of immunogenicity [39], were created, effort was then directed at engineering both the antigen binding domains (affinity maturation, stability) and altering the effector functions (ADCC, clearance rate, CDC) [40,41]. Much antibody engineering effort has also gone into designing mAb-drug conjugates (ADCs) for cancer therapy and other creative ways to enhance the utility of mAb therapeutics, such as bispecific antibodies with unique CDR regions, each arm with a unique target [42–44].

mAb-drug conjugates are biopharmaceutical molecules consisting of a small molecule covalently linked to a monoclonal antibody moiety via a stable cleavage or noncleavable linker. The first two FDA-approved ADCs are humanized mAbs conjugated to antimicrotubule cytotoxic agents. Adcetris (brentuximab vedotin) is composed of a chimeric anti-CD30 mAb conjugated by a protease-cleavable linker to four molecules of monomethylauristatin E (MMAE) and indicated for relapsed/refractory Hodgkin lymphoma and anaplastic large-cell lymphoma [45,46]. Kadcyla (adotrastuzumab emtansine) is comprised of a trastuzumab antibody linked to a tubulin polymerization inhibitor, mertansine, [45,47] for treatment of human estrogen receptor (HER)-positive metastatic breast cancer. Currently, the vast majority of the 29 ADCs in clinical trials use either auristatins or maytansinoids as drug payload [45]. Nonclinical safety evaluation of these hybrid molecules, consisting of a mAb, linker, and small molecule, offers new challenges and is based on a case-by-case scientific approach [48].

The first FDA-approved bispecific antibody was Blincyto (blinatumomab), with one arm engineered to bind CD19 and the other to CD3, and classified as a bispecific T-cell engager (BiTE) molecule for treatment of B-cell lymphoma. This bispecific design brings two cells in close proximity and thus activates T cells to destroy the CD19-positive tumor cell [49,50]. Blincyto is composed of two single-chain variable regions (targeting either CD3 or CD19) that heterodimerize noncovalently to form the active diabody antibody fragment [49]. There are currently over 30 bispecific antibodies in clinical development [44].

These next-generation antibodies, including antibody–drug conjugates, bispecific antibodies, and antibody fragments will bring many novel therapeutic applications into the clinic.

NOMENCLATURE OF MONOCLONAL ANTIBODIES

Therapeutic mAb candidates and products are given generic, or nonproprietary, names based on their class of drug and intended target. This naming scheme is used for both the WHO International Nonproprietery Names (INN) and the US Adopted Names (USAN) for pharmaceuticals (July 2011). In general, word stems are used to identify classes of drugs, and all mAb names end with the stem -mab. Unlike most pharmaceuticals, mAb-antibody nomenclature uses different preceding word parts depending on structure and function, which are called substems. The nomenclature system is summarized in Table 23.2. For example, the name infliximab describes a chimeric mAb therapeutic that targets the immune system.

PRECLINICAL DEVELOPMENT OF MONOCLONAL ANTIBODIES

The preclinical development of mAbs encompasses several important fields of study including ADME (absorption/metabolism/distribution/elimination), pharmacology, pharmacokinetics (PK), tissue cross-reactivity (TCR), toxicology/nonclinical safety evaluation, and bioanalytical development. This section will discuss the roles of these disciplines in the development of mAb therapeutics.

The nonclinical safety evaluation process differs for small- and large-molecule therapeutics, due to the inherent differences in their characteristics, which are summarized in Table 23.3.

The Fate of Monoclonal Antibodies in the Body

The fate of a therapeutic mAb following administration is governed by several key factors including its route of administration, dose, distribution, elimination, and Fc neonatal receptor (FcRn) interaction. Additionally, the size of the target-receptor population, degree of receptor downregulation or shedding, and generation of ADA also play roles in the fate of mAb [36,37,51].

For practical reasons, new mAb development programs often utilize IV administration for initial clinical

TABLE 23.2 Nomenclature of Monoclonal Antibodies

	Target Subsystem			Source Substem		
Prefix	**Old**	**New**	**Meaning**	**Substem**	**Meaning**	**Stem**
Variable-	-anibi-	–	Angiogenesis (inhibitor)	-a-	Rat	-mab
	-ba(c)-	-b(a)-	Bacterium	-e-	Hamster	
	-ci(r)-	-c(i)-	Circulatory system	-i-	Primate	
	-fung-	-f(u)-	Fungus	-o-	Mouse	
	-ki(n)-	-k(i)-	Interleukin	-u-	Human	
	-les-	–	Inflammatory lesion	-xi-	Chimeric (human/-foreign)	
	-li(m)-	-l(i)-	Immune system	-zu-	Humanized	
	-mul-	–	Musculoskeletal system	-Xizu-[a]	Chimeric/-humanized hybrid	
	-neu(u)(r)-	-n(e)-[a]	Nervous system	-axo-	Rat/mouse hybrid	
	-os-	-s(o)-	Bone			
	-toxa-	-tox(a)-	Toxin			
	-co(l)-	-t(u)-	Colon tumor			
	-go(t)-	-t(u)-	Testicular tumor			
	-go(v)-	-t(u)-	Ovarian tumor			
	-ma(r)-	-t(u)-	Mammary tumor			
	-me(l)-	-t(u)-	Melanoma			
	-pr(o)-	-t(u)-	Prostate tumor			
	-tu(m)-	-t(u)-	Miscellaneous tumor			
	-vi(r)-	-v(i)-	Virus			

[a]*Under discussion.*

TABLE 23.3 Differences in Characteristics Between Small-Molecule and Biologic Therapeutics

Characteristic	**Small Molecule**	**Biologic**
Molecular weight	<1000 Da	>1000 Da (mAb ~150,000 Da)
Production	Chemically synthesized	Cell culture, recombinant DNA technology
Route	Generally oral	Generally IV, SC, IM
Metabolism	Generally liver and kidney; pathways well characterized	Catabolized and degraded into amino acids; biotransformation not occurring
Metabolites	Potentially toxic	Typically not toxic
Genotoxicity	Potentially genotoxic – needs testing	Typically not genotoxic – testing not needed
Species limitations	Generally no; 2 species	Potentially yes, 1 species acceptable
Immunogenicity	Generally no; exception hapten protein compounds	Frequent
Food effects	Potential concern	Generally no
Half-life	Shorter	Longer
Drug:drug interaction	Potential concern	Generally no

testing. Development of an IV formulation is often less challenging than developing the more concentrated formulation necessary for SQ or IM administration, and enables a faster launch into the clinical development program. The advantages of IV administration include rapid achievement of high serum concentrations, full systemic availability, and the ability to administer high doses if needed. During the Phase I study, effort is focused on developing a stable, more concentrated formulation that can be administered by SC administration in later clinical studies. The limitation of the IV route is primarily inconvenience, as trained personnel and hospitalization are necessary for drug administration.

Following IV administration, mAbs are initially confined to the circulating vasculature, with initial plasma concentrations consistent with a volume of distribution of ~45–50 mL/kg, approximately equal to the plasma volume. The distribution of mAbs outside the vasculature depends on the rate and extent of mAb extravasation within tissue [37], with a distributional volume of about 0.1 L/kg, approximately equal to the volume of extracellular fluid [12].

Following SC and IM administration, mAbs enter the systemic circulation by absorption into the lymphatic vessels, which tends to be slow due to the low flow rate of lymph [35]. As lymph fluid drains slowly into the vasculature, the absorption of mAbs from the injection site can continue for hours. In general, mAbs administered via extravascular routes exhibit high bioavailability (about 50%) [52,53], with maximal plasma concentrations achieved 1–8 days after SC or IM administration [54–56]. The major shortcoming of these routes is that volumes greater than 2.5 or 5 mL (via SC and IM injection, respectively) cannot be administered due to practical limitations such as mAb solubility (~100 mg/mL) and discomfort at the site of administration [37].

In contrast to small-molecule therapeutics, mAbs are not administered orally. Their oral bioavailability is minimal, due to degradation and poor absorption of mAbs from the gastrointestinal tract. For example, when low-birth-weight human infants were given purified IgG (100–800 mg/kg/day for 5 days) or an IgG-IgA formulation (600 mg/day for 28 days), there was no increase in serum IgG concentrations [57], demonstrating poor oral bioavailability. The therapeutic utility of orally administered mAbs may be limited to local exposure in the gastrointestinal tract for treatment of gastrointestinal infections.

