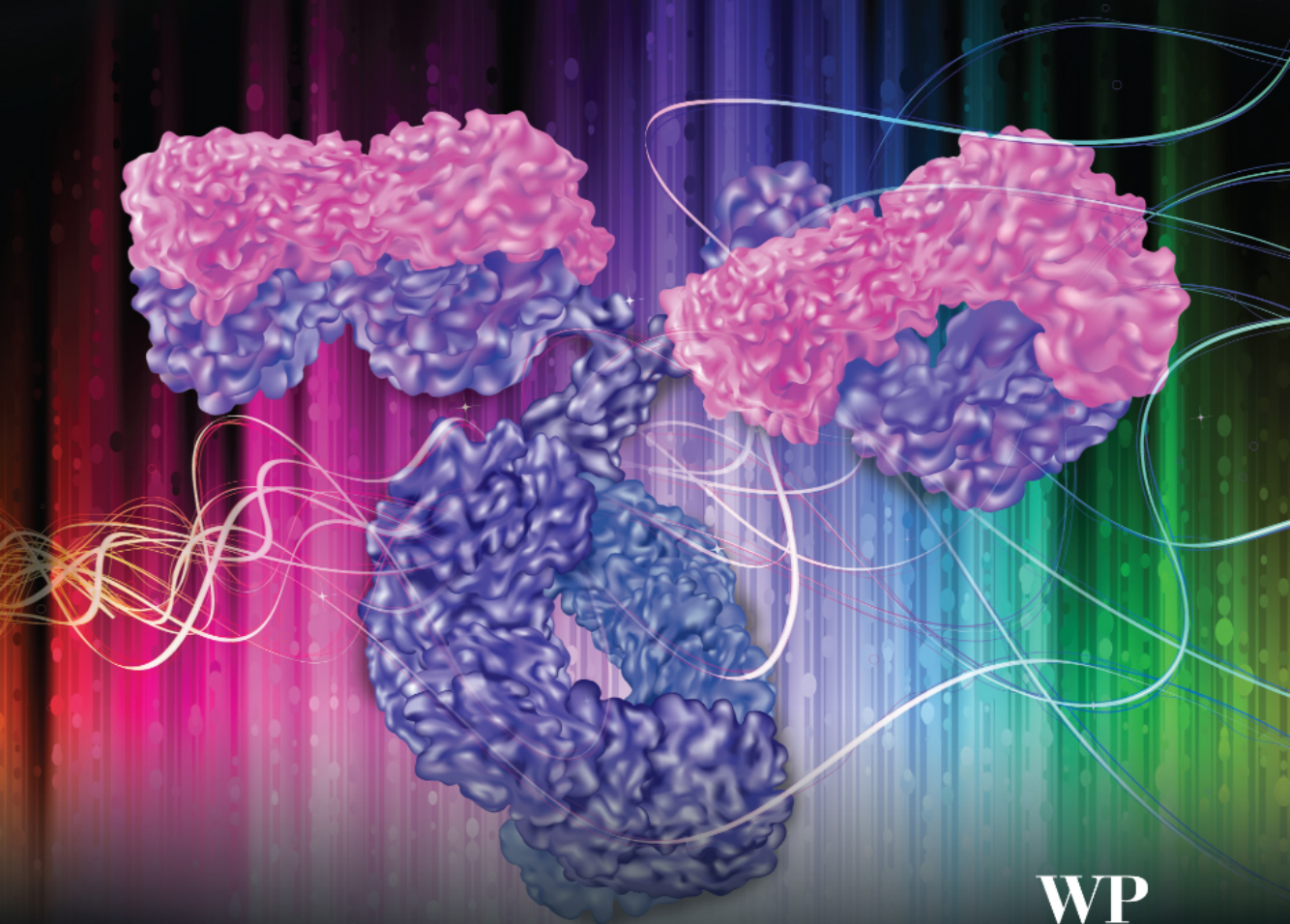


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strongest growth area in the
pharmaceutical industry

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List of acronyms, abbreviations, and definitions

ABC	ATP-binding cassette transporter
ACE	Angiotensin converting enzyme
ADAs	Anti-drug antibodies
ADC	Antibody-drug conjugate
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
ADEPT	Antibody-directed enzyme pro-drug therapy
AFS	Alternatively folded states
AID	Activation-induced (cytidine) deaminase
AIDS	Acquired immune deficiency syndrome
Ala (A)	Alanine
ALL	Acute lymphoblastic leukemia
AMA	American Medical Association
AMD	Age-related macular degeneration
APEx	Anchored periplasmic expression
APC	Antigen presenting cell
API	Active pharmaceutical ingredient
APRs	Aggregation prone regions
Arg (R)	Arginine
ASGP-R	Asialoglycoprotein receptor
Asn (N)	Asparagine
Asp (D)	Aspartate
ATL	Adult T-cell leukemia-lymphoma
AUC	Area under the curve
B ₂₂	2nd virial coefficient, describing a protein's interactions with itself in solvent
BACE	β-secretase
BAFF	B cell activating factor

BBB	Blood–brain barrier
BC	B cell
BCR	B cell receptor
BHK	Baby hamster kidney
BiTE	Bispecific T cell engager
BLA	Biologics license application
BMI	Body mass index
BMS	Bristol-Myers Squibb
BoNT	Botulism neurotoxin
BPCI	Biologics Price Competition and Innovation (Act 2009)
BRAF	Serine/threonine-protein kinase, also known as proto-oncogene B-Raf or v-Raf murine sarcoma viral oncogene homolog B1
BRIC	Brazil, Russia, India, China (countries with rapidly developing economies)
BS	Bispecific
BV	Bivalent
C	Constant (chain)
CAPS	Cryopyrin-associated periodic syndrome
CAT	Cambridge Antibody Technology (now part of MedImmune)
CCL	CC-type chemokine family ligand (e.g. CCL-2)
CCK	Cholecystokinin
CCR	CC-type chemokine family receptor (e.g. CCR-4)
CD	Cluster of differentiation
CDC	Complement-dependent cytotoxicity (or complement-mediated cytotoxicity (CMC))
CDCC	Complement-dependent cellular cytotoxicity
CDR	Complementarity determining regions: six antigen-binding “loops,” three in each of V_H and V_L
CEA	Carcinoembryonic antigen
cGMP	Current good manufacturing practice
C_H	Constant heavy chain, consisting of C_{H1} , hinge, C_{H2} , and C_{H3}
CHO	Chinese hamster ovary (cell line)
CIC	Cross-interaction chromatography
C_κ	Constant kappa light chain
C_λ	Constant lambda light chain
C_L	Constant light chain, consisting of either C_κ or C_λ
CLL	Chronic lymphocytic leukemia

$C_{\max}$	Maximum concentration of drug in serum after administration
C_{μ^+}	Cytoplasmic μ protein
CMV	Cytomegalovirus
CNS	Central nervous system
COGs	Cost of goods
COPD	Chronic obstructive pulmonary disease
CR	Complement receptor
CRS	Cytokine release syndrome
CSR	Class switch recombination
CTLA4	Cytotoxic T-lymphocyte antigen 4
CTP	C-terminal peptide
CV	Cardiovascular (disease)
Cys (C)	Cysteine
CXCR	CXC-type chemokine family receptor (e.g. CXCR-4)
D, D_H	Diversity
Da	Daltons
DAb	Domain antibody of ca. 12 kDa; comprised of either V_H or V_L
DARPin	Designed ankyrin repeat protein
DART	Dual affinity retargeting (molecules)
DC	Dendritic cell
DC-SIGN	Dendritic-cell-specific ICAM-3 grabbing non-integrin (CD209)
DHFR	Dihydrofolate reductase
DLBCL	Diffuse large B cell lymphoma
DM1	$N^{2'}$ -deacetyl- $N^{2'}$ -(3-mercapto-1-oxopropyl)-maytansine
DM4	$N^{2'}$ -deacetyl- $N^{2'}$ -(4-mercapto-4-methyl-1-oxopentyl)-maytansine
DNA	Deoxyribonucleic acid
DPP4	Dipeptidyl peptidase IV
DR	Death receptors (also called TRAIL)
DVD	Dual variable domain
EBV	Epstein-Barr virus
EC	Electron capture
EF1 α	Elongation factor 1 α
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbant assay
ELP	Elastin-like peptide
EMEA	European Medicines Agency
EMP1	Erythropoietin mimetic peptide 1

ENPP3	Ecto-nucleotide pyrophosphatase/phosphodiesterase 3
EO	Eosinophil
EpCAM	Epithelial cell adhesion/activating molecule (CD326)
EPO	Erythropoietin
EP-PCR	Error-prone polymerase chain reaction
ETA	Exotoxin A
EU	European Union
FAb	“Fragment, antigen binding” region of ca. 50 kDa, comprised of C _H 1, V _H , C _L , V _L
FACS	Fluorescence-based cell sorting
FAS	Receptor, also called APO-1, CD95
Fc	“Fragment, crystallizable,” comprised of paired heavy chains from hinge through C _H 3
Fcγ	Fc gamma chain
FcγR	Fc gamma receptor
FcFP	Fc fusion protein
FcR	Fc receptor
FcRn	Neonatal Fc receptor
Fd	Heavy chain portion of FAb fragment, comprised of C _H 1, V _H domains
FDA	Food and Drug Administration (United States)
FIH	First in human (initiation of Phase I clinical studies)
Fn3	Fibronectin domain-3
FOB	Follow-on biologic (also called biosimilar)
FR	Framework region
FSH	Follicle stimulating hormone
Fv	Fragment of an antibody containing only the paired V _H and V _L domains
FW	Framework (non-CDR sequences in variable chains)
G-CSF	Granulocyte colony-stimulating factor
GERD	Gastro-esophageal reflux disorder
GITR	Glucocorticoid-induced tumor necrosis factor (TNF) receptor (aka TNFRSF18)
GFP	Green fluorescent protein
GlcNAc	N-acetyl-glucosamine
Gln (Q)	Glutamine
GLP	Good laboratory practice
GLP-1	Glucagon-like peptide-1

GLP-tox	Good laboratory practice – pre-clinical toxicology (studies)
Glu (E)	Glutamate
Gly (G)	Glycine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good manufacturing practice
GPCR	G-protein coupled receptor
Gp	Glycoprotein
GPI	Glycosylphosphatidyl inositol
GRAS	Generally regarded as safe (FDA designation)
GS	Glutamine synthetase
GSK	GlaxoSmithKline
GVHD	Graft vs. host disease
H ₂ L ₂	Two heavy chains, two light chains configuration
HAART	Highly active anti-retroviral therapy
HACA	Human anti-chimeric antibodies (anti-drug response)
HAHA	Human anti-human (or humanized) antibodies (anti-drug response)
HAMA	Human anti-mouse antibodies (anti-drug response)
HAP	Homo-amino acid polymer
HAT	Hypoxanthine-aminopterin-thymidine (selective growth medium for hybridomas)
HC	Heavy chain
HCab	Heavy chain (only) antibodies
HCG	Human chorionic gonadotropin
HEK	Human embryonic kidney (cell line, i.e. HEK293)
HES	Hydroxy-ethyl starch
HFA	Human framework adaptation
HGF	Hepatocyte growth factor
HGH	Human growth hormone
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HGS	Human Genome Sciences
HIC	Hydrophobic interaction chromatography
His (H)	Histidine
HIV	Human immunodeficiency virus
HKB	Cell line derived from hybrid of kidney and B cells
HLA	Human leukocyte antigen
HPLC	High-performance liquid chromatography
HPRT	Hypoxanthine phosphoribosyltransferase

HPV	Human papilloma virus
HSA	Human serum albumin
HSC	Human string content
HTLV-1	Human T cell leukemia virus 1
HTRP	Hydroxytryptophan
HVL	Hypervariable loop
ICH	International Committee for Harmonization
IEF	Isoelectric focusing
IFN	Interferon (e.g. IFN- α , IFN- γ)
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgAbp	Immunoglobulin A binding protein
IgAN	Immunoglobulin A nephropathy
IgD	Immunoglobulin D
IGF-1R	Insulin-like growth factor receptor-1
IgG	Immunoglobulin G
IGH	Immunoglobulin heavy chain gene locus
IGHC	Immunoglobulin heavy chain constant region gene(s)
IGHD	Immunoglobulin heavy chain diversity gene(s)
IGHJ	Immunoglobulin heavy joining chain gene(s)
IGHV	Immunoglobulin heavy chain variable gene(s)
IgM	Immunoglobulin M
Ig μ	Immunoglobulin mu chain
IgNAR	Immunoglobulin new antigen receptor
IL	Interleukin (e.g. IL-4, IL-6, IL-12, IL-23, etc.)
IM	Intramuscular (administration)
IMGT	ImMunoGene Tics (database: http://www.imgt.org/)
IND	Investigational new drug
IPF	Idiopathic pulmonary fibrosis
Ile (I)	Isoleucine
IRR	Internal rate of return (on investment)
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITP	Idiopathic thrombocytopenic purpura
IV	Intravenous
IVIG	Intravenous immunoglobulin
J	Joining (chain)
J _H	Joining chain for heavy chain assembly

Jκ	Joining chain for kappa chain assembly
Jλ	Joining chain for lambda chain assembly
JAK	Janus kinase
K _a	Association constant (also known as K _{on})
K _d	Dissociation constant (also known as K _{off})
K _D	Affinity constant
kDa	Kilodaltons
KINEXA	Kinetics exclusion assay
KRAS	Kirsten rat sarcoma (RAS) virus transforming oncogene
KYN	Kynurenine
LC	Light chain
Leu (L)	Leucine
LF	Lethal factor
LFA-1	Lymphocyte function associated antigen-1
LH	Lutenizing hormone
LOXL-2	Lysyl oxidase-like-2 (matrix enzyme)
LRP-1	Low-density lipoprotein receptor-related protein 1
LYO	Lyophilized
Lys (K)	Lysine
MA or MAC	Macrophage
MAb	Monoclonal antibody
MABEL	Minimal anticipated biological effect level
MAD	Multiple ascending dose
Man	Mannose
MBL	Mannan-binding lectin
MCB	Master cell bank
MCP-1	Monocyte Chemotactic Protein 1
MCRC	Metastatic colorectal cancer
mc-vcMMAE	Maleimidocaproyl-valine-citrulline- <i>p</i> -aminobenzyloxy carbonyl-monomethyl-auristatin E
MDR	Multi-drug resistant
MED	Minimal effective dose
Met (M)	Methionine
MHC	Major histocompatibility complex
MMAE	Monomethyl-auristatin E
MMAF	Monomethyl-auristatin F
MMP	Matrix metalloproteinase
MO	Monocyte

MOA	Mechanism of action
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MTD	Maximum tolerated dose
MTX	Methotrexate
NABP	Non-antibody binding protein
NBE	New biological entity
Neu5Gc	N-glycolneuraminic acid
NFK	N-formylkynurenine
NGF	Neurotrophic growth factor
NHEJ	Non-homologous end-joining (process)
NICE	National Institute for Health and Clinical Excellence (United Kingdom)
NIH	National Institutes of Health (US)
NK	Natural killer (cell)
NHL	Non-Hodgkin's lymphoma
NHP	Non-human primate
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NOD	Non-obese diabetic (mice)
NOG	NOD/SCID/ γ^{null} (mice, also called NSG mice)
NP	Natural product
NRAS	Rat sarcoma (RAS) transforming oncogene identified in human neuroblastoma
NRTI	Nucleoside reverse transcriptase inhibitor
NSCLC	Non-small cell lung cancer
NSF	National Science Foundation (US)
NSG	NOD/SCID/ γ^{null} (mice, also called NOG mice)
OTR	Organ transplantation rejection
PA	Protective antigen (one component of <i>Bacillus anthracis</i> anthrax toxin)
PAb	Polyclonal antibody
PAS	Proline-alanine-serine (repeated polypeptide oligomer)
PBLs	Peripheral blood leukocytes
PBMCs	Peripheral blood mononuclear cells
PCD	Pre-clinical development; <i>also</i> Programmed cell death
PCR	Polymerase chain reaction
PD	Pharmacodynamic
PD-1	Programmed cell death-1 (surface protein)
PDB	Protein Data Bank

PDL	Protein Design Labs
PE38	<i>Pseudomonas</i> exotoxin fragment of 38 kDa
PEG	Polyethylene glycol
PEI	Polyethylene imine
PER.C6	Human cell line
PET	Positron emission tomography
PGFR	Platelet-derived growth factor receptor
P-gp	P-glycoprotein (also called “multi-drug resistance” MDR1)
Phe (F)	Phenylalanine
PHS	Public Health Service
PI	Protease inhibitor
pIgR	Polymeric immunoglobulin receptor
PIGS	Modeling software (http://arianna.bio.uniroma1.it/pigs/)
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide (mutated in several human cancers)
PK	Pharmacokinetics
PK-PD	Pharmakokinetics-pharmacodynamics
PMN	Polymorphonuclear (cells)
PNH	Paroxysmal nocturnal hemoglobinuria
POC	Proof of concept
POS	Probability of success
Pre-BCR	Pre-B cell receptor (complex on surface of immature B cells)
Pro (P)	Proline
PSA	Polysialic acid
PSMA	Prostate specific membrane antigen
ΨLC	Pseudo-light chain
PTCL	Peripheral T-cell lymphoma
PTRS	Probability of technical and regulatory success
PYY	Peptide YY, 36-amino acid gut peptide involved in satiety
QALY	Quality-adjusted life-years
Qp	Specific productivity
RA	Rheumatoid arthritis
RAG	Recombination activating gene
RAGE	Receptor for advanced glycation end products
RANKL	Receptor activator of nuclear factor kappa-B ligand
R&D	Research and development
RCC	Renal cell carcinoma
rFIXFc	Recombinant Factor IX Fc fusion protein

RIC	Radioimmunoconjugate
RIPs	Ribosome-inactivating proteins (e.g. bouganin, gelonin, ricin)
ROI	Return on investment
RSS	Recombination signal sequence
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAD	Single ascending dose (in Phase I clinical trials)
SALCL	Systemic anaplastic large cell lymphoma
SAP	Spatial-aggregation-propensity
SARS	Severe acute respiratory syndrome
SAS	Solvent accessible surface
SC	Subcutaneous (administration)
SC	Secretory chain (part of secretory IgA derived from plgR)
scAb	Single chain antibody
scFv	Single chain Fv fragment, consisting of V _H and V _L fused with a linker, ca. 25 kDa
SCID	Severe combined immunodeficiency
SDR	Specificity-determining residue
SDRU	Specificity-determining residue usage
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SEED	Strand-exchange engineered domain
SE-HPLC	Size exclusion-high performance liquid chromatography
SELEX	Systematic evolution of ligands by exponential enrichment
SeqAR	Sequence-activity-relationships
Ser (S)	Serine
SFDA	Food and Drug Administration (China)
SH	Sulfhydryl
SHM	Somatic hypermutation
SIC	Self-interaction chromatography
sIg ⁺	Surface-anchored Ig expression
sIgA	Secretory IgA
SIGN-R1	Specific ICAM-3 grabbing non-integrin-related 1
siRNA	Short interfering ribonucleic acid
SLAM	Selected lymphocyte antibody method
SLE	Systemic lupus erythematosus
SM	Small molecules
SMCC	Succinimidyl-4-(<i>N</i> -maleimidomethyl)cyclohexane-1-carboxylate (linker)

SMIP	Small immunomodulatory pharmaceutical
SN-38	Highly potent irinotecan derivative
SPDB	<i>N</i> -Succinimidyl 4-(2-pyridyldithio)butyrate (linker)
SPECT	Single-photon emission computer tomography
SPP	<i>N</i> -succinimidyl-4-(2-pyridyldithio)pentanoate (linker)
SPR	Surface plasmon resonance
SREs	Skeletal-related events
SV40	Simian virus 40, a polyomavirus found in monkeys and humans
SVMP	Snake venom metalloproteinase
SWOT	Strengths, weaknesses, opportunities, threats (analysis)
TandAb	Tandem antibody dimers
TC	T cell
TD	T cell-dependent
T-DM1	Trastuzumab-DM1
TdT	Terminal deoxynucleotidyl transferase
TGN	Tegenero (company that developed anti-CD28 TGN1412)
Th1	T helper-1 cell types, responses, cytokine profiles (pro-inflammatory)
Th2	T helper-2 cell types, responses, cytokine profiles (anti-inflammatory)
Thr (T)	Threonine
TI	T cell-independent
TNALP	Tissue non-specific alkaline phosphatase
TNF	Tumor necrosis factor
TPO	Thrombopoietin
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand receptors 1 and 2
TRIM	Tripartite motif-containing (as in TRIM21)
Trp (W)	Tryptophan
TSH	Thyroid stimulating hormone
TV	Tetravalent
TWEAK	TNF- α -like weak inducer of apoptosis (receptor)
Tyr (Y)	Tyrosine
uPA	Urokinase-type plasminogen activator
US	United States
USAN	United States Adopted Names

Val (V)	Valine
vcMMAE	Valine-citrulline- <i>p</i> -aminobenzyloxycarbonyl-monomethylauristatin E
VEGF	Vascular endothelial growth factor
VEGF-R2	Vascular endothelial growth factor receptor-2
V _H	Variable heavy chain fragment, ca. 12 kDa
V _{HH}	Single V _H domain antibody fragment
V _K	Variable kappa light chain
V _L	Variable light chain fragment, ca. 12 kDa
V _λ	Variable lambda light chain
V _{NAR}	Variable new antigen receptor
V _{preB}	Pseudo-light chain segment
V _{ss}	Volume of distribution at steady state
vWF	von Willibrand factor
WAM	Web of Antibody Modeling (http://antibody.bath.ac.uk)
WCB	Working cell bank
Wnt	Signaling receptor and pathway in macrophages and adipocytes regulating cell proliferation, differentiation, and developmental processes
XTEN	Half-life extension peptide from Amunix
YTE	Elongated half-life mutant IgG from MedImmune

Note: Amino acid residues are depicted throughout this book by either their three-letter (e.g. Gly, Ser, Leu) or single-letter (e.g. G, S, L) designations, depending on the length of the sequence shown or the context in which it is used.

Foreword

Professor Roy Jefferis

I have enjoyed a long career in scientific research during which time the horizons of immunology have so broadened that a single-author text rarely offers depth or authority. However, in this book Bill Strohl has provided just such a broad and authoritative sweep. It is a tour de force comprised of 19 chapters (151,309 words), including 59 tables, 1,620 references, 43 figures and 12 pages of acronyms, abbreviations, and definitions. Such statistics might suggest a useful reference book that one would reach for as and when needing to check a defined topic; however, Bill has provided a comprehensive text that flows, critically evaluating and linking together data and conclusions reported by individual investigators. The target audience is graduate-level students, researchers in the industry, particularly smaller biotechnology companies, and others within the biopharmaceutical business who would have an interest in biologics in general. The audience will owe a debt of gratitude to Bill for his insight and the labor that he will save them.

Maybe a key to the easy flow of the text is the fact that, actually, it is not a single-author book but a unique project brought to fruition by a husband and wife team. The figures are original and have been created by Lila Strohl who is a Certified Medical Illustrator and a member of the Association of Medical Illustrators. Lila has worked for more than 20 years in the medical illustration field in several medical institutions and is currently President of Biomedscapes. I imagine, therefore, that Bill and Lila had many long conversations leading to the generation of each figure and that those conversations contributed to the ease with which the narrative and figures combine to present specialist material. Bill worked in academia from 1980 to 1997, rising to Full Professor in the Department of Microbiology and the Program of Biochemistry at Ohio State University. During this time he consulted with many companies and in 1997 moved to Merck to head up Natural Products Microbiology before being asked to start a new

department in the field of recombinant monoclonal antibodies. In 2008 he was appointed leader of Antibody Drug Discovery at Centocor (J&J) and in 2009 was named VP and Head, Biologics Research, in the J&J Biotechnology Center of Excellence. Bill has over 100 publications, several patents, has served as guest editor for special editions of journals and has edited two books.

The breadth of Bill's experience and position is reflected in his summary of the recent past, present and future markets, principally in the US. The future is discussed not only as a continuation of current trends, extrapolated forward, but also taking into account political developments that may influence costs, profit and investment. For example, it is suggested that the Biologics Price Competition and Innovation Act of 2009 (BPCI Act) passed by the US Congress is changing the environment and will continue to do so over the next decade. In this context I note that some years ago concern was expressed by biopharmaceutical companies for pronouncements and decisions made by the UK organization the National Institute for Health and Clinical Excellence (NICE). This body acts in the interests of the patient and the National Health Service (NHS) using cost/benefit criteria when making recommendations on funding for drugs within the NHS. This has resulted in a substantial driving down of the cost of drugs to the NHS and NICE is increasingly being used as a "yardstick" for drug pricing, within and outside of the UK; a recent study, reported in the current issue of *Pharmacotherapy*, found that the cost for drugs used to lower cholesterol (statins) is more than 400% higher in the United States than in the UK.

The text opens with a general introduction to biologics and monoclonal antibodies (MAbs) and moves on to discuss discovery and development of mAbs and Fc fusion proteins (FcFP), their present market and future potential. Being "in the business" Bill has access to data that are difficult to find elsewhere, unless purchasing very expensive market surveys offered by specialist financial analysts. The figures given are impressive; thus the value of sales for MAbs and FcFPs for 2010 is put at ~ \$50bn and is projected to grow to \$70–90bn by 2015.

The chapter on antibody structure-function goes well beyond the usual level of discussion. The flexibility afforded by the hinge regions of each subclass is emphasized while noting that there is evidence to suggest that FAb structure can influence Fc effector activities and vice versa. This could be a result of flexibility, if the FAb arm folds back to make non-covalent interactions with the Fc, rather than an allosteric mechanism transmitted through the hinge region. Flexibility is further explored in discussion of the antigen binding site structure and the induced fit versus the preformed

binding site model. These discussions can inform us on the engineering of antibodies generated and selected by current “conventional” protocols and, by extension, on the selection of candidate antibodies revealed by next-generation sequencing techniques.

While the antigen specificity of an antibody is frequently regarded – and presented – as its defining feature, the interactions of antibody-antigen complexes with other elements of the immune system is given rightful prominence. There are a multiplicity of ligands that bind the IgG-Fc, both endogenous, to enhance immune protection, and exogenous. The latter can aid evasion of immune protection (bacterial proteins) and/or subvert the immune system to enhance infectivity (viral pseudo Fc receptors). The endogenous Fc receptors (FcR) and the C1q component of complement are comprehensively reviewed and the relatively new TRIM21 receptor is included; perhaps DC-SIGN should be included since it is reported to specifically bind sialylated IgG-Fc. The affinity and stoichiometry of the interaction between IgG and its ligands is of interest and points to selection in evolution operating at two distinct regions of the IgG-Fc, at the hinge proximal region and at the C_H2/C_H3 interface region. These discussions set the scene for consideration of the effector activities contributing to the efficacy of individual antibody therapeutics and possible targets for engineering.

The four IgG, IgM, IgA and IgE isotypes are introduced in outline and subsequently individually and in some depth. This sets a platform for detailed review of attempts to maximize or minimize biologic functions by IgG-Fc protein engineering and optimize the efficacy of an antibody therapeutic for a given disease indication. However, it is emphasized that the impact on efficacy may vary between individual patients due to polymorphisms within interacting ligands. Improvements in clinical outcome and “cost of treatment” (CoT) will result from stratifying patients according to polymorphisms shown to affect outcomes. Therapeutic antibodies have been particularly successful in the elimination of cancer cells, through the activation of IgG-Fc effector functions; however, these activities may be ablated or significantly reduced for aglycosylated IgG antibodies. This highlights the importance of glycosylation per se and, given the heterogeneity of glycoforms, that individual glycoforms may vary in efficacy. Second/third-generation antibody glycoforms showing enhanced tumor cell killing abilities are entering the clinic. For other disease indications minimal effector function activation may be indicated. Aglycosylated antibodies can be generated by engineering out the glycosylation site, at Asn297, for production in mammalian cells, yeasts, plants, etc. or by production in bacteria. Selected glycoforms may be

generated following cell engineering of production vehicles, e.g. “knocking in” or “knocking out” selected glycosyl transferases.

Exploitation of antigen binding specificity is further explored in a comprehensive review of the properties of a multiplicity of antibody fragments. This field has benefitted from innovative academic studies that have both exploited and advanced genetic engineering possibilities and, following a long gestation, have entered the clinic. One important objective has been to target solid tumors for which penetration may be favored for smaller molecular entities. In other possible applications multi-specific antibody products may be more efficacious, achieved either by combinations of antibodies targeting different epitopes on the same antigen or bispecific antibody constructs that can form a bridge between the antigen and effector molecules or cell bound receptors. Increasingly, therapeutic antibodies are administered in combination with small molecule drugs; however, increased target specificity is anticipated for antibody-drug conjugates that limits bystander toxicity. In other approaches the long half life of the IgG-Fc is exploited to increase the period of exposure to small or large molecule drugs.

Many challenges remain and the future will prove to be as demanding and exciting as the recent past. It is to be hoped that returns on capital investment for the production of first- and second-generation therapeutics might allow for reduced “cost of goods” (CoG); however, this may not contribute significantly to “cost of treatment” (CoT). Delivery of therapeutic antibody in acute disease may continue to require a clinical setting but repeated treatments for chronic diseases may be achieved by self-administration, with attendant reduction in CoT. Self-administration requires antibody to be formulated at high concentrations while maintaining an acceptable shelf life, stability, and absence of aggregate formation, which can predispose to immunogenicity. Assessment of antibody activities prior to “first in man” procedures requires testing, initially in mouse or rat models and subsequently in non-human primates. The strengths and weaknesses of these heterologous systems are discussed together with the development of transgenic animal models. This is an ongoing activity and we can anticipate a quickening of these approaches.

The final chapter reiterates some of the points made previously but with extension to future needs and developments. It particularly refers to the discovery of new targets. Bill’s position within the industry provides for authoritative insight and he informs us that “large pharma have decided to abandon the area of target discovery, with the intention of leaving this part of the business to academic investigators and small biotechnology companies.” The consequence of such decisions is further discussed in the

light of restricted government funding of academic research and the scarcity of venture capital funds. I look forward to the publication of this book, to keep it near at hand both as a source book and to follow future developments within this fascinating field.

Thank you Bill and Lila.

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UK

Preface

The field of monoclonal antibodies has been with us since the pioneering work of Caesar Milstein and Georges Köhler in the mid-1970s for generating hybridomas capable of continually producing monospecific antibodies against an antigen. The first commercialization of antibodies, which ushered in the age of therapeutic monoclonal antibodies, the topic of this book, began in June 1986, with the approval by the US Food and Drug Administration of Orthoclone OKT3[®], a fully murine antibody targeting CD3, for use as an immunosuppressive agent in organ transplantation. This first and thus transformative antibody was developed and commercialized by Ortho Biotech, a subsidiary of Johnson & Johnson. While Orthoclone OKT3[®] led the path towards commercialization of monoclonal antibodies, it was not without its problems. Being completely murine, it induced a significant anti-antibody response against it, and targeting CD3 with a highly active Fc, it fostered a potentially dangerous cytokine storm. In improving OKT3 through humanization to reduce its immunogenicity and muting of its Fc effector functionality to reduce the cytokine storm, researchers at Ortho Biotech and other labs led the way towards the kind of antibody engineering we have today. The second significant breakthrough in antibody engineering was the generation of the first recombinant therapeutic antibody, ReoPro[®], a chimeric FAb fragment binding glycoprotein gpIIb/IIIa on platelets that blocks platelet aggregation and reduces cardiac ischemic complications. This advance came from the laboratories at Centocor, a small biotechnology company originally founded on the west side of Philadelphia, which was acquired in 1999 by Johnson & Johnson. Centocor discovered and developed ReoPro[®] and entered into an agreement with Eli Lilly for distribution and marketing. This allowed Centocor scientists to then focus on the development of a novel anti-TNF- α antibody, Remicade[®], which was not only the first anti-TNF- α antibody, but the first antibody to be prescribed for an immune-mediated disease (Crohn's disease) of any kind. Remicade[®] is now approved for 16 different indications and has a 2011 worldwide market value of more than \$5 billion, leading the industry as perhaps the best example of a "pipeline in a drug." Finally, the first truly bispecific antibody and the first

fully human antibody developed from the use of the Medarex transgenic mouse, Stelara[®], which targets the cytokines IL-12 and IL-23, was developed by Centocor R&D and approved for marketing in 2009 for treatment of psoriasis. There are two links between all of these examples. The first is that each represents a truly innovative new medicine that has helped to pioneer a new era in therapeutic monoclonal antibodies. The second link is that all four examples are antibodies developed and marketed by companies associated with Johnson & Johnson, a long-time leader, along with Genentech, Novartis, and others, in the field of therapeutic monoclonal antibodies. For the past few years, Bill Strohl has been blessed to be an employee of Centocor R&D, which is now part of Janssen R&D LLC, one of the family of pharmaceutical companies of Johnson & Johnson, and part of the tradition of innovative antibody development that the company has established. This book is derived from experiences in the field, discussions with many colleagues at Johnson & Johnson and elsewhere, and borne out of a love for science by both authors. The opinions expressed in this book, however, are specifically those of the authors and not those of Janssen R&D or Johnson & Johnson.

It is not possible to write a book such as this without significant help, encouragement, and discussion. To that end, we offer our sincere thanks to the following colleagues for their efforts in editing, explaining difficult concepts, and batting around ideas. Thank you to all: Marian Nakada, for reviews on Chapters 1, 2, 19, and comments on the entire manuscript; Ken Dow, for legal review of the entire manuscript; Gary Gilliland, for review of Chapter 3; Ellen Chi, for review of Chapter 4; Mike Ryczyn, for review of Chapter 5; Jeffrey Luo, for review of Chapter 6; Bernie Scallon, for review of Chapter 7; Gordon Powers, for review of Chapter 8; Ray Sweet, for review of Chapter 9; Omid Vafa, for review of Chapter 10; T. Shantha Raju, for review of Chapter 11; Jill Carton, for review of Chapter 12; Mike Naso, for review of Chapter 13; Chichi Huang, for review of Chapter 14; Jennifer Nemeth-Seay, for review of Chapter 15; Yiqing Feng, for review of Chapter 16; Randy Brezski, for review of Chapter 17; and Steve Lang, for review of Chapter 18. We also thank Roy Jefferis for many significant comments on several chapters of the book, Renee DesJarlais, for making the chemical structures in Figure 15.2; Gary Gilliland, for making the structures in Figures 1.1, 3.1, and 12.1; and Joshua Strohl, for significant contributions to the detailed references. Finally, our heartfelt thanks go to our incredible sons, Justin Strohl and Joshua Strohl, for their patience with both their mother and father working so hard on this book for over a year.

Bill and Lila Strohl, March 2012
Bridgewater, NJ

About the authors

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Dr William R. Strohl received his BS degree in Biology from Central Michigan University in 1975 and his PhD in Microbiology from Louisiana State University in 1980; he also worked as a guest researcher at the GBF in Braunschweig, Germany in 1978–9. From 1980 to 1997 he rose from Assistant to Full Professor in the Department of Microbiology and the Program of Biochemistry at Ohio State University, Columbus, OH where he pursued the molecular biology and biochemistry of polyketide biosynthesis pathways, particularly doxorubicin, in actinomycetes, and the physiology of *E. coli* in computer-controlled high cell density fermentations. As part of the latter endeavor, Dr Strohl designed, implemented, and equipped a P2 fermentation facility at Ohio State University (in 1994). As a faculty member at Ohio State, Dr Strohl consulted with over a dozen different companies in the area of biotechnology. Dr Strohl moved to Merck in 1997 to head up Natural Products Microbiology, which he did for four years before being asked to start a new department in the field of recombinant monoclonal antibodies. From 2001 to 2008, Dr Strohl was a leader in Merck's efforts to develop therapeutic monoclonal antibodies, as well as in-licensing of therapeutic targets and technologies associated with monoclonal antibodies. As part of this effort, Dr Strohl was a key scientific leader in the acquisition and integration of Abmaxis and GlycoFi into the Merck Biologics organization. In April 2008, Dr Strohl was named leader of Antibody Drug Discovery at Centocor (J&J). Concomitant with the evolution of Biologics at Johnson & Johnson to serve additional therapeutic areas beyond Immunology and Oncology, in 2009 Dr Strohl was named as VP and Head, Biologics Research, in the J&J Biotechnology Center of Excellence. Dr Strohl has over 100 publications and several patents, has served as guest editor for special editions of journals, and has edited two books.

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Ms Lila M. Strohl obtained her BS degree in Allied Health Professions from the College of Medicine, Ohio State University. She received a Certificate in Medical Illustration from the School of Allied Medical Professions, OSU. She has worked for more than 20 years in the medical illustration field, first as Head of Medical Illustration at St Anthony Medical Center, Columbus, Ohio, then as a Medical Illustrator in the Department of Biomedical Communications, Ohio State University Medical Center, and then as owner of Medcom Graphics, an independent contracting company. Ms Strohl is a Certified Medical Illustrator (CMI) and a longtime member of the Association of Medical Illustrators (AMI). Ms Strohl has contributed artwork to several books and journal articles, and has done freelance work for over a dozen different companies. Currently, Lila Strohl is President of Biomedscapes, a company focusing on medically and biologically oriented art.

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Introduction to biologics and monoclonal antibodies

Abstract: The first recombinant protein therapeutic was Humulin®, recombinant human insulin, which was licensed in 1982. Today, there are four different types of biologics, including replacement protein therapeutics with enzymatic or regulatory activity, protein therapeutics with binding specific targets, protein-based prophylactic vaccines, and protein diagnostics. Monoclonal antibodies and Fc fusion proteins are the major forms of therapeutic proteins possessing binding activities. Monoclonal antibodies have two major functional domains, the FAb arms, which are responsible for binding the antigens, and the Fc domain, which interacts with the immune system. The potency of a monoclonal antibody depends on the strength of binding, the epitopes to which the antibodies bind, and the Fc activity. Monoclonal antibodies are named according to the sources from which they were obtained as well as the human component of the antibody.

Keywords: biologics, protein therapeutics, vaccines, insulin, small molecules, monoclonal antibodies, Fc fusion proteins, naming of therapeutic antibodies

1.1 Introduction

Emil von Behring, considered to be the father of immunology and winner of the first Nobel Prize in Medicine and Physiology in 1901, and Shibasaburo Kitasato, eventual founder of Japan's famed Kitasato Institute, were the first to discover that a substance in blood was capable of neutralizing diphtheria toxin (Behring and Kitasato, 1890). This substance was subsequently named "*Antikörper*", or "antibodies", and was determined to have specificity for one toxin over another. In the following years, the terms "*Antisomatogen*" and "*Immunkörperbildner*" also were coined to

describe the material that induced the formation of the *Antikörper*. The term “antigen” eventually arose from the combination of those names (Schroeder and Cavacini, 2010), and thus the foundation of immunology consisting of an antigen and its cognate antibody was laid for the next century of antibody research.

The field of genetically engineered therapeutic monoclonal antibodies (MAbs), the topic of this book, has been built on the shoulders of many inventions over decades of research, but two key discoveries in the mid-1970s stand out as seminal events that laid the groundwork for this field to exist as it does today. When Stanley Cohen, Herbert Boyer, and their colleagues made the first recombinant DNA molecules in 1973 by isolating a bacterial plasmid, cutting it site-specifically with the restriction endonuclease *EcoRI*, inserting foreign DNA into it, and then reforming the modified plasmid with DNA ligase (Cohen et al., 1973), they started a revolution that has changed the medical world in ways likely envisioned by only the wildest imaginations at that point in time. Virtually every new drug developed by the pharmaceutical industry today has been derived by a process that includes cloning and expression of the drug target, and in many cases, X-ray crystallographic analysis of the drug and recombinant target together. Likewise, the entire field of monoclonal antibodies derives from a key event, i.e. the discovery by Köhler and Milstein (1975) that murine B cells could be fused with murine myeloma cells to produce single fusion cell lines (hybridomas), which produce antibodies with a single, unique specificity (i.e. monoclonal antibodies). The field of genetically engineered MAbs, which marries standard molecular biology with antibody technologies, can be traced directly back to those two seminal papers.

Now, more than 35 years later, the field of genetically engineered MAbs, using technologies descended from both Cohen et al. (1973) and Köhler and Milstein (1975), is a rapidly maturing field in which hundreds of researchers in academia and in dozens of biotechnology and biopharmaceutical companies are engaged. Genetically engineered therapeutic antibodies have now been marketed for over a quarter century, starting with the United States Food and Drug Administration (FDA) approval of ReoPro® in December of 1984. Since that approval, an additional 39 recombinant therapeutic antibodies and related Fc fusion proteins (FcFPs) have been approved for marketing, with hundreds more now in clinical trials to follow them. This book describes the current status of therapeutic antibody engineering as well as the path to get to where we are today.

It also attempts to address the major issues, challenges, and opportunities we will face as we push forward into the second decade of the twenty-first century.

1.2 Definitions of biologics

Biologics have been defined by the FDA as “a virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, or analogous product, or arsphenanaine, or derivative of arsphenamine (or any other trivalent organic arsenic compound), applicable to the prevention, treatment or cure of a disease or condition of human beings” (Public Health Services Act 42 USC § 262(i)). While this definition by statute is now antiquated, it does provide a broad guidance as to what may be included in the term “biologic.” Clearly, chemical compounds like arsphenines that were historically considered as biologics no longer fit the description. Leader et al. (2008) recently summarized the field of biologics, placing biopharmaceutical proteins into four major categories:

1. protein therapeutics with enzymatic or regulatory activity (e.g. replacement therapies such as insulin, growth hormone, Factor IX, β -glucocerebrosidase);
2. protein therapeutics with special targeting activity (e.g. MABs or other binding proteins, including FcFPs, that bind specific therapeutic targets. Two examples are the anti-tumor necrosis factor (TNF)- α MAB, Remicade®, and the anti-TNF- α/β FcFP, Enbrel®);
3. protein-based prophylactic vaccines (e.g. human papilloma virus (HPV) vaccine made using virus-like particles containing HPV major capsid protein L1); and
4. protein diagnostics (e.g. biomarkers such as glucagon, and imaging agents such as technetium- or indium-conjugated antibodies) (Leader et al., 2008).

Figure 1.1 shows the fundamental structure of an immunoglobulin G (IgG), and a typical FcFP, etanercept, as compared with the non-antibody biologic, human growth hormone (HGH), the peptide hormone insulin, and the small molecule penicillin G. This book will focus largely on therapeutic MABs and FcFPs, with some reference to the therapeutic protein history and markets as they relate to the development and acceptance of therapeutic

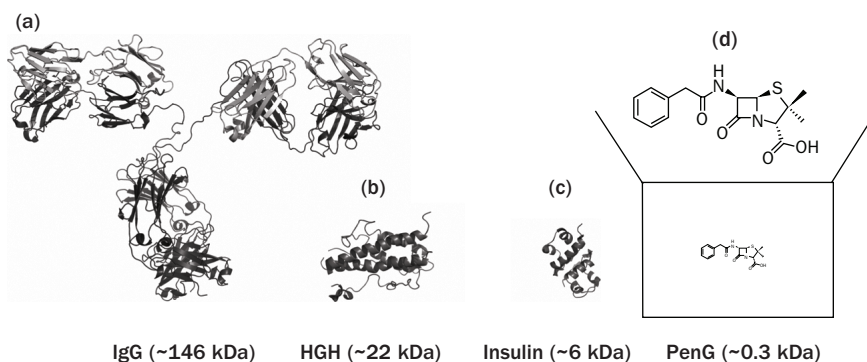


Figure 1.1 IgG structure and size vs therapeutic protein vs small molecule

Comparison of the overall size and structures of: **A** an IgG (146 kDa) (mouse IgG_{2a}, PDBid, 1igt) (Harris et al., 1997); **B** human growth hormone (22 kDa) (PDBid, 1hgu) (Chantalat et al., 1995); **C** insulin (5.8 kDa) (PDBid, 1trz) (Ciszak and Smith, 1994); and **D** penicillin G (0.3 kDa).

MAbs by the industry. Table 1.1 defines some of the most significant similarities and differences between non-MAb biologics, MAbs, small molecules, and traditional vaccines. The key differences between biologics and small molecules lie in the types of targets addressed, molecular size and complexity, inherent toxicities, off-target activities, drug metabolism, pharmacokinetics, route of administration, method of manufacturing, and product homogeneity (Table 1.1). Biologics can be subdivided into three major categories: monoclonal antibody (MAb) products, non-MAb products, and vaccines. Since vaccines are used to stimulate an immune response and, at least historically, have been typically developed for prophylactic use rather than therapeutic use (Table 1.1), they will not be addressed further here.

The key differences between MAbs and non-MAb biologics are that the latter largely are typically replacements for natural proteins, enzymes, hormones, or peptides that no longer function (or are not present) as desired, resulting in a disease state. Administration of the non-MAb biologic is intended to exert a pharmacological effect similar to the missing protein function to bring the patient back to a more normal state. Non-MAb biologics include insulins and insulin analogues, growth hormones, epoetins, colony-stimulating factors, enzymes, peptides, and similar products.

Table 1.1 General properties of non-MAb biologics, MABs, small molecules, and traditional vaccines

Property	Type of prophylactic or therapeutic intervention			
	Biologic (non-antibody)	Monoclonal antibody	Small molecule	Traditional vaccine
Composition	Typically hormones or enzymes; sometimes can be natural or mimetic peptides	Antibody-protein	Synthetic organic compound or organic, non-protein natural product	Protein, peptide (usually conjugated to protein), carbohydrate; usually with adjuvant
Molecular weight	Generally >700 Da	Generally ~150,000 Da	Generally <700 Da	Generally >700 Da
Mode of activity	Usually a replacement for a missing activity (natural peptide, protein, or enzyme)	Binds to extracellular targets to antagonize, agonize, or deplete them	Binds to intracellular or extracellular targets to antagonize or agonize them	Provokes a specific immune response to provide prophylaxis against disease-causing entity
Production	By cells (typically bacterial, yeast, or mammalian)	By cells (typically mammalian)	Chemical synthesis	Typically by cells
Chemical homogeneity of product	Can be heterogeneous, especially if glycosylated	Typically heterogeneous, especially glycan portion	Very homogeneous	Very heterogeneous
Product defined by:	Biological activity; biochemical analysis; by the process used to make it	Biological activity; biochemical analysis; by the process used to make it	Chemically	Immunological activity; by the process used to make it
Target specificity	High	Very high	Tends to be lower	Specific to epitopes used in vaccine
Delivery	Injected, IV or SC	Injected, IV or SC	Usually oral, sometimes injected IV	Injected SC or IM, oral, intranasal

Table 1.1 General properties of non-MAb biologics, MAbs, small molecules, and traditional vaccines (*cont'd*)

Property	Type of prophylactic or therapeutic intervention			
	Biologic (non-antibody)	Monoclonal antibody	Small molecule	Traditional vaccine
Half-life	Hours to days	Usually > week but < month	Usually several hours to 1 day	Results in long-term immunological memory
Current example	Epoetin	Remicade® (infliximab)	Lipitor® (atorvastatin)	Gardasil® (HPV vaccine)
Examples of typical indications	Anemia, neutropenia, diabetes	Rheumatoid arthritis, psoriasis, cancer	Cholesterol lowering, allergy, hypertension, gastrointestinal disorders, depression, osteoporosis, cancer	Prophylaxis against viral and bacterial diseases

Abbreviations: Da: Daltons; HPV: human papilloma virus; IM: intramuscular; IV: intravenous; SC: subcutaneous.

1.3 Recombinant protein therapeutics

The recombinant monoclonal antibody field has grown out of a wide variety of different technologies and therapeutic approaches, as summarized by Strohl (2009a). One key advance that led to the ability to produce and market recombinant monoclonal antibodies was the engineering, expression, and production of recombinant human proteins as therapeutics. Recombinant human protein therapeutics largely fall into three subgroups: (1) hormones and blood factors; (2) enzymes; and (3) cytokines. In these cases, the recombinant human product replaces a missing or dysfunctional protein in the patient. The most well-known case is that of Type I diabetes, in which the body mounts an immunological attack against its own insulin-producing beta-cells, resulting in the inability to transport and metabolize blood glucose (Luo et al., 2010b). In 1923, Eli Lilly developed a commercial process for the production of a heterologous insulin product, called Iletin®, for the treatment of Type I diabetes. The insulin was obtained and purified

from the pancreas of livestock animals such as pigs and cows (Shook, 2007). Since the mature forms of porcine and bovine insulin are one and three amino acids different from human insulin, respectively, some patients developed antibodies against the heterologous products. Taking advantage of the recombinant DNA revolution that took place in the early 1970s, Genentech came up with a process for producing recombinant human insulin in *Escherichia coli* (Goeddel et al., 1979). Eli Lilly licensed the recombinant human insulin product from Genentech and developed it clinically, obtaining marketing approval on 30 October 1982, for Humulin®, for the first recombinant human protein to reach the market (Strohl, 2009a). Since Humulin® was a fully human protein, it eliminated the problems with immune responses associated with the use of heterologous insulins from farm animals. In the 28 years since Humulin® was first approved for marketing in the US, more than 165 therapeutic proteins have been marketed for a broad array of indications. Although a few of these such as α -anti-trypsin, α -1-proteinase, and intravenous immunoglobulin (IVIG) (see Chapter 13) are still purified from human serum, the vast majority of marketed therapeutic biologics are derived from recombinant sources, requiring reliable and consistent cell-based expression and manufacturing platforms (Strohl, 2009a).

1.4 MAbs and Fc fusion proteins (FcFPs)

MAbs and FcFPs function in a fundamentally different manner to biologics used as replacements. MAbs are immunoglobulin G (IgG) molecules that mimic the function of a natural IgG in the body, which is to bind to pathogens or foreign entities to effectively neutralize and clear those entities. FcFPs are proteins consisting of the Fc moiety of an IgG fused with a natural or modified protein (e.g. receptor exodomain, cytokine, or natural binding protein) that can bind another protein (see Chapter 14). Similar to natural IgGs, MAbs and FcFPs function to bind extracellular targets, cells, or pathogens to neutralize them, block their function, remove them from circulation, or otherwise modulate or mimic their activity. In this way, MAbs and FcFPs function in a manner similar to small molecules in that they are pharmacologically active agents intended to modify a disease state by pharmacologically altering components of the disease state itself. Examples of this include the neutralization of inflammatory cytokines such as TNF- α or interleukin (IL)-1 β to result in immunosuppression, or to deplete pathogenic cell types.

1.5 General anatomy of a therapeutic IgG MAb

Figure 1.2 shows the general anatomy of a therapeutic IgG MAb, the isoform that has been used for all therapeutic antibodies approved for marketing to date. A therapeutic IgG (currently limited to IgG₁, IgG₂, and IgG₄ isotypes, and variations thereof), which has a molecular mass of about 146 kDa, is comprised of a standard H₂L₂ structure in which the heavy chain (HC) has approximately 446–454 amino acid residues (depending on the length of the variable chain sequences). The HC of a therapeutic IgG is comprised of four domains (V_H, C_H1, C_H2, C_H3), each with about 100–120 amino acid residues, with a flexible hinge region of 11–14 residues connecting C_H1 with C_H2. The length of the hinge depends on which isotype (IgG₁, IgG₂, or IgG₄) is used. The light chain (LC), which may belong to either a kappa (κ) chain or lambda (λ) chain family, contains about 208–214 amino acid residues, depending on the length of the variable chain sequences and which family is used. The LC is comprised of two sub-domains, the V_L (either Vκ or Vλ), which is typically in the range of 107–122 amino acid residues, and the C_L, which is 102 amino acid residues for Cκ or 103 residues for Cλ. Thus the two heavy chains are about 49–50 kDa each, and the two light chains are about 23–24 kDa each, giving a typical therapeutic MAb a nominal molecular weight of about 146 kDa.

The sequences responsible for binding antigen with high affinity and specificity are three loops each in the V_H and V_L chains (six loops in total), called the complementarity-determining regions (CDRs). These sequences are described in greater detail in Chapter 5.

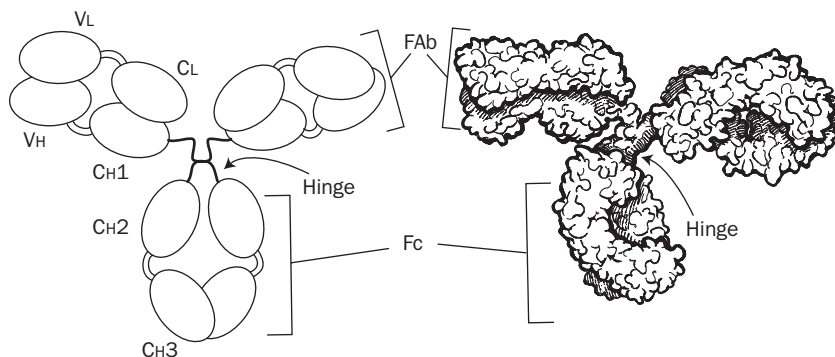


Figure 1.2 Anatomy of a monoclonal antibody (MAb)

Simple model of an IgG antibody on the left, showing the major domains (V_L, C_L, V_H, C_H1, hinge, C_H2, and C_H3) compared with an artistic rendering of an antibody based on structural analyses (right).

At a high level, there are four significant features that are built into the discovery and development of a therapeutic IgG-based MAb (Figure 1.3):

1. The variable V_H and V_L domains bind to a particular antigen at a specific epitope, i.e. the binding site on the target. These antigen binding sequences of the antibody are defined as the paratope.
2. The V_H and V_L chains bind to that epitope with a specificity and affinity defined by their sequences and folding; because there are two Fab arms, avidity toward insoluble targets is a powerful weapon to increase the potency of the MAb.
3. The Fc of the antibody, comprised of paired hinge- C_H2 - C_H3 domains, bind to various Fc receptors (FcRs) and blood proteins (e.g. complement components) to activate the immune system to neutralize the target, as well as increase the half-life of the antibodies in serum to weeks.
4. Since the constant regions are by definition “not variable,” the sequences of the V_H and V_L from MAb to MAb of the same isotype will dictate how well the antibody behaves as a potential commercial molecule, i.e. its solubility, proper folding, biochemical stability, lack of aggregation, *in vivo* half-life, and immunogenicity.

The features noted above give antibodies some definable characteristics, such as the absence of off-target activity and thus generally low to no non-mechanism-based toxicity, the ability to clear soluble proteins from the serum, the ability to kill cells that they target, the inability to cross the blood-brain barrier to any significant extent, the inability to enter cellular

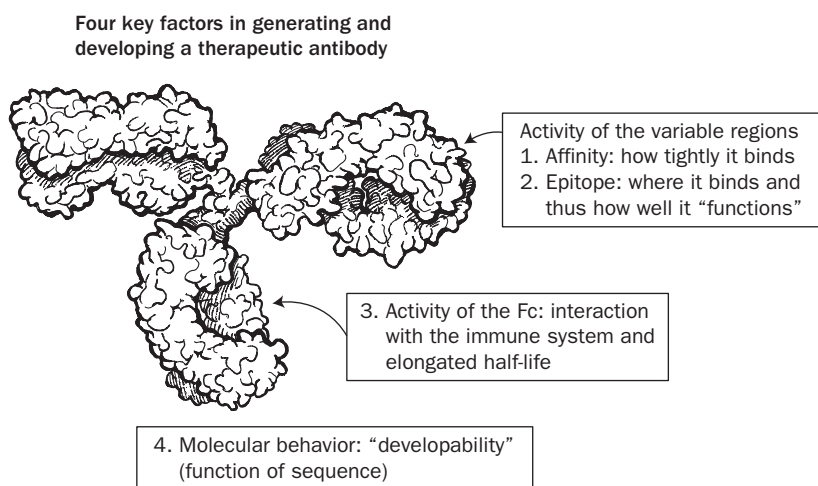


Figure 1.3 Four key factors to consider when developing a therapeutic antibody

cytoplasm, only moderate tissue penetration due to their size and radius, and clearance mediated primarily by the liver and cells of the reticuloendothelial system. Below is a list of general high-level characteristics of IgG MABs, most of which will be discussed in greater detail throughout this book:

- protein – IgG has $\alpha_2\beta_2$ (e.g. H_2L_2) configuration with a size of ~146 kDa;
- ability to inhibit protein–protein interactions on cell-bound, extracellular, and serum targets (e.g. receptors, peptides, hormones, cytokines, exo-enzymes, etc.);
- avidity effects allow high affinity to targets (matured down to <10 pM);
- long half-life in serum (nominally ~14–21 days for IgG₁, IgG₂, IgG₄);
- ability to mediate:
 - antibody-dependent cellular cytotoxicity (ADCC);
 - complement-dependent cytotoxicity (CDC);
 - antibody-dependent cellular phagocytosis (ADCP);
 - antibody-mediated immune complex formation with clearance;
 - or a completely silent Fc with none of the above activities;
- lack of blood–brain barrier penetration (e.g. peripheral targets only – reports suggest that only about 0.1–0.6% of serum antibodies cross the blood–brain barrier);
- ability to penetrate some tissues ($V_{ss} = \sim$ blood volume);
- relatively shallow peak-to-trough ratio;
- clearance mediated largely by the liver and cells of the reticuloendothelial system (too large for kidney);
- little or no off-target activity or drug metabolism-related issues;
- little or no non-mechanism-mediated toxicity;
- minimal drug–drug interactions (although some are known).

1.6 Naming convention for antibodies from different sources

MABs are also defined by the source of the amino acid sequences they contain. The first marketed antibody, muromonab-CD3 (Orthoclone OKT3®), was a fully mouse-derived antibody (Figure 1.4). Later, the mouse variable chains, V_H and V_L , were grafted onto human constant chains to produce “chimeric” antibodies (Figure 1.4) (Morrison et al., 1984), which reduced the foreign amino acid sequence content from 100% to about 33%. Rituximab (Rituxan®), infliximab (Remicade®), and cetuximab (Erbix®) are examples of marketed chimeric antibodies. Note that each of their generic names end in the suffix “-ximab.”

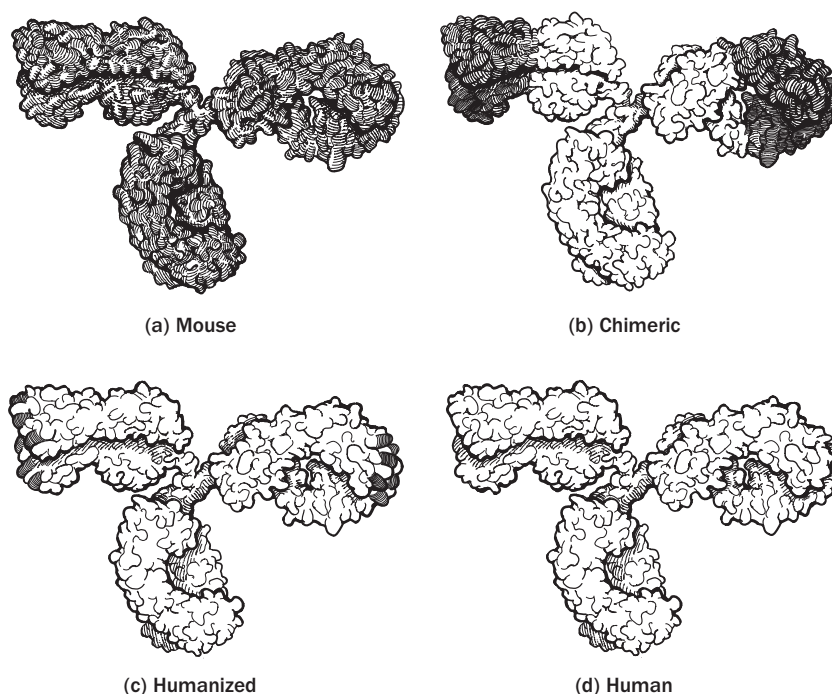


Figure 1.4 Mouse vs chimeric vs humanized vs human MABs

Drawings of IgG antibodies and the source of their amino acid content. (a) Murine antibody with 100% mouse residues. (b) Chimeric antibody with human constant chains and mouse V_H and V_L chains, resulting in antibodies that typically have >70% human sequences. (c) Humanized antibody with human constant chains and framework regions with mouse CDRs engrafted into the variable chains, resulting in antibodies that typically have >95% human sequences. (d) Fully human antibodies in which nearly 100% of the amino acid residues are derived from human antibody genes.

In efforts to reduce non-human sequences further, “humanized” antibodies were made by grafting just the complementarity-determining regions (CDRs), which are six loops in the V_H and V_L chains (three loops in each chain – see Chapter 3) totaling approximately 60 amino acid residues, from the mouse antibodies into human frameworks, followed by back-mutating key framework residues to help support proper orientation of the CDR loops in the humanized antibody (Jones et al., 1986; Queen et al., 1989; Co and Queen, 1991). These antibodies, which generally contain >95% human residues (Figure 1.4), have been demonstrated to have, for the most part, reduced immunogenicity in the clinic. Omalizumab (Xolair®), trastuzumab (Herceptin®) and bevacizumab (Avastin®) are examples of humanized antibodies on the market. Note that each of their generic names end in the suffix “-zumab.”

Finally, antibodies derived completely from human antibody genes (Figure 1.4), either through phage display libraries (Osbourne et al., 2005), from transgenic mice containing human antibody genes in place of their own (Green, 1999; Lonberg, 2005, 2009), or by direct cloning of human antibody genes from human B cells (Wild et al., 2007; Mouquet et al., 2010; Wrammert et al., 2011), have been made. These generally provoke very low anti-antibody immune response rates in patients. Adalimumab (Humira®), panitumumab (Vectabix®), ustekinumab (Stelara®), and golimumab (Simponi®) are examples of fully human antibodies that have been successfully marketed. Note that each of their names ends in the suffix “-umab.”

The generic names for the antibodies mentioned above are not random. A naming system was developed in the early 1990s by the American Medical Association (AMA, 2007) for giving monoclonal antibodies generic names (Table 1.2). This system is used by both the World Health Organization’s

Table 1.2 Current naming conventions for MABs

Prefix	Target		Source of antibody	Suffix
Variable	Non-tumor target	Immune (-li[m]-)	Human (-u-)	-mab
		Interleukin (-k[in]-)		
		Cardiovascular (-ci[r]-)	Mouse (-o-)	
		Neurologic (-neu[r]-)		
		Musculoskeletal (-mu[l]-)	Rat (-a-)	
		Bone (-o[s]-)		
		Viral (-vi[r]-)	Hamster (-e-)	
		Bacterial (-ba[c]-)		
		Fungal (-fu[ng]-)	Primate (-i-)	
		Infectious lesions (-le[s]-)		
	Tumor target	Toxin (-tox[a]-)	Chimeric (-xi-)	
		Colon (-co[l]-)		
		Mammary (-ma[r]-)	Humanized (-zu-)	
		Melanoma (-me[l]-)		
		Ovary (-go[v]-)	Rat/murine hybrid (-axo-)	
		Prostate (-pr[o]-)		
		Testis (-go[t]-)	Combination of humanized and chimeric chains (-xizu-)	
		Miscellaneous tumor (-tu[m]-)		

International Nonproprietary Names and United States Adopted Names (USAN). The naming protocols were streamlined in 2008 after the International Nonproprietary Name Working Group met, and a document has been made available summarizing their recommendations which can be found at: <http://www.who.int/medicines/services/inn/Generalpoliciesfor monoclonalantibodies2009.pdf>.

The names are essentially split into four parts: (1) a unique prefix; (2) affix letters identifying the type of target; (3) affix letters indicating the original source of the variable chains, e.g. human, mouse, etc. as well as how those V-regions are engineered, as noted above, e.g. chimeric, humanized, or fully human (Figure 1.4); and (iv) the suffix “mab” (Table 1.2). Hence, for golimumab, “go” serves as the unique prefix, “lim” refers to an immune system target, “u” refers to the fact that it contains fully human sequences, and then “mab.”

These names also are used for antibody fragments such as FAbs (e.g. ranibizumab), scFvs, and domain antibodies. An additional word may be included in the name to describe a small molecule or toxin conjugate, e.g. gemtuzumab ozogamicin, whereby “ozogamicin” refers to the calicheamicin (small molecule toxin) conjugate. If the MAb is conjugated to a radioisotope, the name of the isotope precedes the name of the antibody, e.g. “technetium Tc 99m biciromab.”

The name for Ortho Biotech’s anti-CD3 monoclonal antibody, muromonab-CD3, was given prior to the development of antibody naming convention, so its name does not follow the convention. Muromonab-CD3 stands for “*murine monoclonal antibody targeting CD3*.”

Value proposition for therapeutic monoclonal antibodies and Fc fusion proteins

Abstract: Biologics in general, and more specifically monoclonal antibodies and Fc fusion proteins, pose a very attractive value proposition. The value of the MAb and FcFP market has grown from just about \$2 billion to nearly \$57 billion in just ten years, and continues to grow at one of the fastest rates in the pharmaceutical business. Currently, MAbs and FcFPs against just five targets, CD20, TNF- α , VEGF, EGFR, HER2, comprise 88% of the current value of the total MAb and FcFP market. By 2016, eight of the top ten pharmaceuticals by sales will be biologics, and of these, seven will be MAbs or FcFPs. To date there have been 30 IgG MAbs, 3 FAb fragments, and 7 FcFPs (40 in total) approved for marketing in the major markets. Due to the size of the current Phase III pipeline, this number is expected to grow to over 60 within the next 4–5 years, which will further strengthen the market for these biologics.

Keywords: probability of success (POS), probability of technical and regulatory success (PTRS), market value, targets, return on investment, therapeutic areas

2.1 Overview of discovery and development of therapeutic MAbs and FcFPs

Therapeutic MAbs and FcFPs are typically discovered by finding binders to the human target that, if possible, also bind to the same target from mice, rats and non-human primates (see Chapter 17). The binding protein, whether MAb or FcFP, is then genetically engineered to optimize its affinity, its humanness (i.e. content of human-like primary sequences), and its biophysical properties. The details of this process are described throughout

the remainder of this book and will not be further detailed here. What will be described in this section is the overall, high-level process, time, and funding required for discovery and development of a typical MAb or FcFP.

Table 2.1 shows the normal stages and typical timelines to discover and develop innovator therapeutic MAbs. Industry-wide, it takes about 12–15 years from the initiation of discovery to FDA approval. The discovery phase takes about 36–45 months and results in the generation of a single molecule (often with a few back-up molecules with similar or comparable characteristics) that possesses the affinity, degree of humanness, solubility, biochemical stability, and lack of aggregation desired in a development candidate. Also, the biology and efficacy of the lead molecule is generally understood quite well as detailed by multiple *in vitro* assay systems and *in vivo* models. By this point, the target to which the MAb or FcFP binds is generally validated pre-clinically, at least as well as the models allow. It is generally accepted, however, that pre-clinical animal models are marginal at best in predicting human response in most disease states, so it is often the full body of biological and pharmacological evidence, including data derived from animal knock-out models and human genetics (i.e. in which polymorphisms predictably either lead to a disease state or to a disease-resistant state (e.g. Schuelke et al., 2004; Loughlin et al., 2004; Yue et al., 2006)), that gives enough confidence to take the biologic forward into development. Approximately half of the MAb or Fc fusion programs for which discovery programs are formally initiated result in a bona fide candidate for pre-clinical development. Usually, the reasons for drop-out in this phase relate to the validity (or lack thereof) of the target.

Pre-clinical development, including submission and approval of the investigational new drug (IND) or similar application, typically requires another 21–29 months. Thus it generally takes from four to six years to get to the first-in-human (FIH) stage (Table 2.1). In the pre-clinical development phase, two major events need to take place. First, the cell line generating the potential commercial MAb or FcFP needs to be generated and optimized for manufacturing in bioreactors. Examples of this process are described in more detail in Chapter 18. A critical part of this process is the development of detailed, validated methods for analyzing the purity, concentration, potency, immunogenicity, and structural integrity of the MAb or FcFP being developed.

The second major function in pre-clinical development is to generate GLP (good laboratory practice) non-clinical toxicology data to support the investigational new drug (IND) filing and proposed Phase I clinical trials. The GLP toxicology studies will help to define the potential safety issues

Table 2.1 Typical timelines required to discover and develop innovator therapeutic MAb and success rates*

Phase	Activity in phase	Typical timeline	POS*
Initiation of target research	Design, expression and purification of key reagents for assays; development of <i>in vitro</i> screens; early validation experiments	18 months	~51% (Paul et al., 2011)
Hit and lead generation	Generation and identification of initial biologic lead from protein, library, or immunization strategy	6–9 months	
Lead optimization	Optimization of protein or antibody lead via protein engineering; expression; characterization of lead quality	12–18 months	
Pre-clinical development	Development candidate selection; cell line generation; production and release of GLP-toxicology and Phase I API*; formulation; analytical development; non-GLP (good laboratory practice) and GLP-toxicology; and generation of investigational new drug (IND) documentation	20–28 months	86% (industry average)
IND	FDA evaluation of IND package	1 month	
Phase I clinical trials	Typically, 20–80 healthy volunteers and/or patients; safety, immunogenicity, and pharmacokinetic data obtained	20–22 months	84%
Phase II clinical trials	Typically, 100–300 patients**; Phase IIa is generally proof-of-concept trial for evaluation of efficacy. This is the stage where most MAb and FcFPs fail for lack of efficacy, generally caused by an insufficient understanding of the target biology in humans; Phase IIb is generally dose ranging for Phase III trials, as well as evaluation of different indications	24–30 months	53%

Table 2.1 Typical timelines required to discover and develop innovator therapeutic MABs and success rates* (*cont'd*)

Phase	Activity in phase	Typical timeline	POS*
Phase III clinical trials	Typically 1,000–3,000 patients**; confirmation of efficacy for specific doses and uses; safety in broader population and over longer duration; pharmacokinetics; immunogenicity; registration trials	31–42 months	74%
Registration	FDA review and evaluation of BLA	12–15 months	96%
Total	Initiation of target research to FDA approval	144–183 months (12.0–15.3 years; average ~13.7 years)	13–14% from portfolio entry; 27% from entry into pre-clinical development

* Combination of data from Reichert (2008), Paul et al. (2010), and DiMasi et al. (2010), and http://www.imgt.org/IMGTmedical/Overview_of_Drug_Development.pdf, as well as other industry sources. Note that the POS data are based on expectations of current MABs in the pipeline, most of which are humanized or human IgGs, shown by Reichert (2008) to possess a higher PTRS (~30%) than “all MABs” (which would include failures of IgMs, bispecifics, and other forms).

** The number of patients given here for these trials is a broad estimate. The actual number will depend on the number of patients required to obtain the data desired such as proof-of-concept (POS) in Phase IIa, efficacy with doses used, e.g. Phase II, and safety database and broad-based efficacy in Phase III.

related to administration of the MAB or FcFP in humans (Lynch et al., 2009; Stebbings et al., 2009) as well as to help estimate what safe doses in humans should be (Muller and Brennan, 2009; Muller et al., 2009). Thus far, the vast majority of MABs and FcFPs that enter formal pre-clinical development pass and move into Phase I clinical studies. While different probabilities of success (POS) values are given by various authors for this phase, they are all in the 75–95% range; for the purposes of this book, the authors assume that 86% of all MABs and FcFPs entering formal pre-clinical development will succeed in moving on to Phase I clinical trials. This value is far greater than what is found for small synthetic or natural product molecules, which typically only have a 62–69% chance of passing through the formal pre-clinical development phase (Paul et al., 2010). The reasons for this are well known: most small molecules that fail formal pre-clinical development do so because they either are toxic, they are actively

metabolized, their metabolites are toxic, they possess undesirable off-target activities, they are not orally bioavailable, or they are insoluble or possess other formulation problems (Table 2.2).

Clinical studies on biologics require approximately 6.3–7.3 years, and registration usually takes slightly more than a year on average to complete (Table 2.1). This is consistent with the data from Reichert (2008, 2010), who showed that the average time for marketed MAbs to progress through clinical trials was 85 months (~7.1 years). Antibodies for cancer indications take on average about six months longer to develop clinically than for those treating non-oncology indications (Reichert, 2010). In Phase I trials, a total of 20–80 healthy volunteers are typically dosed with the MAb or FcFP in a single ascending dose (SAD) trial typically followed by a multi-dose ascending dose trial (MAD). For severe diseases including oncological, patients are sometimes included in Phase I studies, which is very different from what occurs in small molecule trials, in which patients are seldom

Table 2.2 Key differences in issues encountered with early development of small molecules versus MAbs and FcFPs

Parameter	Small molecules	MAbs and FcFPs
Drug metabolism	+	–
P450 interaction	+	–
Off-target activities	+	–
Chemical class toxicities	+	–
Oral bioavailability	+	–*
High attrition rate in pre-clinical GLP toxicology studies due to toxicity	+	–
Pharmacokinetics	Variable and difficult to predict	Long, usually predictable
Drug-drug interactions	+	+/–
On-target toxicity	+	+
Aggregation/lack of solubility	+/–	+
Immunogenicity	–	+
Cell culture expression (quantity and product quality)	–	+
Purification issues	–	+

* Since MAbs and FcFPs are not orally bioavailable, they are not developed for indications in which they must be orally bioavailable to be marketable.

treated in Phase I studies. The outcomes that typically are used to advance a MAb or FcFP beyond Phase I are safety (or lack thereof), immunogenicity, and pharmacokinetics (PK). Since most MAbs and FcFPs have proven to be safe in Phase I trials, ~84% have been continued in development past this phase. The one very notable exception is Tegenaro TGN1412, an anti-CD28 agonist MAb that generated a severe hyper-immunostimulatory response in healthy volunteers in 2006 (Stebbing et al., 2007; Stebbings et al., 2009). That incident has stimulated a significant review and modification of the approaches, strategies to calculate initial dose levels in humans, trial design and safety criteria used for Phase I clinical trials of MAbs and FcFPs (Stebbing et al., 2007; Stebbings et al., 2009; Muller and Brennan, 2009; Muller et al., 2009). Occasionally, data can be obtained from Phase I clinical trials that are either indicative of clinical efficacy or that yield biomarker evidence that is consistent with potential clinical efficacy. As discussed later in this chapter, these findings are significant when they occur, because they assist in making difficult funding decisions that need to be made before clinical proof-of-concept (POC) studies are generally completed.

Phase IIa clinical trials are typically considered as POC studies, in which efficacy for a specific indication in a relatively small population must be achieved to continue development. Phase IIb trials are usually a combination of dose-ranging for Phase III studies as well as primary trials in which the efficacy of the MAb or FcFP is tested for multiple different indications. In total, typically 100–300 patients are dosed with the MAb or FcFP in Phase II (http://www.imgt.org/IMGTmedical/Overview_of_Drug_Development.pdf), usually enough to obtain statistically significant data for efficacy and to observe immunogenicity trends. Since the reason for failure for most antibodies and FcFPs that fail clinically is lack of efficacy, only 53% of MAbs and FcFPs entering Phase II studies have progressed into Phase III (Table 2.1). Not including manufacturing and release of the test biologics and long-term non-clinical safety studies that generally take place around Phase II, Phase II clinical studies usually require about two years to complete.

Phase III clinical trials for MAbs and FcFPs, which are generally considered as registration trials, should be large enough (sometimes >1,000 patients) to obtain efficacy, toxicity and immunogenicity data across the broader population to support the filing of a BLA (Biologics License Application) or similar registration documents. Phase III clinical trials usually require around three years for completion, but may require longer timelines for difficult development programs and certain indications with longer end-points.

The total costs for the discovery, development, and launch of novel ethical pharmaceutical drugs have been estimated recently to be about \$1.2 billion (La Merie, 2011) to \$1.7 billion (Kola, 2008), depending on the size and complexity of the clinical development program as well as the size of the launch package. The cost to discover and develop MAb and FcFPs should be in this same range, although the actual timing for the development costs for biologics versus synthetic small molecules is remarkably different. For development of biologics, there are two major “cost-cliffs” that occur due to the need to manufacture, purify, and release biologic drug lots derived from cell-based cultures. The first of these cost-cliffs occurs prior to non-clinical GLP toxicology, the point at which active pharmaceutical ingredient (API) needs to be produced for the GLP-toxicology studies and Phase I studies. The second of these cost-cliffs occurs when the API for the larger Phase IIb and Phase III clinical studies needs to be generated, analyzed, and released. This usually occurs sometime during the Phase I clinical trials, so the decision for spending the large sums of money to generate a test drug for late-stage clinical trials often needs to be made prior to having solid POC data in hand. This puts extraordinary pressure on developing biomarker or other “indicative” assays that can be used in Phase I clinical studies to obtain data at least suggestive of success or failure.

The overall PTRS (Probability of Technical and Regulatory Success) for discovery and development of therapeutic MAb and FcFPs today is in the range of 13–14%. This number, however, includes the discovery phase in which approximately half of the programs are dropped due to target-related issues rather than to the biologic itself (Table 2.1). Thus a better measure of biologics success is the PTRS from entry into pre-clinical development to approval by the regulatory authorities. Overall, therapeutic MAb entering pre-clinical development have a low rate of attrition in pre-clinical development (86% POS) and Phase I clinical trials (84% POS), indicating that safety, tolerability, and pharmacokinetics are generally not major issues for therapeutic MAb. The lowest POS is found in Phase II, where nearly half of all therapeutic MAb candidates drop out, mostly due to lack of efficacy as determined in Phase IIa POC trials. As mentioned previously, the decision to spend significant funds to generate API for the later Phase IIb and Phase III clinical trials is usually made prior to having Phase IIa POC data in hand. Considering that only about half of those therapeutic MAb candidates will succeed in moving on to those larger clinical trials, this represents a significant decision challenge in the development of therapeutic MAb.

Several groups have come up with different numbers for this success rate (Reichert, 2008), partly due to the fact that there are still a relatively limited number of therapeutic MAb and FcFPs on the market. Table 2.1 shows

numbers based on the most recent industry-wide data, indicating that, on average, therapeutic MABs and FcFPs have about a 28% success rate from entry into pre-clinical development to marketing approval. This is very consistent with data shown by Reichert (2008) for humanized and human antibodies, for which she calculated a 30% PTRS from initiation of Phase I through approval. Using Reichert's clinical humanized MAB success data (Reichert, 2008) and our pre-clinical development POS of 86% (Table 2.1), that combination would result in 26% PTRS from initiation of pre-clinical development to marketing approval. As mentioned above, the overall PTRS for development of a therapeutic MAB from initiation of pre-clinical development is about 28% (Table 2.1). This compares favorably with the PTRS for development of small molecules, which is typically characterized to be in the 7–10% range (Reichert, 2008; DiMasi et al., 2010). The most recent data by DiMasi et al. (2010) suggest that the PTRS for small molecules from entry into Phase I to market is 13.2% (Phase I, 63%; Phase II, 38%; Phase III, 61%; registration, 91%). When this is added to the industry-wide rate of about 62–69% to progress from entry into pre-clinical studies to FIH (Paul et al., 2010), due largely to toxicology, oral bioavailability, and drug metabolism issues (Table 2.2), it results in a PTRS for small molecules from entry into pre-clinical development to marketing approval of only about 8.2–9.1%. Based on these numbers, therapeutic MABs entering pre-clinical development have about a threefold greater chance of being marketed than small molecules at the same phase.

The inverse of these numbers indicates the large number of molecules that fail. Thus for small molecules, every single successfully marketed drug represents a total of about 12 that were initially taken into pre-clinical development. On the other hand, each successful MAB or FcFP represents about four taken into formal development. Since nearly half of all small molecules entering formal pre-clinical development do not reach Phase I, and even fewer (39%) will reach Phase II, small molecule discovery programs typically have between one and three back-up programs, depending on the priority of the targeted biology. In general, there may be as many as two to four small molecules taken into development for any given target, thus increasing the overall development cost for the success of the program. On the other hand, since MABs and FcFPs entering pre-clinical development have a very high chance to go into Phase II (about 72% overall), few of these programs have formal back-up strategies. Usually, there are back-up MABs or FcFPs that are held in reserve in case there are unforeseen issues with the primary molecule in development, but these are not typically taken forward into development unless the lead suffers a significant setback.

Overall, the costs for the failed drug candidates that fall out during the development pathway must be recouped through the few successes, putting additional pressure on the industry. These cost pressures, in turn, will likely result in a greater drop-out rate for new therapeutic MAb and FcFPs due to management funding decisions as result of the intense competition on targets. Thus similar to what is found in the small molecule world, highly successful therapeutic MAb and FcFPs will soon be limited to those that are either first-in-class or best-in-class. It is expected, therefore, that the PTRS for MAb and FcFPs will decline substantially over the next decade, not due to toxicities or failures in the clinic, but rather due to business and prioritization decisions to stop clinical development because of the competitive position. The first prominent example of this has already been seen. In December 2009, Roche announced that they were halting clinical development of their fully human anti-IGF1-R MAb, R-1507, based on a business decision due to the intense competition on that target.

David et al. (2009) recently modeled the expected return on investment (ROI) for the ethical pharmaceutical business and demonstrated that the current internal rate of return (IRR) for small molecules was ~7.5%, as compared with an IRR of ~12% for small molecules in the 1997–2001 period. This suggests that it is more difficult and more costly to discover and develop small molecules than in past years, something that is well known throughout the industry (Paul et al., 2010). What makes this number more intriguing, however, is that David et al. (2009) pointed out that this figure of 7.5% is below the current cost of capital, which they estimated to be 9.5%. The authors suggested that increases in efficiencies and shortening of timelines in small molecule development could help to minimize the gap. Biologics, on the other hand, currently provide an IRR of 13%, a hefty 3.5% above cost of capital, mostly due to higher peak sales and less erosion upon patent expiry (David et al., 2009). While the authors argued for increases in efficiencies, shortening of timelines, and earlier no-go decisions on non-efficacious small molecules to help increase the value of small molecule approaches, the clear message of their data was that there is a solid business case for increasing the discovery and development of biologics within large pharmaceutical organizations.

2.2 Market for MAb and FcFPs

The healthcare industry has undergone some substantial changes over the last decade, including a significant increase in the overall ethical prescription drug market, from \$132 billion in 2000 to over \$602 billion in 2010.

Additionally, the third-party payor system has come under significant pressure, and the Biologics Price Competition and Innovation Act of 2009 (BPCI Act) passed by the United States Congress will change the environment even more as we head into the second decade of the second millennium AD. Therapeutic proteins, either as human protein replacement therapies or as binding proteins targeting protein–protein interactions, occupy niches not applicable to small molecule intervention. These large molecules already have had an immense impact on modern medicine, bringing novel, life-saving therapies to critically ill patients, and their impact is expected to increase substantially over the next decade.

One very significant change in the pharmaceutical industry from the year 2001 is the incredible rise in biologics as novel therapeutics. Ten years ago, nine of the top ten selling prescription pharmaceutical drugs were small molecules, led by Lipitor® (atorvastatin), Prilosec® (omeprazole), Zocor® (simvastatin), and Norvasc® (amlodipine besylate) (Table 2.3). The only biologics to make the top ten in 2001 was Procrit/Eprex, epoetins used to treat anemia. Note that the indications treated by those top ten pharmaceuticals in 2000 were depression and mood swings (2 drugs), cholesterol (2 drugs), and ulcers or gastro-esophageal reflux disorder (GERD) (2 drugs). In 2001, all ethical therapeutic biologics combined made up only a \$26.8 billion market. The top selling biologics were the epoetins Procrit/Eprex and Epogen, which combined to sell for about \$6.7 billion (Figure 2.1). The entire market for MAbs and FcFPs was less than \$3 billion, selling less than the interferons, insulin-related peptides, and anti-fibrinolytics (Figure 2.1). In 2001, the MAbs and FcFPs that had hit the market in the 1997–1998 timeframe, including Remicade®, Rituxan®, Enbrel®, and Synagis®, were still just getting off the ground in terms of sales.

By 2010, however, all biologics combined for sales of more than \$107 bn, more than 300% growth in eight years, driven by the rapid increase in the market for MAbs and FcFPs (Figure 2.1) (La Merie, 2011). Additionally, five therapeutic proteins (Remicade®, Enbrel®, Avastin®, Humira®, Herceptin®) reached the top 10 in worldwide drug sales by 2009 (Table 2.3). These five MAbs and FcFPs were worth a combined \$35 billion in 2011, about 62% of the total value of all MAb and FcFP products (Table 2.4). The market for therapeutic MAbs has been estimated by various business groups to grow at an annual rate from 9% (Goodman, 2009) to 15% (Hiller, 2009) over the next several years. Market Research News (<http://news.wooeb.com/NewsStory.aspx?id=706876>), for example, projects a 12.4% CAGR for therapeutic MAbs from 2010 to 2015, while La Merie has forecasted a growth rate of 9.5% until 2015. Figure 2.1 shows that from 2005 to 2010, the MAb and Fc fusion market grew by approximately

Table 2.3 Top ten prescription drugs in year listed

Rank	2001*			2009**			Projected 2016***		
	Drug name	Sales (\$bn)	Indication	Drug name	Sales (\$bn)	Indication	Drug name	Projected sales (\$bn)	Indication
1	Lipitor	\$6.4	Cholesterol lowering	Lipitor	\$12.5	Cholesterol lowering	Humira ^{a, b}	\$10.0	RA
2	Prilosec	\$5.6	Ulcers, GERD	Plavix/Iscover	\$9.8	Thrombosis	Avastin ^{a, b}	\$8.0	Cancer
3	Zocor	\$5.2	Cholesterol lowering	Advair/Seretide	\$7.8	Asthma/COPD	Enbrel ^{a, b}	\$7.0	RA
4	Norvasc	\$3.6	Hypertension	Remicade ^{a, b}	\$7.2	RA	Rituxan ^{a, b}	\$6.0	Cancer
5	Procrit/Eprex ^a	\$3.4	Anemia	Enbrel ^{a, b}	\$6.6	RA	Crestor	\$6.0	Cholesterol lowering
6	Claritin	\$3.2	Allergy	Diovan	\$6.0	Hypertension	Herceptin ^{a, b}	\$6.0	Cancer
7	Celebrex	\$3.1	Pain, arthritis	Avastin ^{a, b}	\$5.7	Cancer	Remicade ^{a, b}	\$5.0	RA
8	Zyprexa	\$3.1	Psychosis, schizophrenia	Humira ^{a, b}	\$5.6	RA	Lantus ^a	\$5.0	Diabetes
9	Prevacid	\$3.0	Ulcers, GERD	Herceptin ^{a, b}	\$4.9	Cancer	Seretide/Advair	\$5.0	Asthma
10	Paxil	\$2.7	Depression	Crestor	\$4.8	Cholesterol lowering	Prolia ^{a, b}	\$5.0	Osteoporosis

Abbreviations: COPD: chronic obstructive pulmonary disease; GERD: gastro-esophageal reflux disorder; RA: rheumatoid arthritis.

* Med Ad News (2003).

** EvaluatePharma (2010) and eKnowledgebase.

***EvaluatePharma (2010).

^a Biologic.

^b MAb or FcFP.

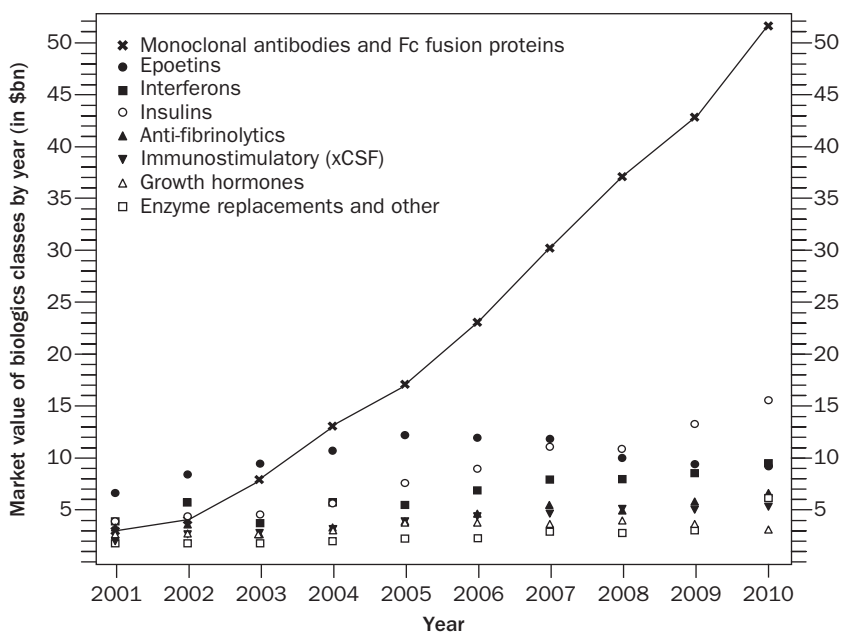


Figure 2.1 Growth of MAb and Fc fusion protein market from 2001 to 2010

The MAb and Fc fusion protein market has grown from ~\$3 billion to >\$50 billion from 2001 to 2010, while the other major biologics such as epoetins, interferons, insulins, anti-fibrinolytics, immunostimulatory factors (i.e. G-CSF, GM-CSF) growth hormones, and enzyme replacements have either remained relatively flat or have had minor growth.

Table 2.4 Market values in 2011 of top five targets for MABs and FcFPs

Target	Marketed MABs and FcFPs for target	Total 2011 value of market for target
TNF- α	Remicade [®] , Enbrel [®] , Humira [®] , Simponi [®] , Cimzia [®]	\$24 bn
VEGF	Avastin [®] , Lucentis [®]	\$9.6 bn
CD20	Rituxan [®] , Arzerra [®] , Zevalin [®]	\$6.7 bn
HER2	Herceptin [®]	\$5.8 bn
EGFR	Erbix [®] , Vectibix [®]	\$2.3 bn
Total 2011 value of top five targets		\$48.4 bn
Total MAB and FcFP market in 2011*		\$56.7 bn

* Calculated from data presented in Table 2.6. These data exclude the 2011 market values of Orthoclone OKT3[®], Zenapax[®], Simulect[®], Campath H1[®], and Bexxar[®], for which no data were available (however, all of these MABs were of low market value in 2011). The data also exclude Raptiva[®], which was withdrawn in 2009. Data calculated from: La Merie (2012).

\$7 billion/year, indicating both solid growth but an overall declining CAGR. These data combined suggest that the MAB and Fc fusion market should be worth in the range of \$75–90 billion by 2015.

As of August 2011, there are at least 396 publicly announced, commercially funded MABs and FcFPs either on the market or in clinical trials (Table 2.5). Figure 2.2 shows the overall combined regulatory approval history of MAB and FcFPs in major markets from 1994 to August 2011. By 2011, 39 MABs and FcFPs (including those that later were removed from the market) have been approved for marketing either in the United States or European Union (Table 2.5). Another 36 are post-Phase IIb or in Phase III clinical trials (Table 2.5). With a 71% POS for antibodies transitioning from successful Phase II trials to market approval, 26 of those late-stage clinical

Table 2.5 Current marketed and clinical candidate therapeutic MABs and FcFPs as a function of the primary therapeutic area for which they are indicated*

Therapeutic area	Marketed (includes those removed from market)	Phase III + post-Phase IIb	Phase I + Phase II	Total
Oncology	12	22	158	192 (48%)
Immunology	22	6	98	126 (32%)
CVMEB**	2	3	22	27 (7%)
Infectious diseases	1	2	20	23 (6%)
Neurosciences (not including pain)	0	2	13	15 (4%)
Ophthalmological	2	0	5	7 (2%)
Osteoarthritis and pain	0	1	5	6 (2%)
Total	39	36	321	396

* Data derived from 39 marketed, 36 Phase III, and 321 Phase I and II MAB and FcFPs in a database, based on information from company websites, La Merie Biologics (La Merie Business Intelligence, Inc.), http://www.drugs.com/drug_information.html, Prous Integrity, and published information, continually updated by one of the authors (WRS). The data focus on company-sponsored clinical trials. Date of data lock for this table was August 20, 2011.

** Abbreviation: CVMEB: cardiovascular, metabolic, endocrine, bone, and blood.

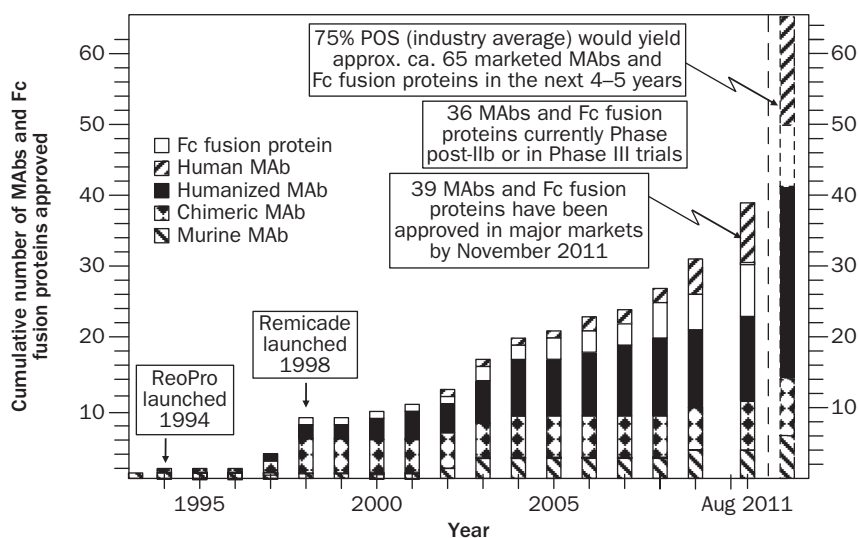


Figure 2.2 Growth in number of approved MABs and Fc fusion products

Number of marketed MABs and Fc fusion proteins approved in major markets from 1993 to November 2011 as a function of type (murine MAB, chimeric MAB, humanized MAB, human MAB, Fc fusion protein (FcFP)). The last column projects approvals over the next 4–5 years based on current MABs and FcFPs in advanced clinical trials.

MABs and FcFPs should be successful in reaching the market, probably by the 2013–14 timeframe. Thus, by 2014, over 60 MABs and FcFPs will have been approved at one point or another for marketing in the major markets (Figure 2.2). Reichert (2011) has recently written a nice overview of many of the MABs and FcFPs in advanced stage clinical trials.

Another 141 MABs and FcFPs are currently in commercially funded Phase II clinical trials (W.R. Strohl, unpublished data), with a combined PTRS of ca. 38% from Phase II to successful marketing approval. This would suggest that about 53 of these Phase II MABs and FcFPs will achieve marketing approval, probably by the ~2018 timeframe. Thus, by 2018, it is expected that more than 115 innovative MABs and FcFPs will be on the market, not including biosimilars.

The trend should continue, as evidenced by the fact that for MABs alone, an average of 45–50 new first-in-human (FIH) studies were initiated per year in the 2007–9 timeframe (Reichert, 2010). Given the timeline and the PTRS from entry into Phase I to market (Table 2.1), it is expected that the 40 open INDs in 2009 should result in at least 10 BLAs for innovative MABs filed in 2018–19. In 2009, a total of 32 new molecules were approved by regulatory agencies, 13 of which were biologics (all classes;

only five MAbs were approved by the FDA in 2009). EvaluatePharma has recently noted that Humira® sales are expected to reach \$8.7 billion in 2012, which should make it the largest selling drug in 2012 and beyond, since Lipitor® lost market exclusivity in November 2011. By 2014, it has been estimated that 50% of the top 100 ethical pharmaceutical drugs (as measured by sales) will be biologics, and by 2016, it has been projected that eight of the top 10 selling prescription drugs will be therapeutic proteins (EvaluatePharma, 2010), seven of which should be MAbs, led by Humira® (Table 2.3). The other biologic, Lantus®, is a long-acting insulin. Thus the enormous promise that was predicted for therapeutic proteins and monoclonal antibodies in the early 2000s will soon be realized (Carter, 2006; Riley, 2006; Leader et al., 2008; Dimitrov and Marks, 2008).

2.3 Currently and recently approved MAbs and FcFPs

As of June 2012, 40 MAbs and FcFPs have been approved in the major markets starting in 1986 with the approval in the US of Muromonab-CD3 (Orthoclone OKT3®) (Table 2.6). Panorex®, which was approved only in Germany in 1994 and then withdrawn from the market after a few years, is not included in this table. Similarly, MAbs and FcFPs currently approved only in the current minor markets such as Korea, India, and China have not been included in this tabulation.

Of these 40, four have been discontinued or withdrawn from the market, and one of those, Tysabri®, has been reinstated. The breakdown of these 40 protein therapeutics is: 30 IgG MAbs, three FAb fragments, and seven FcFPs. Of the 33 MAbs and FAb fragments, 23 were originally derived from murine hybridomas, eight were derived from hybridomas from transgenic mice producing human antibodies, and two were derived from human antibody phage display libraries.

The predominance of murine hybridomas as the source of the V-regions for marketed MAbs largely reflects the evolution of MAb technologies at the time when those therapeutic MAb programs were initiated. As might be expected, there is a trend towards V-region sources other than murine hybridomas. Of the last 11 MAbs approved, six were derived from transgenic mice producing human antibodies, four came from traditional murine hybridomas, and one was from a human antibody phage display library. As more sophisticated antibody discovery and engineering technologies are developed and integrated into the therapeutic MAb discovery process, there will be a broader range of potential V-region sources, as described in Chapter 5.

Table 2.6 Current and recently marketed monoclonal antibodies and fusion proteins*

	US trade name (generic name)	Company	Approval date (US) ^a	Target	Major indication	Protein format	Antibody source	2011 sales
1.	Orthoclone OKT3 [®] (Muromonab-CD3)	Ortho Biotech (now Janssen Biotech, Inc.)	06/19/1986; discontinued in 2011	CD3 on T cells	OTR	Murine IgG _{2a}	Hybridoma	NA (now withdrawn)
2.	ReoPro [®] (Abciximab)	Centocor (now Janssen Biotech, Inc.) / Eli Lilly	12/22/1994	gPIIb /IIIa on platelets	CVD	Chimeric FAb, derived proteolytically from IgG ₁	Hybridoma	\$173 m
3.	Rituxan [®] (Rituximab)	Biogen Idec / Genentech	11/26/1997	CD20 on B cells	NHL, RA	IgG ₁ κ, chimeric	Hybridoma	\$6,596 m
4.	Zenapax [®] (Dacizumab)	Abbott (PDL / Roche)	12/10/1997	IL-2Rα (CD25; tac)	OTR	IgG ₁ , humanized	Hybridoma	NI
5.	Synagis [®] (Palivizumab)	MedImmune	06/19/1998	RSV F-protein	RSV (infant)	IgG ₁ κ, chimeric	Hybridoma	\$975 m
6.	Remicade [®] (Infliximab)	Centocor (now Janssen Biotech, Inc.)	08/24/1998	TNF-α	RA, Crohns	IgG ₁ κ, chimeric	Hybridoma	\$7,157 m
7.	Herceptin [®] (Trastuzumab)	Genentech	09/25/1998	HER2/Neu	Breast cancer	IgG ₁ κ, Humanized	Hybridoma	\$5,770 m
8.	Enbrel [®] (Etanercept)	Immunex (now Amgen)	11/02/1998	TNF-α; TNF-β	RA	FcFP; IgG ₁ -Fc fused to TNFR p75 exodomain	Recombinant Fc fusion	\$7,867 m
9.	Simulect [®] (Basiliximab)	Novartis	12/05/1998	IL-2Rα (CD25; tac)	OTR	IgG ₁ κ, chimeric	Hybridoma	NI

10.	Mylotarg® (Gentuzumab ozogamicin)	Wyeth (now Pfizer)	05/17/2000; voluntarily withdrawn from market on 6/21/2010	CD33	Leukemia	Humanized IgG ₄ κ- ozogamicin conjugate	Hybridoma	NA (now withdrawn)
11.	Campath-1H® (Alemtuzumab)	Genzyme	05/07/2001	CD52 on B- and T cells	Leukemia	IgG ₁ κ, humanized	Hybridoma	NI
12.	Zevalin® (Ibritumomab tiuxetan)	Biogen / Idec	02/19/2002	CD20 on B cells	NHL	Murine IgG ₁ κ radio- conjugate, ⁹⁰ Y or ¹¹¹ In	Hybridoma	\$28 m
13.	Humira® (Adalimumab)	CAT, Abbott	12/31/2002	TNF-α	RA, Crohn's disease	IgG ₁ κ, human	Phage display	\$7,932 m
14.	Amevive® (Alefacept)	Biogen	01/30/2003	CD2 – inhibits CD2-LFA-3 interaction on activated T cells	Psoriasis	FcFP; IgG ₁ Fc fused to CD2- binding domain of LFA-3	Recombinant Fc fusion	\$5 m
15.	Xolair® (Omalizumab)	Genentech	06/20/2003	IgE	Asthma	IgG ₁ κ, humanized	Hybridoma	\$1,140 m
16.	Bexxar® (Tositumomab-1131)	Corixa	06/27/2003	CD20 on B cells	NHL	Murine IgG _{2a} /λ- ¹³¹ I	Hybridoma	NI
17.	Raptiva® (Efalizumab)	Genentech	10/27/2003; withdrawn 4/8/2009	CD11a, α-sub- unit of LFA-1	Psoriasis	IgG ₁ κ, humanized	Hybridoma	NA

Table 2.6 Current and recently marketed monoclonal antibodies and fusion proteins* (*cont'd*)

	US trade name (generic name)	Company	Approval date (US) ^a	Target	Major indication	Protein format	Antibody source	2011 sales
18.	Erbix [®] (Cetuximab)	ImClone / BMS	02/12/2004	EGFR (HER1, c-ErbB-1)	Colorectal cancer	IgG ₁ κ, chimeric	Hybridoma	\$1,821 m
19.	Avastin [®] (Bevacizumab)	Genentech	02/26/2004	VEGF	Colorectal cancer	IgG ₁ , humanized	Hybridoma	\$5,813 m
20.	Tysabri [®] (Natalizumab)	Biogen / Elan	11/23/2004; withdrawn Feb, 2005; reinstated Oct, 2006	α4 subunit of α4α1 or α4α7 (VLA4)	Multiple sclerosis	IgG ₄ κ, humanized	Hybridoma	\$1,500 m
21.	Orencia [®] (Abatacept)	BMS	12/23/2005	CD80/CD86	RA	CTLA4-FcFP- modified IgG ₁ Fc	Recombinant Fc fusion	\$917 m
22.	Lucentis [®] (Ranibizumab)	Genentech / Novartis	06/30/2006	VEGF-A	Wet AMD	Humanized IgG ₁ κ FAb fragment	Hybridoma	\$3,723 m
23.	Vectibix [®] (Panitumumab)	Amgen	09/27/2006	EGFR (HER1, c-ErbB-1)	Colorectal cancer	Human IgG ₂ κ	Transgenic humanized mouse	\$525 m
24.	Soliris [®] (Eculizumab)	Alexion Pharma	03/16/2007	Complement C5	PNH	Humanized IgG _{2/4} modified Fc	Hybridoma	\$783 m
25.	Arcalyst [®] (Rilonacept)	Regeneron	02/27/2008	IL-1β, IL-1α, IL-1RA	CAPS, Muckle Wells Syndrome	FcFP; IgG ₁ Fc- fusion protein with IL-1R & IL-1AP fused in-line	Recombinant Fc fusion	\$20 m

26.	Nplate® (Romiplostim, AMG-531)	Amgen	08/22/2008	TPO-R	Thrombo cytopenia	FcFP; aglucosyl IgG ₁ Fc-peptide fusion (peptibody)	Recombinant Fc fusion	\$297 m
27.	Simponi® (Golimumab; CNTO-148)	Centocor (now Janssen Biotech, Inc.)	04/23/2009	TNF-α	RA	Human IgG ₁	Medarex transgenic mouse	\$674 m
28.	Stelara™ (Ustekinumab; CNTO-1275)	Centocor (now Janssen Biotech, Inc.)	9/25/2009	P40 subunit of IL-12 & IL-23	Psoriasis	Human IgG ₁	Medarex transgenic mouse	\$738 m
29.	Actemra® (RoActemra in EU) (Tocilizumab)	Roche / Chugai	1/09/2010	IL-6R	Castle- mans disease; RA	IgG ₁ , humanized	Hybridoma	\$679 m
30.	Removab® (Catumaxomab)	Fresenius / Trion	EU only 4/23/09	EpCAM and CD3	Malignant ascites; cancer	Rat IgG _{2b} -mouse IgG _{2a} hybrid	Hybridoma	\$5 m
31.	Cimzia® (Certolizumab pegol)	UCB/Schwartz	05/14/2009	TNF-α	RA	PEGylated humanized FAb	Hybridoma	\$412 m
32.	Illaris® (Canakinumab)	Novartis	06/19/2009	IL-1β	CAPS	Human IgG ₁ k	Medarex transgenic mouse	\$48 m
33.	Arzerra™ (Ofatumumab)	GenMab / Glaxo- SmithKline	10/26/2009	CD20 on B cells	B-cell chronic leukemia; NHL, RA	Humanized IgG ₁ k	Medarex transgenic mouse	\$71 m

Table 2.6 Current and recently marketed monoclonal antibodies and fusion proteins* (*cont'd*)

	US trade name (generic name)	Company	Approval date (US) ^a	Target	Major indication	Protein format	Antibody source	2011 sales
34.	Prolia™ and XGEVA™ (Denosumab)	Amgen / GlaxoSmithKline (EU)	6/01/2010 (11/19/10 for XGEVA™)	RANK-ligand	Osteoporosis; cancer support ^b	Human IgG ₂	Abgenix transgenic mouse	\$554 m for both indications
35.	Benlysta™ (Belimumab)	GlaxoSmithKline / HGS	3/09/2011	BLyS	Lupus (SLE)	Human IgG ₁ λ	Phage human antibody library	\$59 m
36.	Yervoy™ (Ipilimumab)	Medarex / Bristol-Myers Squibb	3/25/2011	CTLA4	Malignant melanoma	Human IgG ₁ k	Transgenic humanized mouse	\$360 m
37.	Nuliojix™ (Belatacept)	Bristol-Myers Squibb	6/16/2011	CD80/86	Renal transplantation	CTLA-4 modified IgG ₁ Fc fusion LEA (higher affinity than abatacept)	Recombinant Fc fusion	\$3 m
38.	ADCETRIS™ (brentuximab vedotin)	Seattle Genetics / Takeda	8/19/2011	CD30	Hodgkin lymphoma; SALCL	Chimeric IgG ₁ -conjugated to auristatin derivative	Hybridoma	\$43 m

39.	Eylea™ VEGF trap-eye	Bayer-Schering Pharma / Regeneron	11/18/2011	VEGF	Wet AMD; central retinal vein occlusion	IgG ₁ -Fc IgG ₁ -Fc fusion	Exodomain 1 of human VEGFR1 & 2 of VEGFR2 – Fc fusion	\$25 m
40.	Perjeta™ (Pertuzumab)	Genentech	6/8/2012	HER2 dimerization	HER2-positive breast cancer	Humanized IgG ₁	Hybridoma	NA

Abbreviations: AMD: age-related macular degeneration; CAPS: cryopyrin-associated periodic syndrome; CV: cardiovascular disease; IV: intravenous; LYO: lyophilized; NA: not applicable; ND: not disclosed; NHL: non-Hodgkin's lymphoma; NI: no information; OTR: organ transplantation rejection; PNH: paroxysmal nocturnal hemoglobinuria; RA: rheumatoid arthritis; RSV: respiratory syncytial virus; SALCL: systemic anaplastic large cell lymphoma; SC: subcutaneous; SLE: systemic lupus erythematosus; TNF: tumor necrosis factor; TPO-R: thrombopoietin receptor; VEGF: vascular endothelial growth factor.

* Updated, July 6, 2012. Data obtained from Prescribing Information released by the manufacturers, company websites, Prous Science Integrity; La Merie (2012). Prescribing information: Actemra® prescribing information, 2011; Adcetris® prescribing information, 2012; Amevive® prescribing information, 2011; Arcalyst® prescribing information, 2009; Arzerra® prescribing information, 2011; Avastin® prescribing information, 2012; Benlysta® prescribing information, 2012; Bexxar® prescribing information, 2012; Campath® prescribing information, 2009; Cimzia® prescribing information, 2011; Enbrel® prescribing information, 2011; Erbitux® prescribing information, 2012; Herceptin® prescribing information, 2010; Humira® prescribing information, 2012; Ilaris® prescribing information, 2012; Lucentis® prescribing information, 2010; Mylotarg® prescribing information, 2010; Nplate® prescribing information, 2012; Orendia® prescribing information, 2011; Orthoclone OKT3® prescribing information 2011; Prolia® prescribing information, 2012; Raptiva® prescribing information, 2005; Remicade® prescribing information, 2011; Removab® product information (no date included); ReoPro® prescribing information, 2005; Rituxan® prescribing information, 2012; Simponi® prescribing information, 2011; Simulect® prescribing information, 2005; Soliris® prescribing information, 2011; Stelara® prescribing information, 2012; Synagis® prescribing information, 2012; Tysabri® prescribing information, 2012; Vectibix® prescribing information, 2012; Xolair® prescribing information, 2010; Yervoy® prescribing information, 2011; Zenapax® prescribing information, 2005; Zevalin® prescribing information, 2011.

** Conjugate is ozogamicin, a catecheamycin (natural product cytotoxin).

*** Suspended February 28, 2005; reinstated under specified conditions.

a Unless otherwise stated.

b XGEVA is approved for prevention of skeletal-related events (SREs) (bone fractures and bone pain) in patients with cancer that has metastasized to bone and caused bone damage.

Antibody structure–function relationships

Abstract: Antibodies are comprised of two FAb regions and an Fc domain which are connected together by the relatively unstructured hinge. There are four isotypes of the human IgG, including IgG₁, IgG₂, IgG₃, and IgG₄, all of which have unique hinge sequences that confer both unique physical and physiological features. The domains themselves are built from the highly conserved immunoglobulin fold which itself is built from packed anti-parallel β -sheets connected by loops. There are currently over 1,300 structures of FAbs, Fvs, and scFvs in the public databases, allowing for sophisticated modeling and analysis of antibodies. These analyses, coupled with experimental approaches, have resulted in identification of the hypervariable loops in the Fv that are responsible for binding the targets, the complementarity determining regions (CDRs), and the exact paratopes that are specifically responsible for binding the epitopes on the targets. As natural antibodies are matured, their variable chains have the capabilities of evolving their shape to bind different kinds of targets such as proteins, peptides, and haptens.

Keywords: immunoglobulin (Ig), Ig fold, hinge, variable sequences/chains, constant sequences/chains, Fc (crystallizable fragment), complementarity determining regions (CDRs), trastuzumab, antigen combining site, Kabat, Chothia, single chain-Fv (scFv)

3.1 Introduction

Antibodies are large, complex, multifunctional proteins that possess several functional domains, each of which plays a significant role in the overall functionality of the molecule. All classes of human antibodies are comprised of Y-shaped monomeric subunits with a basic H₂L₂ structure consisting of a pair of identical heavy chains, each ~50–75 kDa in size, each paired with one of a pair of identical light chains, each of ~25 kDa. The sequence of the

heavy chain defines the class of Ig, such that α , δ , ϵ , γ , and μ heavy chains define the IgA, IgD, IgE, IgG, and IgM classes, respectively, each with a distinct role in the human adaptive immune system. The light chains are either κ or λ isoforms for all classes.

Within this basic H_2L_2 structure, Igs have two major regions, Fc (“fragment, crystallizable”) and FAb (“fragment, antigen binding”), both of which are comprised of domains consisting primarily of β -pleated sheets. The Fc portion of an IgG antibody, which consists of a pair of hinge, C_{H2} , and C_{H3} constant domains, determines a variety of key functions including multiple cell killing mechanisms as well as mechanisms for extending the plasma half-life of the antibody (Dall’Acqua et al., 2006; Roopenian and Akilesh, 2007; Baker et al., 2009; Suzuki et al., 2010). The FAb domains, which function to bind to a given antigen with high affinity and specificity, are comprised of both heavy and light chain components. The heavy chain component of a FAb has the C_{H1} and V_H domains, whereas the light chain component has either κ or λ C_L and V_L domains.

It has long been known that IgGs possess extraordinary flexibility, with the ability of the Fc to “wag” back and forth, of the FAb arms to wave and also rotate on the hinge, and for the FAb elbows to bend (Hanson et al., 1981; Brekke et al., 1995; Roux et al., 1997; Roux, 1999). The hinge region allows (see definition below) for both rotational and “wagging” flexibility, giving the antibody the ability to take on a variety of conformations (Hanson et al., 1981; Roux et al., 1997; Roux, 1999). The overall flexibility of human IgGs, as measured by hinge wag flexibility (Table 3.1) from greatest to least is $IgG_3 > IgG_1 > IgG_4 > IgG_2$ (Roux et al., 1997).

Table 3.1 Structural properties of human IgGs

Property	Isotype			
	IgG ₁	IgG ₂	IgG ₃	IgG ₄
Molecular mass	146	146	170	146
Amino acids in hinge	15	12	62	12
Inter-heavy chain disulfide bonds	2	4	11	2
Average FAb-FAb angle (Roux et al., 1997; Roux, 1999)	117°	127°	136°	128°
FAb-Fc preferred angles (Roux et al., 1997)	107°	99°	87°	98°
Hinge wag flexibility (Roux et al., 1997; Roux, 1999)	±43°	±32°	±53°	±39°
Susceptibility to proteases	+++	+/-	++++	++

The crystal structure of IgG₁ b12 MAb was the first IgG for which the entire structure, including the hinge, was intact and ordered (Saphire et al., 2002). While an IgG antibody is constructed of a seemingly symmetrical H₂L₂ structure, the analysis of b12 demonstrated that the crystal structure of the antibody showed asymmetry of structure, in part due to crystal packing and in part, due to distortion of the hinge (Saphire et al., 2002). Saphire et al. (2002) made a point that crystal structures offer only a “snapshot” of the wide range of conformers that could be adopted by a particular antibody. Sandin et al. (2004) described the structure of a mouse IgG_{2a} molecule as visualized by cryo-electron tomography and compared with X-ray crystal structures. Their results indicated that the IgG in solution appeared as two ellipsoidal FAb arms connected to a heart-shaped Fc. As judged by the wide variety of conformations adopted by the IgG in solution, the FAb arms exhibited exceptional flexibility with respect to the orientation of the Fc. Their results also supported the concept that antibodies adopt many structural forms in solution with apparent constant motion and flexing (Sandin et al., 2004; Bongini et al., 2005).

The constant chains of four human IgG subclasses, IgG₁, IgG₂, IgG₃, and IgG₄, share approximately 95% overall similarity, with the greatest differences, both in amino acid composition and structure, being in the “hinge region” which links the FAb arms with the Fc portion of the antibody (described in more detail below in section 3.2.2). Another significant structural difference between the human IgG isotypes is the placement of the disulfide bond linking the heavy and light chains. This disulfide bond links the carboxy-terminus of the light chain with the cysteine residue at position 219 in IgG₁ (Bongers et al., 1997; Cys 220, EU numbering), at position 127 in IgG₂ (Bagal et al., 2010; Martinez et al., 2008; Cys 131 in EU numbering), or at position 131 (EU numbering) in IgG₄ (Schuurman et al., 2001) of the C_H1 sequence of the heavy chain. Because these positions are very close in the three-dimensional structures, the essential structures of the different isotypes are similar.

3.2 Constant region structure/function

The antibody Fc is the portion of the molecule that interacts broadly with the immune system to help eliminate antigens to which the antibody is bound. As will be described in detail in Chapters 7–9, the Fc of different antibody isotypes interacts with different Fc-gamma receptors (FcγRs), thereby bringing about different biological responses.

3.2.1 Structure of the Fc

The Fc (Fragment, crystallizable) of an IgG consists of a paired set of antibody HC domains, each of which has a C_H2 fused to a C_H3 , which form a structure of about 50 kDa (Figure 3.1). The name “Fragment, crystallizable” (Fc) comes from the fact that after cleavage of serum-derived myeloma IgG fractions with papain, the only fragment that could be crystallized was the paired C_H2 - C_H3 fragment (Porter, 1959; Deisenhofer, 1981). The FAb's were not crystallizable in that effort because the different V-chains from the serum-derived antibodies made the FAb fraction heterogeneous.

Within the Fc, the two C_H3 domains bind each other tightly (albeit less so for IgG₄s; Labrijn et al., 2011), whereas the two C_H2 domains have no direct protein–protein contact with one another. An oligosaccharide is bound to asparagine-297 (N297) within each of the two C_H2 domains, filling part of the space between the two C_H2 s. In some crystal structures, hydrogen bonding has been observed between the two carbohydrate chains, directly and through bridging water molecules (e.g. Krapp et al., 2003; Matsumiya et al., 2007). The oligosaccharide consists of a bi-antennary heptasaccharide structure that is described in more detail in Chapter 11.

While the antibody appears to be a highly segmented molecule, it has been demonstrated that the structure of the Fc can impact the binding of

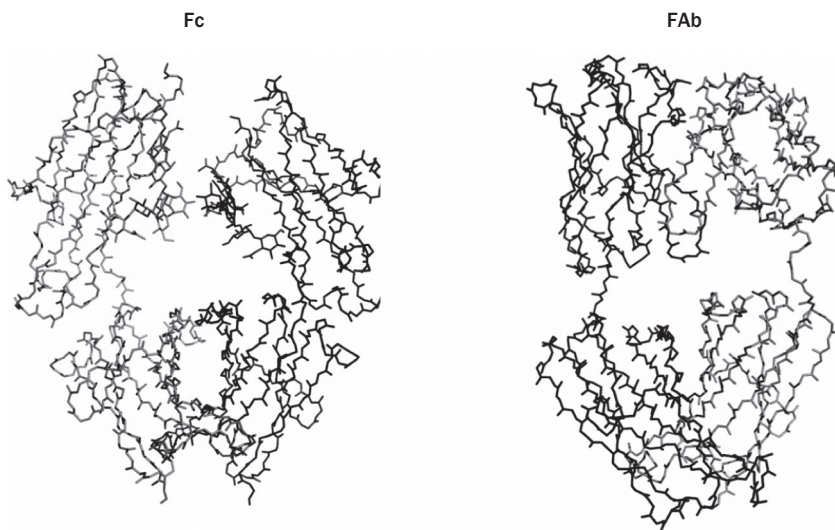


Figure 3.1 Wire structures of an Fc (left) versus a FAb (right)

Both Fc and FAb structures are built using the same basic structures of β -sheets as described in the text.

the FAb to the targeted antigens (Torres et al., 2007; Torres and Casadevall, 2007) and, similarly, that the content of the variable chain in the FAb can impact binding of the Fc to various receptors (Igawa et al., 2010). Recently circular dichroism studies have confirmed significant structural coupling between the FAb arms and the Fc of the IgG (Janda and Casadevall, 2010). Thus the IgG molecule is a highly complex molecule in which the different domains significantly interact, even at long distances.