The metabolism/elimination of mAbs is primarily governed by normal protein catabolism, interaction with the FcRn, target-mediated elimination, and immunogenicity [58]. The metabolic products of mAb catabolism are generally not considered to be a safety risk [12]. mAbs are taken up into cells by processes such as endocytosis/pinocytosis, carrier-mediated or membrane receptor-mediated transport, where they eventually undergo degradation (for a review see Refs. [37,59,60]). Enzymes involved in catabolism are ubiquitous in the body and break down the mAbs into its amino-acid constituents. The receptors involved in uptake can include receptors specific for mAbs, such as members of the Fcγ receptor family or the FcRn receptor, and the membrane-bound therapeutic target receptor. It is the interaction with the FcRn (also known as the Brambell receptor) that plays a major role in the fate and recycling of mAbs (Fig. 23.4).

The mAb is taken up into the endosome, and as the pH of the endosome decreases from 7.4 to 6.0, the affinity of the FcRn for the Fc region of the mAb increases. The contents of the endosome are later sorted into two different endosome vesicles, with one containing the FcRn-mAb complexes and the other containing free mAb. The endosome containing the complexes then fuses with the cell membrane, thus releasing the mAb back into circulation [60]. The free mAb remaining in the endosome is eventually catabolized into smaller peptides in the lysosome.

The FcRn was first discovered in rats, where it was found to transport IgG from the mother's milk across the epithelium of the newborn's gut into its bloodstream [61]. A similar protein, with a comparable function and also a role in recycling IgG to the cell's surface, was later found in humans. Transgenic mice lacking FcRn have been shown to have a 10–15 times faster elimination of IgG than wildtype mice [62,63]. Other immunoglobulin isotypes do not bind the FcRn and, as expected, have shorter half-lives than IgGs. The FcRn structure differs in specificity between species, with the human/humanized mAbs showing poor binding affinity to mouse FcRn receptors. Due to these species differences in FcRn, care should be taken when interpreting PK data following administration of human/humanized mAbs to mice, and should be complemented by binding studies with human FcRn [64].

High levels of circulating mAb or IgG can saturate the FcRn recycling pathway, which can lead to increased clearance of the mAb. Endogenous IgG levels and standard therapeutic dose levels of mAbs administered in the clinic (~0.5–10 mg/kg) are not expected to saturate the FcRn pathway [12]. Higher mAb levels used in toxicology studies can have the potential to saturate the pathway and enhance clearance.

With regard to circulation to the kidney, the majority of mAbs transported into the urinary space of the glomerulus is reabsorbed in proximal tubules and reenters the systemic circulation [37]. Lower-molecule-weight

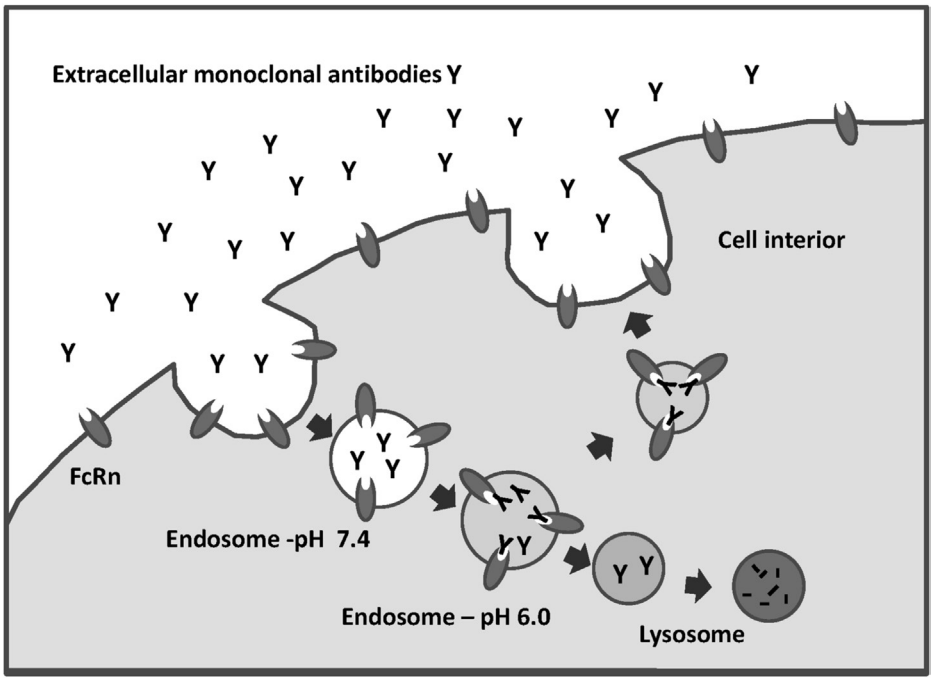

FIGURE 23.4 FcRn-mediated uptake and recycling of monoclonal antibodies (mAbs). Extracellular mAbs are taken into the cell via pinocytosis into endosomes. As the endosome pH decreases (6.0), the mAb binds with the FcRn. The contents of the endosome are sorted upon fusion with lysosomes. Any unbound mAb is released into the lysosome and degraded by proteases. Bound mAb remains in the endosome, and upon fusion with the plasma membrane as well as exposure to higher pH (7.4), is released into the plasma.

fragments (Fab) are filtered by the glomerulus and are subsequently reabsorbed and catabolized by proximal tubule cells. The excretion of intact mAb is a minor component of the overall elimination process, and very little is excreted in the urine or bile.

Therapeutic mAbs, even human/humanized mAbs, can induce an immune response in humans, with the development of an ADA response. This can lead to enhanced clearance of the ADA-bound drug, with a subsequent loss of efficacy depending on the magnitude of the response. The type of immunogenicity that plays a role in mAb clearance will be discussed further in a later section.

Pharmacokinetics, Toxicokinetics, and Pharmacodynamics of Monoclonal Antibody Therapeutics

Pharmacokinetics (PK)/toxicokinetics (TK) and pharmacodynamics (PD) together play an integral role in drug development. Pharmacokinetics evaluation provides information on the exposure profile of a drug in the body over a given period of time following administration, employing multiple blood samplings through the duration of study. These exposure measurements can be used to compare and extrapolate drug exposure across species, to determine the levels of drug required for therapeutic efficacy as well as toxicity, to facilitate selection of an initial safe starting dose for clinical studies, and to estimate safety margins.

Toxicokinetics is the term used to evaluate the relationship between the PK profile and any safety-related findings in a toxicology study. TK is defined as:

> The generation of pharmacokinetic data, either as an integral component in the conduct of nonclinical toxicity studies or in specially designed supportive studies, in order to assess exposure ***ICH S3A*** *[65]*

For toxicology studies, the sampling times are more sparse than found in the PK studies, with blood samples typically taken predose, midway (depending on the duration of the study), and at the end of the study. In rodent toxicology studies, a separate group of animals are often designated as TK animals as to allow multiple sampling times. These data may be helpful in the interpretation of toxicology findings and determining their relevance to potential clinical safety issues.

The International Conference on Harmonization (ICH) S3A guidance document [65] provides a good summary of the goals and objectives of TK evaluation, including describing the levels of systemic exposure achieved in animals, the dose–response relationship and the time course of effects in the toxicology study, and understanding how exposure in toxicology

studies is related to adverse findings. In addition, TK data play a role in assessing the relevance of these findings to clinical safety, supporting the selection of relevant species and treatment regimens in toxicology studies, and facilitating design of the subsequent toxicology studies.

Pharmacodynamics is defined as the desirable and undesirable pharmacological effects of a drug on the body. Integration of PK/TK exposure data in animal studies with PD data on pharmacological or toxicological findings defines the exposure–response relationship (PK/PD), which allows a continuous description of the effect of the drug over time. Understanding the PK/PD relationship is important to anticipate the pharmacological or toxicological behavior of these agents, and is critical for the development of appropriate dose regimens and the planning of future studies. In addition, determining whether a mAb exhibits linear or nonlinear clearance, and whether the clearance is stable or changes over time, is necessary for the determination of safe and effective dosing regimens [12].

An elegant example of defining the PK/PD relationship of a therapeutic mAb was described by Bauer and colleagues [66] for an anti-CD11a mAb, efalizumab (Raptiva), in subjects with psoriasis. CD11a is a subunit of LFA-1, a cell-surface molecule involved in T-cell-mediated immune responses. Blocking the function of CD11a leads to a reduction of T-cell trafficking and has been shown to be effective treatment for psoriasis (although no longer on the market due to safety issues). The humanized IgG1 mAb binds only to chimpanzee and human CD11a, thus the PK/PD evaluations were conducted in the chimpanzee to predict the PK/PD relationship in humans.

Following IV infusion to chimpanzees, efalizumab caused a rapid reduction in the number of CD11a cells detected on the surface of circulating T cells until a new steady-state level of ~20% compared to predose numbers was achieved (Fig. 23.5). CD11a expression remained suppressed until drug levels approached 3 μg/mL and then recovered to predose levels about 7–10 days later.