3.2.2 Antibody hinge

The heavy chains of IgG, IgD, and IgA antibodies possess a hinge region that connects the Fc with the FAb regions and offers both rotational flexibility as well as independent orientation of the FAb with respect to each other and the Fc. The upper hinge (Figure 3.2), which is most proximal to the FAb arms, allows the angle between the FAb arms to vary (i.e. FAb-FAb flexibility), and also allows for rotational flexibility of each individual FAb. The flexibility of the lower hinge region, proximal to the C_H2 domain, dictates the position of the FAb relative to the Fc region (i.e. FAb-Fc flexibility). Hinge-dependent FAb-FAb and FAb-Fc flexibility appear to be important in effector functions such as complement activation and Fc receptor binding (Brekke et al., 1995).

The structure of the hinge has been divided into three regions: the upper hinge, the core hinge, and the lower hinge (see Figure 3.2) (Brekke et al., 1995). The upper hinge provides the FAb with their ability to rotate freely and independently. As can be seen in Figure 3.2, the upper hinge of IgG₂ is

	Upper hinge Papain ↓	Core hinge	Lower hinge Pepsin ↓	Disulfide bonds
HuIgG ₁	EPKSCDKTHT	CPPCP	APELLGGP	2
HuIgG ₂	ERK	CCVE CPPCP	APPVA -GP	4
HuIgG ₄	ESKYGPP	CPSCP	APEFL GGP	2
EU:	216	226	231 238	

Figure 3.2 Human antibody hinge sequences

Comparison of the sequences of the human IgG₁, IgG₂, and IgG₄ hinge regions, with separation of the upper hinge, core hinge, and lower hinge sequences. Differences in sequences in hulgG₂ and hulgG₄ from hulgG₁ are noted in bold, and the number of disulfide bonds within each hinge is noted at the right. The left and right arrows denote the cleavage sites for papain and pepsin, respectively. Numbering based on hulgG₁ according to the EU system is noted at the bottom. Note that the lower hinge is also part of the C_H2 constant domain, as shown in Figure 9.2.

substantially shorter than the upper hinges of IgG₁ and IgG₄, leading to what has been described as “greater stiffness” in the IgG₂ hinge (Roux et al., 1997). The core hinge is the region that covalently links the heavy chains through disulfide bridges. The lower hinge allows for motion of the Fc providing a significant contribution to the binding of the Fc to FcγRs.

The flexibility of the hinge regions has hindered the ability to obtain intact antibody structures, so the data for completely intact IgG₁ structures are based on only the structure of b12 (Saphire et al., 2002), with additional data from Kol (Marquart et al., 1980) and a complex of the Fc fragment with an Fc receptor (Sondermann et al., 2000). Crystallographic studies on intact Kol IgG₁ demonstrated that the upper and core poly-L-proline hinge regions in that antibody were both well ordered and helical in nature, whereas the lower hinge of Kol IgG₁ was disordered (Marquart et al., 1980). The IgG₁ b12 upper hinge, on the other hand, was less ordered and the core poly-L-proline helix was partially unwound, leaving one pair of cys residues unpaired (Saphire et al., 2002). The structure of the IgG₁ lower hinge actually was first observed in the structure of the complex of human FcγRIII and IgG₁ Fc (Sondermann et al., 2000).

The sequence, structure, and flexibility of the hinge, which vary among the human IgG isotypes, help to define the Fc-mediated activity of the antibody. The hinges of IgG₁, IgG₂, IgG₃, and IgG₄ possess 15, 12, 62, and 12 amino acid residues respectively, and form 2, 4, 11, and 2 interchain disulfide bonds respectively (Martinez et al., 2008) (see Table 3.1). The IgG₁ hinge, which includes amino acid residues 216–231, is very flexible, so the FAbs can both rotate about their axes of symmetry and move within a sphere centered at the first of two inter-heavy chain disulfide bridges (Maizels, 2005). IgG₂ has a short hinge with only 12 amino acid residues and four disulfide bridges (Martinez et al., 2008). The lack of a glycine residue in the IgG₂ hinge, coupled with the rigid poly-proline double helix stabilized by two extra disulfide bonds, make the IgG₂ hinge more rigid as compared with the other human isotypes (Brekke et al., 1995).

Human IgG₃ differs from the other IgG isotypes by its long hinge region containing 62 amino acids, including 21 proline and 11 cysteine residues, that form a poly-proline double helix (Martinez et al., 2008). The FAB fragments in IgG₃ are relatively distant from the Fc fragment, giving the molecule the greatest degree of flexibility among human IgGs. The IgG₄ hinge region is shorter than that of IgG₁ and has moderate flexibility. The most significant difference between the human IgG₄ hinge and the hinges of the other human IgGs is that in the IgG₄ hinge the two strands tend to dissociate due to the flexibility of the IgG₄ hinge region around the “Cys-Pro-Ser-Cys”

sequence that favors intrachain disulfide bond formation over interchain disulfide bonding (Schuurman et al., 2001), leading to an equilibrium of intrachain and interchain disulfides that ultimately leads to dissociation (Angal et al., 1993; Aalberse and Schuurman, 2002; van der Neut Kolfshoten et al., 2007). An S228P mutation (EU numbering based on IgG₁ sequence) of the natural IgG₄ hinge has been demonstrated to significantly decrease chain dissociation (Angal et al., 1993; Labrijn et al., 2009).

3.3 FAb structure/function

3.3.1 *The immunoglobulin fold as a structure paradigm*

Both heavy and light constant domains of a FAb are ca. 110 amino acid residues in size that are organized into two tightly packed anti-parallel β -sheets that form what has been aptly termed the “immunoglobulin (Ig) fold” (Poljak et al., 1973). The Ig fold is one of the most widely used folding domains used in proteins and evolutionarily goes back about 750 million years to the origins of sponges (Feige et al., 2010a). In FAb constant domains, C_H1 and C_L, the two anti-parallel β -sheets, are formed from four β -strands, ABED, and three β -strands, CFG, connected by short loops, in a structure often called a “Greek key” barrel (Gilliland et al., 2012). The β -strands B and F are linked via an intra-domain disulfide bridge. Amino acid residues within the β -strands, as well as the connecting loops, are known as framework (FW) residues. FW residues on the complementary faces of the two anti-parallel β -sheets hold the sheets together, while FW residues on the faces of domains that interact with other domains are involved in the domain–domain binding interactions.

The heavy and light variable chains of the FAb also have two anti-parallel β -sheets configured similarly to those found in the constant domain. The β -sheet formed from the four β -strands, ABED, is similar to that found in the constant domain. Different from the constant domains, the other anti-parallel β -sheet of the V-regions has five β -strands, C”C’CFB, instead of three. Two β -strands, C”C’, are inserted between β -strands C and D (Gilliland et al., 2012). Similar to the constant domain, β -strands B and F are linked by an intra-domain disulfide bridge. In general, the variable domains are less compact than the constant domains with longer loops connecting the β -strands. The framework amino acid residues have similar properties and a large number of them are invariant, despite the fact that they arise from a large number of different V-region families.

3.3.2 FAb

This section will focus on the FAb regions of an IgG, particularly the V-regions that make up the antigen binding domains. The overall structure and function of FAb molecules, strategies for the selection of V-regions to make therapeutic IgG antibodies, sources of V-region genes that encode the variable domains, approaches and technologies currently used to obtain the V-region genes, and the optimization of V-regions to generate therapeutic MAb candidates will be discussed in detail.

The fundamental structure of a FAb is shown in Figure 3.1. The FAb contains four domains: the heavy chain variable domain (V_H) linked to constant domain 1 (C_{H1}), and the light chain variable domain (V_L) linked to a constant domain (C_L) (Figure 3.1). The “FAb fragment” (“Fragment, antigen binding”) was originally defined as one of the cleavage products after treatment of rabbit IgG with papain (Porter, 1959), which cleaves above the core hinge (Figure 3.2), resulting in two identical FAb fragments and the intact Fc as products. The FAb fragment is often referred to these days as the FAb arm or the FAb region rather than the more historically used “FAb fragment.” Alternative cleavage of an IgG₁ with pepsin, which cleaves between the two Leu residues in the lower hinge, results in the $F(ab')_2$ fragment, in which two FAb arms are connected via disulfide bridges. Reduction of the disulfide bonds with dithiothreitol or a similar reducing agent will result in the $F(ab')$ fragment, which is ~10 amino acids longer at the C-terminus than the FAb fragment (see also Chapter 12). Since the 1970s, several academic and industrial laboratories have carried out crystallographic studies on both whole antibodies and antibody fragments, leading to a detailed knowledge of antibody structure today (see Stanfield and Wilson, 2009, for review). Due to the hinge region which allows for flexibility of FAbs in relation to the Fc, intact IgGs have proven difficult to crystallize in forms suitable for diffraction studies, and thus, to this time, only a few structures for intact IgGs have been determined (Silverton et al., 1977; Marquart et al., 1980; Rajan et al., 1983; Harris et al., 1992; Harris et al., 1997; Saphire et al., 2001; Guddat et al., 1993). Early on, proteolytic cleavage of IgGs into FAb fragments and Fc (Porter, 1959), and more recently generation and expression of recombinant FAbs and Fc domains, has allowed crystal structures to be obtained of each of the components of IgGs. Currently, over 1,300 structures of FAbs, Fvs, and scFvs, many of which include complexes with haptens, peptides, or proteins, have been deposited in the Protein Data Bank (Berman et al., 2000).

The packing of the light chain (LC, comprised of the V_L and C_L domains) and heavy chain (HC, comprised of V_H and C_{H1} domains) generate the FAb structure (Figure 3.1). The antigen binding site is created by the packing of

the variable domains, V_L and V_H (Ewert et al., 2003). At the top of the barrel are six loops, three each contributed by V_H and V_L , known as the complementarity determining regions (CDRs), that comprise the antigen binding (or combining) site. The anti-parallel C α C α CFB β -sheets pack together in almost parallel fashion to form a barrel-like structure that orients the six CDR loops closely together to generate the antigen combining site. On the other hand, the C_H1 and C_L domains pack in an opposite manner using the surfaces of the ABED β -sheet, which pack tightly in nearly a perpendicular manner.

The variable and constant domains are connected by a sequence, called the “switch region,” linking β -strand F of the variable domain with β -strand A of the constant domain (Stanfield et al., 2006). The switch region allows the V-regions to be oriented at an angle with respect to the constant domains (Gilliland et al., 2012), which is referred to as the elbow angle or elbow bend – this is defined by the angle between the axes relating the two pairs of domains (V_H , V_L and C_H1 , C_L) (Schiffer et al., 1973; Poljak et al., 1974). A recent survey of 536 Fab structures revealed that FAb containing λ light chains had angles ranging from 116° to 226°, whereas FAb with κ light chains had a narrower range of angles, most of which were greater than 180° (Stanfield et al., 2006). This indicates that λ light chains are more flexible, possibly due to the presence of an extra amino acid residue (usually a glycine) present in the switch region associated of λ LCs.

It has been demonstrated that the HC elbow region linking the V_H and C_H1 domains (but not present in the LC) has a highly conserved feature that has been named a “molecular ball-and-socket joint” (Lesk and Chothia, 1988). The ball consists of conserved residues Phe148 and Pro149 in V_H , and the socket is formed by conserved residues Leu/Val11, Thr110, and Ser112 in C_H1 (Lesk and Chothia, 1988; Landolfi et al., 2001). It has been demonstrated that the integrity of the ball-and-socket joint is critical to retention of the biological activity of an antibody when it is made into chimeric and humanized forms (Landolfi et al., 2001). While it was originally proposed that this joint would restrict the elbow angle to a maximum of 180°, other FAb structures (e.g. Love et al., 1993) have reported larger angles in which the ball-and-socket separate, which allows the domains to form elbow angles greater than 180° (Stanfield et al., 2006).

Elbow angle has been demonstrated to play a significant role in the activity of the Type II anti-CD20 MAb, obinutuzumab (GA-101) (Mössner et al., 2010; Niederfellner et al., 2011). During the humanization of the murine antibody, B-Ly1, a Val residue was substituted in the humanized form for Kabat position Leu11 of the murine MAb, which resulted in movement of Phe152 (since the Leu11 residue had held Phe152 in place) and an opening

up of the FAb angle that significantly increased the activity of the resultant humanized MAb (Mössner et al., 2010; Niederfellner et al., 2011). Interestingly, while this change resulted in a substantially more potent antibody, efforts to incorporate that kind of change into other antibodies have thus far resulted in little to no effect (Mössner et al., 2010; Niederfellner et al., 2011).

3.3.3 Fv

The paired V_H and V_L domains comprise the Fv region or fragment, which is the minimal portion of an antibody that maintains the antigen combining site (Figure 3.3). The Fv was first isolated by proteolytic cleavage of an FAb (Inbar et al., 1972) or IgG (Hochman et al., 1973), although Fvs generated

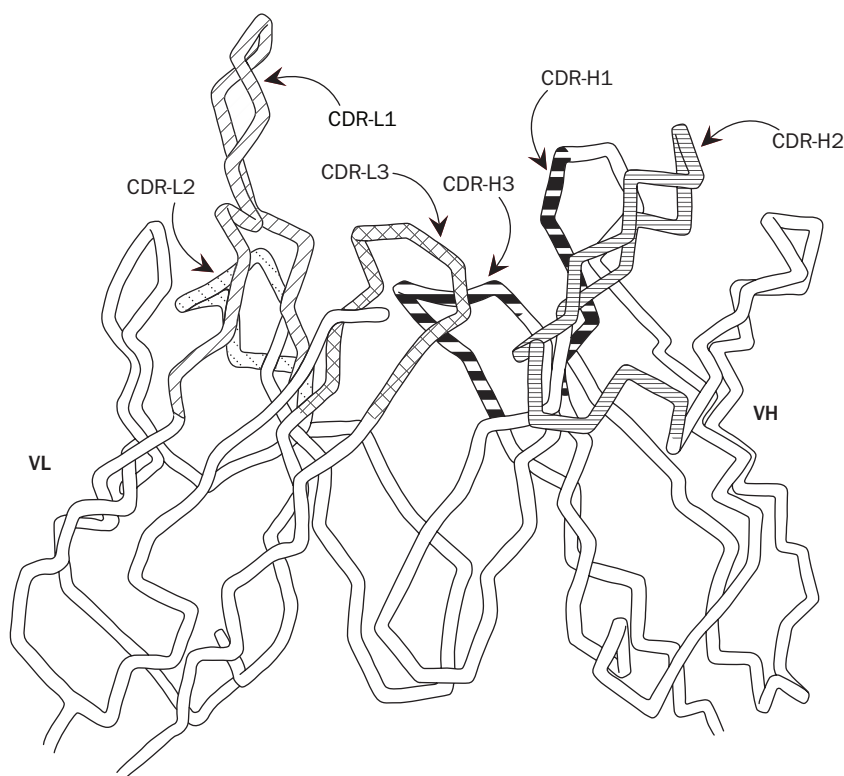


Figure 3.3 Ig fold (Fv)

Drawing of the Ig fold, showing the V_L (left) and V_H (right) domains and the positions of the six CDRs.

in this manner were typically not stable. Functional Fvs were later generated by co-expression of recombinant V_H and V_L genes in *E. coli* (Skerra and Plückthun, 1988); however, these Fvs still were largely unstable and prone to dissociation and aggregation. Since then, several protein engineering approaches have been used to generate stable Fv domains (Wörn and Plückthun, 2001). The most widely used method for stabilizing the Fv is to insert a short, flexible linker (generally ~15 residues) linking the C-terminus of V_L with the N-terminus of the V_H (Bird et al., 1988) or vice versa (Huston et al., 1988), resulting in a single chain Fv (scFv; see Chapter 12). Another approach to stabilize the Fv is to introduce a disulfide bridge between the V_H and V_L domains in either the Fv (Brinkmann et al., 1993) or in an scFv (Young et al., 1995; Zhao et al., 2011). Introduction of the inter-domain disulfide bridge does not alter the antigen combining site relative to that observed in the antibody (Almog et al., 1998; Zhao et al., 2011).

3.3.4 Antigen combining (binding) site and CDRs

The antigen combining site (also called the antigen binding site), or paratope, is defined by the set of amino acid residues that make contact with the antigen. To determine the exact paratope, the experimental structure of the antibody in complex with its corresponding antigen needs to be elucidated by co-crystallography and structural elucidation (Luftig et al., 2006; Covaceuszach et al., 2008; Ekiert et al., 2009; Teplyakov et al., 2009).

V_H and V_L combine by non-covalent association to form the F_v region, which contains the antigen binding or combining site. Each domain contributes three hypervariable loops (HVLs) or CDRs, with CDR-L1, CDR-L2, and CDR-L3 formed by V_L and CDR-H1, CDR-H2, and CDR-H3 by V_H . In the F_v , the two β -sheets and the non-hypervariable loops are referred to as Framework Regions (FRs). CDR-L1 and CDR-H1 HVLs correspond to the residues within the loops connecting β -strands B and C (Gilliland et al., 2012). For CDR-L2 and CDR-H2, the HVLs are formed by the loops connecting β -strands C' and C'', and for CDR-L3 and CDR-H3, the HVLs are formed by the loops connecting β -strands F and G (Gilliland et al., 2012). Due to the large number of different V-regions that can comprise the F_v , both amino acid sequence and length can vary significantly for the HVLs, as will be described later in this chapter.

Chothia and colleagues (Chothia and Lesk 1987; Chothia et al., 1989; Al-Lazikani et al., 1997) analyzed the combining sites of a few FAB fragments and found that the conformations of five of the HVLs had a limited set of main-chain conformations or canonical structures. Prior to

those studies, it had been assumed that each antibody possessed a unique HVL conformation. Thus the concept of a canonical structure was a paradigm shift for the field. A canonical structure was defined by the loop length and conserved residues located in the hypervariable loop and FRs. Based on this model, studies of antibody sequences indicated that only a small fraction of the total number of possible combinations of canonical structures actually occur (Chothia et al., 1992; Tomlinson et al., 1995; Vargas-Madrado et al., 1995). This suggested that structural restrictions at the antigen-binding site may affect antigen recognition.

There is a fundamental difference between the combining sites of natural, polyspecific antibodies and more matured, highly specific antibodies (Thorpe and Brooks, 2007; Zimmerman et al., 2010). Natural antibodies require the ability to recognize multiple epitopes, and thus show remarkable flexibility within the combining sites. On the other hand, as the combining site of an antibody becomes more matured, i.e. gains additional affinity and specificity towards a single epitope, it also becomes significantly more rigid in its structure (Thorpe and Brooks, 2007; Zimmerman et al., 2010). Additionally, Jimenez et al. (2003) suggested that CDR loop length may be correlated with flexibility, with longer loops being more flexible.

In a structural conformation analysis of 17 high-resolution antibody fragments, CDR-L1, CDR-L2, CDR-L3, CDR-H1, and CDR-H2 were all found to have favored canonical structures based on amino acid sequence length and composition (Al-Lazikani et al., 1997). The CDR-H3 loops, on the other hand, have highly variable amino acid sequences and sequence lengths, which results in the absence of true canonical structures. Nevertheless, both the CDR-H3 residues nearest to the framework, as well as residues in the extended loops, have been found to possess defined conformations (Shirai et al., 1996; Morea et al., 1998). Subsequent studies have indicated that CDR loop length is the primary factor in determining the topology of the antigen-binding site (Collis et al., 2003), and therefore is the primary factor determining the canonical structures (Chothia and Lesk, 1987; Al-Lazikani et al., 1997). Recent studies demonstrating that CDR-H3 loops fall into various subcategories (Shirai et al., 1999; North et al., 2011) have helped to improve antibody modeling strategies (Martin and Thornton, 1996; Marcatili et al., 2008; Sivasubramanian et al., 2009; Sircar et al., 2009), as well as providing the first insight into the molecular-level structural mechanisms leading to antibody diversity.

The definition of the CDRs themselves has changed over time (Martin and Allen, 2007). Moreover, there are multiple antibody variable chain numbering schemes (<http://www.bioinf.org.uk/abs/#cdrid>), including those proposed by Kabat (Wu and Kabat, 1970; Kabat et al., 1991),

CDR	Kabat CDR*	Chothia CDR*	Contact range*
CDR-H1	V _H 31-35	V _H 26-32	V _H 30-35
CDR-H2	V _H 50-65	V _H 52-56	V _H 46-55
CDR-H3	V _H 95-102	V _H 95-102	V _H 89-96
CDR-L1	V _L 24-34	V _L 24-34	V _L 24-34
CDR-L2	V _L 50-56	V _L 50-56	V _L 50-56
CDR-L3	V _L 89-97	V _L 89-97	V _L 89-97

Chothia number: 22-23-24-25-26-27-28-29-30-31-31a-31b-32-33-34-35- 36...

Kabat number: 22-23-24-25-26-27-28-29-30-31- 32-33-34-35-35a-35b-36...

Example sequence: C K A S G Y T F T G Y Y M H W

Kabat CDR-H1: _____

Chothia CDR-H1: _____

CDR-H1 contact range: _____

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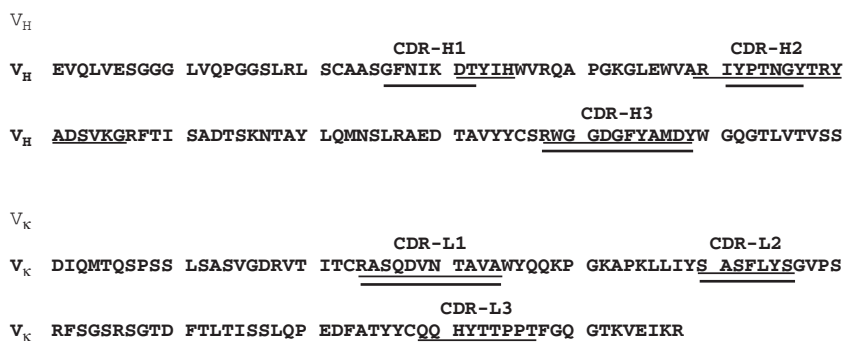


Figure 3.5 Presumed variable chain sequences of trastuzumab (humAb 4D5-8)

Sequence based on data from Carter et al. (1992) with CDRs identified by the Kabat and Chothia definitions. The thin lines represent the location of the CDRs based on the Kabat (sequence-based) definitions, and the thick lines represent the position of CDRs based on the Chothia (structure-based) definitions.

between the Kabat and Chothia CDR definitions and the contact range as defined by MacCallum et al. (1996). Figure 3.4 also demonstrates the key differences in CDR-H1 as defined by the different groups. Figure 3.5 shows example V-sequences with the CDRs defined by both the Kabat (most widely used definition) and Chothia definitions. The following guidance for determining the location of CDRs is modified from Andrew Martin's website (<http://www.bioinf.org.uk/abs/#cdrid>). Figure 3.5, which shows the V_H and V_L chains of trastuzumab (Carter et al., 1992), demonstrates the rules.

- CDR-H1 as defined by Kabat starts nine residues after Cys22 and is followed by Trp36. CDR-H1, as defined by Chothia, generally starts at about residue 26 of the V_H chain, four residues after Cys22, and is typically ~8–10 residues in length. Thus the Kabat CDR-H1 definition is offset from the Chothia definition by about five residues.
- CDR-H2 as defined by Kabat starts 15 residues after the end of CDR-H1 (Kabat definition), is generally 16–19 residues in length, is often preceded by the sequence “LEWIG,” and is usually followed by an Arg or Lys residue. CDR-H2 as defined by Chothia starts two residues after the Kabat CDR-H2 and is generally 6–8 residues in length.
- CDR-H3 occurs 33 residues after the end of the Kabat-defined CDR-H2 and always starts at the third residue after a Cys residue (often Cys-Ala-Arg). CDR-H3 is nearly always followed with a Trp-Gly-xxx-Gly sequence and can be as short as three residues in length or as long as 25 or more residues in length (Figure 3.6 shows human CDR-H3 length distributions).

- CDR-L1 generally starts around residue 24, is typically 10–17 residues in length, and is always flanked by a Cys residue on the N-terminal side and a Trp residue on the C-terminal side.
- CDR-L2 is typically found 16 residues after the end of CDR-L1, is preceded by hydrophobic residues such as Ile-Tyr, Val-Tyr, Ile-Lys, or Ile-Phe, and is seven residues in length.
- CDR-L3 starts 33 residues after the end of CDR-L2, is always preceded by a Cys residue, and is followed by a Phe-Gly-xxx-Gly sequence. CDR-L3s are generally 7–11 residues in length, although some CDR-L3s with as few as three residues have been found.

As can be seen from the descriptions above and in Figures 3.4 and 3.5, the Kabat and Chothia definitions for CDR-H1 and CDR-H2 are different, but the definitions for CDR-H3, CDR-L1, CDR-L2, and CDR-L3 are essentially identical.

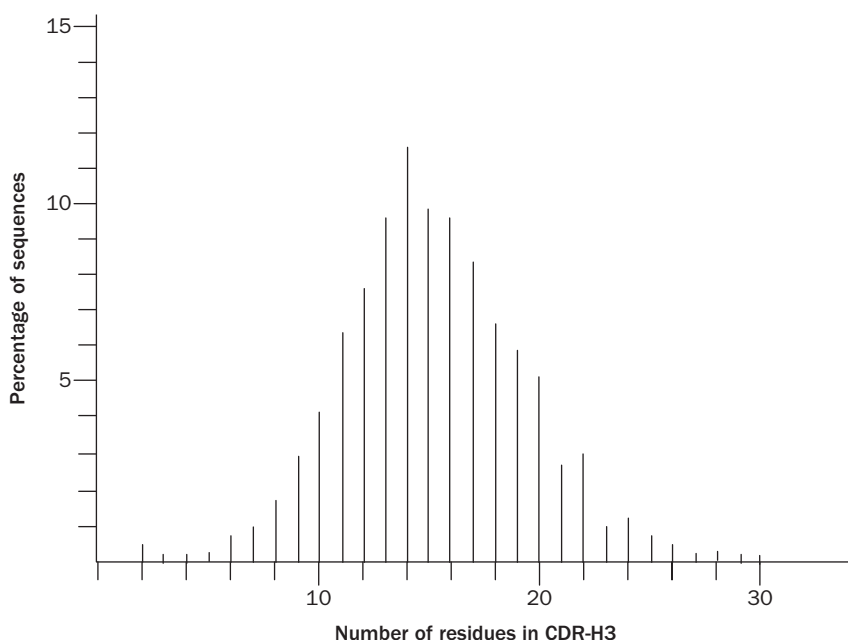


Figure 3.6 Distribution of CDR-H3 lengths in naturally matured human IgGs

Data from Zemlin et al. (2003). Note that CDR-H3 lengths of around 14 amino acid residues are the most prevalent based on that study.

3.3.5 Anatomy of the antigen combining site

It has long been known that the shape of the the Fv combining region can be different, depending on the type of antigen being bound, i.e. hapten versus peptide versus protein (<http://www.bioc.unizh.ch/antibody/Introduction/VirtualSeminars/Institutsseminar97/source/slide16.htm>; Honegger, 2008). Recently, an analysis of 140 antigen-antibody complexes revealed that the CDR-L1 loop is the most variable in terms of responding to the type of antigen. For anti-protein antibodies, CDR-L1 averaged 6–8 residues in length, whereas for anti-peptide or anti-hapten antibodies, CDR-L1 averaged 11–13 residues in length (Raghunathan et al., 2012). There was little difference in the lengths of the other five loops of anti-protein, anti-peptide, and anti-hapten antibodies (Raghunathan et al., 2012). Figure 3.7, which shows a cartoon of the interactions of antibodies, haptens, peptides, and

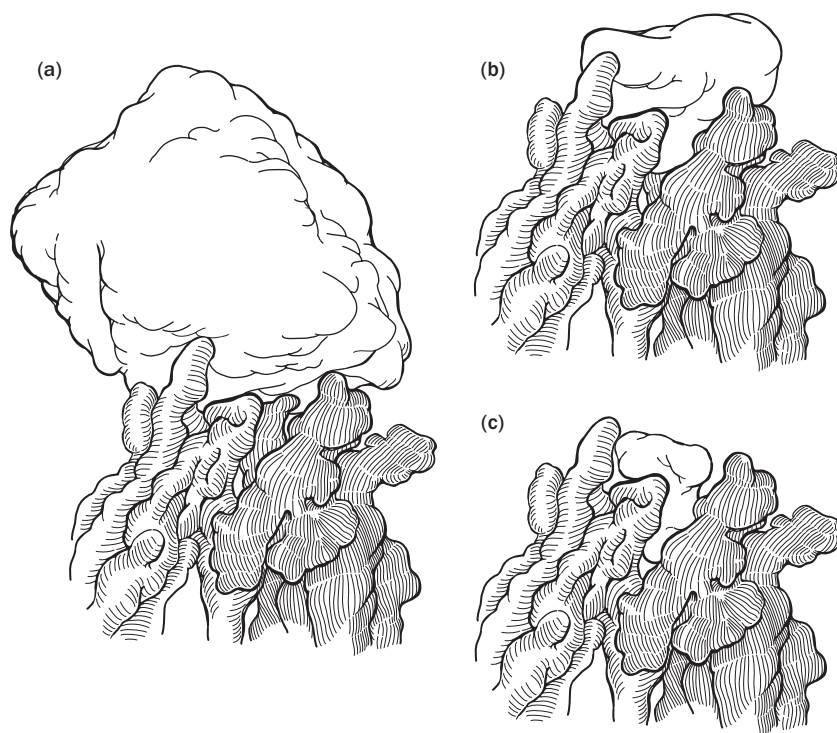


Figure 3.7 Antibody interactions with (a) proteins, (b) peptides, and (c) haptens

Drawing based on data from Honegger (2008) and Raghunathan et al. (2011), which indicate that the CDRs of the antibody provide a flatter surface when binding to proteins, but have deeper grooves with peptides and haptens.

and proteins, demonstrates that the size and shape of the antigen appears to dictate the topography of the antigen-binding site. For example, the binding sites of anti-protein antibodies tend to be flatter, whereas the binding sites for anti-peptide antibodies are grooved, primarily due to the long HVL of CDR-L1. Antibodies binding haptens, on the other hand, have a deeper and smaller antigen-binding site.

As would be expected, the number of CDR residues actually contacting antigens also differs for antibodies recognizing proteins versus peptides and haptens (MacCallum et al., 1996; Almagro, 2004; Raghunathan et al., 2012). The average number of contact residues in V_H for the anti-protein, anti-peptide, and anti-hapten antibodies is 14, 12, and 10, respectively (Raghunathan et al., 2012), and for V_L , are 9, 9, and 7, respectively. The larger number of contacts in V_H as compared to V_L highlights the more critical role for V_H with respect to antigen recognition. On that note, Sherie Morrison once made the point that V_L had minimal impact on most of the properties of an MAb (Montaño and Morrison, 2002). Moreover, antibodies have been found in camelids (Hamers-Castleman et al., 1993) and nurse sharks (Greenberg et al., 1995) that completely lack light chains (see Chapter 12).

Differential patterns for protein, peptide, or hapten binding have also been noted for the Solvent Accessible Surface (SAS), the surface area that becomes buried upon antigen binding, and Specificity-Determining Residues Usage (SDRU), an analysis of the frequency of residues making contact with the antigen (Almagro, 2004; Raghunathan et al., 2012). Anti-protein, anti-peptide, and anti-hapten antibodies have SAS values of $737 \pm 272 \text{ \AA}^2$ (average $\pm$ SD), $544 \pm 158 \text{ \AA}^2$, and $374 \pm 117 \text{ \AA}^2$, respectively.

For protein antigens, the antibody SDRU hotspots are located at the edge of the antigen-binding site (Raghunathan et al., 2012), whereas for peptide antigens the antibody SDRU hotspots are located in the interior of the antigen-binding site but not buried in the V_L : V_H interface. For anti-hapten antibodies, the SDRU hotspots are found in the interior of the antigen-binding site or even buried in the V_L : V_H interface (Raghunathan et al., 2012). Honegger (2008) calculated the approximate contributions of each CDR to the binding of proteins, peptides, and haptens (Table 3.2). The most significant differences were the reduced role for CDR-L2 in peptide and hapten binding as compared with protein binding, and the reduced role of CDR-H2 in hapten binding as compared with peptide and protein binding (Honegger, 2008).

Overall, anti-protein antibodies appear to have a flatter binding site with a greater surface area of interaction, whereas the anti-peptide antibodies tend to have more of a groove as a binding site, and anti-hapten antibodies tend to have a smaller but deeper pocket (Honegger, 2008; Raghunathan et al.,

Table 3.2 Comparison of different binding characteristics for antibodies binding protein, peptide, or hapten antigens*

Characteristic	Characteristics of antibodies recognizing type of antigen		
	Protein	Peptide	Hapten
Average length of CDR-L1	6–8 residues	11–13 residues	11–13 residues
Topography	Flat	Grooved	Small but deep
Average number of contact residues in V_H	14	12	10
Average number of contact residues in V_L	9	9	7
Solvent accessible surface (SAS; area of antibody surface binding to antigen)	$737 \pm 272 \text{ \AA}^2$	$544 \pm 158 \text{ \AA}^2$	$374 \pm 117 \text{ \AA}^2$
Calculated contribution of loops to binding (Honegger, 2008)	H1, 12%; H2, 21%; H3, 28%; L1, 12%; L2, 10%; L3, 16%	H1, 14%; H2, 25%; H3, 24%; L1, 13%; L2, 3%; L3, 21%	H1, 10%; H2, 13%; H3, 35%; L1, 13%; L2, 3%; L3, 26%
Specificity-determining residues usage (SDRU) hotspots	At the edge of the antigen binding site	At the interior of the antigen-binding site, but not buried in the $V_L:V_H$ interface	At the interior of the antigen-binding site or buried in the $V_L:V_H$ interface

* From Raghunathan et al. (2012) and Honegger (2008). Also see Figure 3.7.

2012; Figure 3.7). With the level of predictability of antibody shape correlating with antigen type as shown in Table 3.2, synthetic antibody libraries have been successfully constructed specifically to address the shapes and sizes required for each of these three classes of antigens, e.g. proteins (Almagro et al., 2006), peptides (Cobaugh et al., 2008), and small molecule haptens (Persson et al., 2006).

3.3.6 Antigen binding – lock and key versus induced fit

When antibodies bind antigens, both the antibodies and antigens undergo conformational changes, as revealed by more than a hundred structural determinations of FAbs either alone or complexed with antigen (Wilson and Stanfield, 1994; Davies and Cohen, 1996; Stanfield and Wilson, 2009). Antibody–antigen interactions are often placed into one of two general categories of binding, i.e. the “lock and key” or “the induced-fit” modes (Verdaguer et al., 1996; Jimenez et al., 2003; Sinha and Smith-Gill, 2005; Stanfield et al., 2007; Blackler et al., 2011). In the lock-and-key mode, interaction of the antibody and antigen results in a minimization of conformational changes of the two surfaces as compared with the unbound state. In this mode, the backbone atoms remain in essentially the same conformation as in the unbound state, while amino acid sidechains and solvent atom positions may change (Verdaguer et al., 1996). In the induced-fit mode, on the other hand, the conformations of both backbone atoms and sidechains, especially of the CDR loops, may be changed, sometimes substantially, to result in the best conformation for binding (Verdaguer et al., 1996; Sinha and Smith-Gill, 2005; Stanfield et al., 2007; Blackler et al., 2011). Thorpe and Brooks (2007) found that, in general, the induced-fit paradigm predominantly applied to less mature antibodies, whereas the lock-and-key paradigm becomes more prevalent as an antibody is matured.

CDR-H3, which has no canonical structure, is often found in different conformations in the bound and unbound forms, as determined by structure elucidation of the FAb itself vs the FAb bound to antigen (Sinha and Smith-Gill, 2005). Additionally, the $V_L:V_H$ orientation as well as the elbow angle may change in induced fit binding to antigens. It has been suggested that the induced fit mode of binding introduces plasticity into the antigen combining site that expands antibody diversity beyond that induced solely by amino acid residue changes (Davies and Padlan, 1992). Examples of antibodies for which “induced fit” has been determined include an antibody that neutralizes the foot-and-mouth virus, in which reorientation of the the CDR-H3 results in a significant change in charge distribution (Verdaguer et al., 1996), an antibody recognizing ssDNA, in which there is a 2–4 Å shift in the highly flexible CDR-H3 upon binding to the substrate (Schuermann et al., 2005), induced fit of a shark Ig-new antigen receptor (IgNAR) antibody upon complexation with antigen (Stanfield et al., 2007), and antibodies binding lipopolysaccharide epitopes in which four different conformations of the CDR-H3 have been documented (Blackler et al., 2011). In studying the interaction of an anti-hen egg white lysozyme antibody with its cognate

antigen, Sinha and Smith-Gill (2005) suggested that both lock-and-key and induced-fit mechanisms could apply to different binding interactions within the same antibody-antigen complex. Finally, the concept of induced fit may also apply to the antigen itself, as in the case of the HIV gp120 V3 loop, which has a different structure depending on which of three antibodies binds it (Rosen et al., 2005).

Fundamental technologies for antibody engineering

Abstract: Modern monoclonal antibody technology requires a wide variety of recombinant DNA technologies. These were first developed in the early 1970s and evolved to the very sophisticated methods widely used today. Technologies that have contributed significantly to the generation and optimization of recombinant monoclonal antibodies include DNA cloning, generation of hybridomas, polymerase chain reaction (PCR), various peptide and protein display technologies, and, most recently, deep (or Next Generation) sequencing. These technologies have combined to make the discovery and optimization of recombinant monoclonal antibodies and Fc fusion proteins a standard and highly successful endeavor.

Keywords: hybridomas, phage display, *in vitro* display, yeast display, mammalian cell display, bacterial display, human antibody libraries, B cell development, recombinant technologies, deep sequencing

4.1 Introduction

This chapter deals with the fundamental technologies that have enabled the discovery and engineering of monoclonal antibodies and have been the basis of the therapeutic monoclonal antibody field. Of course, the most essential of these has been hybridoma technology, the development of which opened up the field of therapeutic monoclonal antibody development. Technologies that allowed for the expression of recombinant antibodies, selection of higher affinity antibodies, and display of antibody libraries were additional critical advances for this field. Figure 4.1 gives a general timeline for key events in the development of the recombinant therapeutic MAb and Fc fusion field, some of which are highlighted in this chapter.

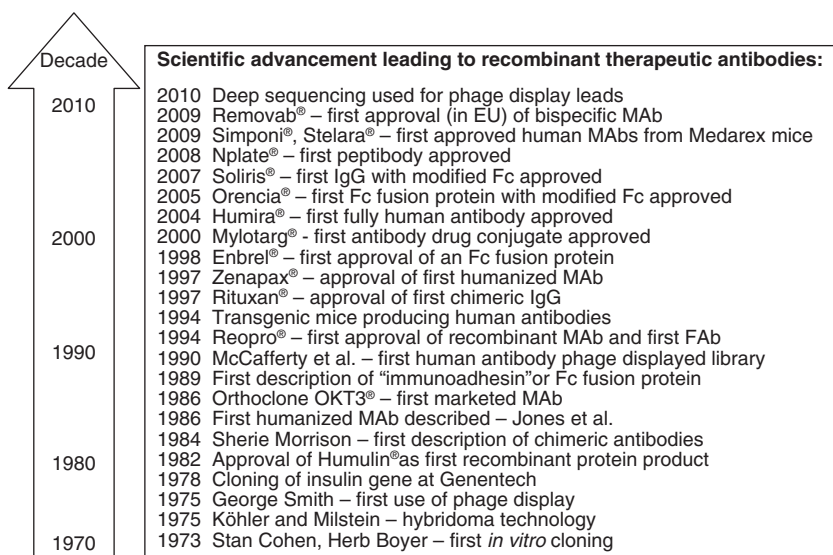


Figure 4.1 Timeline of key scientific breakthroughs for recombinant therapeutic MAbs

Events from 1973 to 2010 leading to the development of the current state of recombinant therapeutic MAbs and Fc fusion proteins.

4.2 Hybridoma technology – the gateway for therapeutic monoclonal antibodies

The development of hybridoma technology, first described by Köhler and Milstein in 1975, was the seminal advancement that led to modern antibody therapeutics based on monoclonal antibodies. A hybridoma, which results from the fusion between an antibody-producing B cell and an immortal cell line such as a myeloma, can provide an infinite source of a specific MAB with defined specificity and affinity. The advent of hybridomas resulted from the convergence in the early 1970s of three technologies: (1) immortal myeloma–myeloma fusions; (2) mortal, antibody-producing B cells; and (3) assays to visualize antibody-producing B cells. César Milstein and his group at the Medical Research Council built the capability to generate myeloma–myeloma fusions secreting antibodies (Cotton and Milstein, 1973; Milstein, 1985). The mouse antibodies produced did not have germline sequences, so they were the result of isotype switching and somatic mutation, albeit to unknown antigens (Alkan, 2004). Schwaber and Cohen (1973) demonstrated that the fusion of an immortal murine myeloma cell

line with human peripheral blood lymphocytes resulted in hybrid cell lines that continuously secreted human immunoglobulin. Thus the groundwork had been laid for specific hybridoma generation. At about the same time, Georges Köhler was assessing antibody diversity produced in response to immunization of mice with a single antigen for his doctoral thesis at the Institute of Immunology in Basel, Switzerland. He was attempting to obtain antibodies from mortal B cells in culture, but with little success due to the extreme fragility and short lifespan of the B cells when cultured. After attending a lecture by Milstein on his myeloid fusions, Köhler asked to join Milstein's lab in Cambridge, and in 1973 he joined as a postdoctoral fellow. In this setting Köhler developed the process for generating fusions between B cells and myelomas that were both immortal and secreted a single antibody recognizing a specific antigen (Köhler and Milstein, 1975).

In 1984 Milstein, Köhler and Jerne received the Nobel Prize in Physiology or Medicine for the discovery of methods to generate hybridomas producing monoclonal antibodies. Köhler and Milstein's decision not to patent their discovery allowed for the royalty and milestone-free use of their hybridoma technology by both academics and industry. Because of its free access, as well as its power to generate highly epitope-specific binding reagents, hybridoma technology quickly became the standard approach for obtaining murine antibodies against specific antigens.

Within two years of Köhler and Milstein receiving the Nobel Prize, the first antibody was approved for marketing in the United States by Ortho Biotech (Johnson & Johnson) for kidney transplant rejection. The murine anti-CD3 antibody, Orthoclone OKT3®, was manufactured by hybridoma culture. OKT3 was one of a series of antibodies isolated by Patrick Kung at Ortho Biotech after immunizing mice followed by screening for antigens on human T cells (OKT family), B cells (OKB family), and myeloid cells (OKM family) (Jill Giles-Komar, personal communication). OKT4 was a second antibody from this research effort to enter the clinic (Moreland et al., 1998).

Hybridoma generation is initiated by immunization of rodents with the antigen of interest mixed with an adjuvant to enhance immune response. A conventional immunization schedule might include primary immunization with antigen in Freund's complete adjuvant, followed by boosts of antigen in incomplete Freund's adjuvant at days 14 and 22 (Staquet et al., 2006). Once sufficient titers against the antigen have been generated, the murine splenocytes are harvested and antibody-producing B cells (or, alternatively, peripheral B cells, B cells from lymph nodes and/or bone marrow) are harvested and fused to an immortal mouse myeloma cell line such as SP2/0, NS0, or FO in the presence of either polyethylene glycol (PEG) (Groves and Morris, 2000; Staquet et al., 2006) or Sendai virus (Pandey, 2010). Unfused

B cells are short-lived and the myeloma cells should not produce antibodies. The fusions, however, result in immortal, antibody-producing hybridomas (Pandey, 2010). For rats, the rat myeloma cell line Y/0 is typically used as the fusion partner (Groves and Morris, 2000; Sib  ril et al., 2006).

In the presence of HAT (hypoxanthine-aminopterin-thymidine) medium, myeloma fusion partners, which lack the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT), are killed (de St Groth and Scheidegger, 1980). The aminopterin blocks nucleotide synthesis and due to lack of HGPRT, unfused myeloma cells are unable to produce nucleotides via salvage pathways. Fused cells, or hybridomas, possess active HGPRT from the B cells and grow in HAT medium, allowing for selective propagation of the newly formed hybridomas (de St Groth and Scheidegger, 1980). After selection, individual hybridoma clones are separated so that each clone produces a single monoclonal antibody with one specificity in a process known as subcloning. Antibodies from individual clones are then assayed for binding to the immunization antigen. Once desired antibodies are found, their V-regions are cloned from the hybridoma lines and made recombinant.

Several technologies have improved the hybridoma generation process (Pandey, 2010). Two recent advancements are the use of type 1 interferons to boost production of antibodies against antigens fused to an Fc (Staquet et al., 2006) and the use of an anti-CD40 agonist antibody to shorten the immunization process as well as increase the number of hybridomas containing unique antibodies (Staquet et al., 2006; Ryczyn et al., 2008). Additionally, technology has been developed to clone antibody V-genes directly from hybridomas to make recombinant IgGs very shortly after the fusion process, shortening timelines and increasing outputs from hybridomas (Crosnier et al., 2010).

The use of hybridoma technology to generate high affinity murine antibodies revolutionized immunology as a field and has led to both the generation of tens of thousands of research tools as well as therapeutic candidate antibodies.

4.3 Key recombinant DNA technologies

As mentioned in the opening, the recombinant DNA revolution began in the early 1970s when Cohen and Boyer and their colleagues demonstrated that foreign DNA could be inserted into a plasmid and expressed in *E. coli* (Cohen et al., 1973). Lasting until the late 1980s, this was a key period in which several seminal technologies were developed that significantly enabled the generation of recombinant human therapeutic antibodies.

These include the isolation and use of restriction enzymes to clone genes into plasmids (Cohen et al., 1973), site directed mutagenesis for protein engineering (Zoller and Smith, 1982; Dalbadie-McFarland et al., 1982), phage display methods (Smith, 1985), and polymerase chain reaction (Mullis et al., 1986; Saiki et al., 1988). In addition to Köhler and Milstein's hybridoma technology mentioned above, key advances in antibody technologies included the sequencing and characterization of human germline antibody genes (Kabat et al., 1987), the expression of antibody genes in *E. coli* (Better et al., 1988; Skerra and Plückthun, 1988) and mammalian cells (Neuberger, 1983; Neuberger and Williams, 1986), and the development of an understanding of the genetics of antibody expression, including the contributions of germline versus somatic mutations as a basis for antibody diversity (Hozumi and Tonegawa, 1976; Early et al., 1980; Gough and Bernard, 1981; Tonegawa, 1983; Silverstein, 2003).

4.4 Generation of chimeric antibodies

Only two commercially marketed monoclonal antibody therapeutics are manufactured using the original cell line from which they were identified, in a non-recombinant form. Orthoclone OKT3® (muromonab CD3), the first antibody ever approved for manufacture and marketing in the US, was produced by hybridoma culture up until 2010, when manufacturing was halted for economic reasons. Removab® (catumaxomab; binds EpCAM and CD3) is produced from culture of a quadroma cell line, which is derived from the three-way fusion of a mouse B-cell, a rat B-cell, and a myeloma cell (Zeidler et al., 1999). Removab® is approved for manufacturing and marketing in the European Union but not in the US. All other antibodies approved for marketing and sales in the US have been re-generated as recombinant molecules and produced in recombinant cell lines.

Three key developments allowed for this transition from hybridoma to recombinant cell culture: (1) the ability to clone out the murine V_H and V_L genes via polymerase chain reaction (PCR) for recombinant expression (Orlandi et al., 1989); (2) the ability to express both heavy and light chain antibody genes in stable human cell lines after transfection (originally called "transfectomas"; Neuberger, 1983; Neuberger and Williams, 1986; Beidler et al., 1988); and (3) construction of chimeric antibodies possessing murine V_H and V_L chains fused onto human constant regions (Morrison et al., 1984; Boulianne et al., 1984). Chimeric IgG antibodies are approximately one-third murine (two subunits each of non-human V_H and V_L) and two-thirds human (two subunits each of human C_H1 , C_L , C_H2 , C_H3).

ReoPro® (abciximab), a chimerized FAb fragment that targets glycoprotein (gp) IIb/IIIa on platelets, was the first chimeric antibody product to be marketed. ReoPro® is first produced by cell culture as a chimeric IgG and then cleaved enzymatically to the FAb. The FAb is re-purified to make the final drug. The first chimeric IgG to be marketed was Rituxan® (rituximab), an anti-CD20 MAb with annual worldwide sales of >\$3 billion. Currently, there are six chimeric antibodies in the world market and two in Phase III clinical trials. With the development of humanization technologies and methods for generating fully human MAbs, both of which appear to decrease immunogenicity issues, fewer chimeric antibodies are being clinically developed.

4.5 Display technologies

In 1985, George Smith (1985) demonstrated that peptides could be fused into protein P3 of the *E. coli* filamentous phage M13 and could be displayed on those phages for binding studies. The key to this and every subsequent display technology is having the binding agent co-purified with the DNA encoding the agent. Thus, if a phage particle with desired binding characteristics to a target protein is co-purified with that protein, the DNA encoding the binding portion is carried along with it within that phage's genome.

In this section, different display technologies that have been used for the generation and screening of naïve libraries as well as for antibody optimization will be presented. While phage display is both the first display procedure developed and also the most widely used, it has limitations, not the least of which has been a complex array of intellectual property claims. Thus a variety of other display methods including yeast (Gai and Wittrup, 2007), bacterial (Harvey et al., 2004), and mammalian cell (Ho et al., 2006; Akamatsu et al., 2007) display have been developed (Hoogenboom, 2005) as well as at least three different approaches for *in vitro* display (c.f. Lipovsek and Plückthun, 2004). All antibody display technologies share common features (Hoogenboom, 2005), including:

- a library of antibody gene sequences;
- linkage of the gene sequence with the displayed antibody;
- a selection (or screening) protocol to identify antibody variants that uniquely bind the target of choice;
- a protocol for amplification of the nucleic acid sequence, either with or without introduction of diversity; and
- production of soluble antibody for functional characterization.

For therapeutic monoclonal antibodies, these types of display systems are typically used for three applications: identification and isolation of target-specific antibodies either from naïve, non-immune, libraries or from libraries derived from immunized animals or genetic engineering of antibodies to optimize them for increased humanness, higher affinity, or improved biophysical characteristics. Display technologies, however, have many uses beyond isolation and optimization of therapeutic antibodies. They have been used to find binders from libraries of non-antibody binding proteins (Strohl and O'Neil, 2012) as well as to find antibody and non-antibody binders to a wide variety of peptides, proteins, small molecules, carbohydrates, and so forth (Bradbury et al., 2011).

4.5.1 Filamentous phage display

As mentioned previously, the display of peptide sequences on the P3 protein of *E. coli* M13 filamentous phage was first described by George Smith in 1985 (Smith, 1985). Phage display quickly advanced to include the display of proteins (Markland et al., 1991), where it made immediate impact as a strong tool for the selection of variants for protein engineering (Bass et al., 1990; Lowman et al., 1991; Markland et al., 1991). To make this a viable approach for monoclonal antibody discovery, however, key technologies such as the efficient cloning of antibody variable genes by PCR (Larrick et al., 1989) and efficient expression of FAb fragments in the *E. coli* periplasm (Better et al., 1988; Plückthun et al., 1990) were both required and developed by the late 1980s (reviewed in Hoogenboom, 2005; Carter, 2006).

Several groups immediately demonstrated the ability to make large antibody libraries in M13 phage (Marks et al., 1991; Barbas et al., 1992; Griffiths et al., 1994; Vaughan et al., 1996). Phage display quickly became a tool not only for the selection of antibody binders but also for the selection of improved binders after mutagenesis of the CDRs and/or framework regions (Gram et al., 1992; Hawkins et al., 1992; Marks et al., 1992a, 1992b). The panning strategies for obtaining binders from large libraries differ from those used for affinity optimization, so the selection process needs to be tailored for the purpose (Hoogenboom, 2005; Dufner et al., 2006). The widespread use of antibody phage display stems from its relative simplicity, durability, and the ability to generate large libraries suitable for both *de novo* discovery and antibody optimization.

The filamentous M13 phage is a positive, single stranded DNA phage that infects only F⁺ *E. coli* strains. M13 is a non-lethal phage, producing

turbid plaques, that contains 11 different proteins encoded by nine genes. The wild-type M13 genome is 6,407 nt long but can be packaged at both much larger and much smaller lengths. The phage coat or external surface is comprised of about 2,700 copies of the 50 residue long protein pVIII (Rodi et al., 2005; Mandecki and Goldman, 2009). The blunt end of the phage is covered by the 32 amino acid long pVII protein, which is buried, and about five copies of the 33 amino acid long protein pIX. The other end is covered by about five copies each of pVI and pIII. Protein pIII, a 406 amino acid long protein, contacts the F-pilus TolA protein to initiate infection and is also the final protein in contact with *E. coli* upon phage release (Mandecki and Goldman, 2009).

Five different M13 surface proteins have been used for display, pIII (N-terminus), pVI (C-terminus), pVII (N-terminus), pVIII (N- or C-terminus), and pIX (N-terminus) (Mandecki and Goldman, 2009). The most widely used protein for display is pIII, or an N-terminally truncated form of pIII. This protein is ideal due to its low copy number (average five copies), its ability to be expressed even when fused with large fragments of foreign proteins, and because incorporation of exogenous proteins into pIII generally does not inhibit the phage cycle (Barbas et al., 1991; Garrard et al., 1991; Rodi et al., 2005; Mandecki and Goldman, 2009). The low copy number of pIII is well suited for selecting high affinity binders (Barbas et al., 1991; Garrard et al., 1991). Development of a phagemid system allowing for monovalent display was an improvement that enabled separation of high affinity binders over high avidity binders (Barbas et al., 1991).

Protein pIX localizes on the blunt end of M13 in an orientation suitable for both protein and peptide display (Gao et al., 1999). Similar to pIII, foreign sequences can be inserted into pIX without disturbing the phage cycle (Gao et al., 1999, 2002) and pIX is present at about five copies per phage, but at the opposite end of the M13 phage. Libraries of scFv (Gao et al., 2002) and FAb (Shi et al., 2010; Tornetta et al., 2010) proteins have been constructed as fusions to pIX demonstrating that pIX can tolerate at least 50 kDa worth of protein insert, similar to pIII. An advantage of pIX over pIII display is that pIX is not involved in the infection process, so phage bound to the target protein can be recovered directly by incubation with host bacteria, which can avoid bias in antibody selections (Tornetta et al., 2010).

There are two general formats used for phage display of antibodies based on where the antibody genes are inserted. In one, the antibody and display protein gene sequences are fused and incorporated directly into the phage genome so that five copies of a pIII-antibody fusion protein are produced.

This format works reasonably well for libraries and general selections but falls short when seeking high affinity binders as these are difficult to distinguish from lower affinity binders that have apparent higher affinity resulting from the avidity effect created by the binding of five copies to the target. In the second format, the display protein-antibody fusion is encoded on a separate plasmid, a phagemid, that is subsequently encapsulated into phage particles upon infection with helper phage. In this case, the number of pIII-fusion proteins in the mature phage is either zero or one (i.e. monovalent display), allowing high affinity binders to be more easily isolated due to the absence of avidity effects (Barbas et al., 1991).

The phage display process for finding antibodies or proteins that bind specifically to a given target has been detailed elsewhere (Rodi et al., 2005; Mandecki and Goldman, 2009) so will only be highlighted here. Selection of phage particles containing target-specific pIII-binding protein (peptide, antibody, etc.) fusions is done in a manner allowing bound phage to be easily separated from unbound phage. Typically the target protein is either immobilized on a surface or beads that can be washed to remove unbound phage. To accomplish this, the target protein is often tagged. When tagged with biotin, the target protein can be bound with very high affinity to streptavidin-coated plates or beads.

After washing away non-specific binders, the high specificity, binding phage particles can be eluted from the target protein or antigen using high ionic strength or low pH buffers. They are then recovered by re-infection or transformation of *E. coli*, producing enriched phage pools. This cycle of bind, wash, elute, and enrich is carried out iteratively and generally enriches significantly for the highest affinity phages after three to five cycles. Individual binding proteins from the enriched pool can then be screened for specific binding by ELISA using phage or soluble antibody fragments generated by suppression or excision of the fused pIII coat protein sequence. The best binders then are usually expressed in the periplasm of *E. coli* (Better et al., 1988; Skerra and Plückthun, 1988) and tested as periplasmic extracts in more specific assays. *E. coli* periplasmic extracts containing antibodies of interest, however, are often too crude for screening in many cell-based functional assays. For these assays the antibodies can be purified using affinity tags such as His or myc tags incorporated into the antibody sequence. Alternatively, the FAb or scFv fragments of interest can be cloned and expressed as IgGs in mammalian or other suitable cells, which can support both cell-based functional assays and *in vivo* studies.

Phage display has many strengths, including robustness, simplicity, and stability of the phage particles. One weakness of phage display is that selection of antibody fragments on phage is biased by expression. Those

fragments that express well as pIII fusion proteins are more likely to dominate the libraries than the highest affinity or best behaved fragments (Dufner et al., 2006; Pavoni et al., 2007). Additionally, some antibodies can be toxic to *E. coli* and accumulate stop codons throughout their coding sequence during the phage display process and be eliminated (Pavoni et al., 2007). Finally, due to phage size and volume, as well as the maximum efficiency of *E. coli* transformation, phage display libraries have been limited to about 10^{11} unique members (Hoogenboom, 2005). Thus it is generally accepted that leads derived from naïve libraries, will need to be affinity matured to make reasonable development candidates.

4.5.2 In vitro display

Due to the phage display library size limitation imposed by the maximum transformation efficiencies of *E. coli*, several efforts have been made since the early 1990s to develop *in vitro* display technologies that would not require *E. coli* transformation. These technologies would allow for the generation and panning of much larger libraries of polypeptide variants ($>10^{13}$) coupled with rapid and efficient sequence diversification using PCR methods.

When Tuerk and Gold (1990) developed methods (termed SELEX, for Systematic Evolution of Ligands by Exponential Enrichment) for generating large libraries of RNA, they suggested those libraries might be used to translate peptides for display on polysomes. Kawasaki (1991) and Mattheakis et al. (1994) independently showed that ribosome display of DNA-encoded libraries could be used to isolate peptides that bound specifically to a target. The key to ribosome display is removing the stop codon from a DNA sequence so that the *in vitro* transcription of that sequence generates a message that remains tethered to the ribosome. The (poly)peptide is translated from the ribosome-tethered message, producing a complex containing both the translated product and the nucleic acid encoding it (Mattheakis et al., 1994; Lipovsek and Plückthun, 2004; Rothe et al., 2006). The library of complexes is then used to pan a target, binders are selected out, the genes are recovered by reverse transcription, and the process is reiterated, usually with the introduction of PCR-generated mutations to enable evolution of the binding capability. The ribosome display process has been described several times (Lipovsek and Plückthun, 2004; Odegrip et al., 2004; Hoogenboom, 2005; He and Khan, 2005; Dufner et al., 2006; Groves et al., 2006; Fukuda et al., 2006; Yan and Xu, 2006; Zahnd et al., 2007).

A second approach called “mRNA display” (Roberts and Szostak, 1997) is also frequently utilized. The key difference in mRNA display compared to ribosome display is covalent attachment of the *in vitro* translated peptide or protein to the mRNA through a puromycin linker (Roberts and Szostak, 1997; Lipovsek and Plückthun, 2004; Rothe et al., 2006). The mRNA and translated product are non-covalently associated in ribosome display. Several other *in vitro* display systems have since been devised and used to display peptides, proteins or single chain antibodies, including *in vitro* virus display (Nemoto et al., 1997), polysome display (Mattheakis et al., 1994), mRNA or covalent display (Roberts and Szostak, 1997), DNA display (Bertschinger and Neri, 2004), and CIS display (Odegrip et al., 2004).

In vitro display of antibodies requires the antibody to be in a form encoded by a single gene. Hence, scFv or domain antibody formats can be used but not FAb fragments. *In vitro* display libraries as large as 10^{12} to 10^{13} have been generated from immunized sources (Hanes et al., 1998) or from synthetic sequences (Hanes et al., 2000) and panned to obtain antibody leads. Additionally, high affinity antibodies have been obtained using *in vitro* display methods for the affinity maturation process (Zahnd et al., 2004). Error-prone PCR (Groves et al., 2006; Fukuda et al., 2006), DNA shuffling (Fukuda et al., 2006), or other non-directed approaches are typically used to generate variants for optimization efforts via *in vitro* display. Thus, for antibodies, the resultant molecules may contain modified amino acids in both the CDRs and framework regions.

Key advantages to the various *in vitro* display technologies include speed, flexibility, library size (which can theoretically reach 10^{15}), protein folding in solution, lack of negative selection pressures (e.g. cell-based expression and secretion), and use of error-prone PCR in each selection round, enhancing the mutation and selection process (Dufner et al., 2006). While potentially very robust, application of *in vitro* display systems has been hindered by technical issues of incomplete translation and RNA instability. Additionally, various intellectual property issues have thus far kept *in vitro* display technologies from being broadly adapted by many groups. Efforts to continually improve and standardize reagents as well as the *in vitro* processes are being made and should translate into greater applicability and use of *in vitro* display technologies in the future (Thom, 2009).

4.5.3 Yeast display

The display of proteins on the surface of yeast cells is another powerful method for connecting a binding protein with the DNA expressing it

(Gai and Wittrup, 2007) and utilizes a process fundamentally similar to other display technologies except that the antibody is bound to a surface protein of yeast. The power of yeast display is primarily in the capability of coupling the display method with multi-color fluorescence-activated cell sorting (FACS), which can measure both antibody expression on the yeast surface and the binding of antigen to it (Boder and Wittrup, 1997; van den Beucken et al., 2003; Gai and Wittrup, 2007). The ability to measure both expression levels and binding precludes false readings of high affinity for certain antibody variants having only enhanced expression (Gai and Wittrup, 2007). Furthermore, the binding properties of the antibody can be discerned directly without the conversion to soluble antibodies due to the versatility of FACS systems (VanAntwerp and Wittrup, 2000). Finally, the eukaryotic protein secretion pathway favors display of only properly folded proteins (Shusta et al., 1999; Rakestraw and Wittrup, 2006), thus potentially selecting against poorly folded antibody variants. The major limitation of yeast display has been library size, which is limited to about 10^7 total variants due to the transformation efficiency of *Saccharomyces cerevisiae*. This limitation was overcome with the development of a FAb display technology using the mating of two different yeast strains, each carrying one of the FAb chains, resulting in a multiplication of the library size (Weaver-Feldhaus et al., 2004; Blaise et al., 2004).

Dane Wittrup and his colleagues developed a yeast display system using *Saccharomyces cerevisiae* for antibody optimization in which a library of mutated scFv fragments was linked to the α -agglutinin Aga2p subunit in a plasmid. The fusion protein is then assembled via a disulfide linkage with a chromosomally expressed Aga1p subunit, leading to cell surface display of the scFv (Boder and Wittrup, 1997; Boder et al., 2000). The incredible power of this yeast display process was demonstrated with the identification of several optimized antibodies having sub-pM affinities by FACS analysis (Boder et al., 2000; Boder and Wittrup, 2000; Gai and Wittrup, 2007). The Aga1p/2p system was modified for efficient FAb display by fusion of the Fd to Aga2p along with co-expression of the light chain from the same vector (van den Beucken et al., 2003; Lin et al., 2003), similar to the approach for FAb display in phage. Yeast FAb display has been used for both antibody optimization and *de novo* discovery of antibodies from a naïve library with $>10^9$ diversity generated through use of the yeast mating system (van den Beucken et al., 2003; Weaver-Feldhaus et al., 2004; Blaise et al., 2004). Thus yeast display has been developed as a great tool for antibody optimization, improving antibody affinity and function (Boder et al., 2000; Rajpal et al., 2005; Zhou et al., 2007), increasing thermal stability and expression properties (Graff et al., 2004), and both mapping epitopes and

redirecting antibody binding to novel epitopes (Siegel et al., 2004; Chao et al., 2004; Weaver-Feldhaus et al., 2005).

Three biotechnology companies, GlycoFi, Adimab, and Alder, have specialized in using yeast to either engineer or produce MABs. Adimab has developed yeast display as a primary tool for sampling their human antibody libraries as well as optimizing MABs for affinity. Primary naïve libraries have been constructed using the yeast mating system approach (Feldhaus et al., 2003; Weaver-Feldhaus et al., 2004), and the optimization strategies are likely to follow those developed by Wittrup and his colleagues (Boder et al., 2000; Boder and Wittrup, 2000; Gai and Wittrup, 2007). GlycoFi and Alder are discussed further in Chapter 18 on cell line development for antibodies.

Finally, one other noteworthy yeast display method is that devised by the Abmaxis team at Merck, in which an adapter-directed system was used to display antibodies on the surface of yeast (Wang et al., 2010a). The advantage of this system is the ability to shuttle antibody sequences between different forms of display including phage and yeast (Wang et al., 2010a).

4.5.4 Mammalian cell display

The most relevant cell type for expression and display of human monoclonal antibodies should theoretically be mammalian cells. However, antibody display by mammalian cells has been hindered by library size limitations and difficulties in obtaining clonality. Nevertheless, several mammalian antibody display systems, all employing some type of FACS sorting similar to that described above for yeast display, have been developed for antibody optimization and discovery from immune and non-immune libraries.

Mammalian antibody display has thus far been used mostly for affinity optimization when small libraries are acceptable or for discovery from smaller, immune-based libraries. The simplest process utilized HEK293 cells transiently transfected with a library of anti-CD22 scFv mutants that were surface displayed and selected for improved affinity by flow cytometry (Ho et al., 2006; Ho and Pastan, 2009). In another example, a Sindbis virus expression system was used in conjunction with flow cytometry to identify antibodies from a scFv library generated from an enriched, antigen-specific B-cell population (Beerli et al., 2008).

One form of mammalian display that has recently been successfully used is the display of full IgGs on the surface of mammalian cells (Akamatsu et al., 2007). An EBV-derived episomal vector for mammalian display of full-length IgG, in combination with magnetic bead and FACS selection, was

utilized to isolate neutralizing antibodies against human and mouse IL-12 from a library constructed from splenocytes of immunized chickens (Akamatsu et al., 2007). Since the eukaryotic secretion system transports the displayed antibody to the mammalian cell surface, this process may favor antibodies with better folding, stability, and solubility features. An Amgen group recently reported the development of a new mammalian antibody display system that displays full-length, functional IgGs (Zhou et al., 2010a). Their system uses recombinase-mediated hot-spot integration to obtain high expression of a single antibody gene per cell coupled with FACS analysis. Use of this system identified full length MABs with 500-fold affinity improvement that should theoretically also have superior folding features.

One mammalian display system that has achieved success in isolating antibodies from non-immune libraries is the vaccinia-based system developed at Vaccinex (Smith and Lauderer, 2009). Secreted antibodies were isolated from libraries constructed using the vaccinia vector system in which independent libraries of heavy and light chains were co-infected at high multiplicity into several independent host cell cultures (Smith and Lauderer, 2009). Antibodies in the culture supernatants were deconvoluted by limiting dilution, yielding at best moderate affinity antibodies that could be further matured by mutagenesis using the same system. A variation of the vaccinia vector system that incorporated surface IgG display and selection using magnetic bead sorting and flow cytometry was also described for antibody optimization (Smith and Lauderer, 2009).

4.5.5 Bacterial display

In theory, surface display of antibodies in bacteria would seem optimal due to high transformation efficiency and the ability to express antibody fragments and even intact, non-glycosylated full-length IgG (Van Blarcom and Harvey, 2009; Mazor et al., 2007, 2009, 2010). With the possibility to select top binders by flow cytometry (Harvey et al., 2004; Van Blarcom and Harvey, 2009), this system would seem ideal. Very few bacterial display systems, however, have proven sufficiently robust to support antibody engineering for a range of antigens, and even fewer have been used successfully to isolate antibodies from naïve libraries. The primary reason is that bacterial cell walls pose additional barriers to expression, secretion, and display on their outer surfaces.

The external peptidoglycan layer of Gram-positive bacteria poses a significantly more complex environment in which to display proteins.

While display protocols have been developed for *Staphylococcus carnosus* (Löfblom et al., 2007; Rockberg et al., 2008; Rockberg et al., 2010; Löfblom, 2011) that have been coupled with FACS analysis in a manner similar to that described above for yeast, this system has not been used widely, perhaps because it is not robust enough for antibody display. This system has, however, been used for affinity ranking of a limited number of protein mutants (Löfblom et al., 2007; Löfblom, 2011) as well as epitope mapping of antibodies against HER2, ephrin-B3, and SATB2 using displayed peptide libraries (Rockberg et al., 2008; Rockberg et al., 2010). With respect to Gram-negative bacteria, the focus has been on *E. coli*, in which transformation is remarkably efficient, typically about 10^7 per μg of DNA (Fregel et al., 2008). External to the cytoplasmic membrane of Gram-negative bacteria are a thin peptidoglycan layer and an outer membrane containing lipopolysaccharide.

Thus far, the most successful bacterial display system is the APEx (for “Anchored Periplasmic Expression”) system first described by Harvey et al. (2004). In the early versions of this system, scFvs were displayed on the cytoplasmic membrane of *E. coli* by fusion either to a lipoprotein A fragment or to the bacteriophage gene 3 coat protein (pIII) (Harvey et al., 2004). Because antibodies are anchored to the cytoplasmic membrane in this display method, the *E. coli* outer membrane must be permeabilized via conversion to sphaeroplasts to allow for the passage of large protein targets into the periplasm. The sphaeroplasts containing the displayed scFvs bound to fluorescently labeled target proteins are then sorted using a FACS system. Permeabilization results in loss of viability, however, requiring recovery of the DNA by PCR rather than by growth of the bacterium. Despite these limitations, scFvs of several antibodies have been affinity optimized using this procedure (Harvey et al., 2004, 2006; Van Blarcom and Harvey, 2009). In a more recent version of the APEx system, full-length, aglycosylated IgGs are expressed, secreted, and then displayed via capture on a Fc binding domain fused to a lipoprotein A fragment bound to the cytoplasmic membrane (Mazor et al., 2007, 2009, 2010). Mazor et al. (2007) used this format to isolate antibodies against *Bacillus anthracis* protective antigen from immune libraries generated in mice with nanomolar K_D affinity. They termed antibodies isolated by this method “E-clonal” antibodies (Mazor et al., 2007; Mazor et al., 2010). In the most recent evolution of this technology, Mazor et al. (2010) demonstrated the display of full IgGs on the surface of filamentous phages as a method to “pre-screen” a library and reduce its size to a level more easily analyzed by FACS upon bacterial display of the pre-screened library.

4.5.6 Deep sequencing approaches to antibody discovery

With the recent advent of next-generation sequencing technologies capable of sequencing thousands to millions of DNA fragments in parallel (Fischer, 2011), it is possible to sequence millions of antibody clones derived from antibody libraries (Ravn et al., 2010) or directly from lymphocytes (Reddy et al., 2010). The potential advantages of this approach for discovering antibodies are that a rich trove of sequence-activity-relationships (SeqAR) can be obtained, helping to drive affinity and biophysical optimization strategies, and that a deeper understanding of antibody development and structure/activity relationships can be gained (Fischer, 2011). “Hits” are identified as high frequency sequences in deep sequencing efforts. This approach may also eliminate the need for screening, which can remove screen-based biases that may mask valuable leads (Fischer, 2011). Finally, deep sequencing approaches may allow for a more significant understanding of human antibody and B cell maturation, possibly leading to discovery of antibody leads by better mining of the human repertoire identified directly from lymphocytes of human donors.

Currently there are three primary next-generation sequencing approaches: the 454 platform, the Illumina/Solexa platform, and the Pacific Biosciences (PACBio) platform. The 454 pyrosequencing platform can generate sequences long enough to get information on the CDRs and much of the framework regions but is limited in general to <100,000 genes. The Illumina platform can deliver up to a million different reads but with each read being only about 100 bp, which is too short to be useful in an antibody discovery program (Fischer, 2011). The 454 platform, however, provides tens of thousands of sequence reads and generates reads long enough to cover an entire V-region. Weinstein et al. (2009) utilized 454 high throughput sequencing to analyze the antibody heavy chain repertoire of 14 zebrafish, obtaining between 28,000 and 112,000 useful sequences per fish, resulting in a significantly greater understanding of antibody development in that organism. Each zebrafish was calculated to have approximately 5,000–6,000 unique heavy chain sequences (Weinstein et al., 2009). The collection of all antibodies in an animal, such as the 5,000 unique heavy chains found in the zebrafish, has been termed the “antibodyome” by Dimitrov (2010).

Third-generation deep sequencing methodologies are just now becoming available as this book goes to press. The most interesting of these may be the PACBio platform, which is based on real-time sequencing of individual nucleotides using a fluorescence readout. This platform can provide both the numbers and sequence lengths desired for deep sequencing of antibody repertoires but currently is very expensive, requires substantial set-up time,

and has higher than desired error rates. Nevertheless, third generation sequencing is anticipated to be more the norm than the exception within a few years.

Certainly, much greater use of these platforms is expected in the future of antibody discovery from varied sources. Deep sequencing has the potential to have a revolutionary impact on antibody discovery. It is not out of the question that many, if not most, antibodies discovered in 2015 and beyond will have been discovered through this type of approach. Companies such as X-Body already base their antibody discovery efforts on deep sequencing. The promise of deep-sequencing approaches is the potential to find antibodies from libraries or B cells that would have been lost in the screening or hybridoma generation process. These losses are estimated to be as high as 80% of the potential binders in some cases, suggesting that deep sequencing could identify better antibodies against a target than are found by traditional methods. Additionally, families of sequences provide built-in sequence-activity relationships that can help with affinity and developability optimization.

4.6 Maturity timelines for biologics technologies

As with any new pharmaceutical industry technology, there is a significant lag between the introduction of a new biotechnology-based technology and its incorporation into new approved drugs. However, in the area of monoclonal antibodies and Fc fusion proteins, this time lag is remarkably short. Below are a few examples of how biotechnology has moved from lab to market in the biologics field. Remarkably, the average time from the first major public description of a new technology and the first example of that technology to reach the marketplace (~11 years – see Table 4.1) is actually shorter than the average time to develop a drug from initiation of discovery (13–14 years – see Table 2.1, Chapter 2). Lonberg (2005) and Strohl (2009a) have shown similar technology timeline progressions.

Examples of technology maturing into marketed biologic products range widely from the initial cloning of genes to antibodies with modified Fc functionality to reduce the risk of immunological complications (Table 4.1). Stan Cohen, Herb Boyer, and colleagues first demonstrated the splicing of DNA using restriction endonucleases in 1974 (Cohen et al., 1974). Genentech immediately utilized this new technology to generate recombinant forms of human proteins such as insulin that were already being sourced from livestock animals. They were able to achieve FDA approval in 1982 for Humulin®, recombinant insulin, requiring only eight

Table 4.1 Time required for recombinant protein technologies to progress from initial discovery to commercialization

Technology	Discovery or first use	First marketed drug from technology	Time from discovery to commercialization
Use of PEGylation to extend half-life of proteins	Abuchowski et al. (1977a, 1977b)	FDA approval of Adagen® in 1990	13 years
Recombinant DNA technology	Cohen, Boyer et al. (1974)	FDA approval of Humulin® in 1982	8 years
Hybridoma technology for generation of monoclonal antibodies	Köhler and Milstein (1975)	FDA approval of Orthoclone OKT3® in 1986	11 years
Chimerization of antibodies	Morrison et al. (1984)	FDA approval of ReoPro® in 1994	10 years
Humanization	Jones et al. (1986)	FDA approval of Zenapax® in 1997	11 years
Fc fusion proteins ("immunoadhesins")	Capon et al. (1989)	FDA approval of Enbrel® in 1998	9 years
Human MAbs from phage displayed libraries	McCafferty et al. (1990)	FDA approval of Humira® in 2002	12 years
Human MAbs from transgenic mice	Lonberg et al. (1994); Green et al. (1994)	FDA approval of Vectibix® in 2006	12 years
Antibodies with modified, muted Fc function (first OKT3-Ala-Ala mutant)	Alegre et al. (1994)	FDA approval of Soliris® in 2007	13 years
Average time from publication on new technology to marketed biologic			~11 years

years to progress from technology discovery to the market. Eleven years after Köhler and Milstein's seminal paper on the generation of hybridomas and MAbs (Köhler and Milstein, 1975) Orthoclone OKT3® became the first murine antibody to be approved in 1986. Abe Abuchowski first published the use of protein PEGylation (PEG, polyethyleneglycol) as

a method to extend the half-life of therapeutic proteins in 1977 (Abuchowski et al., 1977a, 1977b). He subsequently co-founded the biotechnology company Enzon in 1982 to commercialize protein PEGylation. The first commercial PEGylated protein product was Adagen® (PEGylated adenosine deaminase for severe combined immunodeficiency (SCID)), which was approved by the FDA in 1990 and marketed by Enzon.

The generation of chimeric antibodies was first described by Sheri Morrison in 1984 (Morrison et al., 1984; Boulianne et al., 1984). Ten years later the FDA approved ReoPro®, the first chimeric antibody product. The FDA approved Zenapax®, the first humanized antibody, in 1997, 11 years after the first report on humanization of chimeric antibodies by CDR grafting (Jones et al., 1986). McCafferty et al. (1990) initially documented the construction of phage-displayed human antibody libraries in 1990 and in 2002, Humira® became the first approved antibody from such a library. Similarly, Vectibix®, the first antibody from a transgenic humanized mouse, was approved in 2006 by the FDA twelve years after the generation of functional transgenic humanized mice producing human IgGs (Lonberg et al., 1994; Green et al., 1994).

Alegre et al. (1994) generated and tested OKT3 Ala-Ala in 1994. This was the first detailed characterization of an Fc-muted antibody (in which Fc functionality was deliberately reduced to impair interaction with the immune system). In 2007, Soliris® became the first approved antibody with a modified Fc (Soliris® is an IgG_{2/4} hybrid Fc MAb; Rother et al., 2007). The hybrid IgG_{2/4} Fc was used in Soliris® to reduce the potential for immune system interaction (Rother et al., 2007). In these examples, the key technologies required 8–13 years to be incorporated into FDA-approved therapeutic proteins. Considering the average time of 13–14 years to progress a biologic from target research initiation to US FDA approval (Table 2.1), the uptake and application of new technologies in biologics to marketed drugs has been extraordinarily fast.

Several interesting and important technologies introduced many years ago, however, have not yet been utilized in marketed biologics despite much effort having been expended to translate the technologies into drugs. One prime example of this is the human or humanized bispecific antibody targeting two different antigens. There have been many efforts to produce bispecific antibodies (discussed in Chapter 13) going back to 1985 (Paulus, 1985; Brennan et al., 1985), making the technology more than 25 years old with no resulting marketed drug. Removab® is a marketed drug using bispecific principles but lacks the characteristics desired in most modern bispecific antibody designs (see Chapter 13). Similarly, antibody-directed enzyme prodrug therapy (ADEPT) technology, a cytotoxin-targeting

approach discussed further in Chapter 15, was also first conceptualized in 1985 (Bagshawe, 1990, 2009), but has not produced even a late-stage clinical candidate as of 2011. There are likely other similar examples of potentially important technologies either requiring significantly more time to mature than those listed in Table 4.1 or that may never be fully translated into therapeutic drugs.

Sources of antibody variable chains

Abstract: Natural human antibodies are produced and matured by B cells via a highly organized process. Human B cell-derived antibodies are both a great source of potential antibody therapeutics as well as tools to understand the features that make a good, optimized antibody. Therapeutic antibodies can be derived from a variety of sources such as mice, rats, rabbits, non-human primates, or humans, and then engineered to be as human as possible, using information based on natural human antibodies, the genes that produce them, and even B cell biology. Most antibodies on the market or in late-stage clinical trials are currently derived from mouse hybridomas and then humanized.

Keywords: variable chains, B cell maturation, mouse, rat, rabbit, non-human primate, camelids, human B cells, antibody diversity, human antibody libraries, transgenic mice producing human antibodies, heterohybridomas, polymerase chain reaction (PCR)

5.1 Human antibody gene organization

Human Igs consist of heavy and light chains encoded by 14 genes organized into three gene families, a kappa gene family, a lambda gene family and a family of nine heavy chain genes. The nine heavy chain (IGH) genes define five human classes (IgA, IgD, IgE, IgG, IgM) and six subclasses (IgG₁, IgG₂, IgG₃, IgG₄ of IgG, and IgA₁ and IgA₂ of IgA) of human Igs. “Isotype” is defined as the antigenic determinant, or today, by amino acid sequence differentiation, created by the combination of a particular heavy chain class or subclass of Ig with one of the light chain genes kappa (κ) or lambda (λ). “Allotypes” (see Chapter 9), defined as allelic variants within the constant regions that are inherited in a Mendelian fashion, are known to exist for six of the heavy chain loci as well as the κ locus. “Idiotypes” are defined by the unique combination of V_H and V_L which characterize each particular Ig combining site (see Chapter 3).

The variable chains of antibodies essentially define the specificity, affinity, and efficacy of all antibodies, including therapeutic MAbs. Thus an understanding of how the variable chains are organized, expressed, constructed and optimized *in vivo* can provide a sound basis for the engineering of them in the laboratory. Mammals build a diverse repertoire of pre-immune functional heavy chains from a combination of choices of IGHV, IGHD, and IGHJ genes (Marchalonis et al., 1998; Hendricks et al., 2010), although the diversity is very different between species. The V genes, which consist of V_H (heavy chain loci), V_κ , and V_λ (both light chain loci), are typically grouped into families based upon sequence similarity (Schroeder, 2006; Zhu and Yu, 2009).

The human IGH genes, which are found on the long arm of human chromosome 14q32.33 near the telomere, span about 1,250 kbp of DNA. There are a total of 170–176 IGH (immunoglobulin heavy chain) genes, of which 76–84 are thought to be functional (<http://www.imgt.org/LocusView/docs/humanIGHlocus.pl>). The functional genes, i.e. those that can be incorporated into functional Ig proteins, include 38–46 IGHV genes (39 is the number given by many sources) that fall into 6–7 families based on sequence similarity, 23 IGHD genes, 6 IGHJ genes, and 9 IGHC genes. The IGHV genes, which are clustered at the 5' end of the IGH locus, encode the heavy chain variable domain containing framework region 1 (FR1), CDR-1, FR2, CDR-2, and FR3 of the antibody. Downstream of the IGHV (V, for Variable) genes, in order from 5' to 3', are the IGHD (D, for Diversity) gene cluster, the IGHJ (J, for Joining) gene cluster, and finally, the large cluster of 9 IGHC (C, for constant) genes in order: C_μ , C_δ , $C_\gamma3$, $C_\gamma1$, $C\alpha1$, $C_\gamma2$, $C_\gamma4$, C_ϵ , and $C\alpha2$. The IGHC genes encode all of the constant regions that could potentially be incorporated into an antibody.

Each V_H and J_H gene segment in the cluster has an associated “double-turn” recombination signal sequence (2-turn RSS) which contains a highly conserved heptameric sequence followed by a spacer of 23 nucleotides and a conserved nonameric sequence. The spacer size results in two full turns of the DNA that puts the heptamer and nonamer on the same face. Each D_H gene is flanked on each side by a 1-turn RSS in which a spacer of 12-nt yields a single turn between the heptamer and nonamer sequences that line up on the same face of the DNA. The 2-turn RSS sequences cannot recombine with other 2-turn RSS sequences, which eliminates the possibility of VJ combinations (Schroeder and Cavacini, 2010), as will be discussed below.

The κ locus, which spans 1,820 kilobases, is located on the short arm of chromosome at band 2p11.2. The locus contains a total of 82 genes in two large clusters (<http://www.imgt.org/IMGTrepertoire/index.php?section=LocusGenes&repertoire=locus&species=human&group=IGK>), including 76 IGKV gene segments, 5 IGKJ segments, and a single unique IGKC gene,

encoding the kappa constant domain. From 5' to 3', the κ locus contains the two large clusters of IGKV genes, followed by a cluster of five IGKJ genes (IGKJ1-5), which is followed by the gene encoding the constant domain (<http://www.imgt.org/IMGTrepertoire/index.php?section=LocusGenes&repertoire=locus&species=human&group=IGK>). Each IGKV gene encodes FR1, CDR-1, FR2, CDR-2, FR3, and the amino terminus of CDR-3. The IGKJ sequences encode the carboxy-terminus of CDR-3 as well as FR4. Approximately a third of the IGKV genes contain frameshift mutations or stop codons that prevent them from forming functional proteins (Schroeder and Cavacini, 2010). Only about 31 to 36 of the IGKV genes, which fall into six sequence-related groups, are considered to produce functional V_{κ} chains.

The λ locus, which is located on the long arm of chromosome at position 22Q11.2, has a very different architecture from the κ locus described above. The λ locus spans 1,050 kbp and has a total of 87–96 genes, including 73–74 IGLV genes, 7–11 IGLJ genes and 7–11 IGLC genes (http://www.imgt.org/IMGTrepertoire/LocusGenes/locusdesc/human/IGL/Hu_IGLdesc.html). Of these, it has been determined that 29–33 of the IGLV genes, which fall into 10 sequence groupings, can produce functional proteins. Additionally, 4–5 of the IGLJ genes and 4–5 of the IGLC genes are thought to be functional. Structurally, the λ locus contains two large, separate clusters of IGLV genes followed by a cluster of IGLJ and IGLC genes in which each IGLJ gene is followed by, and associated with, its cognate IGLC gene. Additionally, the λ locus encodes the pseudo-light chain (Ψ LC) segments $\lambda 5$ and V_{preB} that together complex with μH to form the pre-B cell receptors (pre-BCR; Schroeder and Cavacini, 2010). Expression of the pre-BCR serves as an antigen-independent checkpoint at which functional Ig H-chain rearrangement is monitored for integrity, followed by clonal expansion and progression of development of properly rearranged $Ig\mu^+$ pre-B cells. Thus the pre-BCR checkpoint has been called a “cell-autonomous proliferation switch” (Hendriks and Middendorp, 2004).

5.2 Antibody gene rearrangement and diversity *in vivo*

In humans, the natural process of antibody assembly and maturation has been characterized in great detail, as reviewed recently by Schroeder and Cavacini (2010). The overall, high-level, assembly process for the heavy chain and light chain gene assembly and translation into an IgG antibody sequence is shown in Figure 5.1. The extraordinary diversity of human antibodies comes from the combination of the assembly of different V_H , D_H , and J_H , V_{κ}

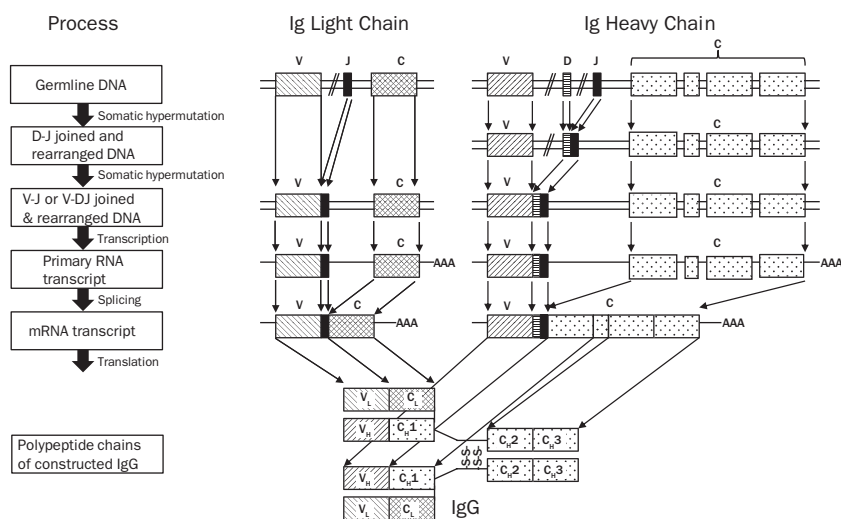


Figure 5.1 Antibody gene and protein assembly *in vivo*

High-level view of antibody gene and protein assembly *in vivo*.

Abbreviations: C: constant chain; D: diversity segment; J: joining chain; V: variable chain. Note that while only single V, J, D, and C regions are shown for simplicity, these are selected from a large number of genes. The major processes leading to the next step in antibody assembly are noted on the left. RNA transcripts are noted by "AAA" designating the poly-A tails.

and J_k segments, or V_k and J_k segments, and pairing of the heavy and light chains, to generate the primary antibody repertoire (Tonegawa, 1983), followed by a series of mutational events to generate greater diversity.

The process of Ig diversification in humans is very complex and hierarchically coupled with the five stages of B cell development in the bone marrow, with each biochemical and genetic stage required for the next step and matched to a cell development stage. This coupling also forms the basis for allelic exclusion that results in one cell producing only one type of antibody. Through interactions with stromal cells, $CD34^+$ stem cells are converted into $CD22^+$, $CD45^{\text{dim}}$, pro-B cells, a process that occurs in the bone marrow and initiates their commitment towards B cell development. The pro-B cells then interact with cells producing IL-7, and through interactions of the IL-7 with its receptor (CD127) on the pro-B cells, the transcriptional machinery that drives Ig gene rearrangement is induced (Perez-Andres et al., 2010). The first stage of the process is the somatic recombination (Tonegawa, 1983) of three gene segments of the heavy chain locus (V_H , D_H , and J_H) to generate a functional variable chain gene. Upon

induction of recombination activating gene 1 (RAG1), RAG2, and terminal deoxynucleotidyl transferase (TdT) in the pro-B cells, the D_H and J_H gene segments are joined first by a RAG-1,2-dependent process (Table 5.1), and TdT directs the random addition of nucleotides in the joining process to increase the diversity (Perez-Andres et al., 2010).

To carry out this process, RAGs 1 and 2, which are expressed at specific stages almost exclusively within developing lymphocytes, introduce a double strand break in the DNA between the gene segment being rearranged and its associated recombination signal sequence (RSS) (Dudley et al., 2005;

Table 5.1 Antibody development *in vivo*

Cell type	Location	Characteristic markers	Antigen dependence	Key Ig development process	Key enzymes involved
Stem cell	Bone marrow	CD34 ⁺	–	–	–
Pro-B cells	Bone marrow	CD22 ⁺ , CD34 ⁺ , CD38 ^{hi} , CD45 ^{dimm} , CD117 ⁺	Independent	DJ _H joining	RAG 1,2; TdT
Pre-BI cells	Bone marrow	CD25 ⁺ , Cμ ⁺ , pre-BCR forms during this stage	Independent	VDJ _H joining	RAG 1,2; TdT
Pre-BII cells	Bone marrow	CD25 ⁺ ; Cμ ⁺	Independent	VJ _L joining	RAG 1,2; TdT
Immature B cell	Peripheral blood	IgM ⁺ , IgD ⁺ , Cμ ⁺ , slg ⁺	Dependent	slgM production	RAG 1,2; TdT
Naïve mature B cell	Bone marrow, serum, spleen, lymph nodes	IgM ⁺ , IgD ⁺ , Cμ ⁺ , slg ⁺ , CD19 ⁺ , CD20 ⁺	Dependent	Unmutated V-regions	–
Germinal center B cell	Lymphoid tissues	slg ⁺ , IgM ⁺ , IgD ⁺ , CD10 ⁺ , class switching	Dependent	SHM, CSR	AID
Memory B cell	Peripheral blood, spleen, lymph nodes	slg ⁺ , CD44 ^{high} , class switching	Dependent	CSR; slgM, slgG, slgA	AID
Plasmablast; plasma cell	Peripheral blood, spleen, lymph nodes	CD38 ^{high}	Dependent	CSR; slgM, slgG, slgA, slgD	AID

Acronyms: BCR: B cell receptor; AID: activation-induced cytidine deaminase; CSR: class switch recombination; Cμ⁺: cytoplasmic μ protein; RAG: recombination activating gene; SHM: somatic hypermutation; slg⁺: surface-anchored Ig expression; TdT: terminal deoxynucleotidyl transferase.

Schroeder and Cavacini, 2010). The mechanistic details for this complex process have been reviewed extensively (Dudley et al., 2005; Soulas-Sprauel et al., 2007; Swanson et al., 2009; Desiderio, 2010; Schroeder and Cavacini, 2010). These breaks are repaired by the common DNA repair processes known as the non-homologous end-joining (NHEJ) process, resulting in precise joins between the RSS ends of the segments but imprecise joins at the coding ends. The coding ends can be further modified by DNA polymerases, nibbled back, or even serve as a substrate for nucleotide addition by TdT. As mentioned above, this step yields additional non-germline encoded diversity at an early stage in the process.

The pro-B cells then express pax-5, which stimulates their conversion into CD19⁺ pre-BI cells (Perez-Andres et al., 2010). At this stage, the IGH DJ joining process is followed by VDJ joining to generate a complete V-chain, which is then spliced onto an Igμ constant chain to form the μH chain (Table 5.1). Cells identified as cytoplasmic Igμ⁺ (also called Cμ⁺) are considered to be pre-BII cells. These cells no longer express the stem-cell marker, CD34. If the rearranged μH chain is able to pair productively with the proteins V_{preB} and λ5, which together comprise the surrogate light chain (SLC, or ΨLC), this complex then forms a pre-B cell receptor (pre-BCR). Expression of the pre-BCR, along with the signaling complex Igβ (CD79,CD79b) on the surface of the pre-BII cells (Table 5.1), results in downregulation of RAG1, RAG2, TdT, and ΨLC generation (Grawunder et al., 1995; Mårtensson et al., 2007), preventing any further H chain recombination, i.e. allelic exclusion. About half of the pre-BII cells express functional pre-BCR, indicating that they have generated a functional VDJ exon that can interact productively with V_{preB} and λ5 to produce a pre-BCR. These pre-BCR⁺ cells interact with the environment to signal for proliferation (Perez-Andres et al., 2011), whereas the other half lacking a functional pre-BCR are subject to apoptosis and, therefore, selection of those cells producing a productive VDJ chain. This stage is considered to be the checkpoint 1 stage in antibody development (Mårtensson et al., 2007).

These pre-BII cells eventually lose the ability to express pre-BCR due to a lack of continued ΨLC synthesis, and upon losing the pre-BCR marker, they cease to proliferate and reactivate the rearrangement machinery including RAG1, RAG2, and TdT (Melchers et al., 2000). This results in the ability of those cells undergoing proliferation to carry out V_L recombination resulting in either a viable V_{Jκ} or V_{Jλ} chain but not both. In humans, rearrangements of the κL allele occur about five times more frequently than rearrangements of the λL allele (Melchers et al., 2000). Generation of a productive κ or λ light chain results in the ability of the cell to express a surface IgM (sIgM), also known as a B cell receptor (BCR), which defines the immature B cell

(Schroeder and Cavacini, 2010). The rearrangement machinery remains active in both the pre-B cell I population as well as in immature B cells, allowing for multiple rounds of light chain rearrangements (Melchers et al., 2000).

Immature B cells that successfully express an sIgM BCR also activate gene expression of the C δ locus, which when combined with alternative splicing, results in the production of IgD possessing the identical V-regions as the BCR. Immature B cells then pass through a negative selection process that results in apoptosis of those B cells making Igs recognizing self antigens, and those that pass this selection process develop into naïve mature B cells (Perez-Andres et al., 2010). IgM⁺ IgD⁺ naïve mature B cells, which comprise about 60–70% of the circulating B lymphocytes (Perez-Andres et al., 2010), then enter the bloodstream and migrate to the periphery where they exist as the majority of B cells in the spleen and secondary lymphoid organs (Schroeder and Cavacini, 2010). Both surface expressed IgD and IgM interact with CD79/CD79b resulting in signaling that helps to control B cell development (Geisberger et al., 2006).

The life span for IgM⁺ IgD⁺ naïve mature B cells, in the absence of antigen stimulation, is only a few days to a week. As originally described in the Burnet and Talmage “clonal selection” theory (Burnet, 1976), those B cells not stimulated by antigen ultimately undergo apoptosis, whereas antigen-stimulated B cells are rescued from the apoptotic pathway by activation. There are two separate antigen stimulation pathways. On one hand, T cell independent stimulation of B cells results in differentiation into short-lived plasma cells that have a limited ability to class-switch. On the other hand, T-dependent antigen stimulation results in a broader array of diversification including somatic hypermutation, affinity maturation, and more extensive class-switching. These T-dependent stimulated cells also differentiate into long-lived memory B cells or plasma cells (Schroeder and Cavacini, 2010). Memory B cells, which have been stimulated by antigen and have undergone significant somatic hypermutation (SHM), make up about 20–30% of all peripheral B lymphocytes (Perez-Andres et al., 2010). Typically about half of these B cells also have undergone class switch recombination (CSR) and produce alternative isotypes, e.g. sIgG, sIgA, on their surface (Perez-Andres et al., 2010). The other half of the memory B cells in circulation only express sIgM and (sometimes) sIgD on their surface (Perez-Andres et al., 2010).

5.3 Sources of antibody diversity

Antibody diversity in humans comes from several stages of Ig development, including both pre-immune repertoire, in which antibodies are developed for

sampling the system for foreign bodies, and the post-immune repertoire, in which antibodies against foreign antigens are selected for and matured. In the pre-immune repertoire, there are four major sources of antibody diversity. The first source of pre-immune diversity is the recombination of V_H , D_H , and J_H chains to build a functional V_H chain, and V_L and V_J (λ or κ) chains to build a functional light chain. To build a functional V_H gene, there is a random assortment in which one of 39 functional V_H genes is coupled with one of 27 functional D genes and six J_H chains. This random assortment of germline encoded genes alone yields greater than 10^4 diversity.

Within the VDJ joining process, however, the junctional diversity created in the recombination process adds infinitely more diversity in four different ways. First, the D genes can be translated in any one of three open reading frames in either direction to yield a total of six possible peptide fragments. Second, during the rearrangement process, a hairpin is formed that when resolved can result in the addition or deletion of N nucleotides (Schroeder and Cavacini, 2010) resulting in added diversity. Third, as mentioned above in the discussion about the VDJ joining mechanism, N nucleotides can be added or deleted as part of the VDJ joining process. In some cases, the coding sequences for several amino acid residues can be lost during the recombination process. Finally, N nucleotides can be added or replaced by the activities of TdT, particularly on either side of the D segment within the VDJ junctions, which make up CDR-H3 in the functional V-region. It has been estimated that these N changes can result in up to $>10^7$ different variations of CDR-H3 (Schroeder and Cavacini, 2010), including CDR lengths ranging from just a few amino acid residues to over 25 (Zemlin et al., 2003) (see Figure 3.6). Combined together, the somatic rearrangements, the combinatorial diversity within chains, the inherent mutagenesis that occurs in the assembly process, and the combination of heavy and light chains result in a potential pre-immune diversity of $>10^{16}$ different antibodies (Schroeder and Cavacini, 2010). Members of this diverse pool of pre-immune immunoglobulins exhibit relatively low affinity binding to given targets (Neuberger and Milstein, 1995) as well as polyreactivity (Schroeder and Cavacini, 2010).

As mentioned above, IgM^+ IgD^+ mature B cells enter the circulation where they may encounter foreign antigens. After exposure of cell-bound IgM to antigen, the antibody genes undergo an affinity maturation process, generating new diversity from which antibodies with higher affinity to the targeted antigen epitope are selected, resulting in more effective binding and elimination of the antigen from circulation during the secondary immune response. Mechanisms for natural affinity maturation of antibodies include somatic hypermutation, which occurs in most mammalian species

and, in certain species such as rabbits and chickens, gene conversion (Maizels, 2005; Zhu and Yu, 2009).

Activation-Induced (Cytidine) Deaminase (AID), a key enzyme expressed in germinal center B cells (Table 5.1), plays a significant role in both somatic hypermutation and class-switch recombination. AID, a single-strand DNA cytidine deaminase, deaminates single-stranded DNA to form dU/dG mismatched base pairs. Repair by uracil DNA glycosylase, which removes the mismatched dU, results in mutation of the synthesis strand. In humans, the affinity maturation process can occur via two different mechanisms (Maizels, 2005). In the first, a mutational hot spot defined by the amino acid motif RGYW (encoded by the sequence: purine-G-pyrimidine-A), which includes the reverse complement of the preferential substrate site for AID (Dörner et al., 1998), is targeted for AID-mediated mutation. The second mutational mechanism utilizes error-prone DNA synthesis leading to basepair mismatching between the template and newly synthesized DNA sequence, resulting in nucleotide changes (Rada et al., 1998; Edry and Melamed, 2007).

These mutational processes, which occur at a rate of ca. 10^{-3} changes per base pair per cell cycle, favor the formation of single-base transitions over transversions at a 3:1 ratio (Betz et al., 1993). Insertions and deletions may also occur, but at a lower rate (Wilson et al., 1998). The mutational frequency of V_H and V_L are similar, both of which follow an exponential distribution resulting in 15–20% of the V regions possessing no amino acid mutations (Tomlinson et al., 1996; Ramirez-Benitez and Almagro, 2001). It has been demonstrated that during the affinity maturation process, the average number of mutations in V_H and V_L are eight and five, respectively (Tomlinson et al., 1996; Ramirez-Benitez and Almagro, 2001; Clark et al., 2006). Although these mutations occur throughout the V domains, approximately half occur in the CDRs making up the antigen binding site, another ca. 33% occur in the framework regions that make up the surface and V_L : V_H interface, and about 17% occur in the core of the V domain (Clark et al., 2006). The antigen binding regions are known to contain mutational hot spots, which explains the generally higher frequency of changes in those residues (Ramirez-Benitez and Almagro, 2001; Clark et al., 2006).

5.4 Class-switch recombination

As mentioned above, nine functional C_H genes lie downstream of the V_H , D_H , and J_H clusters on human chromosome 14q32.33. Each of these nine C_H genes can undergo alternative splicing that can result either in the

production of cell-bound Igs possessing a C-terminal tail that anchors the Ig onto the cell surface, or soluble forms lacking the C-terminal tail. Each functional C_H gene is preceded in the chromosome by a non-translated exon known as the “*I* exon” and repetitive DNA sequences known as the “switch” (Schroeder and Cavacini, 2010). Upon antigen exposure, IgM-producing B cells can respond to cytokines and other signals from T cells or other micro-environmental sources that result in activation of the *I* exon of a particular C_H gene, activating the associated functional gene. This results in a recombination event between the C_μ switch region and the switch region of the activated C_H gene so that the V-regions are fused to a new constant region. This process, called “class switching” or “class switch recombination” (CSR), is a highly ordered, cytokine signal-driven process resulting in Ig isotypes required to clear the foreign antigen, e.g. IgG₁ or IgG₃ for protein antigens, IgG₂ for pathogen polysaccharide antigens, IgA for targeting the mucosal surface, IgE for targeting parasites. More details about the activities of each isotype are described by Strohl (2011) and in Chapter 9.

It has been demonstrated that the enzyme AID, which is primarily expressed in activated B cells found in germinal centers, is required for CSR to occur (Muramatsu et al., 2000). Unchecked or faulty CSR, the mechanism of which is double strand breaks that can lead to genome instability, has been shown to contribute significantly to B cell malignancies (Edry and Melamed, 2007).

5.5 Human variable gene usage

5.5.1 Human V_H , V_κ and V_λ gene expression

A detailed understanding of human V_H and V_L gene construction and use has been very helpful in designing and building synthetic human antibody libraries as well as transgenic mice capable of producing human antibodies. These details also have helped antibody engineers generate and optimize better human antibodies. It turns out that not all human antibody V-genes are expressed equally, leading to a distinct bias of V-genes incorporated into circulating IgGs.

PCR amplification and sequencing of the V_H genes from single B cells of multiple human donors indicate that the V_H families V_H1 , V_H3 , V_H4 , and V_H5 make up most of the correctly assembled and functional V_H segments (Huang et al., 1992; Brezinschek et al., 1998; de Wildt et al., 1999). In one study, for example, the most widely utilized V_H genes (averaged from two individuals) were IGVH3 at ~36%, IGVH1 at ~26%, IGVH4 at ~15%, IGVH5 at ~12%, IGVH2 at ~4%, and IGVH6 at ~3% (Huang et al., 1992).

In another study of only the V_H3 gene family, V_H3 -23, V_H3 -30, and V_H3 -33 were the most widely utilized V_H3 genes at 24%, 16%, and 13%, respectively (Huang et al., 1996). Combined, these data suggest that the single locus, IGHV3-23, accounts for about 9–10% of all functional V_H loci.

In human V_L loci, both κ and λ V regions are derived primarily from families 1, 2, and 3 (Cox et al., 1994; Ignatovich et al., 1997). In terms of the V_κ gene usage, $V_\kappa1$ was used 41% of the time, $V_\kappa3$ 38%, $V_\kappa2$, 10%, and one locus (DPK24) of $V_\kappa4$ 9% (Cox et al., 1994). Gene segment DPK22 (also called $V_\kappa3$ -A27) accounted for 20% of all κ chains expressed (Cox et al., 1994). For the V_λ family, $V_\lambda2$ accounted for about 36–49%, $V_\lambda1$ accounted for another 28–30%, and $V_\lambda3$ another 15–20% (Ignatovich et al., 1997). Very little expression was observed from the other seven V_λ loci (Ignatovich et al., 1997). Within $V_\lambda2$, segment 2a2 was used most predominantly, at 27% (Ignatovich et al., 1997).

Importantly, V_H - V_L gene pairing appears to be a stochastic process in which pairing in both B cells (de Wildt et al., 1999) as well as in recombinant libraries (Glanville et al., 2009) reflect the relative abundance and expression levels of the V_H and V_L gene family members rather than any pre-ordained pairing. As such, the most predominant pairing recovered from a sampling of B cells was $V_\kappa3$ -A27, the highest expressing V_L gene segment, paired with VH3-23, the most highly expressed V_H gene segment (de Wildt et al., 1999).

5.5.2 κ vs λ gene usage

As mentioned above, there are only two major families of light chains, known as V_κ and V_λ . The relative expression of V_κ and V_λ genes is species-dependent. In mice and rabbits, approximately 90–95% of serum Ig contains κ light chains, with λ light chains found only 5–10% of the time (Schroeder, 2006; Mage et al., 2006). On the other hand, the distribution of light chains incorporated into serum Igs by humans is approximately 60% κ and 40% λ (Hood et al., 1967; de Wildt et al., 1999; Farner et al., 1999). These percentages appear to reflect the relative number of V_L genes in each species as shown in Table 5.2.

Interestingly, even within human antibodies, the incorporation of κ or λ chains into an antibody varies quite significantly both with the class of antibody, and even with isotypes within the IgG class (Wingren et al., 2005). The ratio of κ/λ incorporation into human antibodies is (from highest κ/λ ratio to lowest): IgG₄, 8.0/1; IgM, 3.2/1; IgG₁, 2.4/1; IgA₂, 1.6/1; IgA₁, 1.4/1; IgG₃, 1.4/1; IgG₂, 1.1/1; IgD, 0.3/1 (Wingren et al., 2005). This

Table 5.2 Use of genes in formation of serum Igs

Property	Properties of genes in species indicated				
	Human	NHPs	Mouse	Rats	Rabbits
Functional V_H genes	39	45	>150	131	Est'd 100–200
Number of V_H families	7 families	7 families	16 families	13 families	1 family (~200 V_H genes that are ~80% similar)
Mostly highly expressed V_H genes	V_3 , 36% $V_{1/7}$, 26% V_4 , 15% V_5 , 12% V_2 , 4% V_6 , 3%	V_{H3} , 41% V_{H4} , 39% V_{H1} , 14% V_{H5} , 4% V_{H7} , 2%	Nd	Nd	V_{H1} , 100%
Number of J_H genes	6	7	4	5 (4 functional)	5 (J_{H4} and J_{H2} , used 80% and 20%, respectively; others are rarely used)
Number of D_H genes	27	30 (in 6 families)	12–13	21 (14 functional)	12
Number of V_K genes	30	62	90	Nd	Unknown, but large
Number of V_K families	6	3 major	18	Nd	2, V_{K1} and V_{K2} (V_{K1} used primarily)
Usage of V_K chains	60%	Nd	90–95%	Nd	90–95%
Number of J_K genes	5	Nd	4	Nd	Nd
Number of V_λ genes	30–36	52	3	Nd	Very few
Number of V_λ families	11	3 major	nd	Nd	Nd
Usage of V_λ chains	40%	Nd	5–10%	Nd	5–10%
Number of J_λ genes*	4	Nd	2	Nd	nd

Abbreviations: Nd: no data; NHP: non-human primates.

* Each associated with a separate C_λ locus

suggests that during cytokine-mediated class-switching (section 5.4, above), there may be some bias for switching antibodies with κ vs λ light chains.

Historically, most marketed therapeutic MABs have been derived from rodent V-regions isolated from hybridomas so, as expected from the distribution of κ and λ chains used in rodents, most current antibodies sport κ chains (Table 5.2). The notable exception is Bexxar®, which is a murine IgG_{2a} λ . Thus the current marketed antibodies do not truly reflect human usage of light chains. To better reflect human light chain usage in IgGs, human λ genes have been included in *in vitro* display libraries (Marks et al., 1991; Kuroiwa et al., 2000; Söderlind et al., 2000; Rothe et al., 2008) as well as in transgenic mice producing human IgGs (Kuroiwa et al., 2000).

5.6 Variable region selection

5.6.1 Sources of variable regions of current marketed therapeutic MABs

After the generation of mouse hybridomas producing mouse MABs was described by Köhler and Milstein (1975), many academic and industrial labs generated mouse MABs to a wide variety of human antigens. In the early days, many of the MABs obtained were, in fact, IgMs, which were characteristically low affinity and in some cases polyreactive. Others were IgGs that became useful reagents both for studying target biology as well as providing the basis for early MAB drug candidates. One particularly important panel of antibodies was constructed by Patrick Kung and colleagues at Ortho Biotech, a subsidiary of J&J located in Raritan, NJ. Kung and his colleagues generated panels of antibodies against T cells (“OKT” panel), B cells (“OKB”), and myeloid cells (“OKM”) (Kung et al., 1979; J. Giles-Komar, personal communication). One of the early antibodies in this series, OKT3, an IgG that recognized CD3 on T cells, was ultimately developed commercially as a fully mouse antibody produced in the hybridoma cell line from which it was originally isolated. This antibody, named Orthoclone OKT3® (muromonab CD3), became the first MAB to be approved for marketing in the United States. Muromonab CD3 was found to be highly immunogenic in humans (Kimball et al., 1995), eliciting anti-antibody responses in over 80% of patients who received it. In retrospect this is not surprising at all, since muromonab CD3 contained 100% mouse sequences that were foreign to the human immune system. As described in Chapter 6, this led to the process of increasing the humanness of potential commercial antibody candidates via chimerization and eventually via humanization technologies to reduce immune response.

Chimeric mouse-human MAbs are constructed by genetically fusing mouse or rat variable chains onto human constant regions, resulting in antibody that retains about 30–35% of the sequences as murine, with the remainder being human (Morrison et al., 1984). The first marketed chimeric antibody was ReoPro® (abciximab), a FAb fragment of IgG, developed by Centocor (now Janssen Biotech, Inc.) and approved by the FDA in 1994. Because ReoPro® is a FAb fragment, the murine sequences totaled about 50% of the total sequence of the antibody.

Even after chimerization of antibodies became widespread in the late 1980s, it was apparent that immunogenicity would still be an issue. This resulted in the development of humanization technologies by Jones et al. (1986), in which they grafted the CDR loops from the murine antibody into human frameworks to reduce the non-human sequences to less than 10% of the total sequences of an IgG. The first humanized MAb to be approved for marketing was Zenapax® (daclizumab), which was developed by Protein Design Labs to target CD25 (IL-2R α) and approved for marketing in 1997 by the FDA.

5.6.2 Criteria for selection of variable regions

Since it was demonstrated by the 1990s that mouse antibodies could be modified via chimerization and humanization and optimized to generate potential drug candidates, V-genes from a variety of sources have been isolated, chimerized onto human constant chains, and humanized to generate development candidates. Several key factors need to be considered in making the choice of variable chains for a potential therapeutic antibody. The V-regions ultimately are key determinants of the biochemical and biophysical properties of the MAb, including its ability to fold properly, pI, solubility, biochemical stability, and lack of aggregation. Fundamentally, there are four criteria for the source of a successful, non-human starting V-region:

1. The V-regions need to bind to the antigen of choice at an epitope that yields the functionality desired and with enough affinity (e.g. at least ~1–20 nM) to be a reasonable starting point for affinity optimization.
2. They need to possess fundamentally “developable” properties (see Chapter 16 for more details) of solubility, lack of aggregation, and chemical/biochemical stability.
3. They need to be able to be chimerized on a human constant framework, humanized and affinity-optimized without significant detrimental changes in binding or biophysical properties.

4. The process of humanization of the sourced non-human V-region needs to result in an antibody that is as human-like as possible to reduce the possibility of immunogenicity (see Chapter 16 for more detail).

Variable and constant chain amino acid sequences are specific for the species from which they are derived, and are usually recognized as foreign proteins by the immune systems of other species. Thus, when non-human variable chains are incorporated into an antibody intended as a human therapeutic, modifications need to be made to the sequences of those foreign amino acid sequences in order to make them resemble human V-regions as much as possible, i.e. “humanization”, to reduce the potential for humans to mount an immune response against them.

5.6.3 Sources of variable region genes

Fundamentally, over the past two decades, three primary sources of V-chains have been used to construct therapeutic MAbs: (1) immunized rodents, usually either BALB/c mice or transgenic mice producing human antibodies; (2) phage display libraries of human antibodies; and (3) B cells of immunized humans (Lanzavecchia et al., 2007). With the advances in molecular biology and protein engineering techniques over the past two decades, the species from which V_H and V_L genes are sourced for a therapeutic MAb candidate has become less critical because, in most cases, they can be engineered to closely resemble human sequences, thus minimizing potential immune responses against them. The choice of species from which to obtain a V-region is often based upon technical and practical considerations dictated by the available technologies. As such, most of the antibodies on the market and in late-stage clinical trials today are derived from mouse V-genes obtained by traditional hybridoma technology (Table 5.3).

Hybridoma-based systems have been developed for a number of species (e.g. mice, rats, and now rabbits), display-based libraries can be made from any immunized animal (Popkov et al., 2003; Som mavilla et al., 2010), and the direct cloning of Ig genes from the B lymphocytes of immunized animals or humans (Babcook et al., 2006; Tiller et al., 2009; Schüller et al., 2010) can be adapted, in principle, to any species. Beyond strictly technical reasons, choice of species may depend on factors such as the desire for certain species cross-reactivity, or the ability of some species to generate high affinity antibodies to difficult targets such as conjugated peptides or polysaccharides. Including domain antibodies (see Chapter 12), V-regions for potential therapeutic antibody candidates have been sourced from mice, rats, humans, non-human primates, rats, pigs, rabbits, camelids, sharks, and chickens.

Table 5.3 Source of V-regions for marketed and late-stage clinical candidate MAbs*

Source of V-regions	Format	Marketed MAbs	Phase III MAbs	Total
Mouse	100% murine	3	3	6
	Chimeric	7	4	11
	Humanized	11	13	24
Rat	Humanized	0	1	1
Mouse and rat	Mouse-rat hybrid	1	0	1
Rabbit	Humanized	0	0	0
Non-human primates	Primatized	0	1	1
Human	Human antibody libraries displayed on phage	2	5	7
	Transgenic mice producing human antibodies	8	5	13
Mammalian proteins	Fc fusion proteins	7	5	11
Totals		39	36	75

* Data as of August 18, 2011.

5.7 Variable genes from non-human species

5.7.1 Rodents

The mouse has a V_H repertoire of more than 150 V_H genes (depending on the strain) distributed among 15 families, making it both larger and more diverse than the human V_H gene repertoire (Schroeder, 2006). Similar to the mouse, rats have 131 functional V_H genes in 13 families (Hendricks et al., 2010). The V_H families in the mouse and rat tend to be located in clusters of related sequences, unlike humans in which family members are generally dispersed. As mentioned earlier in this chapter, the V_H region is derived from the recombination of V_H genes with D_H and J_H genes. Mice and rats possess 13 and 14 functional D_H genes, respectively, and both mice and rats have four functional J_H genes (Hendricks et al., 2010; Table 5.2).

Mice have approximately 90 functional V_K genes clustered in 18 families as well as four functional J_K genes. In sharp contrast with human and non-human primate λ gene loci, the mouse λ locus has only three V_λ genes

and two C_λ segments, each with a unique J gene (Selsing et al., 1982). The percentage of circulating IgGs in mice possessing λ light chains is approximately 5–10%, which correlates with the substantially reduced number of λ loci in those species.

Of the 32 MAbs currently on the market in the United States, 22 possess V-regions derived from mouse or rat hybridomas (Table 5.3). Mouse V-regions are the source of most of the currently marketed and clinical candidate MAbs, either as 100% murine MAbs, chimerized MAbs, or humanized MAbs (Table 5.3). Murine hybridoma technology is quite robust, and is still used as a major source of V-region genes, as evidenced by the number of Phase I/Phase II candidates that have V-regions originating from mice. The standard mouse myeloma cell lines used as fusion partners to generate mouse hybridomas are SP2/0 and FO. Similar technology to generate rat hybridomas also has been developed, typically using the Y/0 cell line as the fusion partner (Groves and Morris, 2000; Sib  ril et al., 2006). The generation of rat hybridomas is especially useful for cases in which it is desirable to generate MAbs against a human target that also cross-reacts with the mouse target. Rat hybridomas also are useful when mice are either unable to mount a strong immune response to an antigen, or conversely, when immunization of mice with the antigen results in toxicity or death to the mice.