To build the PK/PD model, data on the binding affinity for human and chimpanzee CD11a, number of CD11a target molecules on the surface of T cells, amount of drug needed to saturate the CD11a target molecules, and plasma drug levels over time were used. Two models were developed for predicting the PK profile in humans and for selecting the doses for a study in subjects with psoriasis. One model was based on Michaelis–Menton clearance designed to represent the clearance of efalizumab based on binding to CD11a receptors, which was a saturable process due to the limited number of receptors on the T cells. The other

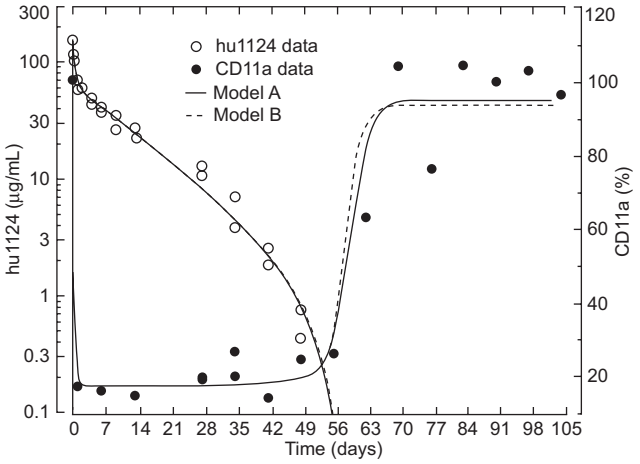

FIGURE 23.5 Michaelis–Menten (Model A) or dynamic receptor-mediated (Model B) clearance of plasma hu1124 and downregulation of CD11a in a nonhuman primate. One animal received 8 mg/kg hu1124 intravenously. The figure shows the hu1124 concentration in μg/mL (*symbols*), and the predicted concentration of hu1124 as a function of time based on the ADAPT-II log-maximum likelihood analyses (*lines*) of Models A or B. Also, percentages of predose CD11a expression (*symbols*), and the predicted %CD11a based on the analyses (*lines*) are shown (Bauer et al. [66]).

model, a dynamic receptor-mediated clearance model, represents clearance of efalizumab based on interaction with a dynamically changing CD11a receptor population. Both models predicted the response seen in the clinical studies, showing that efalizumab clearance accelerates after plasma concentration drops to a level that is subsaturating for CD11a binding (<10 ug/mL). This PK/PD model was further modified for use during the clinical evaluation of efalizumab, and proved to be a valuable tool for predicting the therapeutic response in patients.

The PK/PD evaluation has become an essential tool for enhancing the decision-making process in drug development.

Tissue Cross-Reactivity Studies

Tissue cross-reactivity studies are used to assess the tissue-binding profile of mAbs, evaluating patterns of on-target (CDR-mediated) and off-target (non-CDR-mediated) staining of therapeutic mAbs to tissue cryosections. Tissue cross-reactivity staining can reveal potential targets related to efficacy or toxicity, as well as provide novel information regarding potential sites of expression of the target epitope or related off-target cross-reactive epitopes [67].

Tissue cross-reactivity studies use immunohistochemical staining methods to determine the binding profile of a mAb on a panel of cryosections from normal, healthy tissues. The recommended list from the FDA comprises about 33 different tissues, mounted

on glass slides. The test-article mAb can be used unlabeled or can be labeled using biotin or other reagents, and is applied to the tissue sections and incubated for a predetermined amount of time. There are a series of incubation steps that result in either a fluorescent or chromagenic signal originating from the location of the binding mAb. A board-certified pathologist typically conducts the slide evaluation.

Tissue cross-reactivity can be added to the development plan during the screening phase, prior to the investigational new drug (IND) submission, or later on a case-by-case basis if needed. Using TCR during the screening and selection phase allows discontinuation of any mAb candidate that shows off-target binding to tissues in the CNS or other critical regions. As part of a risk mitigation plan, omitting these mAbs from development will likely save headaches later.

Tissue cross-reactivity can be used to address specific questions asked during development. It can be used to characterize the protein-expression profile of the target ligand as well as its receptor. Tissue cross-reactivity methodology is also available, which can distinguish endogenous antibodies from the human/humanized therapeutic mAb in a tissue section, facilitating understanding of whether the therapeutic mAb or endogenous antibodies are present at a site of inflammation or other site of interest.

The FDA and other international regulatory authorities strongly recommend TCR studies as part of the IND application, and additional studies may be helpful during the course of development. Prior to the ICH S6(R1), TCR studies were recommended to facilitate species selection, and the mAb candidate was tested on full panels of tissues from several species and humans. The new guidance states that immunohistochemical examination of potential binding of mAbs to its target epitope should not be used for selecting relevant species for safety evaluation. TCR studies in toxicology species are considered to have limited value and are not generally recommended.

The presence of positive staining does not equal toxicity, and conversely the absence of positive staining does not necessarily ensure safety. Rather, the data reveal potential sites where the mAb may bind after administration. Membrane staining is more important than cytoplasmic staining for preclinical development, since the potential negative consequences of binding in the cytoplasm is minimal since the mAb cannot access the interior of the cell. The mAb gains access to the cytoplasm only in tissues that are prepared as cryosections. Guidance ICH S6(R1) states that "Binding to areas not typically accessible to biopharmaceuticals in vivo (ie, cytoplasm) might not be relevant." Thus it is possible to focus only on the membrane staining patterns of human tissues for risk-assessment purposes.

The interpretation of therapeutic mAb staining is critical and should always be done in the context of published literature, knowledge of histological and physiological processes, the potential for in vivo distribution, and common sense [67].

Nonclinical Safety Evaluation

Designing the nonclinical safety evaluation program for a therapeutic mAb candidate should be based on a case-by-case, science-driven assessment, taking into account the characteristics of the mAb, the biology of the disease, the consequences of target-molecule inhibition, modulation or activation, and the degree of similarity of the biology of the toxicology species and humans. There are key philosophies in designing nonclinical programs to support first-in-human (FIH) studies, which will be discussed later in this section. Three important regulatory documents are available to provide overall guidance to nonclinical safety evaluation programs for mAbs, and these will also be summarized in this section. They are:

- Guidance for Industry – ICH S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (1997)
- Draft Addendum to ICH S6: Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals S6(R1) (2009)
- M3(R2) Guidance on Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals (2008)

These ICH guidance documents represent the current thinking of the United States, EU, and Japanese regulatory authorities on the nonclinical safety evaluation of mAbs, and the timing of studies to support clinical development. There are many other ICH and national guidance documents that address specific types of toxicology evaluation; however, these three documents provide a good overall picture of the philosophy and thinking of the regulatory authorities, and are a good place to start when designing nonclinical safety evaluation plans.

The goals of nonclinical safety evaluation are to characterize the potential toxicity profile of a new drug, to extrapolate the specific risk of adverse effects to humans, and to provide guidance during clinical development. The toxicology studies can facilitate identification of potential target organs of toxicity and determine whether such toxicity is reversible. These studies also aid in selection of an initial safe dose and subsequent dose-escalation schemes in humans, and identify safety parameters for clinical monitoring of potentially adverse effects (described in more detail in ICH S6(R1) and ICH M3 and reviewed by Refs. [68,69]).

The more that is known about the intended use of the drug in the clinical program, the better the nonclinical safety plan can be designed to support the clinical studies. The plan should parallel to the greatest extent feasible any plans for anticipated clinical use with respect to dose, concentration, route of administration, dosing schedule, and duration. The range of doses selected for study should include at least one dose that is equivalent to and one dose that is a multiple of the highest anticipated clinical dose. Although limited at the start of clinical development, the nonclinical safety studies should be sufficient to characterize any potential toxic effects that might occur under the conditions of the supported clinical trial.

Timing of the nonclinical studies for mAbs is generally similar to the timing expected for small-molecule drug candidates, as summarized in the ICH M3 guidance document.

Animal Care and Use

Nonclinical safety evaluation studies, including toxicology and pharmacokinetics studies, use research animals to characterize the toxicology profile of therapeutic mAbs, as well as small-molecule drugs. These animal studies provide the foundation for ensuring the safety of new drugs intended for human as well as veterinary patients. Great effort has been made over the years to improve and ensure the humane care and use of laboratory animals. The testing laboratories must comply with all applicable laws, regulations, and policies governing the care and use of laboratory animals in accordance with the regulations of the USDA Animal Welfare Act (ie, 9 CFR, Parts 1, 2, and 3) and/or Public Health Service Policy (PHS 2002). Testing laboratories follow the Guide for the Care and Use of Laboratory Animals (2011) [70], which:

> Promotes the humane care and use of laboratory animals by providing information that will enhance animal well-being, the quality of research, and the advancement of scientific knowledge that is relevant to both humans and animals.