Mice and rats typically can induce an immune response to a broad range of human antigens, even if there is significant homology between the rodent and human proteins. On the other hand, mice and rats usually do not induce very strong immune responses to carbohydrate antigens, haptens, or peptides, unless they are conjugated to a protein carrier (Imanishi and M  kel  , 1974). The reason for this is that a strong humoral response requires stimulation by Th2 cells which are induced by protein antigens (Mosmann and Coffman, 1989).

According to the need for T cell help, the immune response is generally characterized as either T-independent (TI) or T-dependent (TD) with the TD response further characterized as either Th1 or Th2. Whether a Th1 or Th2 response is elicited upon challenge with antigen is highly dependent upon the strain of the mouse. BALB/c mice mount a strong Th2 based response to protein antigens and therefore are the typical strain of mouse used to generate hybridomas (Hsieh et al, 1995). Moreover, Th2-driven BALB/c mice are known to produce an IgG₁ response to antigen, whereas Th1-driven C57BL/6 mice generate predominantly an IgG_{2a} response to antigens (Slack, 1987). As mentioned previously, mouse or rat V-genes also may be obtained from the generation of phage-display libraries or by direct cloning of the genes from B cells of the immunized rodents.

5.7.2 Rabbits

Rabbits have only a single family of V_H genes but it includes an estimated 200 V_H members (Mage et al., 2006; Zhu and Yu, 2009; Yu, 2010). Additionally, rabbits possess 12 D_H genes and five J_H genes, although only a small number of the available D_H and J_H segments are used in the rearrangements (Table 5.2). For example, J_H4 and J_H2 are used 80% and 20% of the time, with the other J_H genes utilized only rarely (Zhu and Yu, 2009). Because rabbits only possess one family of V_H genes, they generate only limited V_H gene diversity (Zhu and Yu, 2009; Yu, 2010). Instead, most of the IgH gene diversification in rabbits occurs through gene conversion with closely related V_H donor genes, and use of somatic hypermutation not only in the germinal centers, but also in secondary lymphoid tissues such as the spleen and lymph nodes (Zhu and Yu, 2009). It is known that chickens, similar to rabbits, also utilize gene conversion as an additional mechanism for generating diversity and increasing antibody affinity (Maizels, 2005).

There are two V_κ families in rabbits, $V_\kappa1$, which is widely used, and $V_\kappa2$. The $V_\kappa1$ family members possess an extra disulfide bond which links V_κ Cys₈₀ with C_κ Cys₁₇₁ (Popkov et al., 2003); this extra disulfide bond is not found in $V_\kappa2$ or V_λ . The total number of V_κ genes in rabbits is not yet known, although it is presumed to be quite large (Mage et al., 2006). Significant efforts are now underway to help define the entire κ gene repertoire of rabbits (IMGT database, <http://www.imgt.org/>). In contrast to the mechanisms used to generate rabbit heavy-chain diversity, there is significant sequence diversity among the rabbit V_κ genes, and the $V_\kappa J_\kappa$ rearrangements contribute significantly to rabbit light-chain diversity. Additionally, the resulting functional V_κ genes are further diversified by somatic hypermutation and gene conversions (Zhu and Yu, 2009).

The exact number of λ loci in rabbits has been difficult to determine because the λ gene organization differs among different rabbit strains. Nevertheless, rabbits are thought to possess very few V_λ genes and these appear to be rearranged infrequently, leading to only approximately 10–15% of rabbit serum Igs containing λ -light chains (Zhu and Yu, 2009).

Rabbits are known to respond well to antigens such as peptides and phospho-proteins that typically do not provoke strong immune responses in mice and rats. This differential immune response is thought to be due to the different mechanisms, described above, used by rabbits to rearrange and mature the V region of their antibodies. Additional potential advantages of rabbit MAbs include their typically high affinities, broad epitope distribution, and the possibility of making the same antibodies against both

rodent and human targets so surrogate MABs would not need to be made for pharmacological and safety assessments (Zhu and Yu, 2009).

Rabbit polyclonal antibodies have been used for decades as reagents due to their general high affinity and ability to bind a wide variety of targets. For many years, the development of rabbit hybridoma technology was hampered by the absence of a successful fusion partner. While rabbit hybridomas were first made and characterized in 1995 using the 240-E1 myeloma-like plasmacytoma cell line as the fusion partner (Spieker-Polet et al., 1995), the hybridomas produced using that line proved to be unstable. Recently, Pytela et al. (2009) made derivatives of 240-E1, particularly 240E-W2, which resulted in more stable rabbit hybridomas (Zhu and Yu, 2009). B cells derived from immunized rabbits may also be used to obtain V region genes using phage display (Popkov et al., 2003) or direct cloning approaches. Phage displayed libraries containing rabbit antibody genes also have been made for panning (Rader, 2009).

Several approaches have been used to humanize rabbit V regions (Steinberger et al., 2000; Rader et al., 2000; Zhu and Yu, 2009; Yu et al., 2010; Borrás et al., 2010), including a new approach called “mutational lineage guided humanization” (Zhu and Yu, 2009; Yu, 2010; Yu et al., 2010). There are at least seven different humanized rabbit antibodies today, so this looks to be an increasingly common approach to obtain therapeutic candidates.

In March 2011, an Investigational New Drug (IND) application was filed by Apexigen, Inc. and Simcere Pharmaceutical Group with the China State Food and Drug Administration (SFDA) for Phase I clinical trials on the rabbit humanized anti-VEGF MAb (Yu et al., 2010), APX003 (also known as BD0801), as a potential cancer therapeutic, demonstrating that rabbit-sourced MABs are now serious candidates for development.

5.7.3 Camelids

Camelids, including both old-world camels and new-world llamas, possess an unusual antibody repertoire (De Genst et al., 2006). They possess normal H_2L_2 -type IgG_1 -like antibodies, as well as IgM , IgD , IgE , and IgA antibodies similar to other mammals. What makes them unique, however, was the discovery that they also possess a single heavy-chain antibody repertoire in which each paired chain consists of a V_{HH} domain attached via a hinge to C_{H2} and C_{H3} domains (Hamers-Casterman et al., 1993). Thus far, five γ -chain genes have been found in camels, two of which encode normal $IgGs$ and three of which code for production of heavy-chain

antibodies (HCABs) (Conrath et al., 2003). These novel γ -type HCABs have given rise to a new line of domain antibodies, several of which are currently being tested in clinical trials, as described in Chapter 12.

5.7.4 Non-human primates

The old-world monkeys, *Macaca mulatta* (rhesus macaque) and *Macaca fascicularis* (cynomolgus macaque), are used widely in research due to their overall similarity to humans. Considering this fact, it is noteworthy that macaques have not been used widely as sources of antibody V-genes. The reasons for this are mostly likely due to patents obtained by the company, IDEC, on the use of macaque genes as sources for “primatized” antibodies (Newman et al., 1992; Newman et al., 1997).

Cynomolgus monkeys were shown to have a total of 70 antibody V_H genes, 45 of which have thus far been deemed to be complete, functional genes (Thullier et al., 2010a). Expression of the cynomolgus monkey V_H genes by family is: IGVH3, 41%; IGVH4, 39%, IGVH1, 14%, IGVH5, 4%, and IGVH7, 2% (Druar et al., 2005). The V_H genes have overall ca. 84–97% sequence similarity to their closest human homologues (Thullier et al., 2010a), and 82.3–97.5% similarity if only the framework regions are compared (Thullier et al., 2010a).

Cynomolgus monkeys also contain six J gene segments, five of which are similar to human J-chains, and a sixth known as the J5 β , which appears to be unique to macaques (Druar et al., 2005). There appears to be no NHP equivalent of human J_H2 (Druar et al., 2005). Rhesus monkeys also were shown to contain 30 D_H genes in six families (Link et al., 2002). Interestingly, while the overall germline D_H and J_H sequences of rhesus monkeys are >90% similar to those of humans, it was demonstrated that there are significant differences from humans in the IgM CDR-H3 sequences of adult rhesus monkeys (Link et al., 2005). The adult rhesus monkeys had overall shorter CDR-H3 sequences than humans, whereas the chimpanzee had longer overall CDR-H3 sequences than humans, indicating significant differences among these primate species in N addition and terminal nucleotide loss during the antibody gene rearrangement processes (Link et al., 2005). In humans and chimpanzees, mean CDR-H3 length increased in the development from fetus to adult stage; the macaque CDR-H3 lengths, however, remained the same lengths (~14 residues long) in both fetuses and adults (Link et al., 2005).

Using PCR gene amplification and recovery approaches, Howard et al. (2005a) found that rhesus monkeys have 62 IGKV genes in three subgroups,

IGKV1 (28 genes), IGKV2 (22 genes), and IGKV3 (12 genes) as compared with six human IGKV subgroups. No equivalents of human IGKV families 4–6 were found (Howard et al., 2005a). Using the same approach as with the IGKV genes, Howard et al. (2005b) showed that rhesus monkeys have 52 IGLV genes in four subgroups, IGLV1 (10 genes), IGLV3 (21 genes), IGLV4 (5 genes), and IGLV5 (7 genes) as compared to 11 human IGLV subgroups. No equivalents of human IGLV families 2 and 6–11 were found (Howard et al., 2005b).

There has been some significant discussion about the similarity index between non-human primate and human antibody genes. Overall, macaque antibody V-genes are approximately 84–97% identical to their human counterparts and they have a “germlinity” index of 90% whereas human to human antibody gene comparison gives a germlinity score of about 95% (Pelat and Thullier, 2009). In fact, Pelat et al. (2008) utilized the superhumanization approach of Tan et al. (2002) to humanize a non-human primate antibody against anthrax toxin so that they achieved a germlinity score >97.8% while retaining functional activity and potency. This distinction appears to be important because previous patents have pointed out that non-human primate antibody genes are “indistinguishable” from human antibody genes (Newman et al., 1992; Newman et al., 1997).

IDEC (now Biogen/IDEC) led the path forward for the concept of using non-human primate V-regions in therapeutic antibodies. After immunization of NHPs, they fused the NHP V-regions onto human constant regions to make “primatized” monoclonal antibodies. Two of IDEC’s primatized antibodies are in advanced clinical trials. Galiximab (IDEC-114), an IgG₁λ primatized MAb which targets CD80 (B7-1) with indication for non-Hodgkin’s lymphoma (NHL) (Czuczman et al., 2005), is currently in Phase III clinical trials, and lumiliximab (IDEC-152), an IgG₁κ primatized MAb targeting CD23 (Poole et al., 2005), is currently in Phase II clinical trials for chronic lymphocytic leukemia (CLL).

Non-human primate antibodies can be obtained for therapeutic use by multiple different approaches, including the possibility of making large, broad repertoire libraries constructed from naïve animals, similar to what has been done with human phage display antibody libraries, libraries constructed from immunized animals (Pelat and Thullier, 2009), direct PCR cloning of antibody V-regions from B cells similar to the approach taken to obtain human antibodies from immunized individuals (Babcook et al., 2006), or construction of heterohybridomas between murine myeloma cells and non-human primate splenic lymphocytes (Blancher et al., 1999; Groves and Morris, 2000). It is clear today that non-human primates are an under-utilized source of antibody V-regions for therapeutic antibodies and it is

expected in coming years that this source will be used more broadly once there is more freedom to operate (Thullier et al., 2010b).

Chimpanzees have antibody sequences that are very close to the human counterparts, although they have not been used widely as a source of human-like antibodies because of ethical concerns about immunizing chimpanzees with potentially dangerous antigens. Chimpanzees have been immunized, however, with some experimental human vaccines such as anthrax lethal factor vaccine (Chen et al., 2006). In that case, three anti-lethal factor antibodies were obtained, one of which provided reasonable protection for rats against lethal toxin exposure (Chen et al., 2006). Similarly, anti-polio virus MAbs were obtained from a phage-displayed FAb library made from chimpanzees immunized with a polio vaccine (Chen et al., 2011b). In this case, the IgGs were comprised of the entire chimpanzee light chain and the V_H , and the remainder of the heavy chain was human (Chen et al., 2011b).

5.8 Use of variable genes from humans

The ideal V-regions for building therapeutic antibodies for humans are, of course, from humans. Two approaches to generate human antibodies, ex-human, include construction and panning of phage or yeast displayed human antibody libraries (section 4.8.1) and immunization of transgenic mice expressing human antibody genes (section 4.8.2). Additionally, humans are exposed to a variety of antigens either naturally (e.g. *Staphylococcus aureus*, influenza virus, cancer antigens, autoantigens, etc.) or via active immunization approaches (e.g. influenza virus, anthrax, etc.) targeting specific infectious diseases. The human antibody repertoire therefore contains many possible antibodies that might be harnessed as therapeutics against specific antigens. A variety of approaches have been used to access such antibody genes, including construction of heterohybridomas, human hybridomas, Epstein-Barr virus (EBV)-driven immortalization of lymphocytes, immune-based phage-display libraries, and direct cloning of antibody genes from lymphocytes as described in more detail in section 5.8.3.

5.8.1 Human naïve antibody libraries

Human antibody libraries can be made in virtually any display system, including, for example, in phage display, in *in vitro* display, in yeast

display, in mammalian display, and in bacterial display (see Chapter 4). Over the past twenty years, phage display has been the most widely used system for *de novo* discovery of human antibodies. The libraries have come from many sources including mature human B cells (McCafferty et al., 1990; Clackson et al., 1991; Hoogenboom et al., 1991), pre-immune repertoires (Nissim et al., 1994), synthetic design (Barbas et al., 1992; Knappick et al., 2000), and combinations of genes from B cells and synthetic design (Hoet et al., 2005), to name a few.

Very large antibody libraries can be constructed using multiple “naïve” donors to increase the overall antibody repertoire of the library. A critical observation in the early 1990s was that combinatorial libraries of 10^7 or larger from naïve human donors were able to yield specific antibodies that would bind to human proteins, i.e. “self antibodies” (Clackson et al., 1991; Marks et al., 1991; Hoogenboom et al., 1991). Using PCR methodology to recover human antibody V-region genes from B cells and hybridomas, McCafferty et al. (1990) built a library of antibody genes displayed on the P3 protein of M13 phage. This was the first demonstration that immunization could be bypassed altogether. Many different libraries followed closely on this success, including combinations of designed synthetic sequences and natural diversity. Several large human antibody gene libraries were constructed, using either multiple “naïve” human donors (Marks et al., 1991; Vaughan et al., 1996) or synthetic gene approaches (Barbas et al., 1992; Griffiths et al., 1994). The human antibody library from Cambridge Antibody Technology (CAT, now part of MedImmune, a wholly-owned subsidiary of AstraZeneca), constructed by linking the V_H and V_L chains together to make a scFv construct (Vaughan et al., 1996), became the early prototype for many later libraries. While much of the early success came from phage displayed human antibody libraries, these libraries can be constructed in virtually any display system.

In contrast to the immunization of non-transgenic animals to source antibody leads, displayed libraries yield fully human antibodies and allow for greater design, manipulation, and variation of the target protein as well as the library itself. Additionally, display technologies can be used to isolate antibodies against targets such as peptides and polysaccharides that normally induce very poor immune responses in animals. For example, an anti-HIV MAb was isolated in this manner using the peptide to the hydrophobic pocket of the HIV gp41 fusion protein (Miller et al., 2006). A second advantage offered by display systems is the ability to alter the selection conditions to fit the desired outcomes. Various approaches have been used with display systems to select for higher affinity antibodies from naïve libraries (Chen et al., 2010; Ponsel et al., 2011), for antibodies

against specific epitopes on the target protein (e.g. Ames et al., 1994; Tsui et al., 1996), for proximal interacting proteins (Osbourn et al., 1998), or for antibodies displaying improved stability (Jung et al., 1999; Jespers et al., 2004a; Christ et al., 2007; Famm et al., 2008). Additionally, *in vitro* libraries are also applicable for targets that are toxic to animals and thus not able to be used as *in vivo* immunogens. Finally, *de novo* non-immune antibody libraries have virtually unrestricted sequence space, thus allowing for the construction of antigen binding regions that are either not possible or very rare in nature. This leads to the possibility of obtaining binding antibodies to molecules and epitopes for which natural antibodies from rodents, rabbits, NHPs, humans, or other species may not be generated.

One limitation often observed is that antibody leads derived from *de novo* or pre-immune libraries generally have moderate affinity to the targets, often in the 1–50 nM K_D range (Hoogenboom, 2005; Carter, 2006), so they often require affinity maturation to be useful as potential therapeutics. The reason for this is that there is a practical limitation to the library size and hence diversity that can be constructed. Phage libraries are restricted to about 10^{10} to 10^{11} total diversity by the efficiency of bacterial transformation as well as by the practical limit of phage particle density of about 10^{13} /mL.

A second potential limitation of antibody libraries is the V_H - V_L pairing. For technical reasons, the natural pairings of heavy and light chains found in antibody-producing B cells are not retained as the combinatorial libraries are constructed, leading to a random pairing of V_H and V_L in those *de novo* or naïve human antibody libraries. This could potentially lead to the generation of antibody molecules that have suboptimal solubility or other biophysical characteristics. In a recent analysis of an scFv library derived from human V genes, Glanville et al. (2009) found no evidence for skewed V_H - V_L pairing. In fact, the V_H - V_L pairing correlated with the proportion of the abundance of expression (Glanville et al., 2009).

Another significant issue with antibodies isolated from *in vitro* libraries is that they may possess “unnatural” sequences that can potentially lead to developability issues such as aggregation, lack of solubility, V-region glycosylation sites, and so forth (Jenkins et al., 2008; Voynov et al., 2009; Conley et al., 2011). Hybridoma-based antibodies, on the other hand, are subject to *in vivo* “editing” which tends to reduce problematic antibody properties such as aggregation (Pepinski et al., 2010; Wu et al., 2010). Another difference from hybridoma technology is that the output of most display systems include antibody fragments such as scFvs or FAbs that must be further manipulated genetically to yield complete IgGs which are often required for initial functional evaluation.

Over the past decade, several fully human antibody phage displayed libraries have been constructed using either Fab or scFv formats, including synthetic libraries constructed at MorphoSys (Knappik et al. 2000; Rothe et al., 2008), a Fab-based library made at Dyax (Hoet et al., 2005), and a simple library based on a single framework constructed at Genentech (Fellouse et al., 2007). Several strategies for building large naïve libraries versus focused libraries have been reviewed recently by Hoogenboom (2005), Sidhu and Fellouse (2006), and Mondon et al. (2008). Interestingly, Sidhu and his colleagues have demonstrated that synthetic diversity in CDR-H3 represented either solely or predominantly by just two amino acid residues, serine and tyrosine, is enough to generate strong binders (Fellouse et al., 2007).

Thus far, Humira®, which was approved in 2002 for use in treating rheumatoid arthritis, is the only antibody derived from a phage-displayed human antibody library to be approved by the FDA for therapeutic use. It took approximately 12 years to progress from the first description of phage displayed human antibody library (McCafferty et al., 1990) to approval of Humira® by the FDA in 2002 (Table 4.1). As a side note, however, the heavy and light chains comprising Humira® were not isolated *de novo* from the CAT human antibody library, but instead they were isolated using a “guided selection” approach (see section 6.3.3) in which a human antibody was derived by chain switching from the original murine antibody binder (Osborn et al., 2005).

5.8.2 Transgenic mice producing human antibodies

After it was demonstrated that over three-quarters of patients treated with Orthoclone OKT3® generated an anti-antibody response to the murine MAb (Norman et al., 1993), it was apparent that more human-like antibodies were going to be needed, especially for chronic indications in which multiple dosing was necessary. As previously mentioned, various *in vitro* manipulations were being employed to generate chimeric and humanized monoclonal antibodies.

A successful approach to the generation of human monoclonal antibodies has combined the use of transgenic and hybridoma technologies. Transgenic mice have been generated that contain portions of the human immunoglobulin genetic loci, including heavy and light chains, and can produce either fully human antibodies or antibodies containing human V-regions (Brüggemann et al., 1989a; Fishwild et al., 1996; Neuberger, 1996; Mendez et al., 1997). Only ten years after the invention of the hybridoma by Köhler and Milstein (1975), Alt et al. (1985) first suggested the idea of generating transgenic mice

carrying the genes to make human antibodies. Marianna Brüggemann and her colleagues were the first to generate transgenic mice carrying human antibody genes (Brüggemann et al., 1989a, 1991), although their studies were limited to detecting human IgMs produced by those mice. Two groups were independently successful in developing approaches to generate functional, fully matured human IgGs directly from transgenic mice (Green, 1999; Lonberg, 2005, 2009). Separately, Nils Lonberg and his colleagues at GenPharm (acquired in 1997 by Medarex, which itself was acquired in 2009 by Bristol-Myers Squibb (BMS)), and Aya Jakobovits and colleagues at Cell Genesys (which later spun off Abgenix, which was acquired in 2007 by Amgen) each engineered transgenic mice by blocking the ability of the mice to produce their own murine antibodies, and replacing that function with human antibodies (Lonberg et al., 1994; Green et al., 1994; Green, 1999; Lonberg, 2005, 2009). Immunization of either of these transgenic mouse constructs results in the generation of fully human, affinity matured IgG antibodies by those mice against the antigen (Green, 1999; Lonberg, 2005, 2009).

Scientists at Regneron have developed a second-generation transgenic mouse that produces human V-regions rearranged on mouse constant chains, which has proven to be very successful in generating high-affinity, antigen-specific antibodies after immunization (Dechiara et al., 2009). The key to this approach was the observation that incorporation of the natural mouse Fc, instead of the human Fc used in both the Medarex and Abgenix transgenic mice, resulted in a more robust B cell maturation (<http://www.regeneron.com/velocimmune>). More recently, several new start-up companies, such as OMT, Harbour Antibodies, Ablexis Therapeutics, and Kymab Ltd., are also developing new versions of transgenic rodents producing human antibodies. Thus it is likely that within a few years, this approach will be widespread and available to virtually anyone who needs to make fully human antibodies against human targets.

The principle advantage of these platforms over traditional hybridoma and display approaches includes the ability to immunize the animals to generate high affinity, via *in vivo* affinity maturation, fully human MAbs against human targets. The foreign background facilitates the development of antibodies against human target antigens. Moreover, use of these platforms avoids the need for further engineering to “humanize” the therapeutic candidate, and in most cases the antibodies have sufficiently high affinity to avoid the need for affinity maturation as well.

Attesting to the success of this approach, there are currently six marketed MAbs that were generated using transgenic mice producing human antibodies. The first human antibody to be developed and marketed from one of these transgenic mouse platforms was panitumumab (Vectibix®),

a fully human, anti-EGFR IgG₂ antibody discovered using the Abgenix XenoMouse™ technology and developed by Amgen. Panitumumab was approved in 2006, just twelve years after the publication of the key paper demonstrating the construction of the mice (Strohl, 2009a). The first two human antibodies derived from the Medarex platform to be marketed are the anti-TNF- α MAb golimumab (Simponi®) and the anti-IL-12/23p40 subunit MAb, ustekinumab (Stelara®), both developed by Centocor (now Janssen Biotech, Inc.), a division of Johnson & Johnson, and approved by the US FDA in 2009. Other fully human marketed antibodies derived from transgenic mice include the anti-IL-1 β MAb canakinumab (Ilaris®; Novartis), the anti-CD20 MAb ofatumumab (Arzerra®; GenMAB and GlaxoSmithKline), and the anti-RANK ligand MAb, denosumab (Prolia®; Amgen).

Human antibodies derived from transgenic mice comprise about a quarter of the current Phase III candidates (Strohl, 2009a) and are expected to continue to feed the pipeline. For example, Amgen, which acquired Abgenix in 2005, has more than a dozen fully human antibodies in clinical trials derived from the Abgenix mouse platform (Strohl, 2009a).

5.8.3 Direct human antibody approaches

5.8.3.1 Introduction

There has been significant interest over the years in obtaining antibody leads for potential therapeutic MAb directly from humans, particularly when human neutralizing antibodies are desired against pathogenic molecules, whether they are viruses (Traggiai et al., 2004; Yu et al., 2008; Throsby et al., 2008; Mouquet et al., 2010; Corti et al., 2011), bacteria (Jessup et al., 2000; Baxendale et al., 2007), bacterial toxins (Wild et al., 2003; Wild et al., 2007; Vor dem Esche et al., 2011), or tumor antigens (Liu et al., 1993; Lanzavecchia et al., 2007). Antigen-specific human antibody genes encoding MAb have been isolated directly from humans using a variety of approaches including heterohybridomas, EBV transformation, human hybridomas, and similar approaches. Success using these procedures has been elusive in many cases and overall quite variable. Recent advances, however, have improved the probability of obtaining therapeutic antibody leads directly from humans as described in this section.

One significant limitation to this approach is that it suffers from restrictions on the immunization of humans. Also, the circulating B cell repertoire generally does not produce antibodies against self-proteins, which comprise the majority of targets for human antibody therapeutics. Thus targets for this type of approach are generally limited to either infectious

disease agents, obtained after individuals have been exposed to a particular pathogen, or possibly cancer cells, utilizing B cells from cancer patients who have mounted immune responses to cancer-associated antigens.

Nevertheless, several companies have exploited this approach by isolating antibody V-genes from B cells of immunized or exposed individuals and either making phage displayed libraries to pan for antigen-specific antibodies or to isolate their B cells and immortalize them either by fusion with a suitable immortal cell or using EBV-mediated transformation of either the B cells themselves or their partners. For most human targets, this kind of protocol would not be expected to yield high-affinity, human antigen-specific MABs due to the fact that the human target antigens themselves would not elicit responses due to tolerance. On the other hand, infectious agents and cancer cell targets are potentially amenable to this strategy because their antigens are not recognized as self. Relatively high-affinity, antigen-specific MABs have been obtained to HIV (Posner et al., 1991a), *S. aureus* (Kelly-Quintos et al., 2006), and influenza virus (Throsby et al., 2008) from patients who have been exposed to those pathogens. Several examples are included in Table 5.4. Antibodies targeting tumor-associated antigens also have been obtained from cancer patients (Liu et al., 1993), although they generally have been low-affinity and/or low-specificity MABs (Vollmers and Brändlein, 2009a, 2009b) that lack the desired functional activity (Posner et al., 1991b).

One key to success for obtaining human antibodies directly from humans is the choice of antibody-producing B lymphocytes as donor cells for the V_H and V_L genes. If the MAB genes are obtained from peripherally short-lived antibody-producing plasma cells, they generally need to be retrieved shortly (within a week to ten days) after a booster immunization has been made (Lanzavecchia et al., 2007). On the other hand, human memory B cells can be immortalized with EBV, in which case the resultant antibodies are likely to be fully matured *in vivo* (Lanzavecchia et al., 2007).

Another factor in obtaining antibody genes from human immune cells is the question of whether to obtain the genes in a manner that retains the natural V_H - V_L pairing, as would be obtained by B cell immortalization techniques (Yu et al., 2008) and RT-PCR from isolated B cells (Babcook et al., 2006; Meijer et al., 2006), or to rely on the total repertoire yielding proper activities with non-natural pairings, as would be obtained with libraries (ter Meulen et al., 2006; Throsby et al. 2008).

One final matter of importance for obtaining antibodies against pathogens directly from humans is that these antibodies, which are indicative of the human response to the pathogen, can help to predict vaccine epitopes that might be successful in building subunit vaccines (Lanzavecchia et al., 2007).

Table 5.4 Examples of MAbs derived directly from humans using a variety of approaches

Target	Method used	Result	Reference
Anthrax protective antigen (PA)	FAb libraries constructed from bone marrow and peripheral B cells of vaccinated subjects	IgG	Wild et al. (2003, 2007)
Protective antigen (PA) and lethal factor (LF)	PBLs of UK anthrax vaccine-vaccinated individuals fused with CB-F7 heterohybridoma 6–18 days after vaccination; <i>in vitro</i> stimulation for 3–4 days	IgG ₁ κ	Vor dem Esche et al. (2011)
SARS virus	CpG-stimulated EBV-transformed plasma and IgG ⁺ , CD22 ⁺ memory B cells	IgG	Traggiai et al. (2004)
RSV and influenza H3N2	EBV-transformed B cells + HMMA2.5 myeloma + CpG stimulation + PBMCs	IgGs	Yu et al. (2008)
Polyreactive pneumococcal polysaccharides	Human-mouse heterohybridomas of Pneumovax® or Prevnar® vaccinated individuals	Polyspecific IgM	Baxendale et al. (2007)
Tetanus	Human-mouse heterohybridoma using HMMA2. 11TG/0 fused with bone marrow mononuclear cells	IgG	Posner et al. (1987)
SARS S protein	scFv library derived from infected patient	neutralizing IgG	ter Meulen et al. (2006)
HIV	RT-PCR of antibody genes from peripheral IgG ⁺ blood memory cells	Polyspecific IgMs	Mouquet et al. (2010)
H1N1 influenza	RT-PCR from sorted CD19 ⁺ , CD20 ^{lo/-} , CD3 ⁺ , CD38 ^{hi} , CD27 ^{hi} plasmoblasts	Broadly reactive MAbs	Wrammert et al. (2011)
Influenza M2e protein	RT-PCR from IgG ⁺ memory cells of healthy adults	IgGs	Grande et al. (2010)
Influenza HA1,2	scFv library constructed from CD24 ⁺ , CD27 ⁺ , IgM ⁺ memory cells	IgMs	Throsby et al. (2008)
Vaccinia virus	RT-PCR from CD19 ⁺ , CD38 ⁺ , CD45 ^{med} plasma cells	IgGs	Lantto et al. (2011)
Influenza groups 1 and 2	RT-PCR from IL-6 supplemented (for promotion of survival) plasma cells one week after immunization	IgGs	Corti et al. (2011)
Broad neutralization of influenza viruses	Single-cell RT-PCR from clonal plasmablasts isolated from a vaccinated subject	IgG	Whittle et al. (2011)

Abbreviations: PBLs: peripheral blood lymphocytes; PBMCs: peripheral blood mononuclear cells; RT-PCR: reverse transcriptase-polymerase chain reaction.

5.8.3.2 EBV transformation

Epstein-Barr virus (EBV) infects B lymphocytes through the surface receptor CD21 (complement receptor 2) (Martin et al., 1994a). The first EBV-based human B cell immortalization was attempted in 1977 (Rosen et al., 1977; Steinitz et al., 1977), shortly after the description of mouse hybridomas by Köhler and Milstein in 1975. Those early transformed lymphocyte lines were obtained with very low efficiencies (usually <10%), were unstable, and generally produced only IgM antibodies (Fraussen et al., 2010). Over the years, many improvements have been made to this approach, including the use of CpG as an co-immunostimulant (Traggiai et al., 2004; Fraussen et al., 2010) and the use of sorted, IgG⁺ CD22⁺ peripheral memory B cells (Traggiai et al., 2004; Fraussen et al., 2010). The use of memory B cells eliminates the need for *in vitro* restimulation and provides cells already programmed for long life (Traggiai et al., 2004; Lanzavecchia et al., 2007).

One recent twist to this approach is the generation of human IgG antibodies from EBV-immortalized CD19⁺ B lymphocytes obtained from tonsil tissue (Duvall et al., 2011). The tonsils provide ~200-fold more B lymphocytes as compared with peripheral blood, and the tonsil B lymphocytes were largely CD45⁺ CD19⁺ CD20⁺ B cells, which were characterized to be IgD⁺ IgM⁺ naïve B cells (Duvall et al., 2011). These cells were transformed at high efficiency (~86%) with EBV concentrated from B95-8 cells. The results were libraries of B cells producing IgGs, primarily of the IgG₁ isotype (Duvall et al., 2011), which have now been used to screen for binders against human self-antigens as well as foreign antibodies by the small biotech company Immunologix (now part of Intrexon).

5.8.3.3 Heterohybridomas

From as early as 1987, efforts have been made to utilize murine myeloma-human lymphocyte “heterohybridoma” technology to make human monoclonal antibodies (Posner et al., 1987). Those early heterohybridomas were made using a heteromyeloma cell line constructed by fusing mouse myeloma cell line P3x63Ag8.653 (a mutant of MOPC21) with bone marrow cells from patients suffering with an IgA myeloma (Posner et al., 1987). This cell line was used for fusions with peripheral B lymphocytes from subjects previously vaccinated with a tetanus vaccine to obtain both human IgM and IgG anti-tetanus virus antibodies (Posner et al., 1987).

5.8.3.4 Human hybridomas

Fully human myeloma cells have proven difficult to derive. Early cell lines included U266 myeloma, and RPMI-8226, which was determined to be heterogeneous (Karpas et al., 2001). Karpas et al. (2001) developed the Karpas 707H human myeloma cell line from a myeloma patient by selecting for PEG-resistance and screening for HAT resistance. Unfortunately, other than an initial characterization of human antibodies obtained from fusions with tonsil lymphocytes (Vaisbourd et al., 2001), little has been published on the use of this human myeloma cell line in the past decade.

5.8.3.5 Phage display libraries

Human antibody libraries also can be constructed from immunized people to recover antibodies to a known immunogen (e.g. anthrax toxin) (Wild et al., 2003, 2007) or infectious agent (e.g. SARS virus (ter Meulen et al., 2006); influenza virus (Throsby et al., 2008)). Such libraries are already significantly enriched for target-specific antibodies in the total repertoire, and the antibodies derived from this process have undergone natural affinity maturation and thus are generally of higher affinity. This approach, however, is very limited for human antibodies because antigen-specific B cells tend to be in miniscule quantities in human peripheral blood, as well as the ethical limitations on immunization of humans with non-approved vaccines. Some libraries derived from humans have purposefully focused on pre-immune IgM antibodies to capture greater diversity in the CDR sequence diversity than might be achieved with IgGs (Hoogenboom et al., 1991; Hoet et al., 2005). This approach was recently applied to isolation of cross-subtype specific anti-influenza virus neutralizing antibodies from human IgM⁺ memory B cells (Throsby et al., 2008).

There are now several examples of phage display libraries that have been made from the B lymphocytes of patients with infectious diseases or cancer, or of subjects who have recently been vaccinated with an approved vaccine (Wild et al., 2003, 2007; Throsby et al., 2008). Wild et al. (2003, 2007) developed FAb libraries using B lymphocytes obtained from the bone marrow or blood of human donors who had previously been vaccinated against anthrax. From those libraries, they isolated FAbs specific for protective antigen (PA) of *B. anthracis* toxin that were converted recombinantly to IgGs. ALXN4100, a clinical candidate MAb, came from those efforts (Wild et al., 2007). Similarly, human MAbs targeting both the rabies virus (Kramer et al., 2005) and SARS virus (ter Meulen et al., 2006)

were obtained from scFv libraries constructed from B lymphocytes of vaccinated donors and convalescing patients, respectively.

Throsby et al. (2008) constructed an scFv library for antibodies targeting influenza HA from convalescent patients and seasonally vaccinated subjects. They focused the libraries on CD24⁺ CD27⁺ IgM⁺ memory B cells and obtained several antibodies capable of broadly neutralizing influenza virus (Throsby et al., 2008).

5.8.3.6 RT-PCR

As noted above, obtaining immortalized human B cells has not been the easiest or most successful strategy for accessing the human immune repertoire. Thus several attempts have been made to generate potential therapeutic MABs directly from human peripheral blood mononuclear cells (PBMCs) by direct PCR-based cloning from antibody-producing plasma cells or from cells derived from secondary lymphoid organs, thus bypassing the need for a hybridoma or B cell stabilization approach (Babcook et al., 1996). The prototypical method for RT-PCR capture of Ig genes from B cells is called the “selected lymphocyte antibody method,” or “SLAM” (Babcook et al., 1996). For this approach, IgG-producing memory B cells are isolated, plated out into wells, and individual wells producing IgGs directed against the target of interest are identified using simple binding methods. The expressed V_H and V_L genes are then cloned directly from the positive wells by RT-PCR amplification and cloned either directly into vectors containing constant genes or, alternatively, into small phage-displayed libraries for further genetic optimization. Recently, Grandea et al. (2010) isolated antibodies directed against the influenza virus M2e protein by cloning antibody genes from IgM⁺ memory cells using RT-PCR. Similarly, Mouquet et al. (2010) obtained polyreactive IgM antibodies targeting HIV by cloning the antibody genes by RT-PCR from IgG⁺ circulating memory B cells. Moreover, IgGs targeting vaccinia virus were obtained from the CD19⁺ CD38⁺ CD45^{med} plasma cells from smallpox vaccine-immunized volunteers by RT-PCR (Lantto et al., 2011) with a particular emphasis on retaining V_H - V_L pairing, using a cell sorting and RT-PCR process called Symplex technology as described by Meijer et al. (2006). Also, a broadly neutralizing anti-influenza HA antibody was cloned by RT-PCR from CD138⁺ plasma cells obtained from volunteers a week after seasonal flu vaccine administration or from patients exhibiting symptoms of influenza infection (Corti et al., 2011). In addition, a broadly neutralizing anti-H1N1 MAB targeting the sialic acid binding region of HA1 was isolated by RT-PCR from single plasmablasts from a vaccinated

subject and matured to be a highly potent, broadly neutralizing antibody (Whittle et al., 2011). Finally, a pool of broadly neutralizing anti-HIV antibodies was obtained from HIV-infected patients using an RT-PCR-based approach (Walker et al., 2011a). These antibodies offer new hope for immunotherapeutic approaches to the treatment of HIV infected patients.

As mentioned in section 4.5.6 of Chapter 4, deep sequencing methods may be a potentially disruptive force in the entire pathway for obtaining lead V-genes. Combining the direct RT-PCR approach described here with deep sequencing approaches may be a primary method for obtaining lead antibodies for human antibody discovery programs of the future. This combination of advanced approaches may, in fact, be a disruptive technology in antibody sourcing in the future.

Variable chain engineering – humanization and optimization approaches

Abstract: Once a variable chain has been identified that binds to the desired target, it generally must be optimized to make it more human-like if it is derived from a non-human source, to increase its affinity for the target, and to make it more soluble and less prone to aggregation or biochemical degradation. The processes for humanization include CDR grafting and back-mutation, which was first described in 1986, as well as several modifications of that process and several newer processes. Affinity maturation can be carried out using either random approaches or structure-guided rational approaches. Both methods have been shown to result in high-affinity antibodies.

Keywords: humanization, humanness, CDR grafting, superhumanization, affinity maturation, chimerization, guided selection, error-prone PCR, DNA shuffling, antibody modeling

6.1 Introduction

As mentioned in Chapter 4, several important technologies were critical for the development of methods to genetically engineer modern recombinant therapeutic MAb and FcFPs. Over the past 10–15 years, remarkable progress has been made in the engineering of MAb variable chains to achieve MABs with greater potency, humanness, and biophysical behavior. This chapter discusses the improvement in the V-regions of antibodies, including humanization to increase humanness, and affinity optimization. Chapter 16 describes strategies for optimization to improve biophysical properties.

What makes a perfect V-region? While there is probably no single correct answer for that question, there are several characteristics that are well known and understood, perhaps the four most important of which are degree of humanness, affinity and specificity for the target, and biophysical characteristics. Obviously, the closer an antibody V-region is to looking 100% human to the human immune system, the less chance for anti-antibody responses. Second, affinity has been demonstrated to be an important factor in antibody engineering. The V-regions need to possess a high enough affinity to the antigen to drive the functional biology desired. How high of affinity is high enough actually depends on the concentration, turnover rate, biology, location, and pharmacology of the target, the desired indication and dosing frequency, and several other factors. There are now many descriptions of affinity maturation of antibodies to very low picomolar or even sub-picomolar binding constants (see Table 6.1 and section 6.4). In most cases, 1–30 pM affinity constants are probably strong enough affinity for antibodies binding to soluble antigens. For antibodies binding cell surface targets, the concept of tissue penetration also may be a factor in deciding on the target affinity since it is known that very high affinity binding to cell surface targets may actually decrease tissue penetration, as well as affect internalization rates (Rudnick et al., 2011). The final characteristic required of an optimized V-region is the effect it has on the chemicophysical behavior of the antibody, as will be discussed in detail in Chapter 16.

6.2 Chimerization

Muromonab-CD3 (Orthoclone OKT3®), the first murine antibody to be approved in 1986, was a mouse hybridoma MAb that was identified, developed, and manufactured purely as a product of the hybridoma cell line; hence that antibody was not genetically engineered in any manner whatsoever. Therefore that antibody was, in the truest sense, a direct result of the technology developed by Köhler and Milstein in 1975 (Köhler and Milstein, 1975). The murine antibody muromonab-CD3 elicited an anti-antibody response in 44–89% of patients who received it (Chapter 16), depending on the patient population treated, which limited its use as a therapeutic (Kimball et al., 1995). Thus, from the moment that MAb was approved in 1986, it was recognized that therapeutic antibodies needed to be more human-like to be acceptable for dosing in humans. The first step to making therapeutic antibodies more human-like was the generation of chimeric antibodies, i.e. the cloning of the mouse variable chains onto human constant chains, which was first described by Sherie Morrison in 1984 (Morrison et al., 1984;

Table 6.1 Examples of strategies and methods used for successful affinity maturation of MAbs

Target	MAb name	Maturation strategy	Selection method	Affinity improvement	Final affinity	Reference
HIV-gp120	b4/12	CDR walking	Phage display	420-fold	15 pM	Yang et al. (1995)
c-erbB2	C6.5	Combination of CDR-H3 and CDR-L3 mutants	Phage display	1,230-fold	13 pM	Schier et al. (1996)
FL-bio	4-4-20	EP-PCR and DNA shuffling	Yeast display	10,000-fold	48 pM	Boder et al. (2000)
GCN4	H6	EP-PCR and DNA shuffling	Ribosome display	500-fold	5 pM	Zahnd et al. (2004)
CEA	smE3	EP-PCR and DNA shuffling	Yeast display	285-fold	30 pM	Graff et al. (2004)
Anthrax toxin	14B7	EP-PCR	Bacterial display (APEX)	200-fold	21 pM	Harvey et al. (2004)
RSV	Numax	CDRs mutation	Screening	125-fold	27 pM	Wu et al. (2005)
P FAb	PrP-SC	EP-PCR and DNA shuffling	Ribosome display; screening	300-fold	1 pM	Luginbühl et al. (2006)
IL-13	BAK1	EP-PCR, CDR-3 mutagenesis, CDR walking	Ribosome and phage display	167-fold	81 pM	Thom et al. (2006)
EGFR	Erbtux	Rational design	Screening	9-fold	50 pM	Lippow et al. (2007)
Lysozyme	D44.1	Rational design	Screening	140-fold	30 pM	Lippow et al. (2007)
RAGE	XT-M4	Saturation and random mutagenesis	Ribosome display	110-fold	170 pM	Finlay et al. (2009)
Botulism neurotoxin	BoNT-A,B	Light chain shuffling and yeast mating	Yeast display	42-fold	7 pM	Lou et al. (2010)
PSGL- α (peptide)	Anti-PSGL- α scFv	EP-PCR plus yeast in vivo homologous recombination mutagenesis	Yeast display	~10,000-fold	70 pM	Swers et al. (2011)
IL-15	DISC0100	CDR saturation mutagenesis	Phage display	288-fold (plus 4,000-fold improvement in cellular potency)	52 pM	Lowe et al. (2011b)

Abbreviations: APEX: anchored periplasmic expression; EP-PCR: error-prone polymerase chain reaction.

Boulianne et al., 1984). Chimeric antibodies such as abciximab (ReoPro®), rituximab (Rituxan®), infliximab (Remicade®), and cetuximab (Erbix®), each of which retains approximately a third of their entire sequence as murine, still induce anti-antibody immune responses in about 5–15% of the patients treated with them, again depending on the patient population being treated (Pendley et al., 2003; Hwang and Foote, 2005; Almagro and Strohl, 2009).

6.3 Humanization

The humanization of an antibody is a process designed to increase the “human” content of a V-region originally obtained from non-human sources such as mouse, rat, rabbit, camelid, non-human primate or perhaps another source (see Chapter 5). The simplest way to measure the degree of identity with an authentic human V-region for guiding engineering efforts is to determine the percent sequence identity of a candidate V-region sequence to the sequences of human V-regions readily available in antibody sequence databases (Abhinandan and Martin, 2007). Using sequence-based comparisons of these types, it is possible to guide the engineering of V-regions derived from foreign or synthetic sources to produce molecules that are indistinguishable from “real” human antibody sequences regardless of the V-region source or the technology(s) used for discovery and optimization. The greatest challenge in any humanization protocol is to balance the need to make the most human-like antibody possible with retention of affinity, epitope specificity, and potency (i.e. neutralization or other functional activity). In most humanization approaches, efforts need to be made to recover affinity and potency lost during the humanization process. The next several sections will cover various approaches to humanize antibodies, the goal of each being to increase the humanness of the V-regions to be indistinguishable from authentic human V-regions.

Jones et al. (1986) first described a method for “humanization” of MAbs by grafting the complementary determining regions (CDRs) from murine or other non-human antibodies into human frameworks, followed by adding key back-mutations in the framework sequences required to stabilize the engineered constructs. Queen and his colleagues (Queen et al., 1989; Co and Queen, 1991; Ostberg and Queen, 1995) at Protein Design Labs refined the CDR grafting methods and developed a stepwise detailed process for humanizing antibodies, which has been the standard used for humanization of several antibodies currently on the market or in clinical trials. The anti-CD25 (IL-2R α subunit) MAb, daclizumab (Zenapax®), was the first humanized antibody to be approved for marketing in the United

States. Zenapax[®], which is used to treat transplant rejection, was humanized and developed at Protein Design Labs (now PDL BioPharma) and licensed by Roche. Of the 33 antibodies currently approved by the FDA for therapeutic indications in humans, 13 have been humanized. Examples include Synagis[®], Herceptin[®], Campath[®], Raptiva[®], Xolair[®], Tysabri[®], and Soliris[®], which retain significantly fewer murine amino acid sequences (usually less than 5%) than chimerized MAbs, and typically elicit anti-antibody responses in the range of 0.1–9% (Almagro and Strohl, 2009).

The original approaches for humanization, which utilized variations on the theme of grafting non-human CDR loops into human frameworks followed by manipulation of the frameworks to stabilize those loops, were protected with numerous patents (e.g. Winter, 1993; Queen et al., 1997; Carter et al., 1992). Thus a variety of other approaches, including both rational design and empirical selection strategies (Almagro and Fransson, 2008), have been developed to humanize non-human derived V-regions without grafting the CDR loops. Rational approaches include such strategies as resurfacing to remove B cell epitopes (also called veneering) (Roguska et al., 1994, 1996; Staelens et al., 2006), deimmunization (De Groot et al. 2006), grafting of specificity-determining residues (SDR) (Kashmiri et al., 2005), superhumanization (Hwang et al., 2005), optimization of human string content (Lazar et al., 2007), and germline humanization (Gonzales et al., 2004; Pelat et al., 2008). The one commonality of these methods is that the design phase allows for the construction of just a few humanized variants to be tested for binding, solubility, lack of aggregation, and other properties. If the designed variants prove to be unsatisfactory, then a new design cycle and functional assessment can be initiated. Empirical approaches, on the other hand, utilize a selection paradigm rather than a design-based approach (Almagro and Fransson, 2008; Almagro and Strohl, 2009). The development of these strategies coincided with the improvement of phage display, which allows for the relatively rapid and easy selection of variants from combinatorial libraries of billions of antibody sequences, as described in Chapter 4. Empirical approaches rely greatly on the power of selection techniques to isolate molecules with the desired functional and biophysical profile. One example of an empirical method is framework shuffling (Dall'Acqua et al., 2005; Damschroder et al., 2007), which is based on the generation of a library of humanized antibody variants by combining non-human CDRs with a repertoire of mixed human germline framework regions (FRs), followed by screening for binding to the target antigen. Strategies that combine elements of rational design and empirical selection have also been published recently (Almagro and Fransson, 2008).

6.3.1 CDR grafting, SDR grafting, and superhumanization

Even before the elucidation of the first antibody structures, Wu and Kabat (1970) defined the CDRs as the sequences containing the antigen-binding site or paratope. They identified the CDRs as the sequences containing the highest variability when multiple antibody sequences were aligned. Subsequent structural elucidation of antigen-antibody complexes (Amzel and Poljak, 1979) validated Wu and Kabat's predictions. As described in Chapter 3, the paratope is defined by the CDR residues of an antibody that bind the antigen. Thus, by transferring the paratope from a non-human antibody into a human scaffold, the specificity of the non-human antibody should, in principle, be transferred into a human context and thus a humanized version of the non-human antibody is obtained. Greg Winter and his colleagues at the Medical Research Council did exactly that when they devised a method to humanize antibodies called CDR grafting (Jones et al., 1986). As originally described, CDR grafting consists of the following three steps: (1) determination of the CDR sequences of the non-human antibody determining the target-binding specificity; (2) selection of human framework regions (FRs) into which the non-human CDRs would be grafted; and (3) three-dimensional analysis of the conflicts between the non-human CDRs and human FRs, resulting in back mutations to stabilize the loops to prevent a loss of affinity or structural integrity in the final humanized antibody. One key to the traditional CDR grafting methods is that the residues within the non-human CDRs themselves are not modified. This section describes CDR grafting, as well as modifications of CDR grafting known as SDR grafting and superhumanization.

Since the original description of CDR grafting, there have been many variations on the theme (Jones et al., 1986; Queen et al., 1989; Co and Queen, 1991; Ostberg and Queen, 1995; Tan et al. 2002; Hwang et al., 2005); these will be generalized as follows. The first step in the process is the chimerization, cloning, sequence analysis and functionality analysis of the chimeric antibody to determine that the correct non-human sequences have been cloned intact. Once biologically active non-human V-regions have been chimerized onto a human constant region and analyzed, the six canonical CDRs from the non-human antibody sequence are identified and selected for grafting into a human framework (Queen et al., 1989; Co and Queen, 1991). Typically this is accomplished by lining up the sequence with similar sequences of the same species and determining the CDRs through a combination of published rules (Kirkham and Schroeder, 1994; <http://www.bioinf.org.uk/abs/>) and comparison of the most variable sequences within the antibody gene. The IMGT database (<http://www.imgt.org/>), ABG mouse

antibody gene database (http://www.ibt.unam.mx/vir/V_mice.html), NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), or similar sites can be used to compare a set of mouse V-region sequences with those in the database.

Once the non-human antibody CDRs have been identified, the next step in CDR grafting is to select human V-region sequences that will serve as appropriate FR partner sequences for the non-human CDRs. The human FR sequences utilized in various CDR grafting protocols may include consensus sequences, sequences that have been naturally affinity matured, or human antibody germline sequences. Consensus sequences would be based on the most prevalent residues in each position, creating an artificial sequence combination that is likely not found in nature, and thus has the potential to be immunogenic. *In vivo* affinity matured sequences, on the other hand, are natural products of immune responses that typically carry somatic mutations that may or may not carry the risk of immunogenicity. In efforts to minimize immunogenicity, human germline genes have become more widely used as the source of human sequences (Tan et al., 2002; Gonzales et al., 2004). To select human germline genes to be used as frameworks for humanization, the amino acid sequences of the non-human V_H and V_L chains are compared with their counterpart human framework sequences, compiled at the Immunogenetics Database (IMGT; <http://www.imgt.org/>). Alternatively, one can select the human FR genes based on the sequences of the most similar CDRs rather than similarity of the FRs (Tan et al., 2002).

The new antibody genes are then constructed by incorporating the non-human CDRs into the chosen human FR genes. As would be predicted, the higher the homology between the FRs of the non-human CDR-donor genes and the chosen human V-genes used, particularly of the key residues that help to maintain the structure of the CDRs, the fewer the number of potential incompatibilities between the non-human CDRs and the human FRs, and therefore the fewer the number of expected back-mutations that would be required to stabilize the newly humanized V-regions.

The next step is to utilize three-dimensional antibody modeling to assess the potential incompatibilities between the non-human CDRs and human FRs, as well as the potential impact of designed back-mutations. Possible incompatibilities can occur in residues supporting the structure of the CDRs or in residues at the interface between the V_H and V_L domains. Foote and Winter (1992) showed that mutations in residues directly in contact with CDRs, dubbed “Vernier Residues,” can significantly impact the conformation and positioning of the CDRs and thus impact antibody binding to its target. Similarly, residues involved in the V_H : V_L interface and in CDR-H3 packing have been found to play an important role in supporting the CDRs for optimal binding to the target (Barderas et al., 2008).

Antibody modeling programs, which have been used widely to detect potential incompatibilities such as these, which may be caused by combining non-human CDRs into human FRs, are an integral part of the process to design and construct humanized MAbs (Queen et al., 1989; Co and Queen, 1991; Ostberg and Queen, 1995). This process has been made considerably more efficient over the years as the knowledge base and modeling programs have improved. The discovery of canonical CDR structures (Chothia and Lesk, 1987; Al-Lazikani et al., 1997) and the now large (and continuously growing) number of antibody crystal structures available in the Protein Data Bank (<http://www.rcsb.org/>) have been key contributors to the improvement of antibody models for humanization. A list of humanized antibodies, and the residues modified to correct incompatibilities, has been compiled by Jose Saldanha and can be accessed on the Internet (<http://people.cryst.bbk.ac.uk/~ubcg07s/>). This compilation can serve as a guide when designing back-mutations in CDR grafting protocols. Modeling softwaresuchasPIGS(<http://arianna.bio.uniroma1.it/pigs/>), RosettaAntibody (<http://antibody.graylab.jhu.edu/>), WAM (Web of Antibody Modeling; <http://antibody.bath.ac.uk/>), and Swiss PDB Viewer (<http://spdbv.vital-it.ch/>) are capable of generating models with a reasonable accuracy for the V_L domain, as well as much of the V_H domain (Almagro et al., 2011), although it is still essentially not possible to obtain an accurate conformation of the CDR-H3 loop. Additionally, Abhinandan and Martin (2010) have recently developed an algorithm for identification of the V_H/V_L interface residues that can help to determine which residues are critical for packing. As of March 2011, there were over 1,700 crystal structures of antibodies and antibody fragments, with more than 100 new structures being added each year over the past few years (Juan Carlos Almagro, personal communication), so there is an enormous database from which modeling software can work.

An alternative to the design- and modeling-heavy strategy shown above is to generate combinatorial libraries of variants at the residues identified as potential conflicts, followed by selection of the best variants by using a phage display or a similar selection strategy. Baca et al. (1997) used this approach to humanize MAb A4.6 targeting vascular endothelial growth factor (VEGF). MAb A4.6.1 CDRs were grafted into human $V_{\kappa}1$ and V_H3 FRs and a combinatorial library representing variants in 11 Vernier Residues was constructed. Following phage display selections, an optimized set of mutations was selected that increased the affinity of the MAb over its parent by 125-fold.

Specificity-determining residues (SDR) grafting is another alternative to traditional CDR grafting. Padlan et al. (1995) noted that only about a third of the CDR residues are actually involved in binding a target antigen. This suggested that a better approach to CDR grafting might be to minimize the

non-human sequences being grafted into the human FRs to reduce the potential for immunogenicity. By grafting only those residues that bind the target antigen, i.e. the SDRs, the resultant humanized antibody would be more human-like. Thus, in SDR grafting, only the sequences within the CDRs that are involved in binding the antigen are grafted into a set of human FRs (Tamura et al., 2000). SDRs can be determined by sequence variability or possibly by mutation analysis of the original non-human sequences. The SDR approach, which is described in detail by Kashmiri et al. (2005), has been used successfully to humanize murine antibody COL-1, which binds carcinoembryonic antigen (CEA) (De Pascalis et al., 2002).

“Superhumanization” is a term coined by Foote and colleagues (Tan et al., 2002) as a description of their approach to humanization, which is based on comparison of only the CDRs (Hwang et al., 2005). To carry out superhumanization, the human FRs are selected by their overall similarity to the CDR canonical structure class of the non-human antibody. Those FRs that contain CDRs most closely resembling the non-human CDRs are then selected as FRs as recipients for grafting of the CDRs (Tan et al., 2002). Tan et al. (2002) used superhumanization to humanize the murine MAb 9.3, resulting in about a 30-fold loss in binding activity. Similarly, Hwang et al. (2005) used superhumanization on antibody D1.3 with a sixfold loss in binding activity, which was considerably better than the 70-fold loss in affinity observed with a CDR-grafted version of D1.3. Finally, Hu et al. (2007) used superhumanization approaches to humanize the murine antibody 1A4A1, a neutralizing antibody against the Venezuelan equine encephalitis virus. The humanized 1A4A1 retained both antigen-binding specificity and neutralizing activity. The most recent of these types of approaches is “human framework adaptation” (HFA), which includes two major steps. In the first step, non-human CDRs are grafted onto a matrix of human V_H and V_L germline frameworks and all of the adapted heavy and light chains are paired for evaluation of binding activity. In the second step, specificity-determining residue optimization is carried out starting from the adapted molecule with the best activity (Fransson et al., 2010). Using HFA, Fransson et al. (2010) successfully converted a murine anti-IL-13 antibody to a sequence that was very homologous to human antibodies.

6.3.2 Other humanization methods

An alternative method to the CDR grafting approach described above would be to modify the sequences of the non-human antibody itself rather than grafting the CDRs from it into human FRs. This approach, called

resurfacing or veneering, involves modification of the murine residues that are surface exposed, and thus potential B cell epitopes, to the closest human residues (Roguska et al., 1994, 1996; Delagrave et al., 1999; Daugherty et al., 2004; Staelens et al., 2006). For this approach, the non-human sequences are modified by comparing them with the closest human framework, followed by modeling of the non-human Fv based on the closest structural counterpart in the PDB database to identify surface exposed residues. The surface-exposed residues are then changed to human residues based on the most similar human framework sequence and tested for retention of binding to target antigen. As with CDR grafting, efforts may need to be made to restore affinity lost during the humanization process. Roguska et al. (1996) directly compared antibodies humanized by resurfacing with the same antibodies humanized by standard CDR grafting. They found that the sequences of the antibodies humanized by the two procedures were somewhat different, but the overall humanness and affinities were similar. The anti-CD56 murine MAb N901 was humanized using the resurfacing approach (Roguska et al., 1994) to make Hu-N901, which was then conjugated to a mytansinoid cytotoxic drug to make Hu-N901-DM1. Hu-N901-DM1, now called lorvotuzumab mertansine, is currently in Phase II clinical trials for treatment of small cell lung cancer and multiple myeloma.