All protocols must be reviewed and approved by the testing facility's Institutional Animal Care and Use Committee (IACUC) prior to initiation of a study, and it is recommended to conduct nonclinical safety testing in laboratories that are Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) accredited, where the animal care and use programs have been vetted by veterinarians. Most sponsoring companies and the testing facilities adhere to the 3Rs and make efforts to "reduce, replace, and refine" the use of laboratory animals, and incorporate the industry standards on group sizes for toxicology testing using various species. Ensuring that laboratory animals are treated according to high ethical and scientific standards is paramount in nonclinical safety evaluation testing.

Good Laboratory Practice

The conduct of nonclinical safety studies, especially those utilized for the extrapolation of risk from animals to humans, must conform to the good laboratory practice (GLP) standards (21 CFR Part 58). Good laboratory practice applies to nonclinical studies conducted for the assessment of the safety or efficacy of chemicals (including pharmaceuticals) to humans, animals, and the environment. An internationally recognized definition of GLP can be found on the website for the Medicines and Healthcare Products Regulatory Agency – UK, which defines GLP as:

> Good Laboratory Practice (GLP) embodies a set of principles that provides a framework within which laboratory studies are planned, performed, monitored, recorded, reported and archived. These studies are undertaken to generate data by which the hazards and risks to users, consumers and third parties, including the environment, can be assessed for pharmaceuticals (only preclinical studies), agrochemicals, cosmetics, food additives, feed additives and contaminants, novel foods, biocides, detergents etc…. GLP helps assure regulatory authorities that the data submitted are a true reflection of the results obtained during the study and can therefore be relied upon when making risk/safety assessments.

Conforming to GLP standards in nonclinical safety evaluation studies ensures the uniformity, consistency, reliability, reproducibility, quality, and integrity of the data used for human risk assessment.

Species Selection for Nonclinical Testing

Due to the inherent specificity of mAbs directed to human targets, it is of foremost importance to select animal species for nonclinical safety evaluation that are pharmacologically responsive to the mAb. As one of the primary goals of the nonclinical safety testing is to extrapolate risk from animal data to humans, using animal species that respond in a similar manner to humans will enhance the robustness of the risk assessment process.

Species selection is typically based on information from two major categories, (1) the biology of the target in humans and animals, and (2) information about the activity of the therapeutic mAb in vitro and in pharmacology studies. Early in the research stage of a project, the biology of the target in healthy and disease states in both humans and animals is evaluated, and much effort is focused on understanding the consequences of inhibition and/or activation of the target. Studies from the literature describing the effects of removing the gene of interest (for both ligand and receptor) in cells or mice are also valuable, as this information can be used to gain a sense of the types of safety issues that may be related to inhibiting or activating a particular target and its associated biological pathway. Gene-homology data from the target expressed in human and animals is also typically

evaluated, although species selection is not only made on the basis of homology, as even a single amino-acid change at the binding site of a target can have profound effects on the binding affinity of a mAb. Gene-homology data can be useful in understanding differences in mAb-binding affinity and activity between species.

The binding and functional activities of the therapeutic mAb are characterized in both human and animal species to facilitate species selection. The binding affinity of the mAb to its human and animal species target is typically measured using surface plasmon-resonance techniques. To understand pharmacological similarities or differences between the human and animal species, mAb activity can be characterized in cell lines or primary cell-culture experiments, and the data from these evaluations are most relevant when both ligand and receptor pair are used from the same species. If cells expressing the desired target are not available, transfected cells expressing either human or animal target can be used. In addition, the distribution of expression of the target (and corresponding receptor or ligand) should also be understood in both human and animal species.

The next step is to test the effectiveness of the therapeutic mAb in pharmacology studies in healthy animals and/or disease models. Such studies may be designed to determine receptor occupancy, receptor saturation, and/or pharmacological effects and to assist in the selection of an appropriate animal species for further in vivo pharmacology and toxicology studies. Here the differences in binding affinity and/or functional activity should be taken into account in the design of animal studies, with the goal of having equivalent saturation of receptors in vivo. These studies are the first to administer the therapeutic mAb to animals, and valuable information regarding tolerability and the dose–response relationship can be obtained. Although there are no hard and fast rules about the limits of the differences between binding affinity and functional activity between human and toxicology species, staying within a 10-fold range is a conservative goal.

Two species, generally a rodent and nonrodent, are required. However, due to the specificity of mAbs for their human-target molecules, the therapeutic mAb may not show binding or functional activity with the rodent-target molecule. This situation is quite different than that involving NCEs, which are active in most species and rely heavily on metabolic-profile evaluation for species selection.

If the rodent and nonrodent species show adequate cross-reactivity with the therapeutic mAb candidate, development of the mAb will be easier, especially during the later stages where potential reproductive toxicity is assessed. One recommended strategy is to have a design goal so that the mAb selected for development cross-reacts with both rodent and nonrodent species.

There are alternative pathways to choose based on species cross-reactivity, as reviewed in ICH S6(R1). If only one species is available, then a justification needs to be presented as to why one species is sufficient. In the case where there is no relevant species because the mAb does not interact with the target in any species, then homologous molecules, transgenic animals, and/or animal models of disease can be considered. These options each have their challenges.

For homologous molecules, the same standards for manufacturing the clinical candidate will be needed, and the sponsor will need to justify its relevance, and show that it functions in the same manner as the clinical candidate. Homologous murine mAbs were used to support the development of efalizumab (Raptiva; only cross-reactive in chimpanzee), canakinumab (Ilaris; no cross-reactivity to rodents), tocilizumab (Actemra; no cross-reactivity to rodents), and ustekinumab (Stelara; no cross-reactivity to rodents).

For the transgenic animal model, the main challenge may be the lack of historical data to help interpret any toxicological findings. Is the model understood well enough to be able to distinguish whether a histopathological finding is a rare background event or is test-article-mediated?

The ICH S6(R1) guidance document also covers mAbs and related products directed at foreign targets (eg, bacteria, viruses, toxins) where no endogenous target exists in normal, healthy animals, or humans. Here the recommendation is to evaluate the toxicity profile in an animal model of disease. This option comes with its own set of challenges, including the ability to ascertain which pathological features are related to the disease, and which may be related to toxicity of the mAb. Effort will be needed to understand the natural history of the disease in animals and humans and justify how the animal disease mirrors the human disease. The relevance of a toxicological finding in the disease model to human disease would need to be evaluated.

Another option for the case in which there is no appropriate animal species due to lack of pharmacological activity would be to consider a microdosing study in humans, where anticipated subpharmacological doses are administered in the first-in-study (FIH) study, and these levels are then carefully escalated. Details about this type of clinical testing can be found in ICH S6(R1).

NONCLINICAL SAFETY EVALUATION/ TOXICOLOGY PLANS TO SUPPORT THE FIRST-IN-HUMAN STUDY

For a mAb, the nonclinical safety evaluation program to support the FIH clinical study is often composed of non-GLP proof-of-concept studies, PK studies, dose range-finding/tolerability studies, and GLP TCR- and

GLP IND-enabling toxicology studies. The aim is to obtain as much information as possible from the toxicology studies to help inform the design and potential risks of the FIH study.

Prior to initiating the nonclinical safety program, all available internal (eg, binding, functional activity, pharmacology) data plus literature information (eg, class effects, gene knock-out data) should be reviewed to determine whether there is any anticipated toxicity based on the mechanism of action of the mAb. Biomarkers of pharmacological activity or clinical effectiveness as well as biomarkers of toxicity should be included in the design of the toxicology study, if possible. These markers will allow analysis of their relationship with systemic drug levels, thus facilitating dose selection and safety margin determination. These biomarkers may also play a valuable role in clinical development.

One of the first items to address is species selection, and whether one or two species will be needed for testing. These choices will need to be justified to the regulatory agencies. The optimum situation is to have cross-reactivity in both rodent and nonrodent species. The nonhuman primate (NHP) is often used for safety testing of mAbs because of the close species similarities among NHP and human therapeutic target molecules. Historical data from the NHP are plentiful, due to the number of therapeutic mAbs tested in this species. Dogs, rabbits, and pigs, used extensively in small-molecule nonclinical safety studies, have not been broadly tested with mAbs, but are possible alternatives.

If ample rodent pharmacology studies have been conducted with the mAb and some safety information is available from these studies, selecting doses and designing a non-GLP dose-range-finding study in the rodent is rather straightforward. If the mAb cross-reacts with the nonrodent species, it is prudent to conduct a small proof-of-concept (POC), PK, and/or dose-range-finding/tolerability study in this species to ensure pharmacological activity in vivo as well as acquire a sense of the tolerability of the mAb before embarking on the large and expensive GLP toxicology studies.