Many other strategies have been used to humanize non-human V_H and V_L sequences and make them more human-like. Many of these approaches utilize one form or another of sequence or structure modeling analysis, followed then by making experimental substitutions and determining their effect on the structure (i.e. developability; see Chapter 16) and function of the targeted antibody. One category of these approaches for humanization includes a variety of deimmunization-related approaches, i.e. the use of *in silico* predicted T cell epitopes to deimmunize a murine MAb into its humanized format (Koren et al., 2007; Parker et al., 2010; Bryson et al., 2010). A second related approach to improve the humanness of an antibody, termed “human string content” (HSC), was recently developed by the team at Xencor. This approach uses multiple frameworks and local sequence variation to guide the modification of murine residues to human amino acid residues. This approach is based on the concept that the “human strings,” or short sequences, yield the critical MHC-II binding information and T cell recognition (Lazar et al., 2007). Changes made to the non-human antibody to remove potential T cell epitopes are then modeled to ensure that the three-dimensional structure was retained. Four antibodies were humanized using the human strings approach, with retention of biological activity and in a few cases modest affinity

optimization (Lazar et al., 2007). More recently, the scientists at Xencor went a few steps further in using rational modeling algorithms to convert mouse V-regions into V-regions that were indistinguishable from human V-regions (Bernett et al., 2010). Using their algorithms, they converted the murine precursors to an anti-CD25 MAb, an anti-VEGF MAb, and an anti-TNF- α MAb to virtually human sequences by making 59, 46, and 45 amino acid residue changes, respectively, with retention of the activity exhibited by the murine antibodies (Bernett et al., 2010). One key difference between this approach and many others is that CDR residues also were included among the proposed changes. They utilized two scores to define humanness: (1) an overall similarity score to the closest human germline V_H , V_K , J_H , and J_K sequences; and (2) a measurement of humanness based on potential T cell epitopes of every 9-amino acid residue stretch in their sequence (Bernett et al., 2010).

No matter which approaches are used to humanize antibodies, e.g. CDR-grafting (Jones et al., 1986; Queen et al., 1989; Co and Queen, 1991; Ostberg and Queen, 1995), deimmunization (Jones et al., 2009; Parker et al., 2010), resurfacing/veneering (Roguska et al., 1994), human-adaptation (Fransson et al., 2010), or any other method (e.g. Lazar et al., 2007; Bernett et al., 2010), the goal is to make antibodies containing sequences that are as human-like as possible, while retaining or improving the activity of the parental non-human antibody. Moreover, the optimized antibodies must also retain the expression, folding, solubility and non-aggregation characteristics required for development as therapeutics (see Chapter 16).

6.3.3 Guided selection

Guided selection is an example of an empirical approach that results in the replacement of a murine antibody sequence with a fully human antibody sequence (thus this is not truly “humanization” but chain substitution to result in a fully human antibody). There are three components to guided selection, including: (1) the V-regions of the non-human antibody to be humanized, constructed in either scFv or FAb phage display (or similar display) format; (2) libraries of human V_H and V_L chains in the same format; and (3) a selection method that gauges the activity conferred by the V-regions. In this approach, no assumptions are made on the impact of V-region alterations on the antibody structure.

To carry out guided selection, the V_H chain of the non-human antibody is paired with a library of human V_L chains (or vice versa) to generate a library of candidate non-human: human V-region hybrids. The library is then selected against the target recognized by the non-human antibody to find hybrids that still retain strong binding. The non-human V_H (or V_L if done in that direction) thus serves as a guide or template on which to select human V-regions specific for the same epitope as recognized by the original non-human antibody. Once a non-human V_H : human V_L hybrid is found that binds the target, the human V_L is paired with a library of human V_H chains and the process is repeated, this time resulting with a fully human V_H - V_L paired antibody with the desired activity on the same or similar epitope as bound by the original non-human antibody. If the resultant human antibody does not have the desired affinity, it can be optimized by affinity maturation methods as described later in this chapter. A variation on the approach would be to select non-human: human V_H and V_L hybrid libraries in parallel and then combine the outcome of independent selections to yield a human Fv.

Guided selection was the strategy used to isolate the fully human sequences for Humira®, the first fully human antibody to be approved for marketing. The human antibody sequences were derived from the Cambridge Antibody Therapeutics phage displayed human antibody library, as described in detail by Osbourn et al. (2005).

A potential issue with guided selection is that in the V-region exchange process, it is possible that the resulting human antibody, containing the substituted V-regions, may not recognize the exact same epitope as the original non-human MAb. Epitope drift has, in fact, been observed in guided selection, where the substituted V-regions possessed a subtle change in specificity (Watzka et al., 1998; Beiboer et al., 2000; Kuepper et al., 2005). Beiboer et al. (2000) demonstrated that while an anti-epithelial glycoprotein-2 human antibody derived by guided selection retained most of the properties, including similar affinity and epitope, of the original murine antibody, it possessed a V-region structure substantially different from the original murine antibody. If it is critical to maintain the exact epitope of the non-human antibody, the libraries could be designed around the non-human CDR sequences, especially CDR-H3, the loop that most defines antibody specificity (Wang et al., 2000). On the other hand, multiple CDRs could be retained in the guided selection libraries (Steinberger et al., 2000), although as more non-human CDR sequences are retained, the process becomes more like CDR grafting than true guided selection.

6.4 Affinity optimization

6.4.1 Introduction

Antibody affinity, which is defined as the strength with which an antibody binds to its cognate antigen, is often expressed as an equilibrium dissociation constant (K_D), which is governed by two kinetic constants, i.e. the rates of association (k_{on} or k_a) and dissociation (k_{off} or k_d). The rate of association is defined by the time required for the antibody to bind its cognate antigen, a function which is in part dictated by translational and rotational diffusion and thus the size and shape of the ligand and antibody (Janin, 1997). When comparing antibodies, k_{on} generally remains within a relatively narrow window. The dissociation rate, which is usually given as a half-life constant, defines the time the antibody-antigen complex remains intact. Several methods have been developed to measure these kinetic constants, which can be used to estimate K_D . The most commonly utilized methods used today are Surface Plasmon Resonance (SPR, the most common form of which is BIAcore; Drake et al., 2004; Katsamba et al., 2006), Proteon (Bronner et al., 2010), and Solution Based Kinetics Exclusion Assay (KinExA; Drake et al., 2004). Speed and accuracy often dictate which method is used. When speed is desired, e.g. in screening regimens, higher throughput methods such as the Proteon or higher throughput, next-generation SPR systems are more often used, whereas when high accuracy is required, lower throughput methods such as KinExA may be used. Moreover, the accuracy of these techniques depends upon the K_D range to be measured. Values above ~20 pM can be measured reasonably well by SPR-based methods, while KinExA is often the method of choice for measuring K_D values below 100 pM (Rathanaswami et al., 2005; Luginbühl et al., 2006).

As previously mentioned, an optimized therapeutic MAb should have sequences that are as human as possible, should have high affinity to the target, and should have good biophysical behavior characteristics. An additional characteristic desired in therapeutic MAbs is the ability to bind to the non-human primate target, a property which greatly facilitates *in vivo* pharmacology and especially GLP-toxicology studies as the antibody goes into development (see Chapter 17).

Lead antibodies often lack the affinity desired in a therapeutic MAb. Often times, the humanization process can result in lost affinity to the target. Additionally, the primary leads obtained from phage displayed naïve human antibody libraries often have sub-optimal affinities of 1–50 nM after isolation from the libraries. Finally, when targeting molecules of very low abundance, high affinity is required to achieve the desired potency of the therapeutic

antibody. Thus, whether starting with an antibody sourced from a human phage displayed library, a humanized antibody, or any other starting material, the goal of affinity optimization is to increase the binding affinity of the antibody to its target while maintaining or improving the biophysical characteristics of the resultant engineered product. Affinity optimization has four well-defined components: (1) a strategy to generate libraries of antibody variants; (2) a selection or screening strategy to enable isolation of variants of interest; (3) reasonably rapid screens to determine expression, solubility, and aggregation characteristics of the optimized IgGs; and (4) functional assays to compare functional potency of the optimized full IgG versus the parental MAb.

The first reports of *in vitro* affinity maturation of the variable chains, perhaps the most definitive form of V-region antibody engineering, occurred in 1992 (Foote and Winter, 1992; Gram et al., 1992). Since then, both random and rational mutagenesis strategies have been used widely to design optimization libraries of antibody variants. Rational strategies generate diversity in predefined sequences of the antibody V-region based on some algorithm or scientific rationale, whereas random strategies introduce point mutations, insertions, deletions, or fragment replacements at random locations within the V-region. The most common example of a random approach is the use of error-prone polymerase-chain reaction (EP-PCR) (Neylon, 2004) to generate mutations throughout the V-region. Rational strategies, on the other hand, which include approaches such as CDR walking mutagenesis (Yang et al., 1995) and rational design (Lippow et al. 2007), focus on specific residues within the V-regions (Table 6.1).

The affinity of an antibody for its target is one of the key factors, along with epitope, that defines the functional potency of an antibody. There are now many examples of antibodies being affinity optimized to result in K_D values of 1–10 pM or even sub-pM (Almagro and Strohl, 2009; see also Table 6.1). Boder et al. (2000) used a combination of mutagenesis strategies to mature an anti-hapten antibody by $>10^4$ -fold, resulting in a 48 fM binder, which may be the highest affinity antibody ever generated.

Affinity maturation has been clearly demonstrated to translate into antibodies with greater functional potency, which can potentially impact dose levels and frequency (and hence impact the cost of goods), as well as safety. Considering the intense competition on major targets (e.g. CD20, EGFR, IGF1-R, TNF- α , IL-6, IL-17A, etc.), there is increased demand for higher affinity antibodies with increased potency. The question of how tight is tight enough has been subject to considerable debate and is likely to be target-specific in any case. Additionally, as antibodies are affinity optimized *in vitro*, a major concern is whether the optimization efforts result in epitope drift and/or epitope spread, i.e. loss of specificity (Hu et al., 2009).

Currently, many of the most successful commercial anti-tumor antibodies that directly bind tumor cells (e.g. Herceptin®, Rituxan®) have relatively moderate affinities for their targets in the range of 0.1–3 nM (Carter, 2006). In other cases, high affinity may be a detriment to the efficacy of the antibody. Adams et al. (2001) demonstrated that the affinity of a monovalent scFv antibody was inversely correlated with the ability of the antibody to penetrate tumors, with higher affinity mutants having the poorest tissue penetration. Schmidt and Wittrup (2009), on the other hand, demonstrated that the affinity of full IgGs was not critical once an affinity in the low nM range was achieved. Furthermore, Tang et al. (2007) demonstrated that the affinity of anti-tumor IgGs was directly correlated to the ability of those antibodies to kill tumor cells via antibody-dependent cellular cytotoxicity (ADCC). Finally, the affinity (and hence resident time of the antibody on its target) also may impact the rate of internalization of an antibody. Combined, these studies suggest that there might be a delicate balance in affinity when targeting tumors with antibodies.

On the other hand, there are examples of other commercial antibodies and FcFPs, e.g. the TNF- α binders Remicade®, Enbrel®, and Humira®, for which very high affinity may be required to achieve an optimal product profile (see also Chapter 8). One possibility is that their success depends on this high affinity since, even during excursions, TNF- α is found in the picomolar range (Kaymakcalan et al., 2009).

In the end, affinity for its target is only one factor dictating the quality of an antibody. Other factors that can be just as critical to the success of a therapeutic antibody epitope include biochemical functionality, absence of off-target activity (Hu et al., 2009), Fc functionality, biodistribution and tissue penetration, half-life, solubility, and stability (i.e. lack of aggregation and biochemical degradation).

6.4.2 Random methods for affinity optimization

Error-prone PCR (EP-PCR) is the most commonly used approach to generate libraries of antibodies for affinity and biophysical optimization. EP-PCR utilizes a low-fidelity DNA polymerase such as *Thermus aquaticus* polymerase (Taq-Pol) to introduce errors all along the V gene being amplified. The error rate of Taq-Pol can be enhanced by substituting Mn²⁺ for Mg²⁺, adding propanol, incorporating unbalanced dNTPs, or utilizing unnatural mutagenic bases (Neylon, 2004).

There are limitations in the EP-PCR approach. First, mutations are introduced throughout the V-region, including the framework sequences,

which can potentially result in the creation of neo-epitopes that might be mutagenic, or instability due to poor folding or packing. One strategy to deal with these issues is to back-mutate framework residues to germline sequences after EP-PCR, but this can be a laborious process, especially if there is a high rate of FR mutations. Second, Taq-Pol often gives poor yields, poor mutation rates, and biased mutations (Biles and Connolly, 2004). In an attempt to find an alternative to Taq-Pol, Biles and Connolly (2004) mutated the *Pyrococcus furiosus* polymerase (Pfu-Pol), resulting in a low fidelity mutant enzyme that yields large quantities of amplified product and a high frequency of highly indiscriminate mutations.

Daugherty et al. (2000) investigated the effects of EP-PCR-generated mutation frequency on affinity maturation. Libraries with ~2, ~4, and ~22 mutations per gene were tested and were all found to result in improved affinity, with greatest affinity improvement directly correlated to the error rate (Daugherty et al., 2000). The majority of the mutations leading to higher affinity were found in residues away from the antigen-binding site, consistent with previous studies on antibodies optimized *in vivo* (Wedemayer et al., 1997). While any selection method can be used with EP-PCR, it has been demonstrated that the combination of EP-PCR, high mutagenesis rates, and ribosome display, which allows for significantly larger libraries than, for example, phage display, can result in optimal mutagenesis and affinity maturation (Finlay et al., 2009; Drier and Plückthun, 2011).

DNA shuffling is another random approach that generates antibody variants with multiple mutations. This method is based on pooling random DNA fragments, which are reassembled into a full-length gene by repeated cycles of annealing in the presence of DNA polymerase. Highly homologous fragments prime each other, resulting in recombination and a template switch (Stemmer, 1994; Luginbühl et al., 2006; Lou et al., 2010).

Chodorge et al. (2008) compared EP-PCR with and without a homologous recombination step. While both strategies improved affinity, the EP-PCR plus recombination approach resulted in a larger population of affinity-improved variants. The best mutant, which had a 22-fold increase in affinity, was isolated from the EP-PCR plus recombination-based pool. Interestingly, mutations from the recombined pool exhibited significant cooperative effects, indicating that combinatorial approaches may be used to explore greater areas of sequence space than EP-PCR alone (Chodorge et al., 2008).

Perhaps the most interesting random mutagenesis approach was the use of error-prone PCR plus yeast *in vivo* homologous recombination mutagenesis to drive a 10,000-fold improvement in affinity of an anti-peptide antibody, using yeast display to select for the high affinity variants (Swers et al., 2011).

6.4.3 Rational strategies for affinity optimization

In general, rational strategies for generating libraries of antibodies are advantageous over random mutagenesis approaches due to the greater control over the consequences of mutations introduced into the V-genes. Rational strategies enable greater exploration of sequence space than random methods, resulting in more focused, but more in-depth, mutational approaches that concentrate on key residues within the V-regions.

CDR mutagenesis is perhaps the simplest form of rational affinity optimization. Comparisons of antibody germline genes as well as antibodies that have naturally been affinity matured *in vivo* indicate that while somatic mutations occur throughout the V-gene, most mutations occur in the CDRs (Tomlinson et al., 1996; Ramirez-Benitez and Almagro, 2001). More specifically, CDR-H3 and CDR-L3 are located in the center of the antigen-binding site and play a major role in determining both specificity and affinity of an antibody. As described in Chapter 5, natural antibody diversity is generated by multiple mechanisms in the formation and maturation of CDR-H3 and, to a lesser extent, CDR-L3 (Tonegawa, 1983). Mutagenesis strategies focused on a single CDR, e.g. CDR-H3, allow for the use of saturation mutagenesis approaches (e.g. probing each position with most of the natural amino acids). On the other hand, focused saturation mutagenesis only allows for probing variations in a few positions because the total number of variants that can be generated and tested in libraries is limited by library size (Chapter 4). For example, a library built on NNK diversification introduces 32 codons in every position and thus grows by 32^n for each residue tested. If phage display were used as the method to select the variants, maximum-sized libraries of 10^9 to 10^{10} variants would allow only saturation mutagenesis of 6–7 residues, not even enough to saturate a single complete CDR. Nevertheless, CDR mutagenesis, often focusing on the primary contact CDRs, CDR-H3 and/or CDR-L3, is widely used to affinity optimize antibodies, often with great success (Yang et al., 1995).

On average, the six CDRs of an antibody are about nine residues long, resulting in a total of ~54 “hypervariable” residues. Thus the sequence space represented by just the CDRs is far too great to be exhaustively explored using saturation mutagenesis. In this case, typically CDR-H3 and/or CDR-L3 are mutagenized by replacing each position of the CDR with libraries of 6–18 amino acid residues. In a limited form of this approach, one or two representatives from each class of amino acid (e.g. small non-polar, polar, hydrophobic, basic, acidic) could be used in the randomization process. In the more detailed approach, all residues except

Cys and Met could be used to randomize at each position; however, this would result in libraries too large to feasibly build and test. Yang et al. (1995) devised a strategy they called CDR walking, in which NNK-randomized CDRs were selected in parallel, followed by combination and screening of the best variants to look for synergistic binding improvement. Alternatively, the CDRs can be mutated sequentially, using the best variant at each step as the base on which to optimize the next CDR. The highest affinity binder reported to be obtained through this strategy was 15 pM, resulting in a 420-fold improvement as compared with the affinity of the starting antibody (Table 6.1; Yang et al., 1995). The sequential CDR walking strategy has consistently yielded variants of improved affinity, whereas the combination of independently optimized CDRs has been less predictable and resulted in more modest improvements in affinity. In another example, Schier et al. (1996) improved the affinity of an anti-erbB-2 antibody by more than three orders of magnitude, to 13 pM, by optimization of CDR-L3 and CDR-H3 separately, followed by combining the mutants with improved affinity. Finally, Lowe et al. (2011b) improved an anti-IL-15 MAb 288-fold using CDR saturation mutagenesis with phage display selections (Table 6.1).

As rational strategies become more and more refined, the most significant challenge is to identify key positions to be modified so that maximal diversification can be applied to those specific positions. In the past four to five years, there has been a significantly increased emphasis on the use of antibody structure and modeling to inform affinity maturation strategies. With 1,700 structures available in the Protein Data Bank (PDB; <http://www.rcsb.org/>) as of May 2011, with the ability of structural biology groups to obtain co-crystal structures of antibodies and their cognate antigens increasing rapidly, and with the increased sophistication in modeling programs (Almagro et al., 2011), several approaches have been proposed to incorporate the structural data into affinity optimization design schemes. To date, however, even sophisticated rational design approaches have met with limited success, in particular in the optimization of nM binding antibodies to pM affinities, where discrimination of free-energy becomes a significant factor (Lippow et al., 2007).

Lippow et al. (2007) recently enhanced the affinity of two antibodies down to the pM range using rational design strategies. They used a physics-based energy function modeling approach to test the effect of mutations at 60 different residues within the CDRs to each other amino acid residue, excluding Pro and Cys (10^{75} variants). In a first step, the backbone conformation was fixed and the potential side chain conformations based

on rotamers were calculated, followed by a more rigorous computation protocol that included energy minimization. By combining six designed mutations in one antibody, D44.1, and three in a separate antibody, they were able to achieve moderate pM affinities in two examples (Lippow et al., 2007). This effort provided valuable lessons for future computational methods for affinity maturation, including a focus on the energy function of electrostatic interactions, which they proposed as a better predictor for improved binding. It is expected that as antibody modeling programs become more sophisticated, model-based antibody optimization will become more widely accepted, used, and useful (Almagro et al., 2011).

Antibody interactions with the immune system

Abstract: All antibodies have the ability to interact with components of the immune system as part of their natural mechanisms of action. IgGs, IgAs, and IgMs interact with Fc receptors and complement components, resulting in various immune responses to the targeted antigen. These immune responses are often associated with specific cell types such as macrophages, natural killer (NK) cells, or neutrophils. For IgGs, the critical Fc gamma (γ) receptors are the high affinity activating receptor Fc γ RI, the low affinity activating receptors Fc γ RIIa, Fc γ RIIIa, and Fc γ RIIIb, and the low affinity inhibitory receptor, Fc γ RIIb. Additionally, the Fc of IgGs possesses sequences that interact with the neonatal receptor, FcRn, resulting in recycling of IgGs and long inherent half-life. Interaction with FcRn also can result in transcytosis as well.

Keywords: Fc gamma (γ) receptor, immunoreceptor tyrosine-based activation motif (ITAM), immunoreceptor tyrosine-based inhibitory motif (ITIM), complement, FcRn, Fc gamma chain, antibody half-life, natural killer (NK) cells, macrophages, neutrophils, recycling, transcytosis

7.1 Introduction

Fc receptors are the primary mechanism by which antibodies work with the immune system to help neutralize and eliminate foreign invaders. Most Fc receptors belong to the human Ig receptor superfamily. This chapter describes the most significant Fc receptors and their interactions with potential therapeutic antibodies. Table 7.1 lists human proteins that interact with antibodies and gives a high-level overview of their biology.

Table 7.1 Major human receptors and proteins that interact with human antibodies

Protein	Structure	Location or cell type	Human Ig isotypes that bind in physiologically relevant manner	Function
Fc γ RI (CD64)	$\alpha, \gamma 2$ (contains ITAM)	Macrophages, eosinophils, dendritic cells; IFN- γ or G-CSF-activated neutrophils	IgG ₁ , IgG ₃ , IgG ₄ (K_D ca. 20–30 nM)	High affinity receptor; phagocytosis, activation of respiratory burst; killing
Fc γ RIIa (CD32a)	α (contains ITAM)	Monocytes, macrophages, neutrophils, eosinophils, platelets, dendritic cells, Langerhan's cells	IgG ₁ (K_D ca. 300 nM); IgG ₂ , IgG ₃ , IgG ₄ (K_D ca. 1–10 μ M)	Low affinity receptor; phagocytosis by monocytes, macrophages, neutrophils; degranulation by eosinophils
Fc γ RIIb (CD32b)	α (contains ITIM)	Monocytes, macrophages, neutrophils, eosinophils, B cells, mast cells, basophils, dendritic cells	IgG ₁ , IgG ₃ , IgG ₄ (K_D ca. 5–10 μ M)	Inhibition of stimulation response; key role in B cell development by inhibition of B cell activation and proliferation
Fc γ RIIc (CD32c)	α (contains ITAM)	NK cells	IgG ₃ > IgG ₁ = IgG ₄ > IgG ₂ (K_D ca. 5–10 μ M)	Activation of NK cells; involvement in ADCC
Fc γ RIIIa (CD16a)	$\alpha, \gamma 2$ (contains ITAM)	NK cells, macrophages, dendritic cells, $\gamma\delta$ -T cells, eosinophils, mast cells, Langerhan's cells (and 10% of monocytes)	IgG ₁ , IgG ₃ (K_D ca. 100–800 nM)	Low affinity receptor; antibody dependent cellular cytotoxicity (ADCC) by NK cells; works synergistically with Fc γ RIIc in NK cells

Table 7.1 Major human receptors and proteins that interact with human antibodies (*cont'd*)

Protein	Structure	Location or cell type	Human Ig isotypes that bind in physiologically relevant manner	Function
Fc γ RIIIb (CD16B)	GPI-anchored α	Neutrophils, IFN- γ -induced eosinophils	IgG ₃ (K_D ca. 900–1,000 nM); IgG ₁ (K_D ca. 5 μ M)	Synergistic affects with Fc γ RIIa; no signaling domain
TRIM21	$\alpha 2$ or $\alpha 3$	Haematopoietic cells and other cell types	Monomeric interactions, ca. 200 nM; <i>in vivo</i> interactions probably sub-nM	Assists in targeting Ig-coated viral particles to degradation pathway
FcRn (also known as neonatal receptor; Brambell receptor)	$\alpha\beta$	Epithelial cells, placenta, macrophages, monocytes, dendritic cells	IgG ₁ , IgG ₂ , IgG ₄ (K_D ca. 500–2,000 nM (Suzuki et al., 2010))	Maternal-fetal transfer; recycling of IgGs and serum albumin, resulting in long half-life
Fc α RI (also known as CD89)	$\alpha, \gamma 2$ (contains ITAM)	Monocytes, some macrophages, eosinophils, interstitial dendritic cells, Kupffer cells (neutrophils stimulated with G-CSF)	Monomeric IgA; dimeric sIgA (K_D ca. 176–431 nM)	Neutrophil-mediated ADCC and ADCP of IgA-antigen complexes

Table 7.1 Major human receptors and proteins that interact with human antibodies (*cont'd*)

Protein	Structure	Location or cell type	Human Ig isotypes that bind in physiologically relevant manner	Function
Fc α / μ R	α	Follicular dendritic cells, as well as a subpopulation of pre-germinal B cells (in humans)	Pentameric IgM (K_D ca. 1 nM); dimeric IgA (K_D ca. 10 nM)	IgM binding to Fc α / μ R may negatively regulate humoral responses to T cell independent antigens (Honda et al., 2009)
Fc μ R (also known as FAIM3/TOSO)	α ?	Primarily mature B cells	IgM (K_D ca. 10 nM)	Possible B cell regulation; anti-apoptotic regulator as a balance to FAS activity
Polymeric Ig receptor (pIgR)	α	Mucosal epithelial cells	Pentameric IgM (K_D ca. 37 nM); dimeric IgA (K_D ca. 55 nM) (J-chain required for binding)	Directional transcytosis of dimeric IgAs and pentameric IgMs to mucosal surfaces
Fc ϵ RI	7-trans-membrane receptor	Mast cells, basophils, activated eosinophils	IgE (K_D ~ 3–5 nM)	Trigger allergic response; IgE-dependent eosinophil or monocyte ADCC

Table 7.1 Major human receptors and proteins that interact with human antibodies (*cont'd*)

Protein	Structure	Location or cell type	Human Ig isotypes that bind in physiologically relevant manner	Function
FcεRII (CD23)	C-type lectin	B cells, T cells, monocytes, eosinophils, platelets, follicular dendritic cells, and Langerhan's cells	IgE ($K_D \sim 1\mu\text{M}$)	Regulation of IgE production
MBL (also known as mannose binding lectin or mannan binding lectin)	Na	Serum protein; component of innate immune system	IgG antibodies with G0 glycosylation; dimeric and polymeric IgA; 20% of IgM	Recognition molecule for activation of lectin pathway for complement activation
C1q	Na	Serum protein	IgG ₁ , IgG ₃ , hexameric IgM (ca. 30–80 μM)	Primary binding protein of classical complement cascade

Notes: Na: not applicable.

Abbreviations: α : first protein chain; ADCC: antibody-dependent cellular cytotoxicity; β : second protein chain; γ : Fc gamma chain; IFN- γ : interferon gamma; G-CSF: granulocyte colony-stimulating factor; GPI: glycosylphosphatidylinositol; ITAM: immunoreceptor tyrosine-based activation motif; ITIM: immunoreceptor tyrosine-based inhibition motif; NK: natural killer (cells).

7.2 Human Fc γ receptors

One of the key features of IgG antibodies, which make up all currently marketed therapeutic antibodies, is that they interact with the immune system via the ability of their Fc domains to bind to specific Fc γ receptors on immune cells. This chapter focuses on the receptors and immune modulatory systems with which IgG antibodies interact. Interactions of IgGs with various immune cells can result in phagocytosis and cytokine release,

in the case of monocytes, macrophages, and neutrophils, and degranulation and release of stored immune mediators, as occurs with mast cells, natural killer (NK) cells, and basophils. Eight genes encoding the human FcγRs are located on the long arm of chromosome 1 in locations q21–q23 (Bournazos et al., 2009). In the mouse species, the low affinity FcγRs are encoded by genes found on chromosome 1 whereas the gene encoding the high affinity mouse FcγRI has been mapped to chromosome 3 (Bournazos et al., 2009).

Human immune cells express four activating Fcγ receptors, FcγRI (CD64), FcγRIIa (CD32a), FcγRIIc (CD32c), and FcγRIIIa (CD16a), as well as the glycosylphosphatidyl inositol (GPI)-linked FcγRIIIb (CD16b) (see Figure 7.1) (Takai, 2002; van Sorge et al., 2003; Nimmerjahn and Ravetch, 2006, 2008). FcγRI and FcγRIIIa associate with a gamma chain (FcR-γ – see below) that contains an immunoreceptor tyrosine-based activation motif (ITAM) through which they cascade their activation responses. FcγRIIa and FcγRIIc contain their own intracellular ITAM motif, and FcγRIIIb is not associated with an intracellular activation domain (see Figure 7.1). Additionally, human immune cells also express a single inhibitor receptor, FcγRIIb (CD32b), which functions through its integral immunoreceptor tyrosine-based inhibitory motif (ITIM). The human FcγRs all have similar overall structures and bind IgGs in the same general way (see Figure 7.1), although specific differences clearly exist between both each receptor and each IgG isotype which allow for differential binding and activation kinetics (Sonderman et al., 2001).

As will be discussed below, multiple FcγR polymorphisms that can significantly affect the binding of IgGs have been found. As far as is known, chimpanzees and other non-human primates (NHP) express orthologs for four of the six human FcγRs, including FcγRI, FcγRIIa, FcγRIIb, and FcγRIIIa (Presta and Namenuk, 2005). The IgG-binding patterns of the NHP FcγRIIIa, however, appear to be different from those of the human FcγRIIIa receptor, raising questions as to the interpretation of human IgG data obtained in NHPs (Rogers et al., 2006; see also Chapter 17). A short description of each pertinent human FcγR is given. More information on human FcγRs can be found in various reviews (Clark, 1997; Sonderman et al., 2001; Radaev and Sun, 2001; Ravetch and Bolland, 2001; Takai, 2002; Van Sorge et al., 2003; Woof and Burton, 2004; Nakamura et al., 2005; Nimmerjahn and Ravetch, 2006, 2008; Strohl, 2011).

7.2.1 FcR gamma (γ) chain

The Fc receptor gamma chain (FcR-γ) was originally described as the activation domain component of the high affinity Fcε receptor (FcεR)

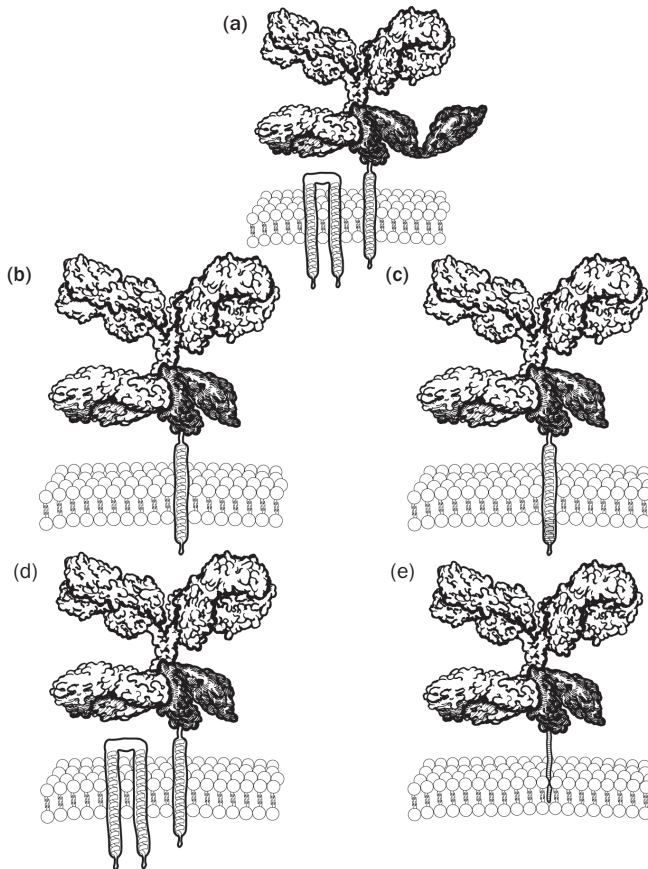


Figure 7.1 Human IgG₁ interaction with human FcγRs

Drawings, based on data from Sonderrmann et al. (2001) and Radaev and Sun (2001), showing: (a) IgG₁ binding to FcγRI (CD64). Note that the binding occurs at the "shoulder" of the IgG and primarily through the first (membrane proximal) domain of the FcγRI (Sonderrmann et al., 2001; Radaev and Sun, 2001). Note the Fc γ chain to the left of FcγRI. (b) IgG₁ binding to FcγRIIa (CD32a). Note that the binding occurs at the "shoulder" of the IgG and primarily through the first (membrane proximal) domain of the FcγRIIa, described by Radaev and Sun (2001) as domain "D2." Note that the ITAM domain of FcγRIIa is an integral part of the intracellular domain of that receptor as described in the text. (c) IgG₁ binding to FcγRIIb (CD32b). The IgG₁ binding to FcγRIIb occurs similarly to that of FcγRIIa as described by Sonderrmann et al. (2001) and Radaev and Sun (2001). Note that the ITIM (inhibitory) domain of FcγRIIb, noted as a darkened region in the drawing, is a part of the intracellular domain of that receptor as described in the text. (d) IgG₁ binding to FcγRIIIa (CD16a). Note that the binding occurs at the "shoulder" of the IgG and primarily through the first (membrane proximal) domain of the FcγRIIIa, described by Radaev and Sun (2001) as domain "D2." Note the Fc γ chain to the left of FcγRIIIa. (e) IgG₁ binding to FcγRIIIb (CD16b). Note that the IgG₁ binding to FcγRIIIb occurs similarly to that of FcγRIIIa as described by Sonderrmann et al. (2001) and Radaev and Sun (2001). Also note that FcγRIIIb is bound to the surface of the cell via a GPI linker which anchors in the lipid bilayer of the membrane and that there is no intracellular domain.

(Küster et al., 1990). It belongs to the family of accessory signal transduction proteins known as the of CD3 ζ /Fc ϵ R1 γ family. A common feature of these proteins is that they are critical for surface expression and the signal transduction function of the proteins with which they associate (van Vugt et al., 1996). Moreover, it has been demonstrated that the CD3 ζ protein can substitute for FcR- γ to generate functional Fc γ RIIIa (Howard et al., 1990). Mature FcR- γ is 68 amino acid residues in length and is found as a disulfide-linked, transmembrane spanning, homodimer of about 15 kDa. Each FcR- γ chain contains two intracellular activating signal motifs with the consensus sequence YxxLx₇YxxL, originally named the “Reth motif” after the discoverer (Scholl and Geha, 1993), and now called the immunoreceptor tyrosine-based activation motif (ITAM), which mediates signal transduction upon Fc γ R engagement (Scholl and Geha, 1993; Van den Herik-Oudijk et al., 1995b; van Vugt et al., 1996). FcR- γ is used as a receptor component for human Fc γ RI, Fc γ RIIIa, and Fc α R1. FcR- γ knockout mice, which do not express any of the activating receptors, Fc γ RI, Fc γ RIII, or Fc γ RIV, are resistant to autoimmune disorders such as glomerulonephritis and induced conditions such as collagen-induced arthritis (Nakamura, 2005; Nimmerjahn and Ravetch, 2008).

7.2.2 Human Fc γ RI

Human Fc γ RI, also known as CD64, is a 72 kDa membrane spanning protein with three extracellular Ig-like domains that functions as a high-affinity IgG receptor (see Table 7.1 and Figure 7.1). In humans, Fc γ RI is constitutively expressed on mononuclear cells including macrophages and dendritic cells, and its expression also can be induced on polymorphonuclear neutrophils by IFN- γ or G-CSF (Kakinoki et al., 2004; van der Poel et al., 2011). In fact, Fc γ RI (CD64) expression on neutrophils has been hypothesized to be a good biomarker for low level and difficult-to-detect bacterial infections (Cid et al., 2010), or other conditions in which IFN- γ might be produced. Distribution of mouse Fc γ RI is very similar to that of human Fc γ RI (Strohl, 2011).

Human Fc γ RI has three extracellular Ig-like domains (see Figure 7.1). Three FCGR1 genes, FCGR1A (locus 1q21.2–21.3), FCGR1B (locus 1p12), and FCGR1C (locus 1q21), have been shown to encode four different transcripts, Ia, Ib1, Ib2, and Ic (van Sorge et al., 2003). Of these transcripts, only FCGR1A produces a receptor whose function has been delineated (Ernst et al., 1992; Concetti and Napolioni, 2010). Due to the presence of

stop codons present in the exons encoding the extracellular domains of FCGRIB1 and FCGRIC, it appears that these genes encode truncated products or soluble forms, the functions of which are currently unknown (van Sorge et al., 2003). FCGRIB2, on the other hand, has been found trapped in the endoplasmic reticulum (van Vugt et al., 1999). While there are no known polymorphisms for human Fc γ RI that affect the binding of human IgG to the receptor, there is a polymorphism (R92X, where X represents a stop codon) that results in the absence of Fc γ RI expression on phagocytes (van de Winkel et al., 1995).

The common FcR- γ chain, containing ITAM, is required both for Fc γ RI cell surface expression and some of its activity (van Vugt et al., 1996; Van Sorge et al., 2003; Nimmerjahn and Ravetch, 2008). Human Fc γ RI associates with the the FcR- γ chain via a conserved set of sequences in its transmembrane domain (Harrison et al., 1995). Human IgG₁, IgG₃, and IgG₄ antibodies bind to human Fc γ RI with a monomeric affinity in the range of 15–35 nM. IgG₂ is the lone human IgG isotype that does not bind Fc γ RI at a measureable level (Bruhns et al., 2009). Due to the high affinity interaction, IgG₁, IgG₃, and IgG₄ bind Fc γ RI in a monomeric manner to elicit downstream signaling (Bruhns et al., 2009; van der Poel et al., 2011). Complexes containing two IgG₁ molecules were shown to bind Fc γ RI with approximately two to threefold higher affinity than monomeric IgG₁, indicating only a modest bump in activity by oligomerization (Luo et al., 2009). Thus, under normal physiological conditions *in vivo*, it is expected that human Fc γ RI would be occupied with a resident monomeric IgG₁, IgG₃, or IgG₄, ready to be displaced by a high-avidity immune complex (Bruhns et al., 2009). To that end, it has been calculated that Fc γ RI is 97.9% occupied with monomeric serum IgGs under normal physiological conditions (van der Poel et al., 2011). Upon opsonization of pathogens or foreign particles with antibodies to form immune complexes, the monomer-occupied Fc γ RI receptors are replaced by the immune complexes which trigger receptor clustering, downstream signaling, and immune cell functionality (van der Poel et al., 2011).

Upon binding IgG, Fc γ R1 activates the Src and Syk family protein tyrosine kinases, e.g. Lyn, Fyn, Fgr, and Hck, resulting in phosphorylation of the ITAM domain associated with the γ chain (Duchemin et al., 1994; van Vugt et al., 1996). It appears, though, that Fc γ R1 signaling is not entirely γ chain-dependent. It was demonstrated that IgG-ligated Fc γ R1 could still trigger IL-6 production even in the absence of γ -chain, and that truncation of the Fc γ R1 α -chain in that experiment resulted in cessation of IL-6 production (Edberg et al., 1999; van Vugt et al., 1999; van der Poel et al., 2011).

7.2.3 Human FcγRIIb

Human FcγRIIb (CD32b) and rodent FcγRII, both of which possess an immunoreceptor tyrosine-based inhibitory motif (ITIM) embedded in their intracellular domain (see Figure 7.1), are the inhibitory Fcγ receptors. Interaction of IgGs with these receptors is important for maintaining the balance of inhibitory and activating signals (Clynes et al., 2000; Baerenwaldt and Nimmerjahn, 2008; Smith and Clatworthy, 2010). Unlike FcγRI, these receptors do not associate with accessory proteins for expression and function. Instead, the ITIM motif, with the unique amino acid sequence “AENTITYSLLMHP” (human sequence shown; Van den Herik-Oudijk et al., 1995a) in the cytoplasmic tail of FcγRIIb, has been demonstrated to be responsible for signal transduction leading to down-regulation of other activation signals in B lymphocytes and mast cells (Smith and Clatworthy, 2010).

FcγRIIb, which is encoded at locus 1q22 in humans (Concetti and Napolioni, 2010), plays an important role in balancing immune response to infection by pathogens. An overabundance of FcγRIIb activity could lead to a reduced immune response and an inability to clear a bacterial infection. Increased FcγRIIb expression has also been linked with a lower T cell-mediated IgG response, and cessation or diminution of autoimmune-related conditions in mouse models of arthritis and systemic lupus erythematosus (SLE) (Brownlie et al., 2008). On the other hand, reduced FcγRIIb activity can result in pathogen-induced, pro-inflammatory, cytokine-mediated septic shock (Clatworthy and Smith, 2004). FcγRIIb polymorphisms also play a role in disease, with decreased expression correlated with predisposition to autoimmunity (Breunis et al., 2008; Brownlie et al., 2008; Smith and Clatworthy, 2010). Perhaps the best studied polymorphism, which is associated with increased prevalence of SLE in people carrying the mutation, is an isoleucine-to-threonine change at position 232 (I232T) within the transmembrane domain of FcγRIIb. This mutation decreases the localization of FcγRIIb to lipid rafts, resulting in reduced signaling through LYN and abnormal SHIP recruitment which ultimately leads to a decrease in FcγRIIb regulation in macrophages and B cells (Floto et al., 2005; Kono et al., 2005).

Both human FcγRIIb and mouse FcγRII are found widely expressed on B cells, mast cells, basophils, macrophages, eosinophils, neutrophils, dendritic cells, and Langerhan's cells (see Table 7.1). Upon antibody binding to FcγRIIb, various cell functions such as cytokine release, B cell activation and proliferation, antibody production by B cells, as well as degranulation of mast cells are inhibited (Baerenwaldt and Nimmerjahn, 2008). These

inhibitor signals help to balance the immune response of the activation FcγRs (Baerenwaldt and Nimmerjahn, 2008). On the other hand, since FcγRIIb is not present on human NK cells (Takai, 2002), it does not appear to play a balancing role in the regulation of NK cell-mediated ADCC in humans (Nakamura et al., 2005).

Impairment or blocking of FcγRII signaling in mice results in inflammatory and autoimmune conditions (Brownlie et al., 2008). Similarly, FcγRII knockout mice exhibit enhanced IgG or IgE-induced anaphylaxis, arthus reactions, and antibody-induced glomerulonephritis (Nakamura et al., 2005). These FcγRII-negative phenotypes demonstrate the key role for murine FcγRII and human FcγRIIb in balancing immune responses (Brownlie et al., 2008; Baerenwaldt and Nimmerjahn, 2008). As expected of this role, overexpression of FcγRIIb on macrophages resulted in greater susceptibility to infection due to a dampening of activating FcγR-mediated responses (Baerenwaldt and Nimmerjahn, 2008).

Li and Ravetch (2011) recently demonstrated a new and completely novel activity for FcγRII in mice, with the presumption of the same activity of FcγRIIb in humans. They showed that an immunomodulatory anti-CD40 antibody binding antigen presenting cells (APCs) was fully activated only when the inhibitory receptor FcγRIIb was co-ligated, which apparently provided a co-stimulatory signal. This surprising result was hypothesized to be due to the release of pro-inflammatory cytokines in the presence of co-stimulation by CD40 agonism and FcγRIIb binding (Smythe and Kershaw, 2011).

FcγRIIb is a component of the B cell complex on B cells and plays a significant role in B cell maturation. Overexpression of FcγRIIb on B cells results in the production of antibodies with higher affinity, demonstrating its important role as a regulator of B cell activation (Baerenwaldt and Nimmerjahn, 2008). Thus it appears that FcγRIIb expression on myeloid cells, which is “anti-inflammatory,” and on B cells, which is pro-immune response, acts to help balance the innate and humoral immune responses.

7.2.4 Human FcγRIIa

Human FcγRIIa (CD32a) is a 40 kDa low-affinity, integral membrane receptor expressed widely on hematopoietic cells, including monocytes, macrophages, B cells, neutrophils, mast cells, and platelets (Takai, 2002; Nimmerjahn and Ravetch, 2008; see Table 7.1). FcγRIIa is absent from lymphocytes, including NK cells, so it does not play a role in NK cell-mediated ADCC.

Fc γ RIIa (human locus 1q23; Concetti and Napolioni, 2010), which shares 93% sequence identity with Fc γ RIIb (Sonderman et al., 2001), is a monomeric receptor in which the ITAM is part of the intracellular domain of the receptor (see Figure 7.1) (Nimmerjahn and Ravetch, 2008). In this case, there are 12 residues separating the two YxxL motifs (YxxLx₁₂YxxL), different from the sequences found in the FcR- γ chain (Van den Herik-Oudijk et al., 1995b). Additionally, it has been demonstrated that the downstream signaling from the human Fc γ RIIa ITAM is qualitatively different from signaling from the FcR- γ ITAM (Van den Herik-Oudijk et al., 1995a, 1995b). FcR- γ ITAM was demonstrated to trigger both IL-2 production and antigen presentation, whereas Fc γ RIIa ITAM did not (Van den Herik-Oudijk et al., 1995b). The Fc γ RIIa ITAM is still capable of signaling, however, which results in cell activation and other activities such as oxidative burst, phagocytosis, and the production of pro-inflammatory cytokines (Hart et al., 2004).

Human IgGs bind Fc γ RIIa with affinities in the μ M range (see Table 7.1). Thus antigen-complexed IgG capable of conferring avidity is required for physiologically significant binding and triggering of receptor activation. Dimeric IgG₁ was reported to bind Fc γ RIIa with 200–800-fold higher apparent affinity than monomeric IgG₁, indicating a significant increase in activity attributable to IgG oligomerization (Luo et al., 2009). It has been demonstrated that Fc γ RIIa is capable of directing macrophage-mediated phagocytosis, based on disease association with its sequence polymorphisms, as well as its activity in *in vitro* functional assays (Richards et al., 2008). There are two polymorphic forms of human Fc γ RIIa, a low-affinity form in which amino acid residue 131 is an arginine (R131), and a higher-affinity form, in which a G to A point mutation results in an R131H substitution (Salmon et al., 1992). The higher affinity form of Fc γ RIIa was discovered when patients determined to be “high responders” to Orthoclone OKT3[®], a murine anti-CD3 IgG_{2a} used for transplantation, were found to possess the H131 mutation of Fc γ RIIa (van der Pol and van de Winkel, 1998). Monocytes from those high-responder patients were shown to exhibit a mitogenic response towards T cells incubated with the anti-CD3 MAb, and it was subsequently shown that the response was correlated with the Fc γ RIIa-H131 phenotype. Importantly, Fc γ RIIa is the only Fc γ R bound by human IgG₂, the primary antibody against repeating epitopes such as bacterial capsular polysaccharides. As such, it has been demonstrated that individuals who are homozygous for the Fc γ RIIa R131 polymorphic form are significantly more susceptible to bacterial infections, especially by microorganisms with polysaccharide capsules such as the pneumococci and meningococci

(Platonov et al., 1998; van der Pol and van de Winkel, 1998; Yee et al., 2000). Additionally, it has been demonstrated that only the higher affinity H131 form of FcγRIIa was associated with complement-independent, PMN-mediated phagocytic activity of encapsulated microorganisms such as *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae* (Sanders et al., 1995). Although there is only a fivefold difference in monomeric affinity of human IgG₂ to FcγRIIa R131 (10 μM) vs FcγRIIa H131 (2 μM) (Strohl, 2011), it is thought that the lower affinity allele does not bind IgG₂ immune complexes efficiently enough to protect against encapsulated microorganisms (Yee et al., 2000). This was demonstrated *ex vivo* as well, whereby phagocytes derived from donors having the homozygous high-affinity FcγRIIa H131 allele phagocytized IgG₂-opsonized particles much more efficiently than phagocytes derived from donors homozygous for the low-affinity FcγRIIa R131 allele (Parren et al., 1992). Under normal physiological conditions, it has been calculated that both polymorphic forms of FcγRIIa on immune cells are about 19–22% saturated with serum IgG, so they are available for cross-linking with immunocomplexes (van der Poel et al., 2011).

It appears that antigen-crosslinked human FcγRIIa and FcγRIIIb work synergistically in generating an optimal phagocytic response by neutrophils, possibly due to the effects of a higher density of FcγRIIIb serving to enhance interactions of IgG-antigen complexes with the available FcγRIIa (van der Pol and van de Winkel, 1998). Moreover, the high-affinity alleles of both FcγRIIa (H131) and FcγRIIIb (NA1) were correlated with optimal phagocytic activity of pneumococci by neutrophils (Jansen et al., 1999). The higher-affinity H131 FcγRIIa also has been correlated with improved therapeutic outcome upon treatment of neuroblastoma with a murine anti-GD2 antibody (Cheung et al., 2006). Finally, several possible correlations have been found between the success of cancer chemotherapy and the polymorphisms of FcγRIIa and FcγRIIIa either independently or together (Weng and Levy, 2003; Zhang et al., 2007; Desjarlais et al., 2007; Musolino et al., 2008; Bibeau et al., 2009). Approximately 24% of Caucasians are homozygous for the higher-affinity H131 allele, 42% are heterozygous for the HR131 alleles, and 34% are homozygous for the lower-affinity R131 allele (Van Schie and Wilson, 2000).

Neither mice nor rats possess a direct equivalent of human FcγRIIa, although mouse FcγRIII is most closely related in sequence and structure (Strohl, 2011; Chapter 17). On the other hand, Lazar and Desjarlais (2009) suggested that mouse FcγRIV is the closest receptor to human FcγRIIa based on the combination of function and cellular distribution.

7.2.5 Human FcγRIIc

FcγRIIc, encoded within human locus 1q23 (Concetti and Napolioni, 2010), is a variant of FcγRIIa and FcγRIIb and is present on monocytes, macrophages, polymorphonuclear cells, and NK cells. It has been found on human NK cells in about 50% of the population (Veri et al., 2007), making it the only FcγR other than FcγRIIIa on human NK cells. The extracellular domain of FcγRIIc is identical to that of FcγRIIb (i.e. the first 254 out of 323 residues are identical between the two proteins), whereas the C-terminus of FcγRIIc has an ITAM in its cytoplasmic tail similar to that found in FcγRIIa (i.e. FcγRIIc shares 90 of 91 C-terminal amino acid residues with FcγRIIa) (Bruhns et al., 2009). Thus it appears that FcγRIIc may have arisen as a genetic recombination of FcγRIIa and FcγRIIb (Nimmerjahn and Ravetch, 2008).

Due to the presence of its activating ITAM domain, it is thought that FcγRIIc may function as a co-activation receptor along with FcγRIIIa (Ernst et al., 2002; Nimmerjahn and Ravetch, 2008). There are multiple polymorphisms of FcγRIIc having differing levels of activation activity that may affect NK cell activation by IgG antibodies (Ernst et al., 2002). Recent data suggest that an increase in the copy number of the gene encoding FcγRIIc is directly correlated with an increase in the incidence of immune thrombocytopenic purpura (IPT) (Breunis et al., 2008). Additionally, it was demonstrated that FcγRIIc on NK cells can actually trigger stronger ADCC than FcγRIIIa (Breunis et al., 2008). Thus the impact of FcγRIIc on NK cell-mediated ADCC activity may be dramatically underestimated. If broadly confirmed, this could alter the impression that engineering IgGs for improvement of FcγRIIIa binding alone, such as occurs with low or no fucosylated antibodies (see Chapter 11), is enough to maximize ADCC. On the other hand, Fc mutations designed to improve IgG binding to multiple Fcγ receptors (e.g. S298A, E333A, K334A (Shields et al., 2001); S239D, I332E (Lazar et al., 2006; Horton et al. 2008; Zalevsky et al., 2009); see Chapter 10), which would be expected to improve binding to FcγRIIc as well, might actually provide the maximum ADCC capability.

7.2.6 Human FcγRIIIa

Human FcγRIIIa, also known as CD16a, is a ca. 28 kDa membrane-spanning protein with two extracellular Ig-like domains that have low affinity for monomeric IgG. FcγRIIIa is found widely on myeloid cells such as macrophages, dendritic cells, eosinophils, and mast cells, as well as on

NK cells, but it is not expressed by neutrophils (Strohl, 2011). About 10% of blood human monocytes express FcγRIIIa, and this subset resembles tissue macrophages. CD16-negative monocytes are typically considered “classic” monocytes (Gordon and Taylor, 2005).

Human FcγRIIIa is encoded by one of the two nearly identical FCGR3 genes, FCGR3A, found on chromosome 1q23 (Concetti and Napolioni, 2010). Analogous to FcγRI, FcγRIIIa requires the ITAM-containing FcR γ-chain for both stable cell surface expression and activity (Van Sorge et al., 2003; Nimmerjahn and Ravetch, 2008). FcγRIIIa on NK cells can associate with the T cell receptor ζ-chain, instead of FcR-γ as an alternative mechanism for signaling (Anderson et al., 1990). Under normal physiological conditions, it has been calculated that FcγRIIIa on immune cells are about 21–26% saturated with serum IgG, so they are available for cross-linking with immunocomplexes (van der Poel et al., 2011).

Human FcγRIIIa on NK cells is the key receptor (perhaps along with FcγRIIc, as noted above; Breunis et al., 2008) involved in triggering antibody-dependent cell-mediated cytotoxicity (ADCC) when bound by antigen-complexed IgG₁ or IgG₃ antibodies. NK-cell mediated ADCC has been demonstrated to be one of the key cell depletion mechanisms in human serum. When human peripheral blood mononuclear cells (PBMCs) were mixed an anti-EpCAM IgG₁ and EpCAM⁺ LS180 cells, NK cells bearing FcγRIIIa mediated most of the targeted lysis of the target cells. When the NK cells were depleted or when FcγRIIIa was blocked by an antibody, most of the PBMC-mediated LS180 cell lysis was absent, indicating the key role played by FcγRIIIa-bearing NK cells (Richards et al., 2008).

Human FcγRIIIa occurs in two major forms, the higher affinity V158 polymorphic variant and a lower affinity F158 polymorphic variant (Wu et al., 1997; Nimmerjahn and Ravetch, 2006; Bournazos et al., 2009). Individuals who are homozygous for the higher affinity V158 allele have been shown to respond significantly better to several anti-cancer therapeutic antibodies (e.g. Rituxan®, Herceptin®, Erbitux®) that target receptors than individuals who are either heterozygous VF158 or homozygous for the lower-affinity homozygous F158 polymorphic variant (Cartron et al., 2002; van Sorge et al., 2003; Weng and Levy, 2003; Desjarlais et al., 2007; Zhang et al., 2007; Musolino et al., 2008). Chong et al. (2006) analyzed the FcγRIIIa allelic distributions across several populations. Using their composite data, 12% of the total individuals tested (n = 560) were homozygous for the high-affinity V158 receptor, 46% (n = 2,200) were heterozygous, and 43% (n = 2,051) were homozygous for the lower-affinity F158 receptor. The significance of this is that if a normal IgG is used to

treat a cancer cell surface target, only about 12% of the population will likely benefit from ADCC activity. Engineering into IgG₁ a higher affinity for FcγRIIIa, which is discussed in detail in Chapters 10 and 11, should allow for a potentially much broader population of individuals to benefit from the ADCC activity of the modified therapeutic MAb.

7.2.7 Human FcγRIIIb

Human FcγRIIIb (CD16b) is a glycosylphosphatidyl inositol (GPI)-anchored receptor of ca. 26 kDa that contains two Ig-like extracellular domains and is found on neutrophils, basophils, IFN-γ-induced eosinophils, a subpopulation of T cells, immature thymocytes, and placental trophoblasts (Ravetch and Perussia, 1989; Scallon et al., 1989; Takai, 2002; Nimmerjahn and Ravetch, 2008; Meknache et al., 2009). Human FcγRIIIb is encoded by the second of the two nearly identical FCGR3 genes, FCGR3B, found on chromosome 1q23 (Concetti and Napolioni, 2010). FcγRIIIa and FcγRIIIb are highly similar, with only 6–8 amino acid residue differences; additionally, FcγRIIIb contains a stop codon 21 residues upstream of the stop codon found in FcγRIIIa, presumably allowing it to be processed to contain the GPI anchor (Scallon et al., 1989; Ravetch and Perussia, 1989). Non-human primates, including chimpanzees, mice, rats, and other lower species do not appear to possess a structural or functional equivalent of FcγRIIIb.

FcγRIIIb does not interact with an FcR γ-chain, nor does it contain its own signaling motif, so it does not appear to signal directly like the other FcγRs. As such, it is generally believed that FcγRIIIb functions by synergizing with other FcγRs such as FcγRIIa (Green et al., 1997; Ebel et al., 2001). FcγRIIIb also has been demonstrated to interact with β2 integrins such as Mac-1 (CD11b/CD18), as well as other low-affinity FcγRs, on the surface of neutrophils, resulting in binding to immune complexes, the effect of which triggers degranulation, respiratory burst activity, and phagocytosis (Coxon et al., 2001). Recently, it has been proposed that ligation of FcγRIIIb results in the activation of a unique signaling pathway via Syk, PI3K and MEK-independent nuclear-restricted phosphorylation of ERK and Elk-1 (García-García et al., 2009). Also, Rivas-Fuentes et al. (2010) recently demonstrated that FcγRIIIb on neutrophils could catalyze modest phagocytosis on its own by a mechanism that could be blocked by srk family kinase (i.e. Syk, PI 3-K, ERK) inhibitors. That mechanism, however, was considerably less active than FcγRIIa-driven phagocytosis by the same neutrophils (Rivas-Fuentes et al., 2010).

Three different isoforms of FcγRIIIb, NA1, NA2, and SH, have been found (Steffenson et al., 1999; Bruhns et al., 2009). In the Caucasian population, 14% are homozygous NA1/NA1, 48% are heterozygous NA1/NA2, and 38% are homozygous NA2/NA2 (Van Schie and Wilson, 2000). Of these, only the NA1 form has been correlated with neutrophil-mediated phagocytosis (Jansen et al., 1999). Interestingly, the NA1 form also is the only polymorphism of FcγRIIIb to be associated with certain autoinflammatory diseases such as idiopathic pulmonary fibrosis (IPF; Bournazos et al., 2010). Whereas FcγRIIIb NA1 had been shown in earlier studies to bind both IgG₁ and IgG₃ and confer phagocytic activity better than the NA2 isoform (Bredius et al., 1994), recent biochemical analyses demonstrated that the binding of IgG₁ (~5 μM range) and IgG₃ (~1 μM range) did not vary significantly between the receptor isoforms (Bruhns et al., 2009). Thus there are still aspects of this biology that need to be sorted out. Clearly, though, FcγRIIIb is a low-affinity receptor for IgG₁ and IgG₃. *In vitro* dimerization of IgG₁ resulted in 200–800-fold higher affinity binding to FcγRIIIb as compared with monomeric IgG₁, indicating a substantial avidity effect by oligomerization (Luo et al., 2009).

Neutrophils, which act as the first line of defense against invading pathogens, utilize phagocytosis, oxidative burst, degranulation and release of anti-microbial compounds, and production of pro-inflammatory cytokines to kill the pathogens. FcγRIIIb and FcγRIIa are both constitutively expressed on neutrophils, and it has been demonstrated that both receptors must be crosslinked by antibody-pathogen immune complexes to initiate phagocytosis and cytokine release by neutrophils (Jakus et al., 2008).

7.2.8 TRIM21

Tripartite motif-containing 21 (TRIM21; also known as Ro52), a member of the large TRIM protein family that is largely involved in innate immunity, is normally an intracellular protein that also has recently been demonstrated to be a receptor for IgGs in both humans and rodents (Keeble et al., 2008). TRIM21 is a multi-domain, trimeric protein with each chain consisting of RING, B Box, coiled-coil, and PRYSPRY domains that binds all IgG species with a monomeric K_D of about 200 nM. The receptor binds IgG in a 2 : 1 stoichiometry, with one TRIM21 molecule binding each half of the IgG Fc domain (James et al., 2007). *In vivo*, however, it appears that TRIM21 acts as a dimer and binds IgG in a 1 : 1 stoichiometry with sub-nM affinity (Mallery et al., 2010).

TRIM21 has been shown to form pathogenic immune complexes, as well as being a major autoantigen in rheumatoid arthritis, systemic lupus erythematosus, and Sjögren's syndrome (Keeble et al., 2008). Nevertheless, it was demonstrated recently that TRIM21 also can bind monomeric IgG through the conserved "PRYSPRY" domain (James et al., 2007). While several mechanisms for TRIM21 activity have been hypothesized, including the targeting of apoptotic cells for phagocytosis (Keeble et al., 2008), Mallery et al. (2010) demonstrated that TRIM21 targets internalized antibody-coated viruses for degradation by autoubiquitination of TRIM21. IgM also was demonstrated to bind TRIM21, albeit at a lower affinity (K_D , 16.8 μ M) than IgG, and functions to neutralize IgM-coated viruses (Mallery et al., 2010). Thus TRIM21 assists IgG and IgM to become part of an "intracellular humoral immunity" pathway (Mallery et al., 2010).

7.3 FcRn and its effect on MAb and FcFP half-life

The average half-life of endogenous human IgG₁, IgG₂, and IgG₄ antibodies in circulation is 21 days (Morell et al., 1970). The half-life of a therapeutic antibody or Fc fusion protein, on the other hand, depends on many factors, including whether the target is soluble or membrane-bound (often yielding very different clearance mechanisms – see Table 7.2), target turnover, dosing frequency, route of administration, anti-antibody immune responses, differential affinity for FcRn at pHs 6.0 and 7.4 (Suzuki et al., 2010), and the overall pI of the antibody (Igawa et al., 2010). Recycling by FcRn, as described later in this section, provides the elongated half-life of MAbs and Fc fusion proteins as compared with other serum proteins of similar size (see Figure 7.2). The nominal half-life values for several antibodies and FcFPs are provided in Table 7.2. As can be observed in this table, the average half-life of the 11 MAbs targeting soluble proteins is about 17.6 days, whereas the half-life of 13 MAbs targeting membrane-bound proteins is about 10.2 days (Table 7.2; note that the data for FcFPs, which typically have shorter half-life values than MAbs (Suzuki et al., 2010), were not included in these averages). The half-life values for MAbs binding membrane-bound targets are not linear with respect to concentration, and are often dose-dependent, with higher doses showing longer half-lives due to proportionally less clearance attributable to target binding. While these values can change depending on what sample set is used, the key theme is that antibodies targeting soluble protein targets have, on average, longer half-life values in human serum, as discussed further in Chapter 8. The

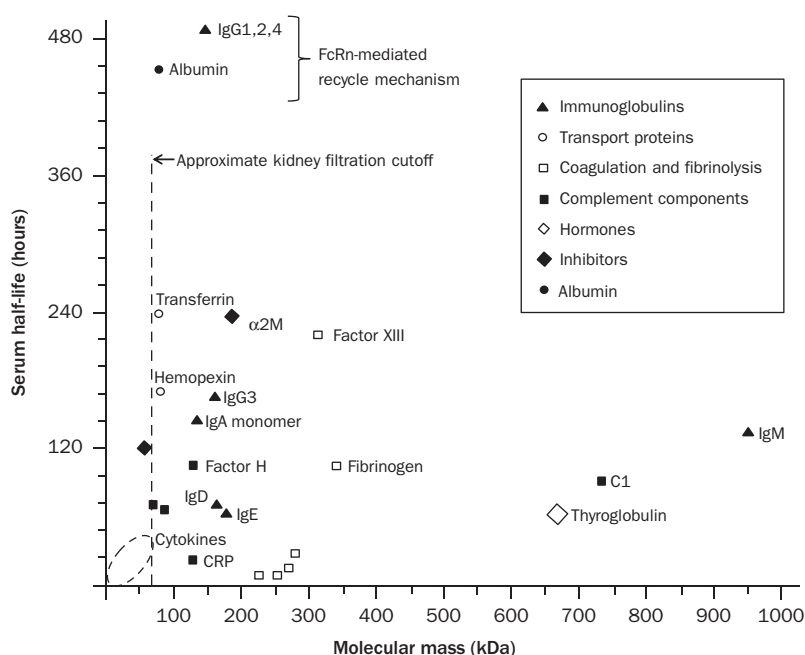


Figure 7.2 Plasma half-life of human serum proteins (in hours) as a function of size (in kDa)

The approximate limit for kidney filtration (~70 kDa) is denoted by a dotted line. Note that IgGs 1, 2, and 4 as well as human serum albumin have very long half-life values due to recycling via FcRn. Also, note the relatively long half-life of transferrin. (Redrawn and modified from Kontermann (2009).

average clearance value for endogenous human antibodies is 2–4 mL/d/kg (Yang et al., 2010). With some exceptions, the clearance values for most of the therapeutic MAbs described in Table 7.2 fall within this range.

The neonatal Fc receptor (FcRn), alternatively known as the Brambell receptor (named for F.W.R. Brambell, who hypothesized a recycling mechanism for IgGs (Brambell, 1966)), is a type I heterodimeric receptor comprised of a 45 kDa, transmembrane-anchored α -chain with a short cytoplasmic tail, and a 17 kDa β -chain known as β -2 microglobulin (Ghetie and Ward, 2000; Roopenian and Akilesh, 2007; Baker et al., 2009). FcRn, which is widely expressed in vascular epithelial cells, endothelial cells, placental syncytiotrophoblasts, intestinal epithelial cells, mammary epithelial cells, monocytes, macrophages, dendritic cells, and polymorphonuclear leukocytes, is most closely related to the major histocompatibility complex (MHC) class I receptors (Baker et al., 2009). Nevertheless, the classic peptide groove present in MHC class I receptors is collapsed in FcRn, making it unlikely that FcRn functions to directly

Table 7.2 Half-life in humans of example therapeutic and clinical candidate MABs and FcFPs

US trade name (generic name)	Molecular target and location (M or S)	Major indication	Protein format	Mean half-life*	Receptor- mediated clearance	Clearance (mL/d/kg)
Ilaris® (Canakinumab)	IL-1 β (S)	CAPS	Human IgG ₁ κ	26 d	NA	2.4
Xolair® (Omalizumab)	IgE (S)	Asthma	IgG ₁ κ ; humanized	26 d	NA	2.4
Prolia™ (Denosumab)	RANK-ligand (S)	Osteoporosis	Human IgG ₂	25.4 d	NA	–
Stelara™ (Ustekinumab)	P40 subunit of IL-12 & IL-23 (S)	Psoriasis	Human IgG ₁	21.6 d	NA	1.9–2.2
Avastin® (Bevacizumab)	VEGF (S)	Colorectal cancer	IgG ₁ ; humanized	20 d	NA	2.9–3.8
Benlysta™ (Belimumab)	BLyS (S)	Lupus (SLE)	Human IgG ₁ λ	19.4 d	NA	3.1
Humira® (Adalimumab)	TNF- α (S)	RA, Crohns	IgG ₁ κ ; human	14 d	NA	4.1
Simponi® (Golimumab)	TNF- α (S)	RA	Human IgG ₁	14 d	NA	4.8–6.7
Remicade® (Infliximab)	TNF- α (S)	Crohns, RA	IgG ₁ κ ; chimeric	7.7–9.5 d	NA	–
Enbrel® (Etanercept)	TNF- α , TNF- β (S)	RA	IgG ₁ -Fc fused to p75 exo-domain of TNFR	4.2 d	NA	2.2
Arcalyst® (Rilonacept)	IL-1 β , IL-1 α , IL-1RA (S)	CAPS, Muckle Wells syndrome	IgG ₁ Fc-fusion protein with IL-1R & IL-1ACP	7.6 d	NA	12.5

Soliris® (Eculizumab)	Complement C5 (S)	PNH (reduce hemolysis)	Humanized IgG _{2/4} hybrid Modified Fc	11.3 d	NA	7.4
Rituxan® (Rituximab)	CD20 – B cells (M)	NHL, RA	IgG ₁ κ, chimeric	22 d	Not apparent	4.8
Zenapax® (Daclizumab)	IL-2Rα (CD25) (M)	OTR	IgG ₁ , humanized	20 d	Yes	–
Herceptin® (Trastuzumab)	HER2/Neu (M)	Breast cancer	IgG ₁ κ, humanized	5.8 d; 20–28 d	Yes	ND
Orencia® (Abatacept)	CD80/CD86 (M)	RA	CTLA4-FcFP-modified Fc	13.1–16.7 d	ND	5.5
Yervoy™ (Ipilimumab)	CTLA4 (M)	Malignant melanoma	Human IgG ₁ κ	14.7 d	ND	5.3
Azerra™ (Ofatumumab)	CD20 (M)	CLL, NHL, RA	Humanized IgG ₁ κ	14 d	Not apparent	ND
Amevive® (Alefacept)	CD2 (M)	Psoriasis	LFA-3 – IgG ₁ FcFP	11.3 d	ND	ND
Tysabri® (Natalizumab)	α4 subunit of α4β1 or α4β7 (M)	Multiple sclerosis	IgG ₄ κ, humanized	11 d	ND	5.5
Perjeta™ (Pertuzumab)	Pan-Her (M)	Breast cancer	IgG ₁ , humanized	10 d	Yes	ND
Vectibix® (Panitumumab)	EGFR (M)	Colorectal cancer	Human IgG ₂ κ	7.5 d	ND	4.8
Simulect® (Basiliximab)	IL-2Rα (CD25) (M)	OTR	IgG ₁ κ, chimeric	7.2 d	ND	ND
Actemra® (Tocilizumab)	IL-6R (M)	Castleman's disease; RA	IgG ₁ , humanized	6.3 d	Yes	7.0

Table 7.2 Half-life in humans of example therapeutic and clinical candidate MABs and FcFPs (*cont'd*)

US trade name (generic name)	Molecular target and location (M or S)	Major indication	Protein format	Mean half-life*	Receptor- mediated clearance	Clearance (mL/d/kg)
Raptiva® (Efalizumab)	CD11a, α-sub-unit of LFA-1 (M)	Psoriasis	IgG ₁ κ; humanized	5.5 d	Probably	24
Zalutumumab® (HuMax EGFR)	EGFR (M)	Head and neck cancer	Human IgG ₁ κ	5.0 d	Yes	ND
Erbixux® (Cetuximab)	EGFR (M)	Colorectal cancer	IgG ₁ κ; chimeric	4.7 d	Yes, rapid	ND
Campath-1H® (Alemtuzumab)	CD52 on B- and T-cells (M)	Leukemia	IgG ₁ κ; humanized	1–6 d	Yes, rapid	ND
Nplate® (Romiplostim)	TPO-R (M)	Thrombo- cytopenia	Fc-peptide fusion ("peptibody")	3.5 d	ND	ND
Averages	MAbs against 11 soluble targets (excluding FcFPs)			17.6 d	–	
	MAbs against 9 membrane bound targets (excluding Herceptin® [conflicting data] and FcFPs)			10.2 d	–	
Endogenous IgG (average half-life of human IgG ₁ , IgG ₂ , and IgG ₄)				21 d**		2–4

Abbreviations: AMD: age-related macular degeneration; CAPS: cryopyrin-associated periodic syndrome; CV: cardiovascular disease; IV: intravenous; LYO: lyophilized; M: membrane bound; OTR: organ transplantation rejection; NA: not applicable; ND: no data; NHL: non-Hodgkin's lymphoma; PNH: paroxysmal nocturnal hemoglobinuria; RA: rheumatoid arthritis; RSV: respiratory syncytial virus; S: soluble; SC: subcutaneous; TNF: tissue necrosis factor.

* Most of the values for marketed products are derived from the prescribing information sheets provided by the manufacturer, often from data obtain in Phase I clinical trials. Steady state kinetics based on multi-dosing are sometimes different. Data for clinical candidate MABs and FcFPs are from publication of Phase I clinical trial data.

** Morell et al. (1970).

present peptides (Roopenian and Akilesh, 2007). FcRn carries out multiple functions, including maternal–fetal IgG transfer, homeostasis of both IgGs and serum albumin, and internalization of antigens by macrophages for eventual presentation (Ghetie and Ward, 2000, 2002; Roopenian and Akilesh, 2007; Cervenak et al., 2011). Some important recent papers and reviews in this area include Dall’Acqua et al. (2006), Roopenian and Akilesh (2007), Baker et al. (2009), Suzuki et al. (2010), Igawa et al. (2010), Yeung et al. (2009, 2010), and Cervernak et al. (2011).

At pH <6.5 FcRn binds the Fc regions of IgG₁, IgG₂, and IgG₄ antibodies with ~500–2,400 nM range of affinities, whereas at pH 7.4, FcRn does not bind the IgGs (Dall’Acqua et al., 2006; Yeung et al., 2009, 2010; Suzuki et al., 2010). This allows for a mechanism by which IgGs are rescued from lysosomal degradation following uptake into cells. Specifically, IgGs are non-specifically pinocytosed into intracellular vesicles, which ultimately fuse with acidic endosomes (see Figure 7.3). At the acidic endosomal pH, FcRn binding to IgGs occurs in a 2: 1 ratio, in which one molecule of FcRn

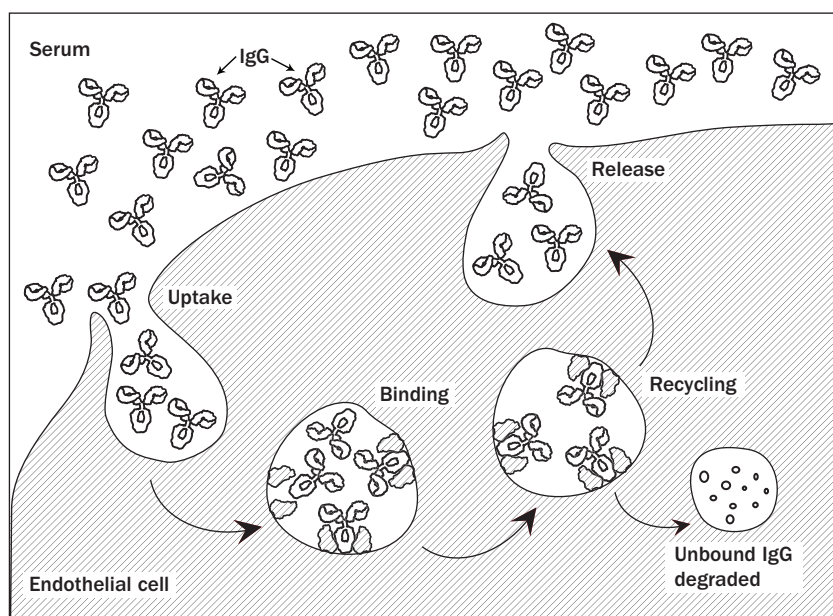


Figure 7.3 FcRn-mediated recycling

IgGs are pinocytosed into vesicles. Upon acidification of the vesicles, the IgGs attach to vesicle-membrane-bound FcRn and are recycled back into circulation by release from FcRn at neutral pH. Those proteins and antibodies that do not bind FcRn in a pH-dependent manner as described in the text enter the lysosomal protein degradation pathway. This recycling mechanism gives IgGs their elongated half-life, which in humans is nominally 2–3 weeks.

binds to each of the Fc halves (Martin and Bjorkman, 1999) (see Figure 7.3). FcRn-bound IgGs are then routed back to the cell surface, where re-exposure to a neutral pH environment prompts release of the IgG, either back into circulation or into the extracellular fluid of the surrounding tissue. The pH shift that the antibody experiences in moving from a vascular medium at pH 7.4 to the acidic endosome with pH of about 6.0 results in protonation of Fc histidine residues, which promotes the interaction of the IgG with FcRn. Histidine has a pKa of approximately pH 6 and is often associated with protein–protein interactions that differ between the acidic endosomal compartment and the surrounding medium (Vaughn and Bjorkman, 1998). The entire binding interface between an IgG and FcRn is about 1870 Å² that includes three key binding regions involving residues 252–257, 307–311, and 433–436 (Martin et al., 2001).

Suzuki et al. (2010) showed a reasonably strong correlation between the affinity of commercial IgGs and Fc fusion proteins to FcRn and their half-life in humans. This correlation only held true if chimeric antibodies and anti-receptor antibodies were not included in the analysis (Suzuki et al. 2010), which makes sense because many anti-receptor antibodies are cleared primarily through internalization of the receptor they bind (see Table 7.2). Since most therapeutic IgG₁ antibodies and Fc fusion proteins with IgG₁ Fc regions have either identical or very similar constant region sequences, the differences in their affinity for FcRn, which can span values of 0.5–3.5 μM K_Ds, must be at least in part due to influences by the variable regions and fusion proteins for IgGs and Fc fusion proteins, respectively (Suzuki et al., 2010). Additionally, Igawa et al. (2010) recently demonstrated that the charge (pI) of the V regions could dramatically affect the half-life of a human IgG. Those MAbs with a lower pI had as much as twice the half-life as the same antibodies with a higher pI (Igawa et al., 2010). Thus not only is the direct interface with FcRn important for binding and thus half-life, but sequences further away from the binding pocket seem to play a significant role as well (Igawa et al., 2010). This is consistent with an analysis of several therapeutic and clinical candidate MAbs that contained the identical Fc sequence (i.e. same allotype), but ranged in half-life values from 10–27 days (W.R. Strohl, unpublished data), indicating that the sequence of the V regions has a significant impact on the half-life of the antibody.

Whereas pinocytosed proteins incapable of binding FcRn (or IgGs that lose out in the competition for binding) are targeted for degradation via the lysosomal pathway, the FcRn-bound IgGs are translocated back to the cell surface and, upon exposure to physiological pH at which they no longer bind FcRn, released back into the serum (Roopenian and Akilesh, 2007;

Ghetie and Ward, 2000, 2002) (see Figure 7.3). This is the mechanism that confers a half-life on human IgG₁, IgG₂, and IgG₄ molecules in the 14–21 d range. IgG₃, which has an altered residue in the otherwise conserved FcRn binding domain (see Chapter 9), does not bind FcRn as well and thus has a significantly shorter half-life of just ~5–7.5 days (Morell et al., 1970; Kim et al., 1999). Similarly, all forms of IgA, IgM, IgD, and IgE are incapable of binding FcRn.

As mentioned above, FcRn also binds to serum albumin in a pH-dependent manner and recycles albumin back into serum in a manner similar to its recycling of IgG (Chaudhury et al., 2003). Albumin, however, binds in a 1 : 1 stoichiometry to a different epitope on FcRn (Baker et al., 2009), and does not directly compete with IgGs for the receptor (Chaudhury et al., 2006). It is worth noting that Chaudhury et al. (2003) calculated that for every molecule of IgG recycled into circulation by FcRn, nearly 700 molecules of albumin are recycled; thus it is clear that FcRn plays a significant physiological role in both IgG and albumin homeostasis, the two most abundant proteins in human serum.

Under normal physiological conditions, the majority of FcRn in polarized epithelial cells is located in subapical (in rodents) or basolateral (in humans) vesicles (McCarthy et al., 2000, 2001; Baker et al., 2009). While in humans the primary route of maternal to fetal transfer occurs in the basolateral to apical direction, FcRn also has been shown to mediate the bidirectional transcytosis of IgGs across epithelial cell surfaces (Dickinson et al., 1999; McCarthy et al., 2000; Claypool et al., 2004) (see Figure 7.4). FcRn also has been demonstrated to carry out the bidirectional translocation of IgG across lung epithelium (Spiekermann et al., 2002; Kim et al., 2004), although assays using rat lung demonstrated that the rate of translocation in the apical to basolateral direction was significantly higher than in the opposite direction (Kim et al., 2004). FcRn-mediated transcytosis of IgG from circulation to the mucosal surface of the lung is critical for the activity of Synagis®, which is administered systemically via intramuscular injection to premature infants to protect them from pulmonary infection by respiratory syncytial virus (RSV) (Groothuis et al., 1993). Moreover, certain mucosal secretions contain both sIgA and IgG which function together to aid in host defense (Spiekermann et al., 2002). Spiekermann et al. (2002) suggested that the bidirectional transcytosis of IgGs across mucosal surfaces may allow for immune surveillance and retrieval of luminal antigens which could be processed by immune cells. Yoshida et al. (2004), in fact, demonstrated that FcRn mediated bidirectional translocation of IgGs across the intestinal epithelial layer in FcRn-transgenic mice as part of an immune surveillance mechanism. Finally, FcRn has been proposed

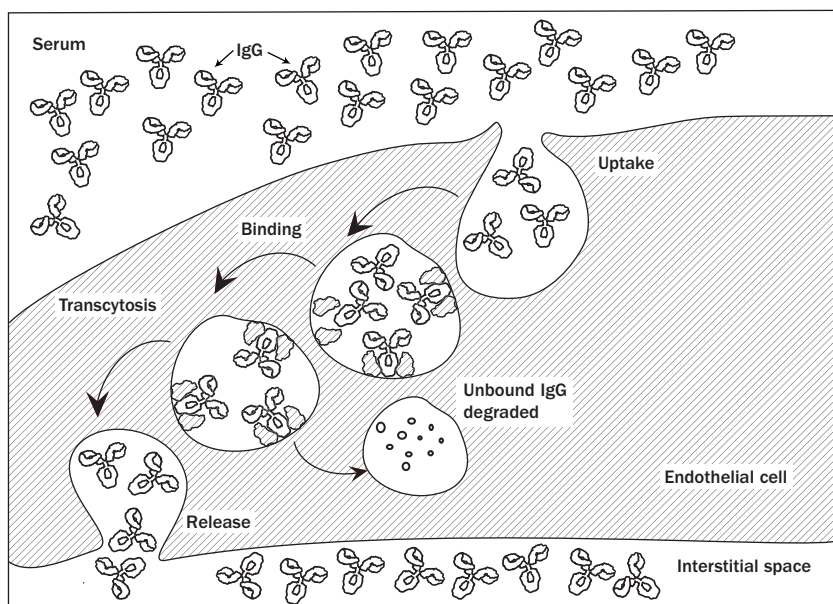


Figure 7.4 FcRn-mediated transcytosis

IgGs are pinocytosed into vesicles. Upon acidification of the vesicles, the IgGs attach to vesicle-membrane-bound FcRn. In this case, rather than being recycled to the same surface, the IgGs are translocated to the opposite surface of an endothelial cell resulting in transcytosis into the interstitial space. The IgGs are released from FcRn on the basolateral side at neutral pH. Those proteins and antibodies that do not bind FcRn in a pH-dependent manner as described in the text enter the lysosomal protein degradation pathway.

to be a primary mechanism by which IgG antibodies can be translocated out of the brain into the serum (Zhang and Pardridge, 2001; Schlachetzki et al., 2002).

Recently, it has been demonstrated by several investigators that FcRn also plays a role in internalization and degradation of IgG-antigen complexes (Vidarsson et al., 2006; Qiao et al., 2008; Mi et al., 2008; Cervenak et al., 2011), leading to the presentation of antigen fragments on the surface of professional antigen presenting cells (Qiao et al., 2008; Mi et al., 2008; Cervenak et al., 2011). Thus FcRn plays a concomitant role in the recycling of monomeric IgGs, while also effectively increasing the ability of antigen presenting cells (APCs) to phagocytose IgG-antigen immune complexes and direct those complexes into lysosomes for degradation (Vidarsson et al., 2006; Qiao et al., 2008; Mi et al., 2008; Cervenak et al., 2011). For the latter mechanism, MHC class II-associated variant chain has been shown to associate with FcRn to help direct

FcRn-IgG-antigen complexes into lysosomal degradation pathways (Ye et al., 2008). These recent findings suggest that the internalization and targeting of IgGs to either the recycling or degradation pathways is more complex than perhaps originally anticipated.

Finally, FcRn is a potential therapeutic target in its own right. Recent studies have shown that interference with the recycling of serum IgG by FcRn with FcRn-binding peptides or other IgG-competitive molecules may serve a therapeutic role for IgG-mediated autoimmune diseases (Sesarman et al., 2010).

7.4 Other Fc receptors of importance

While most of this book focuses on the discovery and development of IgGs as therapeutic monoclonal antibodies, both the IgM and IgA scaffolds have been used to make clinical candidate antibodies. Thus the interaction of those molecules with their cognate Fc receptors is important to the understanding of those molecules. In this section, Fc receptors binding IgM and IgA will be discussed.

7.4.1 *FcαRI*

The primary Fc receptor for IgA is FcαRI (CD89), which is found on monocytes, macrophages, neutrophils, eosinophils, interstitial dendritic cells, and Kupffer cells (Bakema et al., 2006). Human IgA binds human FcαRI with an affinity of about $\sim 2\text{--}5 \times 10^{-7}$ M (Herr et al., 2003). FcαR associates with the ITAM-containing FcR-γ chain for signaling (Bakema et al., 2006; Bakema and van Egmond, 2011).

Neutrophil-mediated phagocytosis by IgA-complexed pathogens has been demonstrated to require cross-linking of FcαRI, similar to that found with antigen-bound IgG cross-linking of low-affinity receptors such as FcγRIIa and FcγRIIIa (Woof and Kerr, 2006). Thus it appears that antigen-complexed IgA activates neutrophil and monocyte phagocytosis better than monomeric forms (Monteiro and van de Winkel, 2003; Woof and Kerr, 2006; Wines and Hogarth, 2006). A single dimeric IgA molecule can bind two FcαRIs, whereby the first IgA-FcαRI interaction has been shown to have an affinity of 176 nM and the second interaction an affinity of 431 nM (Herr et al., 2003). Binding of target-complexed IgA to FcαRI on immune cells triggers multiple responses to deal with invading pathogens,

including ADCC, ADCP, superoxide generation, degranulation, calcium mobilization, cytokine release, and antigen presentation (Herr et al., 2003; Woof and Kerr, 2006; Wines and Hogarth, 2006). Additionally, it has been proposed that another form of Fc α RI, or a similar receptor, may be present on NK cells that could be capable of mediating IgA-dependent, NK-cell mediated cytotoxicity (Mota et al., 2003; Wines and Hogarth, 2006).

It has been shown that in humans, the G248 polymorphism of Fc α RI has been found to be substantially enriched in patients with SLE (Wu et al., 2007b). A functional equivalent of Fc α R has not been found in mice (Takai, 2002; Woof and Kerr, 2006).

7.4.2 Fc α / μ R

Fc α / μ R is a ca. 55 kDa glycoprotein receptor that is expressed on most follicular B cells, follicular dendritic cells, and macrophages, but not on the surfaces of T cells, NK cells, or granulocytes (Shibuya et al., 2000; Kikuno et al., 2007; Honda et al., 2009). The gene encoding Fc α / μ R is located on chromosome 1 (in humans, 1q32.3; in mice, chromosome 1F) near the genes encoding other Fc receptors such as the Fc γ Rs, Fc ϵ RI, and pIgR (Honda et al., 2009). Both IgM and IgA bind Fc α / μ R in a J-chain dependent manner (Ghumra et al., 2009).

IgM binds Fc α / μ R with an affinity of about 1 nM, whereas dimeric IgA binds to Fc α / μ R with a K_D of about 10 nM (Ghumra et al., 2009), both of which are high-affinity interactions for antibodies and their receptors. In mice, Fc α / μ R is constitutively expressed on B cells and macrophages, where it has been hypothesized to function in endocytosis of antigen-complexed IgA (Woof and Kerr, 2006; Ghumra et al., 2009). In humans, it has been demonstrated that the predominant cell types expressing Fc α / μ R are follicular dendritic cells in germinal centers, with a lower expression by IgD⁺ CD38⁺ B cells in pregerminal centers (Kikuno et al., 2007). Recent results using Fc α / μ R-knockout mice suggest that the interaction of IgM and Fc α / μ R results in negative regulation of humoral responses to T cell-independent antigens (Honda et al., 2009).

7.4.3 Fc μ R

The cell-surface receptor TOSO, also known as Fas apoptosis inhibitory molecule 3 (FAIM3) and, more recently, Fc μ R (Kubagawa et al., 2009), has

been hypothesized to be an anti-apoptotic molecule by virtue of its ability to bind the pro-apoptotic Fas-associated death domain with its C-terminal intracellular domain (Pallasch and Wendtner, 2009). Nevertheless, Kubagawa et al. (2009) demonstrated that TOSO/FAIM3 itself was not capable of inhibiting Fas and therefore lacks intrinsic anti-apoptotic activity. They also demonstrated that TOSO/FAIM3 was a strong binder of IgM (Kubagawa et al., 2009).

The gene encoding TOSO/FAIM3 is found on chromosome 1 of both mice and humans, clustered with the genes encoding pIgR and Fc α / μ R (Shima et al., 2010). TOSO/FAIM3 was found to be expressed primarily on B220⁺ IgM⁺ CD19⁺ B cells and CD21⁺ CD35⁺ B cells, but not on T cells, macrophages, or dendritic cells (Shima et al., 2010). It was also demonstrated that IgM⁺ IgD⁺ mature B cells, but not IgM⁺ IgD⁻ immature B cells, expressed this receptor, narrowing the population even further to mature B cells (Shima et al., 2010).

TOSO/FAIM3 has been shown to be capable of binding IgM, but not IgG, and of internalizing IgM-coated beads (Kubagawa et al., 2009; Shima et al., 2010). IgM binds Fc μ R with a 10 nM K_D, which is relatively high affinity for an Ig–FcR interaction (Kubagawa et al., 2009). It is not yet known if the J-chain is required for binding to this receptor, so it is currently uncertain whether J-chain-containing pentameric IgM or J-chain-free hexameric IgM, or both forms, bind to Fc μ R. Additionally, the function of Fc μ R *in vivo* is also not yet fully understood, although it has been hypothesized that Fc μ R might be involved in the regulation of B cell maturation (Shima et al., 2010). It has recently been shown that Fc μ R has an anti-apoptotic function by a novel mechanism involving the adaptor kinase RIP1 and signaling through MAP kinase and NF- κ B (Nguyen et al., 2011). Fc μ R also has been found to be upregulated on lymphocytes of patients with chronic lymphocytic leukemia (CLL) (Hancer et al., 2011).

Additionally, it had been speculated for many years that NK cells might have an Fc μ R (Pricop et al., 1993), although no further data is available on that currently. Based on current evidence, Fc μ R does not appear to be expressed on NK cells.

7.4.4 pIgR

Polymeric Ig receptor (pIgR) is a receptor found on the basolateral surface of epithelial cells that is responsible for transcytosis of J-chain-containing IgA and IgM antibodies across the epithelium to the mucosal surface

(Braathen et al., 2002; Kaetzel, 2005). The pIgR is a type I, transmembrane, glycoprotein receptor that consists of an exodomain with five Ig-like domains, a transmembrane sequence, and an intracellular tail. Binding of IgM and IgA to pIgR is strictly J-chain-dependent (Braathen et al., 2002), so hexameric IgMs do not bind to this receptor. The IgM and IgA affinities for pIgR are ca. 37 nM and 55 nM K_D , respectively, which would make pIgR a high-affinity Fc receptor (Norderhaug et al., 1999).

When an IgA or IgM is transcytosed through the epithelial cell layer, an 80 kDa portion of the pIgR, called the secretory component (SC), is cleaved from the receptor on the apical side. Cleaved SC either becomes a component of secretory IgA (sIgA) molecule after transcytosis of that antibody or it is released as a free protein, as occurs when IgM is translocated (Woof and Kerr, 2006; Wines and Hogarth, 2006).

7.5 Complement activation

The complement cascade, which is a highly complex component of our innate immune system, is comprised of about 30 different proteins, which normally circulate in inactive forms that are activated via proteolytic processing (Gasque, 2004; Morgan et al., 2005; Rickin and Lambris, 2007). The complement cascade, which is an ancient component of the innate immune system, is a primary mechanism used to ward off infections and to mount immune responses against foreign invaders. This cascade has three fundamental activities:

- It defends against microbial pathogens by the generation of opsonins C1q, C3b, iC3b, and C4b, which coat the invading cell and interact with cell surface receptors CR1 (CD35), CR3, and CR4 to help promote phagocytosis. Also, the cascade generates the C5b9 complex, known as the membranolytic complex, which causes cell lysis by formation of a pore in the cell membrane.
- It acts as a bridge between the innate and adaptive immune systems by interacting with CD21 (CR2) on B cells.
- It helps to eliminate antibody immune complexes, apoptotic cells, cell debris, and other products of inflammatory injury to promote healing (Gasque, 2004; Morgan et al., 2005; Rickin and Lambris, 2007).

The complement cascade can be activated by three completely different routes. The classical pathway for complement pathway activation occurs

via the binding of complement protein C1q to certain antibody-antigen immune complexes, whereas the alternative pathway, which accounts for ~80% of all complement activation (Ricklin and Lambris, 2007), occurs via the antibody-independent adsorption of complement factor C3 directly onto the surface of pathogens. The third route for complement activation, the lectin pathway, occurs when bacteria or other microbes bind a “recognition molecule” known as the mannan-binding lectin (MBL; also called mannose-binding lectin, mannan-binding protein, or mannose-binding protein (Sontheimer et al., 2005; Arnold et al., 2006; Ip et al., 2009)), a protein which is remarkably similar in structure to C1q. Activation of the complement cascade by any of these pathways results in proteolytic generation of the pro-inflammatory anaphylatoxins, C3a and C5a, as well as the pore-like membrane attack complex (MAC; comprised of complement components C5b, C6, C7, C8, and C9) that inserts into cell membranes causing lysis (Gasque, 2004)).

The classical pathway for complement activation is initiated by the binding of C1q, a 460 kDa glycoprotein found in human serum at about 70 µg/mL, to antibody-antigen complexes (Kishore and Reid, 2000). Human C1q binds most avidly to the Fc domains of antigen-complexed IgG₁, IgG₃, hexameric IgM, and to a significantly lesser extent with pentameric IgM and IgG₂. Binding of wild-type IgG₁ to C1q has been measured by surface plasmon resonance (SPR) analysis to be 48 nM K_D (Moore et al., 2010). One caveat to any C1q binding experiment, however, is its inherent stickiness, which makes binding data difficult to interpret. In human serum, the strongest activators of the classical pathway were found to be IgG₁ and IgG₃, whereas activation by IgG₂ was minimal and IgG₄ did not activate at all (Seino et al., 1993). Human IgG₁ triggers complement-mediated lysis of target cells better than IgG₃, even though IgG₃ binds C1q better than IgG₁ (Brüggemann et al., 1987). Thus binding C1q alone may not be enough to activate the complement cascade effectively. Also, other factors such as the antibody's epitope and target density are important for C1q-mediated complement activation and cell lysis (Bindon et al., 1990; Lucisano-Valim and Lachman, 1991).

It has been demonstrated that residues D270, K322, P329, and P331 in the human IgG₁ Fc region are the key contact points for binding C1q (Idusogie et al., 2000). Thommesen et al. (2000) also showed K322 of human IgG₃ was the key residue for C1q binding. For murine IgG_{2b}, residues E318, K320, and K322 were found to be the key residues involved in binding murine C1q (Duncan and Winter, 1988), so the only residue in common with the human system is K322 (Idusogie et al., 2000;

Thommesen et al., 2000). The human antibodies IgG₄, IgA, IgE, and IgD do not bind C1q in a physiologically relevant manner (Sontheimer et al., 2005).

The alternate pathway occurs via antibody-independent deposition of complement factor C3 on the antigen. Some antibodies such as human IgG₂ appear to facilitate the alternate pathway (Dillman et al., 1995), although the mechanism for this is unclear. MBL, which is the component of the lectin pathway for complement activation, is a member of the collectin family of proteins produced by hepatocytes. MBL is present in serum at about 1.2 µg/mL (range < 50 ng/mL to 10 µg/mL) (Arnold et al., 2006; Ip et al., 2009). While the lectin pathway for complement activation is usually considered to be antibody-independent, human IgGs with glycoforms terminating in *N*-acetyl-glucosamine (GlcNAc) residues (G0 form) bind MBL in a Ca⁺⁺-dependent manner, and form immune complexes on the surfaces of bacteria, viruses, or other foreign entities (Arnold et al., 2006; Ip et al., 2009). Moreover, having accessible glycans, dimeric human IgA and about 20% of all human IgM species bind MBL and activate the complement cascade via the lectin pathway (Arnold et al., 2006).

Monoclonal antibody targets and mechanisms of action

Abstract: The majority of currently marketed or clinical therapeutic monoclonal antibodies (MAbs) and Fc fusion proteins (FcFPs) are used for indications in oncology or immunology, although in recent years a broader array of disease indications is being targeted by MAbs and FcFPs. The 396 currently known monoclonal antibodies and Fc fusion proteins on the market or in clinical trials are known to bind at least 185 different targets. Of these MAbs and FcFPs, 133 bind the top 16 targets (e.g. CD20, VEGF/VEGF receptor, TNF- α , IL-6/IL-6 receptor, amyloid beta) alone, generating significant competition on those “hot” targets, while the remaining 263 bind 169 different targets. Binding of a therapeutic MAb or FcFP to its cognate target can result in many different potential mechanisms of action (MOAs). Known MOAs include antagonism of a soluble ligand or receptor, blockage of cell–cell interaction, agonism of a receptor, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC), apoptosis, and non-apoptotic programmed cell death. Some MAbs confer multiple MOAs on a target, as exemplified by anti-CD20 MAbs.

Keywords: immunology, oncology, targets, mechanism of action (MOA), receptor, soluble target, antagonism, agonism, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC), apoptosis, programmed cell death, CD20

8.1 Properties of antibody targets

MAbs and FcFPs are good therapeutic choices for some targets, but clearly not for others. So, what makes a good target for MAb therapy? First, the targets must be accessible to IgG therapeutics, meaning that they must exist in the serum, or on cells in the serum, or in vascularized tissues. Table 8.1 describes a set of characteristics that might be included in a “perfect MAb

Table 8.1 General properties of “good” targets for MAb and FcFPs

Desired property of target	Comments
“Extracellular” – receptors, cytokines, hormones	Absolute requirement for target availability
Low concentration in body (low dose)	Affects dosing levels and frequency
Tissue specificity (not widely disseminated is often preferred)	Can affect dosing levels and frequency as well as potential side effects
Low turnover rate	Affects dosing levels and frequency
Soluble (rather than cell-bound) for non-oncology targets, yielding a lower level of target-mediated clearance	For dosing and potential cell depletion considerations of non-oncology based targets; for oncology targets, often cell-bound targets are more desired for cell depletion mechanism of action
Desired dosing frequency longer rather than shorter	Since antibodies have a naturally long half-life in serum, targets for which infrequent dosing is acceptable are best
Targets for indications in which IV, subcutaneous, or IM injections acceptable	This is an absolute requirement, since MABs and FcFPs are not orally bioavailable. Pulmonary administration may also be possible
Target for which small molecules are not an option	If a small molecule against a potential MAb or FcFP target can be developed, it will likely be preferable due to oral dosing, cost-of-goods, and no cold chain requirement
Indication for which high cost is acceptable	Since MABs and FcFPs may have a relatively high cost of goods, the indications must support higher pricing than typically seen with small molecule drugs

or FcFP target.” These criteria describe a soluble protein that is at low concentration that would affect a serious disease for which parenteral dosing is acceptable (see Table 8.1). The problem with developing a set of criteria such as these, though, is that they hold true for very few targets. Interestingly, though, these characteristics essentially hold true for two of the most valuable targets currently addressed by marketed antibodies and FcFPs, TNF- α and VEGF.

Many factors, such as dose, dosing frequency, dosing requirements (chronic versus acute), desired route of administration, pharmaco-economics, potency, and pharmacodynamic effects, enter into the question of what makes a good antibody or Fc-fusion protein target (see Table 8.2).

Table 8.2 Reported dosing frequency and levels for some example marketed MABs and FcFPs*

Antibody or FcFP	Target	Dose level	Dose frequency	IV or SC***	Reported half-life	Reported affinity
Omalizumab (Xolair®)	IgE	150–375 mg (~2–4 mg/kg for normal person**)	Every 2 to 4 weeks	SC	26 d	60–100 pM
Rituximab (Rituxan®)	CD20	37–500 mg/m ² (~10–12 mg per kg for normal person**) for oncology	Every 4 weeks	IV	22 d	8.0 nM
Trastuzumab (Herceptin®)	HER2	4 mg/kg loading dose followed by 2 mg/kg dosing	Weekly	IV	5.8 d (20–28 d at steady state also reported)	100 pM
Tocilizumab (Actemra®)	IL-6R	4–8 mg/kg	Every 4 weeks	IV	6.3 d	2.54 nM
Panitumumab (Avastin®)	VEGF-A	5–10 mg/kg	Every 2 weeks	IV	20 d	0.9–1.8 nM
Adalimumab (Humira®)	TNF- α	40 mg fixed dose (~0.4–0.6 mg/kg for normal person**)	Every 2 weeks	SC	14 d	8.6 pM
Etanercept (Enbrel®)	TNF- α TNF- β	50 mg fixed dose (~0.5–0.7 mg/kg for normal person**)	Weekly	SC	4.2 d	0.4 pM
Golimumab (Simponi®)	TNF- α	50 mg fixed dose (~0.5–0.7 mg/kg for normal person**)	Monthly	SC	14 d	18 pM
Ustekinumab (Stelara®)	IL-12/ IL-23 p40	45–90 mg fixed dose (~0.4–1.3 mg/kg for normal person**)	0, 4 weeks, and then every 12 weeks	SC	21.6 d	Not published
Denosumab (Prolia®)	RANKL	60 mg fixed dose (~0.6–0.9 mg/kg for normal person**)	Every 6 months	SC	25.4 d	3 pM

* Most of these data are derived from the prescribing information for each of these drugs.

** Normal person of 70–100 kg in weight with relatively low BMI.

***IV: intravenous dosing; SC: subcutaneous dosing.

Dosing frequency of marketed MABs and FcFPs range from once every 3–6 months (e.g. ustekinumab and denosumab) to weekly dosing (e.g. trastuzumab) (see Table 8.2). Similarly, dose levels range from a low of a 40–50 mg fixed dose (weekly, biweekly, or monthly, depending on MAB) for the anti-TNF- α MABs up to approximately 1 g doses for some anti-cancer MABs (see Table 8.2). Dosing frequency depends on a variety of factors including the concentration and turnover rate of the target, biodistribution of the target, antibody half-life (see Chapter 10), and potential pharmacodynamic effects that may enhance the biological effects of the antibody beyond its presence in pharmacologically relevant levels, as observed with denosumab, which has a half-life of about four weeks but is only dosed twice per year (Yonemori et al., 2008; Prolia prescribing information, 2012).

Targets that have broad tissue distribution, high total concentration (e.g. >10 ng/mL), and/or high turnover rates may result in the requirement for high doses or more frequent dosing. Also, targets that have a feedback mechanism for their own biosynthesis and/or which are stabilized in the serum by the presence of the binding MAB, such as CCL2 (aka MCP-1) (Haringman et al., 2006) may result in the need for higher or more frequent dosing. In the case of CCL2, it was demonstrated that antibody-mediated targeting of CCL2 dose-dependently stabilized serum CCL2 levels resulting in substantially increased serum CCL2 levels that undermined the activity of the antibody (Haringman et al., 2006).

Additional properties that should be considered in a potential MAB or FcFP target are related to the pharmaco-economics of the targeted disease itself. Since MABs and FcFPs are generally quite expensive, target biology resulting in proposed clinical indications that cannot be supported by highly priced medicines would not result in a favorable rationale for these types of drugs. An example of this might be the use of a MAB or FcFP for the treatment of acne, a market that it is likely would not support parenteral dosing. Thus many of the targets for MABs and FcFPs are for disease indications that are either life threatening or severely debilitating, or for which small molecule approaches have not been particularly efficacious.

Of the 386 current commercialized or clinical candidate MABs, approximately 52% bind cell surface proteins as documented in Table 8.3. Examples of such targets include CD20, EGFR, HER2, IGF-1R, and various integrins. Thus the majority of targets for MABs and FcFPs do not fit the “optimal target profile” outlined in Table 8.1, mostly because the greatest number of MABs and FcFPs marketed or in clinical trials are for cancer targets and these tend to be for cell surface targets (see Table 8.3). Soluble targets such as TNF- α , IL-1 β , IL-6, VEGF, and NGF comprise about 42% of the targets for currently marketed or clinical candidate MABs and FcFPs, with anti-infectious agents making up the remaining 5%.

Table 8.3 Soluble versus cell-bound targets for MAbs and Fc-fusion proteins*

Therapeutic area	Total number of MAbs and FcFPs on the market or in clinical trials for targets indicated by their location			
	Soluble	Cell surface**	Infectious agent	Totals
Oncology	30	162	0	192
Immunology	89	37	0	126
Neurobiology	14	1	0	15
MCVBEP***	29	4	0	33
Ophthalmological	5	2	0	7
Infectious diseases	1	1	21	23
Total number of MAbs/FcFPs in each category	168 (42%)	207 (52%)	21 (5.3%)	396 total MAbs and FcFPs
Total number of unique targets represented by marketed or clinical candidates in all therapeutic areas	69 (37%)	105 (57%)	11 (6%)	185 unique targets

* Data derived from 39 marketed, 36 Phase III, and 321 Phase I and II MAbs and FcFPs in a database, based on information from company websites, La Merie Biologics (La Merie Business Intelligence, Inc.), http://www.drugs.com/drug_information.html, Prous Integrity, and published information, continually updated by one of the authors (WRS). The data focus on company-sponsored clinical trials. Date of data lock for this table was August 18, 2011.

** Most of the cell-surface targets are single-pass membrane targets (i.e. only about 10 of these are GPCR or ion channel targets).

***MCVBEP: metabolic, cardiovascular, blood, endocrinology, pain.

Tables 2.5 and 8.3 summarize the therapeutic areas for which currently marketed and clinical candidate MAbs and FcFPs are indicated. From these data, it is clear that immunology was the major target of the first wave of therapeutic MAbs and FcFPs, while oncology is the current area in which most newer MAbs and FcFPs are directed. Moreover, while the first recombinant MAb ever marketed (ReoPro®) was for a non-oncology/non-immunology indication, very few other early MAbs and FcFPs were developed for indications outside of immunology or oncology. More recently, a broader array of therapeutic indications, including cardiovascular, endocrine, metabolic, and blood indications, are being targeted by therapeutic MAbs and FcFPs.

Table 8.3 points out that the 396 known MAbs and FcFPs that have either been commercialized or are in clinical trials sponsored by industry

are against a total of 185 unique targets. Of those unique targets, about 35 have been validated clinically (W.R. Strohl, unpublished data), and only five (TNF- α , VEGF, CD20, HER2, EGFR – sometimes referred to as the “big 5”) comprised 85% of the total 2011 value of the MAb and FcFP market (see Chapter 2, Table 2.4). Strohl and Knight (2009) showed that the most widely targeted proteins as of mid-2009 were CD20, VEGF/VEGFR, TNF- α , the IL-4/13 pathway, and IGF-1R. Of the 396 MAbs on the market or in clinical development that are mentioned in Tables 2.5 and 8.3, many are focused on the same key targets as noted by Strohl and Knight (2009) (Table 8.4). The target that has seen the greatest increase in the number of candidates since the mid-2009 analysis is amyloid- β , which went from five publicly announced competitive programs in mid-2009 (Strohl and Knight, 2009) to 11 (see Table 8.4) over the past two years. The 16 targets shown in Table 8.4 account for about one-third (133 out of 396) of the total MAbs and FcFPs being studied in publicly available, company-sponsored clinical trials. Most of these targets represent the oncology (CD20, IL-6, VEGF/R, IGF-1R, EGFR, HER, CD19, TRAIL-R2) and immunology (CD20, IL-6, TNF- α , IL-4/13, IL-17A, IFNs, CD3, IL-12/23) therapeutic areas. The hottest targets for indications other than oncology or immunology are amyloid- β for Alzheimer’s disease and neutrophil growth factor (NGF) for pain (see Table 8.4).

One issue that is being raised within the field is whether all of the easy and most obvious targets (cf. Table 8.4) have already been addressed, leaving targets with less well understood biology. Another issue is whether the field is running out of novel antibody and FcFP targets. As shown in Table 8.5, there are about 5,200–7,800 total potential protein targets in the human “exoproteome” for MAbs and FcFPs. These numbers likely exclude splice variants or proteins that might be differentially post-translationally modified under different disease states. Of these potential targets, about 1,620–2,200 are proposed to be secreted proteins, of which 207 are likely to be cytokines, another 47 chemokines, and at least 305 proteins of all types have been documented in serum (Cui et al., 2008). Currently, only 69 soluble targets have been addressed in clinical trials with MAbs or FcFPs (Table 8.3), so if the more conservative number (1,620 soluble proteins) is used, current clinical candidates target only about 4% of the total potential soluble targets.

Rask-Andersen et al. (2011) have documented 435 unique targets of current commercial small molecule and protein therapeutics, of which 69 (16%) are targeted by MAbs and FcFPs (see Table 8.5). Of the total 435 unique targets, cell receptors accounted for 44% (193/435) of all targets currently addressed by pharmaceuticals. A total of 60% of all drugs, including small molecules, MAbs, FcFPs, and non-MAb-derived biologics, are targeted towards

Table 8.4 Known competition for MAb and FcFP targets

Target	Therapeutic area(s)	Phase of development			Totals
		Marketed	Clinical Phase III	Clinical Phase I/II	
CD20	Oncology, inflammation	4	2	6	12
IL-6 and IL-6R	Oncology, inflammation	1	0	10	11
Amyloid- β	Neurosciences	0	2	9	11
VEGF and VEGFR	Oncology, ophthalmology	3	2	6	11
TNF- α	Inflammation	5	0	5	10
IL-4 and/or IL-13	Inflammation, oncology	0	0	10	10
IGF1-R	Oncology	0	0	9	9
EGFR	Oncology	2	3	4	9
HER (HER2,3, pan HER)	Oncology	1	2	5	8
IL-17A	Inflammation	0	1	6	7
CD19	Oncology	0	0	7	7
IFN (α , β , γ) & IFN-R	Inflammation	0	0	7	7
CD3	Inflammation, metabolic	1	2	3	6
NGF	Pain	0	1	4	5
IL-12 and/or IL-23	Inflammation	1	1	3	5
TRAIL-R2	Oncology	0	0	5	5

* Data derived from 39 marketed, 36 Phase III, and 309 Phase I and II MABs and FcFPs in a database, based on information from company websites, La Merie Biologics (La Merie Business Intelligence, Inc.), http://www.drugs.com/drug_information.html, Prous Integrity, and published information, continually updated by one of the authors (WRS). Date of data lock for this table was August 18, 2011.

membrane-bound proteins of all classes (Bakheet and Doig, 2009; Rask-Andersen et al., 2011). As shown in Table 8.3, 52% of all marketed and clinical candidate MABs and FcFPs target membrane bound targets, and 57% of all current MAB and FcFP targets represented by marketed or clinical candidate molecules are membrane proteins. There are an estimated ~3,700 (da Cunha

Table 8.5 Estimated number of potential MAb and FcFP targets

Property	Estimated number	Comments	References
Total number of genes encoding unique proteins in human proteome	20,000–23,000	With up to 10 splice variants per protein and many post-translationally modified, so total number of potential targets is greater than this number	Saviranta and Kallioniemi (2006), Berglund et al. (2008), Uhlén et al. (2010)
Approximate total exoproteome available to MABs and FcFPs as targets	5,200–7,800	Different authors give different numbers based on a variety of methods used to calculate these numbers therefore this is only an estimate based on the best available information in May 2011	Diehn et al. (2006), Fagerberg et al. (2010)
Total number of secreted proteins	1,620–2,200	This is an estimate based on the best available information in May 2011	Chen et al. (2005), Lin et al. (2008)
Total number of unique secreted targets currently represented by marketed or clinical candidate MABs and FcFPs	69	From Table 8.3	W.R. Strohl, internal database, May 28, 2011
Total number of membrane bound proteins	3,700–5,600	This is an estimate based on the best available information in May 2011	Diehn (2006), da Cunha et al. (2009), Fagerberg et al. (2010)
Total number of unique cell membrane targets currently represented by marketed or clinical candidate MABs and FcFPs	105	From Table 8.3	W.R. Strohl, internal database, May 28, 2011

et al., 2009) to ~5,600 (Fagerberg et al., 2010) membrane proteins with exodomains (see Table 8.5). Of these, some 3,000–4,500 are estimated to have single transmembrane domains (single-pass proteins; Fagerberg et al., 2010), another 789–900 are GPCRs (about half of which are involved in the olfactory system leaving ~400–450 GPCRs as potential targets; Fagerberg et al., 2010), a range of 480–930 transporters, and another approximately 500 proteins are cell–cell or cell–matrix adhesions. As noted in Table 8.3, MABs and FcFPs either on the market or in the clinic currently target about 105 unique membrane bound targets. Taking the more conservative number above (3,700; da Cunha et al., 2009), less than 3% of the total available cell surface targets have been addressed by marketed or clinical candidate MABs and FcFPs.

Diaz-Ramos et al. (2011) recently published a paper in which they identified the “immunome,” i.e. a collection of 1,015 proteins that are clearly identified as part of the cell surface repertoire of immune cells. Of this total, 500 were leukocyte receptors, 195 were identified as Ig-superfamily proteins (known to be good antibody targets), 147 were GPCRs, 49 were C-type lectins, and 44 were cytokine receptors (also considered to be possible MAB targets). Sixty percent of these 1,015 possible MAB targets lacked CD (“cluster of differentiation”) names, indicating that they are likely poorly understood.

For small molecules, the total number of potential “druggable” targets has been estimated to be about 3,000 (Bakheet and Doig, 2009; Rask-Andersen et al., 2011), out of the estimated 20,000 total human proteome, which amounts to about 15% of the total proteome (not including splice variants and post-translationally modified proteins). Rask-Andersen et al. (2011) argued that further analysis and subtraction of overlaps with the yeast genome result in 600–1,500 genes that could produce viable pharmacologically relevant targets. If the same percentages were applied to MAB and FcFP targets, the numbers would come to ~120–240 total soluble proteins (of which only 69 have been targeted – see Table 8.3) and ~275–555 cell surface proteins (of which only 105 have been targeted – see Table 8.3). While these data do not account for already known failures or targets currently being addressed in discovery or pre-clinical studies, they do suggest that there are still many targets available for targeting with MABs and FcFPs. The greatest obstacle to most of these, however, is lack of proven biology, which can only be addressed by a maturation of the biological field.

Thirty-six percent of all current drugs marketed today target GPCRs, making them the largest target class addressed by marketed drugs (Rask-Andersen et al., 2011). In recent years, there has been growing interest in using antibodies and FcFPs to target GPCRs that have been refractory to small molecule drugs. In some cases, GPCRs have been addressed using

modifications of their natural peptide ligands, sometimes as peptide-Fc fusion products, as discussed in Chapter 14. The most advanced of these is Eli Lilly's modified GLP-1-FcFP, LY2189265, which is now in Phase II clinical trials for treatment of type II diabetes (Glaesner et al., 2010). Many of those GPCRs have peptides as natural ligands and hence protein–peptide interaction lends itself to possible intervention with a MAb. There are currently at least 10 MAbs and FcFPs in recent or current clinical development that target GPCRs (see Table 8.6). Additionally, there are many

Table 8.6 Examples of clinical candidate MAbs and FcFPs targeting GPCRs

Candidate	Company	Current status (US)	Molecular target	Major indication	Protein format
LY2189265	Eli Lilly	Phase II	GLP-1R	Type II diabetes	Peptide-FcFP
MLN-1202	Millenium	Phase II	CCR2	Scleroderma	Humanized antagonist IgG
PRO-140	Progenics	Phase II	CCR5	HIV-AIDS	Antagonist IgG
AMG-761 (KW-0761)	Amgen/Kyowa Hakko	Phase I	CCR4	Asthma; oncology	Humanized IgG – targets CCR4 ⁺ T cells for depletion
AMG-477	Amgen	Phase I	Glucagon receptor	Type 2 diabetes	Antagonist IgG – clinical program halted
NN8209	Novo Nordisk	Phase I	C5a-R (CD88)	RA	IgG
CVX-096	Pfizer	Phase I	GLP-1R	Diabetes	Peptide mimetic-IgG conjugate
MDX-1338	Medarex/BMS	Phase I	CXCR4	Oncology (leukemias, lymphomas, other)	Human antagonist IgG
ALX-0651	Ablynx	Phase I	CXCR4	Oncology	Nanobody
OMP-18R5	Oncomed	Phase I	Frizzled (Wnt pathway)	Oncology	IgG

reports at meetings and in the literature of MAb being used to target GPCRs, as well as several small biotech companies that have focused their resources on finding ways to target GPCRs with MAbs. Considering that there are at least 400 total potential GPCR targets (Fagerberg et al., 2010), 147 of which are part of the “immunome” (Diaz-Ramos et al., 2011), and only about 25–30 unique GPCRs have been targeted with small molecules, it should be expected that many GPCRs may be excellent MAb targets in the future. At this time, approximately 100 known GPCRs still remain orphans (i.e. no ligand has yet been found for them), so there is still much to be learned about the biology they confer. Much of the difficulty in targeting GPCRs with MAbs has been in obtaining biologically relevant GPCR preparations that could be used for immunizations for panning phage display libraries. Recent advances in GPCR target preparations (Ren et al., 2009), understanding of antibody–GPCR interactions (Paes et al., 2009), and assays for GPCR activities (Heilker et al., 2009; Rajagopal et al., 2010) have made targeting GPCRs with antibodies more feasible. Thus this target class, which for decades has been an important target class for small molecule intervention, is now a very attractive class for MAbs in the future.

8.2 Antibody mechanisms of action

8.2.1 Introduction

Under natural conditions, antibodies are directed, and affinity optimized, against foreign antigens, whether they are soluble macromolecules such as proteins, DNA, lipopolysaccharides, or other molecules; cell-based antigens, which may include cell wall components, proteins, capsular materials, flagella, pili and fimbriae, or other pathogen cell structures; or viral capsid proteins. The job of antibodies *in vivo* is fundamentally to protect the host from pathogens, both by direct killing and clearance mechanisms and through activation of the immune system via their interactions with Fcγ receptors and complement components (see Chapter 7).