The test article should be given by the same route as planned in the Phase I trial, which is usually IV administration, with options of infusion or slow-push bolus injection. Initially information on the stability and solubility of the mAb is sparse and the early formulations will not contain the optimal concentration to be used in later studies. Thus to expedite clinical development, the IV formulation is used initially, and during Phase I clinical studies, a more concentrated SC formulation is developed and tested in later toxicology and clinical studies.

Single-dose toxicology studies are not typically conducted with mAb therapeutics, mainly because the only findings are often related to exaggerated pharmacological responses. Thus with POC and/or PK data in hand, one can proceed directly to a range-finding multiple-dose toxicology study administering the therapeutic mAb IV once weekly for 4 weeks (days 1, 8, 15, 22, and 29) to support most FIH indications (should be determined on a case-by-case basis). The design supports clinical administration of a single mAb dose as well as up to 5 weekly IV doses. The GLP multiple-dose toxicology study is designed on the basis of the results of the range-finding study.

For the design of the GLP repeat-dose toxicology study supporting the FIH study, a vehicle control group and three dose levels of the therapeutic mAb are tested; however, in some cases, two dose levels of therapeutic mAb may be sufficient and for others more than three may be needed. If the PD marker can be estimated with great certainty, then it might be possible to reduce the therapeutic mAb groups to two. If there is a steep dose–response curve based on an expected exaggerated pharmacological effect, more doses may be needed.

The standard endpoints used in toxicology studies of small molecules are also used to test mAbs, including detailed clinical observation, body weight, food consumption, clinical chemistry, hematology, urinalysis, ophthalmology, and macroscopic and microscopic pathology. For studies in NHP, safety pharmacology measurements such as ECG, blood pressure, and CNS assessments are incorporated into the toxicology study to reduce the number of animals needed. If findings in these evaluations show a cause for concern, then additional standalone pharmacology studies may be appropriate.

Based on the mechanism of action of the therapeutic mAb, specific pharmacodynamic endpoints can be incorporated into the design of the toxicology study. For example, denosumab (Prolia), an inhibitor of receptor activator of nuclear factor kappa-B ligand (RANKL), a protein essential for osteoclast formation, function, and survival, showed rapid reduction in serum levels of C-telopeptide, a marker of osteoclast function in cynomolgus monkeys [71], demonstrating the anticipated pharmacological activity of the mAb. Bevacizumab (Avastin), which inhibits vascular endothelial growth factor (VEGF) activity, was shown in repeat-dose IV toxicity studies to cause a dose-related increase in the incidence of physeal dysplasia in the bone-growth plates in young-adult cynomolgus monkeys, thus this type of histopathological finding can be used as a marker for antiangiogenic pharmacological activity in toxicology studies [72].

If the mechanism of action of the therapeutic mAb is immunomodulation, additional endpoints can be incorporated into the study design, such as fluorescence-activated cell sorting analysis to evaluate lymphocyte subpopulations, or immunization with neoantigens (eg, tetanus toxoid, KLH) to assess the T-cell-dependent antibody response (TDAR).

Pharmacokinetics/toxicokinetics sampling should be included. Due to the longer half-lives of mAbs compared to small-molecule therapeutics, the PK sampling times differ considerably. For PK/TK evaluations for nonclinical and clinical studies, analytical methods need to be

developed and validated (for GLP studies) to measure the amount of mAb in serum. These methods may be developed on platforms such as enzyme-linked immunosorbant assay or meso scale discovery.

As human/humanized therapeutic mAbs are regarded as foreign to the animal species tested, an ADA response is anticipated, and an ADA assessment should be included in the study. Typically, predose and end-of-study samples are collected during the study. Although the regulations state that ADA testing may not be needed when pharmacodynamic activity is evident in toxicology studies (ICH S6(R1)), it is prudent to collect the ADA samples during the study and then decide if analysis is warranted or not. Immunogenicity will be discussed further in the following sections.

To select the dose levels for the IND-enabling toxicology study, the first step is to estimate a dose or dose range where clinical efficacy would be expected, which is referred to as the pharmacologically active dose (PAD). This estimation can be based on information collected from in vitro binding assays, receptor occupancy data, cell-culture activity, and PK/PD studies. Physiological-based PK/PD modeling data can also be very useful. The PAD dose/dose range will be used to select the low dose for the toxicology study. Optimally, it would be helpful to have pharmacological activity in the low-dose group for the toxicology study, but at a level low enough not to yield any exaggerated pharmacological or adverse effects.

In practice, selection of a high dose for toxicology studies of mAbs is done on a case-by-case basis. This is a very different process than that for small-molecule therapeutics, where the high dose is related to a maximum tolerated dose (MTD). mAbs are expected to show evidence of exaggerated pharmacodynamic activity but not frank toxicity, as seen with small-molecule therapeutics (although there is always the exception!). The high dose is often selected taking several considerations into account, including the maximal feasible dose (MFD), which is related to the test-article concentration and volume that can be administered to animals, saturation level of target or receptors, and/or an appropriate pharmacologically active dose that has shown signs of exaggerated pharmacology in POC, PK and/or range-finding studies. High-dose selection should have a sound scientific basis related to the activity of the mAb.

The no-observed-adverse-effect level (NOAEL) dose will be determined from the GLP toxicology study, and will play an important role in dose selection for the FIH clinical study.

DOSE SELECTION FOR THE FIH STUDY

The safety of human subjects is the primary concern when selecting a dose for initial clinical studies. Due to the long half-life and subsequent residence time in the body (~5 half-lives) of mAb therapeutics, subjects with the disease of interest rather than healthy volunteers are typically enrolled in the FIH study. The challenge is to select an FIH dose that is initially too low to produce clinical benefit, but will with escalation arrive at one that could potentially provide benefit to the diseased subject. The process for determining the FIH dose for adult subjects in clinical trials is discussed in regulatory guidance documents and reviewed in the literature [73–75]. Two important regulatory documents that provide guidance on selecting the FIH dose are summarized in this section:

- FDA Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers, July 2005
- EMEA/CHMP Draft Guideline on Requirements for First-in-Man Clinical Trials for Potential High-Risk Medicinal Products, March 22, 2007

A case-by-case evaluation is needed to make an FIH dose recommendation for mAb therapeutics. Special considerations need to be taken into account for mAbs with activation of biological processes as their mode of action [74], and for mAbs targeting certain cytokines, chemokines, or other low-level systemically expressed targets where the PAD is expected to be in the microgram/kg range or there is no measureable pharmacodynamic activity.

The first step in FIH dose selection is to determine the maximum recommended starting dose (MSRD), a process that takes into consideration all the available in vitro and in vivo pharmacological, pharmacodynamic, pharmacokinetic, physiological, and toxicological data. The MSRD is calculated from the NOAEL, and needs to be compared with the PAD and the minimal-anticipated-biological-effective-level (MABEL).

The NOAEL is a generally accepted benchmark for safety and is the traditional method used to select FIH doses. For mAbs, it is derived from the GLP repeat-dose toxicity study(ies) designed to support the FIH clinical study. It can be derived from significant findings in toxicological studies, including signs of overt toxicity (eg, clinical signs, macroscopic or microscopic lesions), surrogate markers of toxicity (eg, serum liver enzymes, elevations in serum levels of cellular proteins), or exaggerated pharmacological effects [76,77].

Based on FDA (2005) guidance:

> As a general rule, an adverse effect observed in nonclinical toxicology studies used to define the NOAEL for the purpose of dose-setting should be based on an effect that would be unacceptable if produced by the initial dose of a therapeutic in Phase I clinical trials conducted in healthy volunteers.

If several species were used in the IND-enabling toxicology studies, the most sensitive species should be used to calculate the MSRD.

Once the NOAEL has been determined, it can be converted to a human-equivalent dose (HED) using appropriate scaling factors, if needed. For small-molecule therapeutics, the NOAEL dose is converted to the HED using body-surface area correction factors ($BW^{2/3}$). This conversion is not necessary for mAb therapeutics that show a robust correlation between dose (mg/kg) and plasma drug levels (C_{max} and AUC). According to the FDA guidance (2005), proteins with a molecular weight above 100,000 Da should be normalized to mg/kg for HED calculation. Thus if the NOAEL dose in animals for a mAb is 10 mg/kg, the HED is also 10 mg/kg.

The next step in calculating the MSRD is to apply a safety factor to the HED to increase assurance that the first dose in subjects will not cause adverse effects. A safety factor of 10 is generally used, based on uncertainties in extrapolating from animal data to studies in humans. These uncertainties may include differences in sensitivity to pharmacological activity of the mAb, differences in target affinities or receptor distribution, differences in ADME profiles between animal and human, difficulties in detecting certain types of toxicity in animal studies such as headache or myalgia, and unexpected toxicities.

A factor greater than 10 can be used if there is evidence that this may be necessary. Causes for concern that would support raising the safety factor include a steep dose–response curve, severe toxicities, toxicity without premonitory signs, nonmonitorable toxicity, variable bioavailability, irreversible toxicity, unexplained mortality, large variability in dose or plasma drug levels, nonlinear PK, inadequate dose–response data, novel therapeutic targets, and/or animal models with limited utility [76,77].

Thus to calculate the MSRD, the HED is divided by an appropriate safety factor, typically using a value of 10, but can be increased or decreased based on available data. For a mAb with an HED of 10 mg/kg and using a safety factor of 10, the MSRD would be 1 mg/kg.

Once the MSRD has been determined, it is advisable to compare it to the PAD. This is especially important for mAb therapeutics that may not show any toxicity in the repeat-dose studies, thus resulting in high NOAEL values based on the highest dose tested (~100 mg/kg). For such a case, the NOAEL is 100 mg/kg, the HED is 100 mg/kg, and the MSRD would be 10 mg/kg (using a safety factor of 10), which would be considered a high mAb dose. The PAD, derived from in vitro and/or in vivo pharmacology models, for such a case may be a more sensitive indicator of biological activity and/or potential toxicity than the NOAEL, and thus may support lowering the MSRD.

The second method for FIH dose calculation is the MABEL in humans. This method is an extension of the concepts behind the PAD, and is directed toward potential high-risk medicinal products, which are defined as products where there are concerns that serious adverse effects may occur in the FIH study. The EMEA guidance document (2007) was produced after the tragedy involving the TeGenero monoclonal antibody TGN1412, where the FIH dose caused a cytokine storm following administration to healthy volunteers [78]. The premise here is that potentially high-risk medicinal products may need a more conservative approach to dose selection, as the ability of nonclinical studies to predict safety issues in humans may be reduced if the nature of the target is more specific to humans.

The EMEA guidance (2007) notes that some new drugs should be considered high risk due to concerns arising from particular knowledge, or uncertainties in their mode of action, nature of target, or relevance of animal models. The MABEL dose calculation is a special precaution to minimize the risk of adverse effects. With respect to mode of action, increased risk is associated with the novelty of the target, and the extent of knowledge of its mode of action. A compound with a pleiotropic mechanism that leads to various physiological effects, or one that bypasses normal physiological control mechanisms (eg, CD28 agonists), would be considered high risk. The nature of the target may impact on the risk inherent in FIH dosing, thus the biology of the target, and downstream effects in human species, and the biology and PD effects in both normal and diseased states needs consideration. The relevance of the animal models in predicting the risk to humans needs evaluation. If the available animal models are of limited relevance to predict PD and toxic effects, then the product should be considered high risk.

To calculate the MABEL, all relevant in vitro and in vivo pharmacological, PK/PD, and toxicity data need to be taken into account and integrated into a PK/PD modeling approach. A safety factor is then applied, defaulting to 10. This can be increased or decreased on a case-by-case basis, by considering available data. As with the PAD, the MABEL dose may be a more sensitive method for determining potential toxicity than the NOAEL. When the methods (NOAEL, PAD, MABEL) give different estimates of the FIH dose, the lowest value should be used.

REPEAT-DOSE TOXICOLOGY STUDIES BEYOND FIH

Once the Phase I study is under way, many other nonclinical development activities are necessary, all governed by the clinical development plan and timing of clinical studies. As development of a mAb therapeutic starts with the Phase I study, safety and signs of biological activity and/or clinical efficacy support the investment in further nonclinical studies, and with an acceptable safety profile in the nonclinical studies,

TABLE 23.4 ICH Recommended Duration of Repeat-Dose Toxicity Studies to Support the Conduct of Clinical Trials

Maximum Duration of Clinical Trial	Recommended Minimum Duration of Repeat-Dose Toxicity Studies to Support Clinical Trials	
	Rodents	Nonrodents
Up to 2 weeks	2 weeks	2 weeks
Between 2 weeks and 6 months	Same as clinical trial	Same as clinical trial
>6 months	6 months	9 months (6 months for mAbs is acceptable)

TABLE 23.5 ICH Recommended Duration of Repeated-Dose Toxicity Studies to Support Marketing

Duration of Indicated Treatment	Rodent	Nonrodent
Up to 2 weeks	1 month	1 month
>2 weeks to 1 month	3 months	3 months
>1 month to 3 months	6 months	6 months
>3 months	6 months	9 months (6 months for mAbs is acceptable)

further investment in clinical development follows. This careful, step-wise approach balances risk and investment related to nonclinical and clinical studies.

The ICH M3 and S6(R1) provide guidance regarding the timing of nonclinical studies needed to support clinical activity (Tables 23.4 and 23.5). Table 23.4 summarizes the duration of repeat-dose toxicity studies for small- and large-molecule therapeutics needed to enable similar durations in clinical testing. In general, clinical studies of certain durations should be supported by repeat-dose toxicology studies of at least an equivalent duration. For mAb therapeutics, 6-month rodent and/or nonrodent (based on species cross-reactivity) studies will generally support clinical studies of greater than 9-month duration.

Table 23.5 summarizes the recommended duration of repeat-dose toxicity studies needed to support the marketing authorization for small- and large-molecule therapeutics. For chronic use of mAbs, 6-month repeat-dose studies are adequate for marketing authorization (ICH S6(R1)).

The need to continue using two species beyond the IND-enabling studies is determined on a case-by-case basis, by considering the pharmacology/toxicology profile. If the toxicological findings from the 1-month studies are similar in both species, then longer-term studies in one species are usually considered sufficient, and the rodent species should be used, unless there is a reason for using the nonrodent species (ICH S6(R1)).

The dosing schedule in the animal studies generally varies between once 1 week and once 1 month, based on the half-life of the mAb, the duration of pharmacodynamic effect, and any clinical indications. It uses the route of administration to be used in the clinic.

The initial nonclinical studies generally include recovery periods. These are periods of time after dosing has stopped, to assess recovery from toxicity or PD effects, with the objective of assessing animals after the recovery period to evaluate the reversibility of any effects, but not delayed toxicity. Demonstration of complete recovery from these effects is generally not necessary. The regulatory guidance documents (ICH S6(R1)) note that the addition of a recovery period is not appropriate if the goal is just to assess immunogenicity (low levels of drug at the end of a recovery period allow improved detection of ADA). The decision regarding whether or not to include recovery groups in the subchronic and chronic toxicity studies will need to be made on a case-by-case basis, determined by the pharmacodynamic and toxicity parameters as well as recovery observed in the 1-month studies.

IMMUNOGENICITY OF MONOCLONAL ANTIBODIES

The evolution in antibody engineering has ultimately led to the production of fully human mAbs that are significantly less immunogenic than the early mouse mAbs. For example, the immune response to the murine mAb, muromonab, resulted in its clearance from the circulation after about 2 weeks of treatment [19], whereas human therapeutic mAbs, such as denosumab, can be successfully administered on a chronic basis to postmenopausal patients with osteoporosis.

The assessment of the immunogenicity of mAbs is typically included in nonclinical safety evaluation studies, and is standard practice in clinical studies, but the objectives of assessing the presence of ADA in these studies are somewhat different. The purpose of the former is to gain a thorough understanding of levels of systemic exposure to the mAb over the duration of the study and to aid in interpretation of the study. In a robust immune response, the ADA can bind to the mAb therapeutic, resulting in increased clearance of the drug. Alternatively, the ADA interferes with the binding of the mAb to its therapeutic target, thus possibly masking pharmacological or toxicological effects, making it challenging to assess the safety of the mAb. If no toxicity or PD activity is observed, then it is important to have data showing a lack of neutralizing antibodies as well as PK data showing drug presence, since these data can support the argument that exposure was adequately assessed in the toxicology study [79]. For this reason, at the onset of a

toxicology program, ADA samples should be collected from animals for possible future analysis.

For clinical studies, the objective of ADA assessment is to evaluate clearance of the drug, possible immunotoxicity, and to determine whether the presence of ADA leads to a reduction in efficacy of the therapeutic mAb.

Testing for ADA generally follows a tiered approach (reviewed by the FDA Immunogenicity Testing Draft Guidance, 2009; [80]), including a screening assay, a confirmatory assay (where the unlabeled mAb therapeutic is used to compete with ADA binding), and a titer assay. Characterization of ADA-neutralizing activity is needed on a case-by-case basis for toxicology studies, mainly to aid in the interpretation of the study, if needed, and is expected to be conducted for clinical studies. The bioanalytical field continues to develop new methods to measure and characterize ADA.

The immune response to human or humanized mAbs is expected to be greater in animals than in humans, due to the differences in the mAb protein structure between the different species. Thus any immunogenicity in animals is expected to overestimate the immune response anticipated in humans. For this reason, immunogenicity data from animal studies is not used to predict the immune response to a mAb therapeutic in humans (for a review see Refs. [79,81,82]).

The generation of ADA may have little or no impact on the PK/PD and/or toxicity of a therapeutic mAb; however, in some cases, circulating ADA-mAb complexes may lead to hypersensitivity reactions (eg, Type III reactions, according to Coombs and Gell classification), or deposition of ADA-mAb complexes in the kidney, possibly leading to glomerular nephritis. The potential pathogenic properties of ADA-mAb complexes are incompletely understood, but have been found to depend on a ratio of the concentrations of ADA to therapeutic mAb, as well as the overall amount of antibody in the circulation [83,84].

IMMUNOTOXICITY

The evaluation of potential immunotoxicity in new therapeutic mAbs is critical, especially since a large number of the mAbs marketed intentionally target the immune system. The ICH S8 (Guidance for Industry – S8 Immunotoxicity Studies for Human Pharmaceuticals, [85]) provides regulatory guidance on nonclinical testing approaches to identify potentially immunotoxic drugs, and a weight-of-evidence approach to decision-making for immunotoxicity testing. As per ICH S8, immunotoxicity is defined as unintended immunosuppression or enhancement.

The potential for immunotoxicity is assessed in a tiered manner, starting with general toxicology studies. Signs of immunotoxicity can be ascertained by gross pathology and organ-weight assessment, histopathological evaluation of the spleen, lymph nodes, and bone marrow as well as assessment of clinical pathology parameters (hematology and clinical chemistry). Findings indicating immunotoxicity can be further investigated in studies designed to address specific questions.

If immunomodulation of the immune system is anticipated from the mechanism of action of a mAb, special evaluations such as T-cell-dependent antibody response (TDAR) and/or immunophenotyping, using immunohistochemistry or flow cytometry methods, can be incorporated into the toxicology studies. With immunophenotyping, the lymphocyte subpopulations (absolute values and percentages) can be examined.

Other immune function assays available include natural killer cell activity assays, macrophage/neutrophil function assays, and cell-mediated immunity/delayed-type hypersensitivity (DTH) assays. Host-resistance assays, which can evaluate a wide range of pathogens (eg, *Listeria monocytogenes, Streptococcus pneumonia*), are also available, if warranted [86]. In vitro studies are typically conducted to assess the potential for ADCC and CDC.

As many of the marketed mAbs targeting the human immune system are pharmacologically active only in nonhuman primates, this species has been used extensively in the assessment of immunotoxicity risk [87]. Several reviews on immunotoxicity testing of immunomodulatory biological therapeutics are available [88,89].

For mAbs where immunosuppression or immunostimulation is anticipated from their mechanism of action, special attention is needed to understand the biological consequences of inhibiting or activating pathways involving the immune system, both in the toxicology species and in humans, in order to assess risk. The TeGenero case is a very unfortunate example in which rodent and NHP toxicology data did not predict the activity of an immunomodulatory superagonist anti-CD28 mAb in humans [78]. This mAb, referred to as TGN1412, was administered as a single dose to six healthy volunteers (0.1 mg/kg dose), and a cytokine-release syndrome or "cytokine storm" occurred, involving multiorgan failure. All patients were transferred to intensive care, where at least four suffered multiple organ dysfunction. Retrospective analysis of the FIH dose has implied that a careful analysis of all the available in vitro and in vivo immunological and pharmacological data [90] would have dictated the selection of a lower FIH dose. This tragedy has resulted in renewed awareness of the need to assess all data associated with a new mAb therapeutic, plus others with similar mechanism of action, when extrapolating risk from animals to humans [91]. A new EMEA Guideline to assist with the process of FIH dose selection has also been published [92–94].

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY EVALUATION

Evaluation of the potential of a new drug to cause harm to the fertility, reproductive, and/or developmental processes in animals is critical to the progression of clinical development. Although the general concepts for testing are the same in the ICH countries (the US, EU, and Japan), the timing of when the data are needed to support the different stages of clinical development varies among the countries. Regulatory guidance documents recommended for these reproductive system assessments for mAbs are listed in the following, and their content will be summarized in this section:

- ICH Harmonised Tripartite Guideline – Detection of Toxicity to Reproduction for Medicinal Products & Toxicity to Male Fertility, S5(R2), 2005
- ICH S6(R1)

Although mAbs and small-molecule therapeutics undergo similar nonclinical testing to determine their potential toxicity to the reproductive system, mAbs generally are associated with lower risk. Small-molecule therapeutics are capable of easily crossing cell membranes and gaining access to the interior of the cell. This enables travel across the placenta and access to the developing embryo/fetus, as well as postnatal access to offspring through maternal milk. For mAbs, transport across placenta is generally via the FcRn, allowing access to the embryo/fetus at times that are largely dependent on the expression profile of the maternal FcRn. mAbs have been found in fetal serum and also maternal milk.

Small-molecule therapeutics are also metabolized, which can produce metabolites with greater toxicity than the parent molecule, thus supporting the necessity of testing reproductive system toxicity in two species, due to the variability in metabolism between species. For mAbs, metabolism results in degradation into amino acids, which do not present the same risk as small-molecule metabolites.

The nonclinical safety assessment is:

> Expected to cover all stages of development from conception to sexual maturity, and to allow detection of immediate and latent effects of exposure by following the animals through one complete life cycle (ie, from conception in one generation through conception in the following generation). (ICH S5(R2))

Traditionally, toxicology evaluation studies assessing reproduction have been divided into three parts: Segment I (male and female fertility and early embryonic development), Segment II [embryo-fetal development (EFD)], and Segment III (pre-/postnatal development). Segment I generally covers premating through implantation, Segment II studies cover implantation through the end of pregnancy, and Segment III studies cover closure of the hard palate to weaning and sexual maturity of the F1 pups.

For a new mAb therapeutic, only pharmacologically active species should be used in the evaluation of potential toxicity to the reproductive system. This is where the challenge arises, since traditional rodent and rabbit species may not demonstrate cross-reactivity with the mAb, so the sponsor may need to use NHP or other nontraditional species, and employ creative approaches to safety evaluation of the reproductive system.

Due to issues with cross-reactivity, the NHP has become a more common species for reproductive toxicity assessment, and the traditional Segment I–III studies have been modified in design to facilitate its use and to reduce the number of animals studied. The term enhanced pre-/postnatal development study (ePPD) has emerged for NHP studies and combines fertility, EFD, and postnatal evaluations. Several reviews are available on reproductive toxicity testing with NHP [95,96].

Traditional study designs can be modified based on an understanding of species specificity, the nature of the product, mechanism of action, immunogenicity, and/or PK behavior and embryo-fetal exposure. Once again, a case-by-case science-based assessment will be needed to design these studies. This is especially the case for NHP studies, since they are more complex in nature than the traditional rodent and rabbit studies.

Fertility

For mAb therapeutics, ICH S6(R1) states that for products where rodents are a relevant species, an assessment of fertility can be made in this species, and the fertility studies for small-molecule and biologic therapeutics in rodents are similar in nature.

For female and male fertility assessment in rodents, during the premating period, females are administered the therapeutic mAb 2 weeks prior to dosing and males administered mAb 4 weeks prior to mating. Treatment continues throughout mating at least through implantation for females and study termination for males. The assessments include a general macroscopic evaluation at necropsy, preservation of tissues with any findings as well as the reproductive organs for possible future evaluation, numerical assessment of corpora lutea and implantation sites in the uterus, and counting of live and dead conceptus. Sperm analysis can be done to assess potential effects on spermatogenesis.

Based on ICH S6(R1), when the NHP is the only pharmacologically relevant species, the potential effects on male and female fertility can be assessed by standard histopathological evaluation and assessment of menstrual cyclicity in the repeat-dose toxicity studies of at least 3 months' duration using sexually mature NHPs. If there is a specific cause for concern, specialized assessments

such as sperm count, sperm morphology/motility, testicular volume, and male or female reproductive hormone levels should be evaluated in the repeat-dose toxicity study.

If there is cause for concern regarding potential effects on conception/implantation based on the pharmacological activity of a mAb, this can be investigated experimentally by using a homologous product or transgenic model to assess potential effects on conception or implantation.

When considering timing to support clinical studies, according to ICH M3(R1) men and women can be enrolled in Phases I and II clinical studies before male and female fertility studies have taken place, since evaluation of the male and female reproductive organs has been performed in the repeat-dose toxicity studies. Nonclinical male and female fertility studies should be completed prior to the initiation of Phase III clinical trials, or other large-scale or long-duration studies.

Embryo-Fetal Development

Study designs for small-molecule and mAb therapeutics are similar for EFD studies using rodents and rabbits, with dosing occurring during the period of major organogenesis. The situation is more challenging if there is no species cross-reactivity with the rodent or rabbit, if NHP are used, or if other models need to be developed.

If NHP or other novel models are used, it is important to understand the reproductive biology of the species being tested, the course of development of specific systems of interest (eg, immunological system development), the pharmacological and possible toxicological endpoints to build into the study design for evaluation, PK sampling to understand maternal exposure, and if possible, PK sampling of the offspring. For the NHP, assessment of pregnancy outcome at natural delivery is typically performed, evaluating offspring viability, survival, external malfunction, skeletal effects, and visceral morphology at necropsy.

Based on ICH S6(R1), one species can be sufficient for EFD evaluation if more than one relevant species exists, provided there is a scientific rationale supporting species selection. For other mAbs such as natalizumab (Tysabri; developed prior to ICH S6(R1)), only the guinea pig and NHP showed pharmacological activity with the mAb and were used in the reproductive toxicity assessment. The guinea pig, a nontraditional species for EFD studies, was evaluated for feasibility and then used for assessment of potential toxicity to embryo-fetal development [97] as well as fertility [98].

For products that are only pharmacologically active in NHP, one well-designed study that includes dosing from day 20 of gestation to birth can be considered (ICH S6(R1)). This is referred to as an enhanced pre-/postnatal development (ePPND) study. It evaluates all the stages in traditional EFD (Segment II) and pre-/postnatal (Segment III) studies, but uses fewer animals, since the parameters from the two studies are combined into one. It is also possible to evaluate potential effects on EFD and postnatal development using alternative study designs or a homologous product in rodents.

For efalizumab (Raptiva), which showed species cross-reactivity only in the chimpanzee and human, a homologous mAb was developed that cross-reacted with the mouse, and the mouse model was used for EFD and fertility assessment [99].

There is room for flexibility and creativity in the designs of these studies based on the characteristics of the mAb, intended pharmacological activity, potential toxicity, and duration of intended effect, as long as the study design is based on a science-driven rationale.

In the United States, assessment of EFD should be conducted prior to enrolment of women of childbearing potential (WOCBP) using precautions to prevent pregnancy in Phase III studies. In the EU and Japan, definitive nonclinical EFD toxicity studies should be completed before exposure of WOCBP.

Reproductive toxicology studies might not be warranted when the weight of evidence (eg, mechanism of action, phenotypic data from KO mice, class effects) suggests that there will be an adverse effect on pregnancy outcome, and these data possibly could provide adequate information to communicate risk.

Pre-/Postnatal Development Studies

The objective of pre-/postnatal development studies is to assess the potential toxicity of a new drug to development occurring during the period preceding birth, the neonatal stage, and development and sexual function of the F1 pups. Female animals are exposed to the mAb from implantation to the end of lactation. Species selection will be dependent on mAb pharmacological activity. The traditional species is the rodent (rat). If the NHP is the only species available for testing, consideration should be given to combining the endpoints with those in the EFD, if possible (see ePPND study previously mentioned).

For maternal animals, endpoints typically include clinical observations, body weights, food consumption, duration of pregnancy and parturition, number of implantations, and any abnormalities. For the offspring, observations include viability of offspring at birth, body weight at birth, pre- and postweaning survival and growth/body weight, maturation, and fertility. Physical development, sensory functions, and reflexes and behavior are also evaluated.

In the United States, EU and Japan, the pre-/postnatal development study should be conducted prior to marketing authorization.

CARCINOGENICITY

Based on ICH S1A, carcinogenicity assessment is anticipated to support clinical development and marketing authorization of small-molecule therapeutics intended for treating chronic (greater than 6 months of continuous treatment duration) diseases, and traditionally encompasses 2-year carcinogenicity bioassays in both mice and rats [100]. In some instances, a 6-month transgenic mouse study may be substituted for the 2-year mouse study, based on the mechanism of action of the drug and associated scientific rationale. For mAb therapeutics, which are not metabolized to reactive metabolites, cannot access the cell interior, and do not come in contact or cause damage to DNA, the genotoxic potential is considered to be low, thus supporting the rationale for not conducting the standard battery of genotoxicity tests. For these reasons as well as issues where the mAb is not pharmacologically active in rodent species, or is active but generates a robust immunogenicity response resulting in mAb clearance from the systemic circulation, the traditional rodent carcinogenicity bioassays are not conducted. However, there is a theoretical concern that immunomodulatory mAb therapeutics may affect normal immune surveillance mechanisms and result in increase in the incidence or proliferation rate, or a specific type of neoplasm or group of neoplasms.

Regulatory guidance documents that should be reviewed on this subject include ICH S6(R1) and S1A (Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals). The ICH S1A is most relevant for small-molecule carcinogenicity evaluation, but does recognize the challenges for evaluating mAbs as well as other biological therapeutics. Thus an assessment of the necessity for evaluating potential tumorigenicity risk should be conducted on a case-by-case basis, taking into account the intended clinical population and treatment duration [100].

To determine whether additional studies are needed to assess tumorigenicity risk, a weight-of-evidence assessment is recommended, taking into account data from the published literature (information on transgenic animals, knock-out models, human genetic disease, drug class effects, target biology) as well as data on the mAb from in vitro studies, chronic toxicology studies, and clinical data.

If the weight of evidence suggests a concern about carcinogenic/tumorigenic potential, additional nonclinical studies could be conducted that may mitigate this concern. In some cases, the available data can be considered sufficient to address carcinogenic potential and inform clinical risk without warranting additional nonclinical studies. For example, immunomodulators and growth factors pose a potential carcinogenic risk that can be evaluated by postmarketing clinical surveillance more effectively than further clinical studies (FDA ICH S6(R1)). Vahle and colleagues [101] reviewed past and current practices regarding carcinogenicity assessments of biological therapeutics and provided recommendations for carcinogenicity assessments. Of the 21 mAbs reviewed, none were tested in 2-year rodent bioassays. One (mouse homolog; efalizumab) was tested in the p53(+/+) transgenic model, and showed a negative response; one (mouse homolog; infliximab) was tested in a 6-month repeat-dose study with negative tumorigenicity results; and one (natalizumab) was tested in in vitro proliferation assays and in a tumor xenograft model, with negative results for proliferation and tumor growth rate/metastasis, respectively.

For assessing the carcinogenicity risk of a mAb therapeutic, rodent 2-year bioassays or short-term tumorigenicity studies with rodent homologs are generally of limited value and generally are not recommended by regulatory authorities.

DRUG INTERACTIONS

As mAbs are not metabolized by cytochrome (CYP) 450 enzymes, they have not been expected to play an important role in drug–drug interactions in the clinic, and have not historically been evaluated for such effects. Recently, however, it has been shown that therapeutic proteins, including mAbs, may impact the disposition of drugs metabolized by CYP 450 (Ref. [102], reviewed by Ref. [103]). mAbs and other therapeutic proteins that target pathways reducing inflammation and/or infection have been shown to affect CYP 450 enzyme expression, likely resulting from changes in transcription factor activity for CYP enzyme expression or changes in CYP enzyme activity due to altered immune status [103,104].

This is an evolving area of study, and mAbs-targeting cytokines, interferons, and growth factors may need to undergo assessment. In vitro studies investigating CYP 450 inhibition and/or induction may be relevant on a case-by-case basis, and further evaluation in the clinical studies may be warranted.

PARTNERSHIP IN MAB DEVELOPMENT

Advancing a new mAb therapeutic from the benchtop to the clinic requires the concerted efforts of many people in numerous different fields of study and expertise, and successful development is based on good communication and partnerships between the sponsoring company, regulatory agencies, as well as the contract research organizations involved in many nonclinical and clinical studies, with the ultimate goal of providing safe and effective new medicines for patients.

SUMMARY

The use of antibodies for disease treatment has come a long way since the early days of serum therapy for patients with diphtheria, with the origin of Ehrlich's concept of medicines serving as magic bullets to target disease. Together with the growing fields of molecular biology and antibody engineering, humanized and fully human therapeutic mAbs with less risk of immunogenicity are available for treatment of serious diseases. The understanding and practice of nonclinical safety evaluation of mAbs has advanced together with this growth in technology and remains a case-by-case, science-driven assessment.

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