Therapeutic IgG antibodies are multifunctional proteins that bind an antigen through the activity of the FAb chains, while also binding complement components and Fcγ receptors through the activity of the Fc and hinge regions. IgG antibodies can interact with their target and the immune system to carry out at least seven distinct mechanisms of action, four of which include cell-killing mechanisms: (1) direct blocking of ligand-receptor interaction (see Figure 8.1 and Table 8.7 below); (2) agonist activity on a receptor to trigger downstream signaling

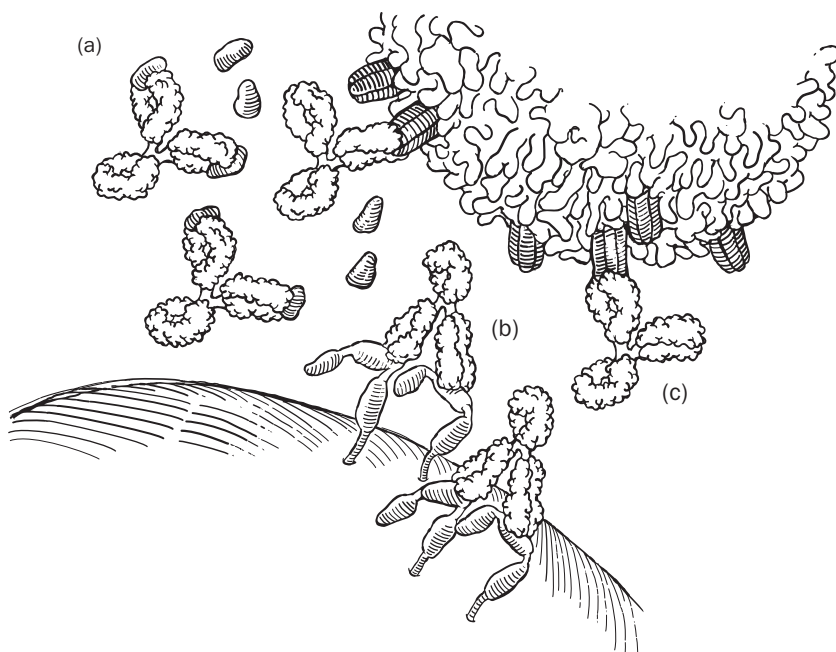


Figure 8.1 Antibody mechanism of action (MOA) – blocking/antagonism

Drawing showing antagonism occurring by binding to the ligand (a) to prevent it from binding to its cognate receptor, or to the receptor (b) to prevent the ligand from binding. Alternatively, an antibody may bind to a cell-bound ligand (or receptor) (c) to prevent that protein from binding its cognate partner on another cell.

(see Figure 8.2 below); (3) antibody-dependent cellular cytotoxicity (ADCC) (see Figure 8.3 below); (4) antibody-dependent cellular phagocytosis (ADCP) (see Figure 8.4 below); (5) complement-dependent cytotoxicity (CDC) (see Figure 8.5 below); (6) direct cell killing by induction of apoptosis; (7) receptor internalization and down-modulation (see Table 8.4); and (8) inhibition of enzyme activity. One key to differentiation of the different cell-killing activities are the assays that delineate the cell-killing mechanism of action, most of which require multiple assay approaches to determine correctly (Kepp et al., 2011).

Jiang et al. (2011) have reviewed marketed and clinical stage MAbs and have categorized their activities with respect to cell killing as Class I MAbs (those that bind target cells and kill them via Fc-mediated activities); Class II MAbs (those that bind target cells and block function but do not kill or deplete the target cells); and Class III (MAbs that bind soluble antigens and typically neutralize their receptor-binding activities). They have proposed criteria for assessing Fc-effector function mediated activities throughout the development process (Jiang et al., 2011).

Table 8.7 Examples of MAb and FcFPs used to block cell–cell interactions

MAb or FcFP	Stage of development	Target	Molecular structure and function	Primary function
Alefacept (Amevive®)	Marketed	CD2	CD2-binding domain of LFA-3 fused to IgG ₁ Fc; inhibits CD2-LFA-3 interaction on activated T cells	Blocks activated T cells
Abatacept (Orencia®)	Marketed	CD80 and CD86	CTLA4-FcFP-modified Fc; blocks CD80 and CD86 on B cells from interacting with CD28 on T cells	Co-stimulation inhibitor; blocks T cell activation
Ipilimumab (Yervoy™)	Marketed	CTLA4 (CD152)	Anti-CTLA4 MAb blocks CTLA4 on helper T cells from interacting with CD80/CD86 on B cells	Augments T cell activation and proliferation
BMS-936558 (MDX-1106)	Phase I	PD-1 (CD279)	Anti-PD-1 MAb blocks T cell negative regulator PD-1 from interacting with PD-L1 on B cells and other cell types	Augments T cell activation and proliferation
MDX-1105	Phase I	PD-L1 (CD274)	Anti-PD-L1 MAb blocks PD-L1 on B cells and tissue cells from interacting with negative T cell regulator, PD-1	Enhances T cell immune responses
ALXN6000	Phase I	CD200	Anti-CD200 MAb blocks CD200 from interacting with myeloid cells containing CD200R, thereby decreasing immune tolerance	Enhancement of immune surveillance

Abbreviations: CTLA4: cytotoxic T-lymphocyte antigen 4; PD-1: programmed cell death 1.

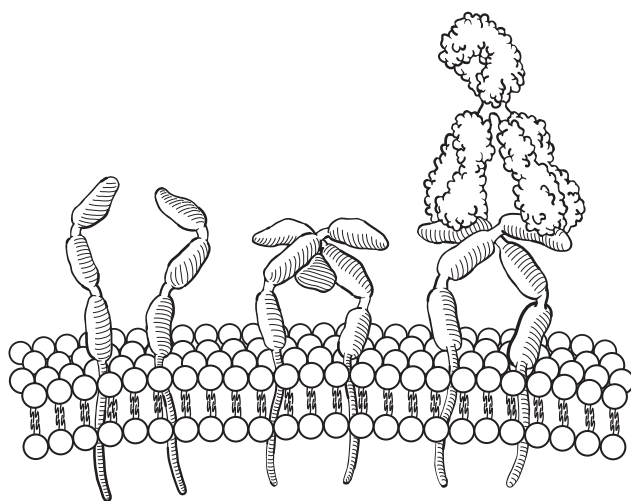


Figure 8.2 Antibody mechanism of action (MOA) – agonism

Antibody-mediated agonist activity can occur in multiple ways, including binding to a receptor in a manner similar to its natural ligand (not shown) or by dimerizing receptors similar to the way they dimerize in a ligand-dependent manner (shown here). The left drawing shows the two receptor chains unbound by ligand, the center drawing shows those same chains now dimerized by the presence of ligand, and the drawing on the right shows the same dimerization, but mediated by an antibody rather than by the natural ligand.

8.2.2 Antagonists (blockers)

The simplest form of antibody activity occurs when the antibody binds a soluble ligand, a cell-bound ligand, or a cell receptor, and blocks the binding of the ligand to the receptor, thereby disrupting the downstream signaling mediated by that receptor–ligand interaction (see Figure 8.1). An example of this activity is the binding of an antibody to the cytokine TNF- α , stopping it from signaling through the TNF- α receptor (Kaymakcalan et al., 2009). Infliximab (Remicade®), adalimumab (Humira®), and golimumab (Simponi®) are examples of IgG₁ antibodies that function by this mechanism.

Antibodies that bind a cell receptor in a non-agonistic manner and block ligand binding and activation of downstream signaling pathways include the anti-EGFR antibodies, cetuximab (Erbix®) and panitumumab (Vectibix®), the anti-HER2 MAb trastuzumab (Herceptin®), and several other marketed and clinical candidate antibodies. Finally, cell–cell interactions typically require a cell-bound ligand on one cell and cell-bound receptor on another cell to engage, resulting in either activation or down-regulation of either or both of the interacting cells, depending on the pathway. In B cell activation

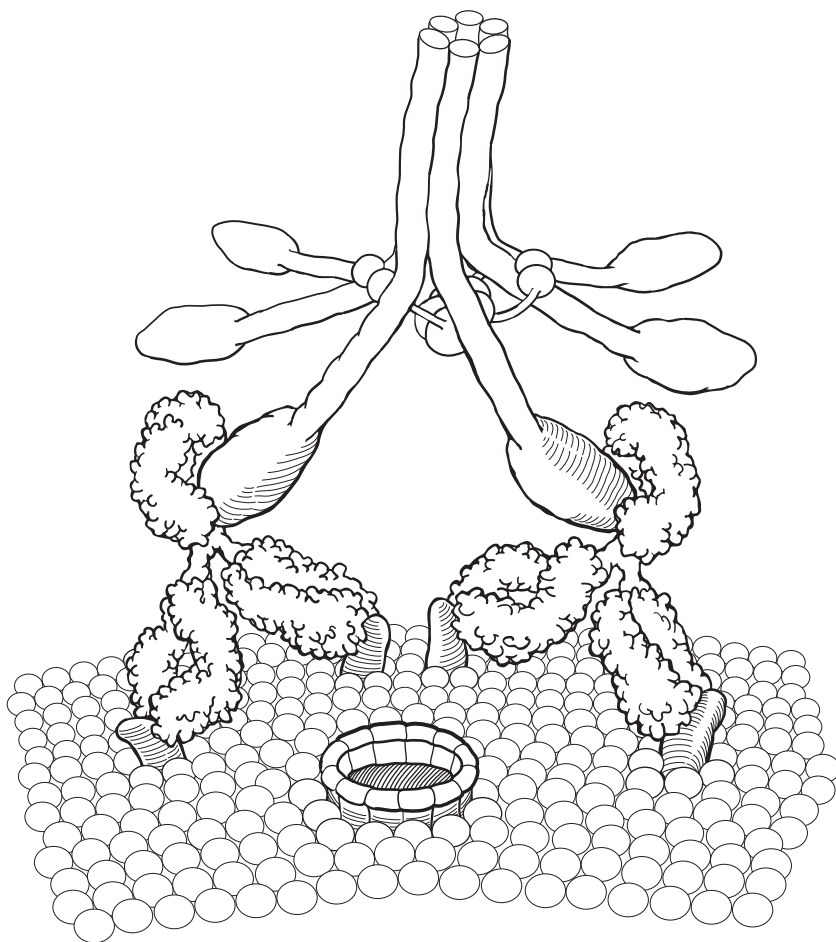


Figure 8.3 Antibody mechanism of action (MOA) – complement-dependent cytotoxicity (CDC)

This drawing shows two IgGs bound to cell surface proteins linked by two chains of a C1q molecule, which initiates the complement activation cascade resulting in cell lysis. A membranolytic pore, consisting of the C5b9 complex, is also shown.

mediated by T cells, this interaction requires both the interaction of a primary ligand/receptor pair and a co-stimulatory ligand/receptor pair. MAbs can potentially be directed against any of these cell-bound targets on either side of the pathway to block the cell–cell interaction from occurring. Alternatively, FcFPs can be used in this manner by fusion of an exodomain of one of the cell–surface pairs to an Fc to block the interaction. Table 8.7 gives some examples of MAbs and FcFPs used to block cell–cell interactions.

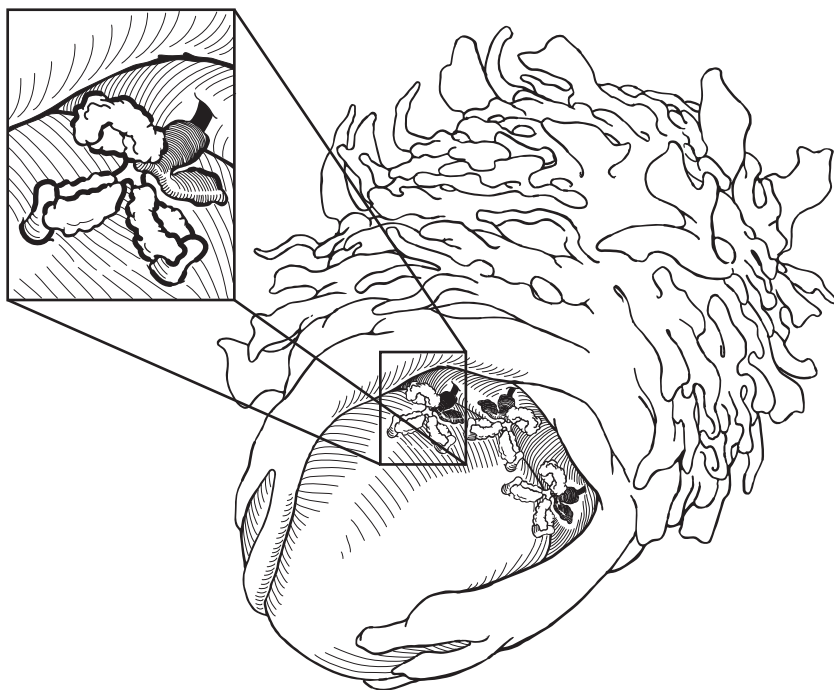


Figure 8.4 Antibody mechanism of action (MOA) – antibody-dependent cellular phagocytosis (ADCP)

This drawing depicts a target cell in the process of being phagocytosed by a macrophage in an antibody-dependent manner. The IgGs binding to proteins on the surface of the targeted cell also bind Fc γ Rs present on the surface of the macrophage (see enlargement), resulting in triggering ADCP of the targeted cell.

8.2.3 Agonists

Antibodies binding to cell surface receptors can antagonize those receptors, as mentioned in the previous section, but, alternatively, could act as agonists of those receptors, essentially replacing the activity of the normal ligand (Taylor et al., 1987; Soos et al., 1992; Rowlinson et al., 1998; Moog-Lutz et al., 2005; Wilson et al., 2011). Oftentimes, in screening for antibodies against cell-surface receptor targets, representatives of both antagonist and agonist antibodies are isolated and can be differentiated by the combination of their epitope, binding affinity, potential for dimerization, or other aspects of their binding profiles (Taylor et al., 1987; Soos et al., 1992; Moog-Lutz et al., 2005). The agonist activity can occur when the antibody binds the receptor in a manner that mimics the binding of the

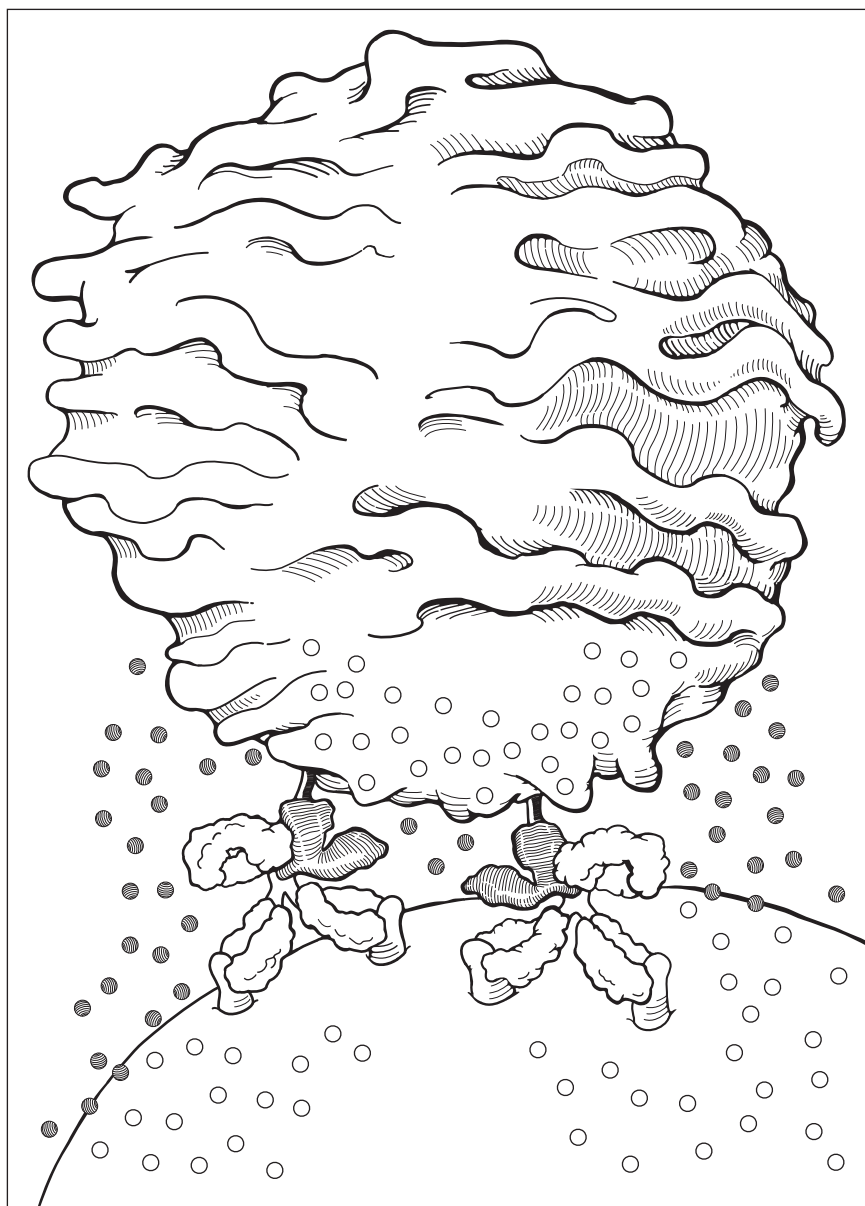


Figure 8.5 Antibody mechanism of action (MOA) – antibody-dependent cellular cytotoxicity (ADCC)

In this drawing, the IgGs bind to the surface of the target cell and their Fcs bind Fc γ RIIIa on the surface of an NK cell, which triggers the release of cytokines, progranulin, and other cytolytic factors which cause the cytotoxicity of the targeted cell.

natural ligand, resulting in antibody-mediated downstream signaling or agonism (see Figure 8.2) (Wilson et al., 2011). Antibody-mediated agonist activity can occur when the two Fab arms of an IgG each bind to a half-receptor of a homodimeric receptor pair and cause the receptors to dimerize, or cross-link, in a way that mimics the activity of the natural ligand (Schreiber et al., 1983; Prat et al., 1998; Rowlinson et al., 1998). It has been observed, however, that cross-linking alone often does not yield full agonism and that other factors such as conformational changes may be required to achieve full agonism (Prat et al., 1998; Rowlinson et al., 1998). Recently, it was shown that cross-linking of agonist MABs to Fc γ Rs on the target cells may also contribute to the agonist activity of the MAB (Wilson et al., 2011). Alternatively, several MAB agonists in clinical and pre-clinical trials target TNF family members, including the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 (also called death receptors (DR) 4 and 5, respectively) which initiate programmed cell death via the extrinsic apoptotic pathway (Jin and El-Deiry, 2005; Camidge, 2008; Dobson et al., 2009; Lavrik, 2010).

There are currently at least 11 MABs in clinical trials that are intended to function as receptor agonists, five of which are against the same target, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-R2 (see Table 8.8). All but one of these clinical candidate agonist MABs are for oncology indications. Most of those are intended either to induce apoptosis in cancer cells (e.g. anti-TRAIL-R1 and TRAIL-R2 MABs) (Jin and El-Deiry, 2005; Dobson et al., 2009; Lavrik, 2010) or to modulate the immune system (e.g. anti-CD40 MAB; Piconese et al., 2008) as an immunostimulator against cancer cells. Li and Ravetch (2011) recently demonstrated that an immunomodulatory anti-CD40 antibody binding antigen presenting cells (APCs) were fully activated only when the inhibitory receptor Fc γ RIIb was co-ligated, providing a co-stimulatory signal.

The single non-oncology agonist MAB is the anti-Fas (CD95) IgM MAB ARG098 intended for direct injection into joints for treatment of RA (Odani-Kawabata et al., 2010). In this case, the anti-Fas MAB was reconfigured as an IgM to take advantage of the cross-linking ability of the pentameric/hexameric IgM to agonize Fas on the surface of Fas-expressing RA synoviocytes resulting in induction of apoptosis (Odani-Kawabata et al., 2010).

Besides these clinical candidates, there are several additional agonist antibodies being studied in discovery and pre-clinical studies for possible development. These include, for example:

Table 8.8 Examples of agonist MABs in clinical trials

US trade name (generic name)	Company	Current status (US)	Molecular target	Major Indication	Protein format
CP-870893	Pfizer	Phase II	CD40	Oncology	IgG
Mapatumumab (HGS-ETR1)	HGS	Phase II	DR4 (TRAIL- R1) (CD261)	Oncology	IgG
Apolizumab (Hu1D10)	NCI/PDL	Phase II	HLA-DR beta-chain epitope	NHL, other oncology	Humanized IgG ₁
BMS-663513	BMS	Phase I/II	CD137	Oncology	Human IgG
Drozitumab (PRO95780; anti-DR5; rhuMAb DR5)	Genentech	Phase II	DR5 (TRAIL- R2) (CD262)	Oncology	Human IgG
Conatumumab AMG-655 (XG1- 048)	Amgen	Phase I	DR5 (TRAIL-2) (CD262)	Pancreatic cancer	Human IgG _{1,k}
Tigatuzumab CS-1008	Daiichi Sankyo	Phase I	DR5 (TRAIL- 2) (CD262)	Oncology	Humanized IgG
Lexatumumab (HGS-ETR2)	GSK, HGS	Phase I	DR5 (TRAIL- R2) (CD262)	Solid tumors	Human IgG
LBY-135	Novartis	Phase I	DR5 (TRAIL- R2) (CD262)	Solid tumors	Chimeric IgG
ARG098	Argenes	Phase I	Fas (Apo-1; CD95)	RA	IgM
TRX518	Tolerx	Phase I	GITR	Oncology – to be used with vaccines	Humanized IgG ₁

Abbreviations: BMS: Bristol-Myers Squibb; DR: death receptor; GITR: glucocorticoid induced TNF receptor; GSK: GlaxoSmithKline; HGS: Human Genome Sciences; HLA-DR: Human Leukocyte Antigen DR-1; NCI: National Cancer Institute; NHL: non-Hodgkin's lymphoma; PDL: Protein Design Labs; RA: rheumatoid arthritis; TRAIL: TNF-related apoptosis-inducing ligand.

- an anti-CD47 (integrin-associated thrombospondin receptor) agonist MAb for the treatment of breast cancer (Manna and Frazier, 2004);
- an anti-CD137 MAb as an immunostimulatory treatment for cancer (Palazón et al., 2011);
- an agonist MAb against the glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR, or TNFRSF18) which was shown to

regulate the infiltration of T regulatory (Treg) cells into tumors (Cohen et al., 2010);

- an anti-OX40 (CD134) MAb that both inhibited Treg cells as well as provided immunostimulatory signals to kill cancer cells (Piconese et al., 2008);
- an anti-MET (hepatocyte growth factor (HGF) receptor) agonist MAb for the protection of cardiac cells from apoptosis (Pietronave et al., 2010);
- an anti-Fn14 (TNF- α -like weak inducer of apoptosis (TWEAK) receptor) agonist MAb that has been shown to induce apoptosis in tumor cells in which Fn14 was upregulated (Michaelson et al., 2011).

The most infamous example of an agonist antibody was the Tegenero TGN1412, an anti-CD28 “superagonist” MAb that induced cytokine storms (Walker et al., 2011b) in several healthy volunteers when administered the antibody in Phase I clinical trials (Stebbins et al., 2009). The TGN1412 incident has changed the paradigm for how to calculate initial doses of potential immunomodulatory MAbs for first-in-human (FIH) Phase I single-dose escalation trials to a MABEL (minimal anticipated biological effect level) approach. The MABEL approach for assessing initial FIH dosing is based on estimations of doses to achieve low receptor occupancy (Muller and Brennan, 2009).

8.2.4 Opsonization process

Invading pathogenic cells or viruses are recognized by the innate immune system, which triggers an adaptive response. The first step in attacking the invading entities is to recognize and coat them with opsonins, which are defined as immune proteins such as antibodies or complement fragments that attach to foreign material, microorganisms, or other objects to enhance their phagocytosis. The primary antibodies that interact with foreign cells are polyreactive, low-affinity IgM antibodies produced by B1 cells (Vollmers and Brändlein, 2006; Baxendale et al., 2007; Holodick et al., 2010; Griffen et al., 2011). As the adaptive immune system becomes activated and B cells develop toward specific antigens on the invading foreign body (see Chapter 5), antigen-directed somatic hypermutation and class switching provide affinity-matured IgGs directed against those antigens. Opsonization is initiated by the binding of opsonins, e.g. C1q, C3b, iC3b, C4a, from the complement system, and/or antibodies that recognize surface antigens on the pathogen. The degree of opsonization is dependent on the number of

antigens recognized, total antigen density, and the affinity of the targeted IgGs (Jefferis, 2007; Moore et al., 2010). The opsonized apoptotic cell, entity, or foreign antigens, opsonized with IgGs of various isotypes and complement opsonins, typically form aggregates which are highly susceptible to immune interaction. The interaction with leukocytes is largely driven by the predominant antibody isotype as well as the presence and concentration of immune cells recruited to the local environment. The resulting immune cell recruitment may result in phagocytosis if monocytes, macrophages, or neutrophils are the primary cells recruited, release of granzymes and other killing factors if NK cells or neutrophils are recruited, and release of pro-inflammatory cytokines in nearly all cases. In the sections below, the specific functions that occur when particular cell types are recruited are discussed in more detail.

8.2.5 CDC

The classical complement pathway (see section 7.5) actually invokes multiple mechanisms of action that can help to clear a targeted antigen. The first is the well-known CDC pathway, in which activation of the complement cascade by IgG₁ or IgG₃ results in a series of reactions culminating in the formation of the membrane attack complex (MAC), which forms a pore through the membrane of the targeted cell. This MAC-mediated, immune cell-independent process results in the killing of the target cell through the disruption of its membrane (Gasque, 2004; Morgan et al., 2005; Jefferis, 2009b, 2009c; Moore et al., 2010). The process by which this occurs is a complex and highly regulated biochemical cascade, as described in detail by Gasque (2004), Morgan et al. (2005), and Ricklin and Lambris (2007). The circulating C1 complex is comprised of C1q, C1r, and C1s. As shown in Figure 8.3, C1q has six “heads” that look like a bunch of tulips, each head of which can bind an IgG. At least two of these heads must be occupied to induce the required conformation for deposition of the components C1r and C1s, a requirement which can be met only in immune complexes and not by circulating monomeric IgG (Jefferis, 2009b). IgG₁ or IgG₃ binding to C1q activates C1 to become a protease which cleaves complement component C4 into C4a and C4b. C4a, which is an anaphylatoxin, activates inflammatory immune cells, whereas C4b binds to C1, resulting in the formation of a protease that cleaves C2 into C2a and C2b. Ultimately, the pathway results in the proteolytic generation of components C3a and C3b by C3 convertases, as well as C5a and C5b. Formation of C3b exposes thioester groups that attach to the surface of invading bacteria or apoptotic

cells to opsonize them. This is a key step in the complement cascade that ultimately leads to elimination of invading pathogens as well as apoptotic host cells (Ricklin and Lambris, 2007). C3a and C5a, which are pro-inflammatory anaphylatoxins, bind to their cognate receptors, C3aR and C5aR, respectively, resulting in activation of the immune system (Gasque, 2004; Ricklin and Lambris, 2007). Similarly, the generation of C5b contributes that component to the membrane attack complex (MAC, which is comprised of complement components C5b, C6, C7, C8, and C9), which inserts into the cell membranes of bacteria and apoptotic cells and forms a pore that causes lysis (Gasque, 2004).

Other than classical CDC-mediated killing of pathogens, the complement pathway can kill through complement receptor (CR)-dependent phagocytosis and cytotoxicity, also sometimes referred to as complement-dependent cellular cytotoxicity (CDCC). These processes involve opsonized target cells and CR1, CR3, and/or CR4. The first of these cellular processes is an analog of the FcγR-dependent mechanisms of similar name, except that the effector function is mediated directly by the binding of CR to opsonin (Gelderman et al., 2004; Moore et al., 2010). In this case, the anaphylatoxin C5a acts as both a chemoattractant for immune cells, particularly neutrophils, as well as an inducer of activation FcγR expression in macrophages and enhancement of FcγR-mediated cell killing activity (Shushakova et al., 2002; Godau et al., 2004; Moore et al., 2010). A key mechanism by which the complement cascade can kill target cells is via CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18)-mediated phagocytosis of target cells opsonized with opsonins such as C3b, iC3b, and C4b (Gelderman et al., 2004; Moore et al., 2010). CR1 is widely expressed on myeloid cells, CR3 is expressed on neutrophils, NK cells and monocytes, and CR4 is expressed on macrophages. IgG-FcγR-mediated phagocytosis, as described below, significantly enhances this process.

8.2.6 ADCP

As mentioned above, target cells opsonized with C component C3b are recognized by complement receptors CR3 (CD11b/CD18), found on a variety of circulating monocytes, neutrophils, and NK cells, and CR4 (CD11c/CD18), which is found on tissue macrophages (Gasque, 2004). The interaction of CR3 and CR4 with C3b-opsonized bacteria and apoptotic cells helps to promote cellular phagocytosis of the pathogens or apoptotic cells. This process is stimulated greatly in the presence of antibodies that recognize the pathogen or apoptotic cell and also bind to

receptors found on the surface of phagocytic cells (e.g. FcγRI, FcγRIIa, FcγRIIIa; Indik et al., 1995). Thus antibody-dependent cellular phagocytosis (ADCP) is a process by which the opsonized cells are engulfed, in an Fc-dependent manner, primarily by macrophages, monocytes, and neutrophils (Figure 8.4).

The Fcγ receptors on those cell types that drive phagocytosis include FcγRI, FcγRIIa, and FcγRIIIa on monocytes and macrophages (Indik et al., 1995). It was recently demonstrated that aglycosyl IgG₁ against a cell-surface antigen was capable of triggering both phagocytosis of that targeted cell as well as cytokine release by the macrophages (Nesspor et al., 2012). Since aglycosyl IgG₁ binds only FcγRI, it is clear that signaling only through the high-affinity receptor FcγRI can result in both cytokine release and macrophage/monocyte-driven phagocytosis. On the other hand, Richards et al. (2008) demonstrated that FcγRIIa was the key receptor driving phagocytosis under the conditions used in their experiments. It is likely, then, that both FcγRI and FcγRIIa contribute to IgG-mediated phagocytosis *in vivo*. The ADCP process is not a passive one. Upon engagement of FcγRs with an opsonized target, FcγR ITAMs are phosphorylated, Lyn and Src kinases are activated, FcγRs are clustered on the surface of the cell, apparently to increase the avidity of binding to the target, and engulfment then takes place (Sobota et al., 2005).

Additionally, dendritic cells (DCs), which are potent antigen-presenting cells, also are known to possess activating Fcγ receptors (FcγRI, FcγRIIa, FcγRIIIa) and have been demonstrated to carry out phagocytosis (Kalergis and Ravetch, 2002; Melief, 2008; Correale et al., 2011). Thus it is thought that perhaps both macrophages and DCs, and perhaps other antigen presenting cells (APCs), have the capability to engulf foreign cells, viruses, and even cancer cells, digest them, and present peptide fragments to T cells as a bridge between the innate immune system and the adaptive immune response (Kalergis and Ravetch, 2002; Melief, 2008; Correale et al., 2011). It is also believed that antibodies may act as a stimulus to DCs to phagocytose target cells and present antigens to stimulate the adaptive immune response (Kalergis and Ravetch, 2002; Melief, 2008).

As mentioned above, neutrophils are active in ADCP as well. Activation of neutrophils via cross-linking Fcγ receptors by immune complexes of antibody-opsonized cells results in a variety of defense mechanisms, including phagocytosis, respiratory burst, degranulation and release of microbicidal molecules, and production of pro-inflammatory cytokines (García-García et al., 2009). The neutrophil activating Fcγ receptors that help mediate these signals are FcγRIIa and FcγRIIIb. Also, when neutrophils become activated *in vitro* by interferon-γ (Hoffmeyer et al., 1997; Rivas-Fuentes

et al., 2010) or *in vivo* by G-CSF (Repp et al., 1991; Valerius et al., 1993), they also express the high-affinity receptor, Fc γ RI, which also may be involved in neutrophil-mediated ADCP. García-García et al. (2009) sorted out the activities of Fc γ RIIa and Fc γ RIIIb in stimulated neutrophils and found that cross-linking of Fc γ RIIa activated Syk, PI3K, and ERK, resulting in phagocytosis, whereas specific cross-linking of Fc γ RIIIb resulted in activation of NF Elk-1 and nuclear ERK, indicating that the two receptors function differently. Rivas-Fuentes et al. (2010) went further to show that Fc γ RIIa and Fc γ RIIIb each could drive phagocytosis that could be blocked by Src family (e.g. Syk, PI3K, ERK) kinase inhibitors, but that Fc γ RI on interferon- γ stimulated neutrophils could not mediate phagocytosis. It is thought that Fc γ RI may help to drive respiratory burst and other pro-inflammatory functions in neutrophils (Rivas-Fuentes et al., 2010).

8.2.7 ADCC

Antibody-dependent cellular cytotoxicity (ADCC) is the process by which opsonized cells or foreign entities are lysed as a result of their interaction with immune cells. In theory, any immune cell capable of secreting cytotoxic proteins or chemicals could potentially drive ADCC, but in practice, the three major cell types known for catalyzing ADCC are CD56⁺, CD3⁻ natural killer (NK) cells (Bryceson et al., 2011), neutrophils (Peipp et al., 2008; Schneider-Merck et al., 2010), and monocytes (Schneider-Merck et al., 2010).

Upon opsonization of a foreign cell or entity with IgG₁ or IgG₃ antibodies, the immune complexes may bind NK cells through the Fc receptors Fc γ RIIIa and Fc γ RIIc, which can result in NK cell activation, the degranulation of lytic granulae, and the release of cytolytic perforin, apoptosis-inducing granzymes (serine proteinases), and apoptosis-inducing FAS ligand (Bryceson et al., 2011). Perforin is a cytolytic, non-specific, pore-forming protein, somewhat similar to complement factor C9, which is secreted by stimulated NK cells and cytotoxic T cells (Tschopp et al., 1986) (see Figure 8.5).

Neutrophils also are known to carry out non-phagocytic ADCC which is thought to be driven by Fc γ RI and Fc γ RIIa on their surface (van der Kolk et al., 2002; Schneider-Merck et al., 2010). Whether Fc γ RIIIb is involved or not in neutrophil-mediated ADCC has not yet been determined (Peipp et al., 2008). In fact, there has been the suggestion that Fc γ RIIIb may compete for antibody occupancy on neutrophils with the other low-affinity activating receptor, Fc γ RIIa, thereby potentially acting in a negative manner with respect to driving neutrophil-mediated ADCC (Peipp et al., 2008).

Monocytes also have been shown to carry out non-phagocytic ADCC in the presence of either IgG₁ (zalutumumab) or IgG₂ (panitumumab) targeting EGFR (Schneider-Merck et al., 2010). It was apparent that FcγRIIIa was the key receptor on monocytes for triggering the monocyte-driven ADCC in those experiments because both IgG₁ and IgG₂ MABs were capable of eliciting the response (Schneider-Merck et al., 2010). Alternatively, Watanabe et al. (1999) demonstrated that a bispecific antibody MDX-210 targeting both HER2/neu and CD64 (FcγRI) simultaneously could drive monocyte-dependent ADCC and ADCP, suggesting that FcγRI could, under some conditions, help mediate ADCC.

Finally, eosinophils have been shown to kill large parasites, which are too large to be phagocytosed, via ADCC. The parasites are opsonized with IgE, which binds FcεRI on the eosinophils with high affinity (see Chapter 7), resulting in degranulation and lysis of the parasite. This biology has been used as the basis for the engineering of an anti-folate receptor-α IgE MAB for treatment of ovarian cancer (Karagiannis et al., 2007, 2008; Rudman et al., 2011). Monocytes bound by this antibody were shown to carry out both ADCP, via FcεRII (CD23), and ADCC, via FcεRI, of target ovarian carcinoma cells (Karagiannis et al., 2007, 2008). This success led Karagiannis et al. (2009) to construct and test an anti-HER2 IgE-based MAB for increased effector functionality.

8.2.8 *Direct cell killing via apoptotic or lysosomal programmed cell death*

It is known that antibodies can kill cells directly, either through induction of the intrinsic apoptosis pathway (Shan et al., 1998, 2000; Jin and El-Deiry, 2005; Lavrik, 2010), or potentially through some other direct killing mechanism such as homotypic cell aggregation and lysosomally related, non-apoptotic programmed cell death (PCD) (Ivanov et al., 2009; Golay et al., 2010; Beers et al., 2010; Niederfellner et al., 2011). Other than directly triggering the extrinsic apoptotic pathway via pro-apoptotic signaling receptors such as Fas (Odani-Kawabata et al., 2010), it is not yet certain exactly how, mechanistically, MABs kill cells via apoptosis. While it is clear that antibody-mediated induction of apoptosis is at least one form of PCD (Shan et al., 1998, 2000), it also has been demonstrated that a second, non-apoptotic, form of antibody-mediated PCD can occur (Ivanov et al., 2009; Golay, 2010; Beers et al., 2010; Niederfellner et al., 2011; Alduaij and Illidge, 2011). It has been determined recently that the

Fc-independent direct killing of B cells by Type II MAbs such as tositumomab or GA-101, which was once thought to be apoptosis, occurs by a non-apoptotic PCD mechanism (Golay, 2010; Beers et al., 2010; Niederfellner et al., 2011). This alternative PCD mechanism has been demonstrated to be related to homotypic aggregation, changes in actin reorganization, and lysosomal disruption which releases cathepsin B into the cytoplasm causing the cell death; this process has been shown to be fully independent of bcl2 and caspases (Ivanov et al., 2009; Golay et al., 2010; Beers et al., 2010; Niederfellner et al., 2011; Alduaij and Illidge, 2011). One major key for determining if the MOA includes apoptosis by either the intrinsic or extrinsic pathway is having multiple assays supporting the hypothesis, since single apoptosis-related parameters also may be present in other MOAs (Kepp et al., 2011), and FACS analyses may be skewed due to the presence of cell aggregation caused by homotypic adhesion (Ivanov et al., 2009; Golay et al., 2010; Beers et al., 2010).

8.2.9 Internalization and down-modulation

One mechanism by which therapeutic MAbs directed against cell surface targets can function is to induce internalization and turnover of those receptors. In some cases, this activity results in a down-modulation of the targeted receptor cell surface copy number, which in itself could yield a potential therapeutic effect (Pedersen et al., 2010), or minimally, a biomarker surrogate for biological function. However, not all MAbs that bind cell surface targets induce internalization of those targets. For example, it does not appear that some anti-CD20 antibodies induce significant internalization and turnover of CD20 on B cells (see Table 7.2).

There are several therapeutic MAbs either on the market or in late-stage clinical trials that bind cell surface receptors (see Table 7.2), some of which induce internalization of those receptors (Rowinsky et al., 2004; Ng et al., 2005; Mould et al., 2007). One key physiological ramification of this activity, however, is the receptor mediated clearance of those therapeutic MAbs can often outcompete FcRn-mediated recycling of those MAbs. In that case, the major route of clearance at lower doses of the antibodies becomes the receptor-mediated internalization route. Generally, at high doses, the receptor-mediated clearance mechanisms have been saturated and clearance then becomes a function of normal antibody clearance pathways (Keizer et al., 2010). In fact, non-linearity with respect to dosing is a hallmark of the pharmacokinetics of MAbs cleared by target-mediated internalization (Keizer et al., 2010).

There is a general correlation between the half-life of a receptor-binding MAb in the clinic and its ability to induce internalization (see Table 7.2). As noted previously, the average half-life of receptor-binding antibodies was considerably lower than the average half-life of several MAbs targeting soluble proteins (see Table 7.2). Some specific examples of this include:

- efalizumab (Raptiva®), which is internalized and has a half-life in humans of ~5.5 d, which is non-linear with respect to dose (Ng et al., 2005);
- alemtuzumab (Campath®), which is rapidly internalized and has a half-life in humans in the range of 1–6 d, which was difficult to measure both because it had non-linear elimination and rapidly depleted the target cells, after which the elimination rate decreased (Mould et al., 2007; Campath® prescribing information, 2009);
- panitumumab (Vectibix®), which is rapidly internalized (Rowinsky et al., 2004) and has a half-life in humans of 7.5 d (Vectibix® prescribing information, 2012);
- anti-HM1.24, which is very rapidly internalized and has a half-life at high doses in cynomolgus monkeys of only 28.6 h (Amano et al., 2010);
- Sym004, which is comprised of a pair of anti-EGFR MAbs that induce very rapid internalization and degradation of EGFR, resulting in very strong *in vivo* efficacy in animal models (Pedersen et al., 2010); and
- several clinical candidate anti-IGF-1R MAbs, which demonstrate receptor internalization, receptor down-regulation (which may be important both therapeutically as well as for biomarker purposes) and, except for figitumumab (Olmos et al., 2010), short half-life values (Burtrum et al., 2003; Hofmann and García-Echeverría, 2005; Goetsch and Corvaia, 2009; Broussas et al., 2009).

As described in Chapter 15, one of the key characteristics required for an antibody to be used as part of an antibody-drug conjugate (ADC) is the ability to induce rapid internalization of the receptor it targets. As such, several researchers have set up screens to isolate antibodies that induce high receptor-MAb complex internalization rates (Becerril et al., 1999; Poul et al., 2000; Kuo et al., 2009; Poul, 2009; Zhou et al., 2010b).

8.2.10 Inhibition of enzymatic activity

For many years it was thought that antibodies would not make good inhibitors of enzymes. There are now several examples in which MAbs

have been shown to inhibit enzymatic activity, either directly by competition with substrate for the binding site or allosterically by indirect inhibition caused by modulation of the enzyme structure. A few examples of these will be described here.

Several MABs have been isolated that bind directly to the active sites of enzymes, including:

- MAb 6A10, which directly inhibited the active site of carbonic anhydrase XII in the nM range (Battke et al., 2011);
- two MABs (clones 3 and 6) that were able to completely and specifically neutralize the activity of the snake venom metalloproteinase (SVMP) BaP1 but not other snake venoms (Fernandes et al., 2010);
- a murine MAB mU1 that was shown to completely neutralize the activity of urokinase-type plasminogen activator (uPA) (Jögi et al., 2010); and
- two MABs (F2-1-11, G8-25-5) that inhibited both the gelatinase and collagenase activities of matrix metalloproteinase 2 (MMP2) in a potent and highly specific manner (Emara and Wozniak, 2010).

Other MABs appear to inhibit their enzyme targets allosterically. These include examples such as: MAB AB0023, which allosterically inhibited the matrix enzyme lysyl oxidase-like-2 (LOXL2) in the nM range (Barry-Hamilton et al., 2010); and MAB 1A11, which bound to the D and F loops of β -secretase (BACE) to inhibit BACE cleavage of intact amyloid precursor protein (APP), but did not directly occupy the active site (Zhou et al., 2011). Finally, there is at least one example of an antibody that bound the substrate of an enzyme, thereby protecting that substrate from cleavage: Boddapati et al. (2011) isolated an scFv that indirectly blocked β -secretase (BACE) activity by binding to amyloid precursor protein at the substrate recognition site for BACE, thereby blocking the ability of the enzyme to access its target.

The key to this section is not to carry out an exhaustive review of MABs targeting enzymes, but to demonstrate that MABs have the capability to inhibit enzymes with high specificity. Thus, if accessible (extracellular or cell-surface) enzymes need to be inhibited as part of a therapeutic approach, MABs should be considered as one possible route to achieve that end. Examples of such enzymes might include matrix metalloproteinases (MMPs), for which potent, specific, and non-toxic small molecule inhibitors are very difficult to find. One potential limitation for that approach, however, may be that IgGs are sensitive to proteolytic cleavage by some MMPs (Gearing et al., 2002).

8.3 CD20 – example of a target for which multiple MOAs apply

As mentioned throughout this chapter, the mechanism of action (MOA) for antibody functionality can fall under various categories such as antagonism, agonism, or cell killing via ADCC, ADCP, CDC, apoptosis PCD, or lysosomal-related PCD. Usually, a therapeutic antibody exerts multiple MOAs to achieve its overall efficacy, especially if the antibody has Fc-mediated activities that engage different aspects of the immune system. It also turns out that antibodies with the same isotype, similar affinities, and binding to similar, albeit not identical, epitopes may have very different MOAs. Detailed understanding of the MOA should allow for a greater stratification of the patient population and an understanding of which antibody with which MOA will best fit a particular disease as described below.

Antibody mechanism of action is perhaps best characterized for anti-CD20 antibodies. CD20 is a 35 kDa integral membrane protein that is expressed in tetramers (Cragg, 2011) on the surface of normal and malignant B cells, but not on precursor B cells or on other tissues. CD20 has two exposed loops, a small loop of eight amino acid residues towards the N-terminus of the protein, and a larger 40 amino acid residue loop more towards the C-terminus of the protein (see Figure 8.6; Teeling et al., 2006).

There are at least 12 different anti-CD20 antibodies targeted for lymphoma and rheumatoid arthritis indications (see Table 2.7) on the market or in clinical trials (see Table 8.4), several of which have been characterized in significant detail. Rituxan® (rituximab), Zevalin® (ibritumomab tiuxetan), Bexxar® (tositumomab-I131; B-1 MAb), and Arzerra® (ofatumumab; HuMax CD20) are all anti-CD20 antibodies that have been approved by the FDA for marketing. All of these antibodies target CD20 to deplete B cells (see Table 8.9).

The mechanisms for cell death mediated by these antibodies vary quite considerably, but generally fall into two categories, Type I and Type II (Bello and Sotomayor, 2007; Glennie et al., 2007; Beers et al., 2008; Beers et al., 2010; Oflazoglu and Audoly, 2010; Niederfellner et al., 2011). Type I anti-CD20 antibodies have largely been defined as those capable of driving the sequestration of CD20 into lipid rafts (Deans et al., 2002; Cragg et al., 2003; Beers et al., 2010), activate CDC (Cragg et al., 2003; Beers et al., 2010), and require FcγR activity for depletion mechanisms (de Haij et al., 2010). Significantly, the ability of a Type I MAb to effect translocation of CD20 into lipid rafts was correlated with the ability to activate the complement pathway (Cragg et al., 2003; Beers et al., 2010). Finally, Type I anti-CD20 MAbs have recently been demonstrated to down-modulate CD20 on the surface of B cells to a much greater degree than Type II MAbs (~90% versus ~20% losses

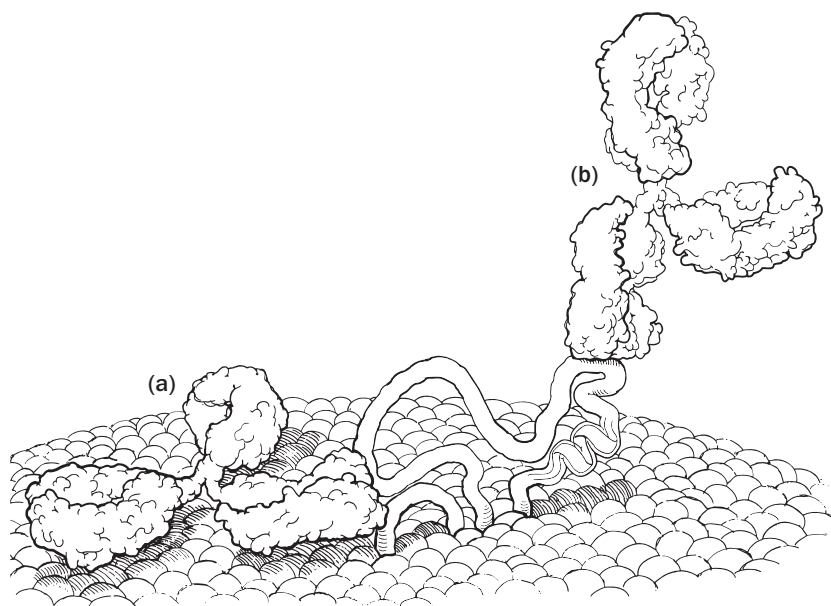


Figure 8.6 Anti-CD20 antibodies binding CD20 on the surface of B cells

Drawing depicting the binding of ofatumumab (a) to the N-terminal portion of CD20 (the small extracellular loop and a few residues on the larger loop) versus the binding of rituximab (b) and other anti-CD20 antibodies which bind CD20 towards the C-terminal portion of the larger loop. The drawing is made from data presented in several references, including Teeling et al. (2006), Du et al. (2008), Du et al. (2009).

for Type I and Type II MABs, respectively) (Beers et al., 2010). Rituximab, ocrelizumab, veltuzumab, PRO-131921, and LY2469298 (AME-133v) are all examples of Type I anti-CD20 MABs (see Table 8.9), with rituximab being the prototype. These antibodies target the larger C-terminal loop of CD20 around residues 168–175 (Teeling et al., 2006; Du et al., 2008; Niederfellner et al., 2011), whereas another Type I MAB, ofatumumab, binds to a membrane-proximal, discontinuous epitope including the small, N-terminal small loop as well as a few residues on the N-terminal end of the large loop (Teeling et al., 2006; Du et al., 2009; Niederfellner et al., 2011; see Figure 8.6). The cell killing activity of Type I MABs rituximab and ofatumumab require Fc receptor ITAM signaling for their activity, indicating that ADCC is likely to be the primary MOA for B cell depletion by these MABs (de Haij et al., 2010). For example, follicular lymphoma patients possessing the low-affinity version of Fc γ RIIIa (158F/F) have been found to respond more poorly to Rituxan[®] than patients possessing the high affinity form of the receptor, homozygous Fc γ RIIIa 158V/V (Cartron

Table 8.9 Antibodies targeting CD20

Name of molecule	Company	Status	Type	Format	Primary MOAs used to deplete CD20* cells
Rituxan® (Rituximab)	Biogen/ Idec/ Genentech	FDA approved 11/26/1997	I	IgG ₁ κ, chimeric	ADCC, CDC; down-modulation of CD20 on B cell surface
Zevalin® (Ibritumomab tiuxetan)	Biogen/ Idec	FDA approved 02/19/2002	I	Murine IgG ₁ κ; ⁹⁰ Y or ¹¹¹ In radioconjugate	Localized irradiation
Bexxar® (Tositumomab- I131; B1)	Corixa	FDA approved 06/27/2003	II	Murine IgG _{2a} λ; ¹³¹ I radio- conjugate	Localized irradiation; PCD
Arzerra® (Ofatumumab; HuMax CD20; 2F2)	GenMab/ Glaxo- SmithKline	FDA approved 10/26/2009	I	Human IgG ₁ ; targets N- terminus of CD20	Like rituximab but with stronger CDC activity
Ocrelizumab (2nd gen. anti- CD20; 2H7; PRO-70769; R-1594)	Genentech	Phase III (NHL)	I	Humanized IgG ₁	Like rituximab but with increased binding to FcγRIIIa yielding higher ADCC; lower CDC
Veltuzumab (Ha-20; IMMU- 106)	Immuno- medics	Phase I/II	I	Humanized IgG ₁	Like rituximab but higher affinity for CD20; dosed subcutaneously
PRO-131921 (3rd generation ant-CD20)	Genentech	Phase I/II (possibly terminated)	I	Humanized IgG ₁ ; Fc- modified ocrelizumab	Like rituximab but increased binding to FcγRIIIa yielding enhanced ADCC
LY2469298 (AME-133v)	Mentrik Biotech, LLC (acquired from Eli Lilly)	Phase I/II	I	Humanized IgG ₁ with modified Fc	Like rituximab but higher affinity for CD20 and increased binding to FcγRIIIa yielding enhanced ADCC

Table 8.9 Antibodies targeting CD20 (*cont'd*)

Name of molecule	Company	Status	Type	Format	Primary MOAs used to deplete CD20 ⁺ cells
Obinutuzumab GA-101 (R-7159)	Roche	Phase II	II	Humanized IgG ₁ ; non-fucosylated Fc	Increased binding to FcγRIIIa yielding enhanced ADCC; programmed cell death (PCD)
LFB-R603 Anti-CD20	LFB	Phase II	Unk.	Chimeric IgG ₁ ; low fucose	Increased binding to FcγRIIIa yielding enhanced ADCC
BM-ca	Academic	Pre-clinical	I/II	Humanized IgG	Mixed MOA: CDC, redistribution of CD20 into lipid rafts; PCD, apoptosis

Abbreviations: ADCC: antibody-dependent cellular cytotoxicity; CDC: complement-dependent cytotoxicity; MOA: mechanism of action; PCD: non-apoptotic programmed cell death.

* Data accumulated and summarized from Teeling et al. (2006), Glennie et al. (2007), Bello and Sotomayor (2007), Beers et al. (2008), Beers et al. (2010), Ofiazoglu and Audoly (2010), Alduaij and Illidge (2011), and Niederfellner et al. (2011).

et al., 2002; Weng et al., 2003; Ghielmini et al., 2005; see Table 10.2, Chapter 10). These data support ADCC as a significant component of the MOA for Rituxan[®] efficacy against follicular lymphoma.

Type II anti-CD20 MABs do not promote redistribution of CD20 into lipid rafts; they generally do not activate the complement pathway to a great extent, but rather kill cells by a combination of PCD and ADCC (Bello and Sotomayor, 2007; Glennie et al., 2007; Beers et al., 2008; Alduaij et al., 2011; Alduaij and Illidge, 2011; Niederfellner et al., 2011). The mechanism for PCD elicited by Type II MABs is not bcl2- and caspase-3 dependent apoptosis, as was once thought (Glennie et al., 2007; Beers et al., 2008), but instead involves homotypical aggregation, actin reorganization, lysosomal degradation and release of cathepsin B into the cytoplasm (Beers et al., 2010; Niederfellner et al., 2011; Alduaij et al., 2011; Alduaij and Illidge, 2011). While Type II antibodies often mediate strong ADCC, they clearly do not rely on ADCC as the only mechanism of killing target cells. Tositumomab is the prototypical Type II anti-CD20 MAB, whereas obinutuzumab (GA-101) is the most well studied of this

group (Niederfellner et al., 2011). Obinutuzumab has been shown to have 1.5–2.5-fold greater cell depletion activity than rituximab in side-by-side assays, with PCD and ADCC each contributing nearly 50% of the killing activity (Patz et al., 2010; Mössner et al., 2010). A significant fraction of the cell-depleting activity of GA-101 is retained by a F(Ab')₂ version of the antibody, most of which is ascribed to the PCD MOA of this Type II MAb (Alduaij et al., 2011). A final aspect that differentiates Type II from Type I anti-CD20 MAbs is that the Type II MAbs do not appear to down-modulate CD20 on the surface of B cells to the same degree as Type I MAbs (Beers et al., 2010). Since PCD contributes significantly to the activity of Type II anti-CD20 MAbs and they do not down-modulate CD20, one concept that has been proposed is to use this type of antibody to treat patients who have the low-affinity version of FcγRIIIa (158F/F) or patients with CLL, where the FcγRIIIa polymorphism did not correlate with efficacy (Farag et al., 2004). Indeed, GA-101 (obinutuzumab) was significantly better than rituximab in depleting B cells in *ex vivo* assays using blood from patients with CLL (Patz et al., 2010). Obinutuzumab, however, was also significantly more potent than rituximab in mediating the degranulation of NK cells (Bologna et al., 2011) and γδ T cells (Braza et al., 2011), indicating that Fc activity is critical for the full depleting activity of this MAb (Alduaij and Illidge, 2011). Finally, obinutuzumab also demonstrated significant superiority over rituximab in follicular lymphoma xenograft animal models, whether dosed alone or in combination with cyclophosphamide (Dalle et al., 2011).

Niederfellner et al. (2011) have recently made significant advances in the understanding of what makes a Type I anti-CD20 MAb versus a Type II MAb. While the gross epitopes are highly related and overlapping, the fine epitopes are subtly but significantly different. The Type I MAbs such as rituximab bind an epitope defined by residues 168–175 in the large C-terminal loop of CD20, whereas Type II MAbs tended to bind slightly to the C-terminal side of that epitope, spanning amino acid residues 172–178 (Niederfellner et al., 2011). Interestingly, while the conformation of the CD20 peptide epitope of Type I rituximab and Type II obinutuzumab (GA-101) was remarkably similar in crystal structures, the angle of binding was clearly different (Niederfellner et al., 2011). Confocal microscopy and tomography suggested three significant differences in binding CD20 between the two MAbs: first, rituximab and other Type I MAbs appear to bind twice as many sites on B cells as Type II MAbs (Niederfellner et al., 2011; Cragg, 2011); second, Type I MAbs only bind closed conformations of CD20 whereas Type II MAbs bind both open and closed conformations, and possibly cause the opening of closed-conformation CD20 molecules (Cragg, 2011); and third, Type I MAbs appear to stabilize highly dynamic, possibly

inter-tetrameric (Cragg, 2011), populations of CD20 that coalesce on the surface of the cells (e.g. migration into lipid rafts), whereas Type II MAbs appear to stabilize static, possibly exclusively intra-tetrameric (Cragg, 2011), CD20 complexes, resulting in homotypic aggregation, actin rearrangement, and lysosomally directed PCD (Niederfellner et al., 2011; Cragg, 2011).

Interestingly, a new anti-CD20 MAb called BM-ca has recently been described that has characteristics of both Type I and Type II anti-CD20 MAbs (Nishida et al., 2008, 2011). This antibody directs the redistribution of CD20 into lipid rafts and activates the complement cascade, both of which activities are common to Type I MAbs, but also induces homotypic aggregation and killing by non-apoptotic PCD, a characteristic of Type II MAbs (Nishida et al., 2011). BM-ca was found to be significantly more potent than rituximab for ADCC, CDC, and PCD killing (Nishida et al., 2008).

In summary, this section has pointed out some critical issues in studying the efficacy and MOAs of antibodies against their targets. First, in the case of rituximab versus ofatumumab, epitope mapping did not predict Type I versus Type II activity, whereas fine mapping and tomograms of the antibodies interacting with the target on the cell surface demonstrated some significant differences in binding angle, clustering, and complexation, which apparently account for the significant differences in MOA (Niederfellner et al., 2011; Cragg, 2011).

Therapeutic antibody classes

Abstract: Natural antibody classes in humans include IgA (two isotypes), IgD, IgE, IgG (four isotypes), and IgM (two major forms, pentameric and hexameric). Of these, all current marketed MABs and Fc fusion proteins are of the IgG classes. Human IgG₁, and to a lesser degree human IgG₂ and IgG₄, have been used as scaffolds for therapeutic monoclonal antibodies (MABs). Antibodies of the human IgG₁ and IgG₃ isotypes bind all Fcγ receptors (FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa, FcγRIIIb), whereas IgG₄ binds primarily FcγRI and very weakly to the other FcγRs, and IgG₂ binds primarily FcγRIIa. These different binding characteristics give these isotypes very different immune-mediated activities, which have been used to help optimize antibody activity. Some efforts have been made to use IgM and IgA scaffolds for therapeutic MABs, although none of these have yet reached the market.

Keywords: IgG, isotypes, Fc, Fcγ receptors, IgA, IgM, IgE, affinity, allotypes

9.1 Human antibody overview

In the late 1930s, Tiselius and Kabat used electrophoresis to fractionate the proteins of serum from rabbits into albumin, α-globulin, β-globulin, and γ-globulin fractions (Tiselius and Kabat, 1939). They then immunized those rabbits with hen ovalbumin and demonstrated that the γ-globulin fraction was increased substantially, thereby identifying that fraction as the “antibody” fraction. This was the first time “antibodies,” which had been known since Emil von Behring’s early work in the 1890s, were identified as a particular protein fraction. Sizing columns were then used to separate the γ-globulin fraction into the heavy fraction, subsequently identified as IgM, “regular fraction,” which was subsequently shown to contain IgA, IgE, IgD, and IgG, and the light fraction, which was later identified as light chain dimers (Li et al., 2000).

All five classes (or isotypes) of human immunoglobulins (Igs), IgA, IgD, IgE, IgG, IgM (see Figure 9.1) are comprised of a basic monomeric H₂L₂

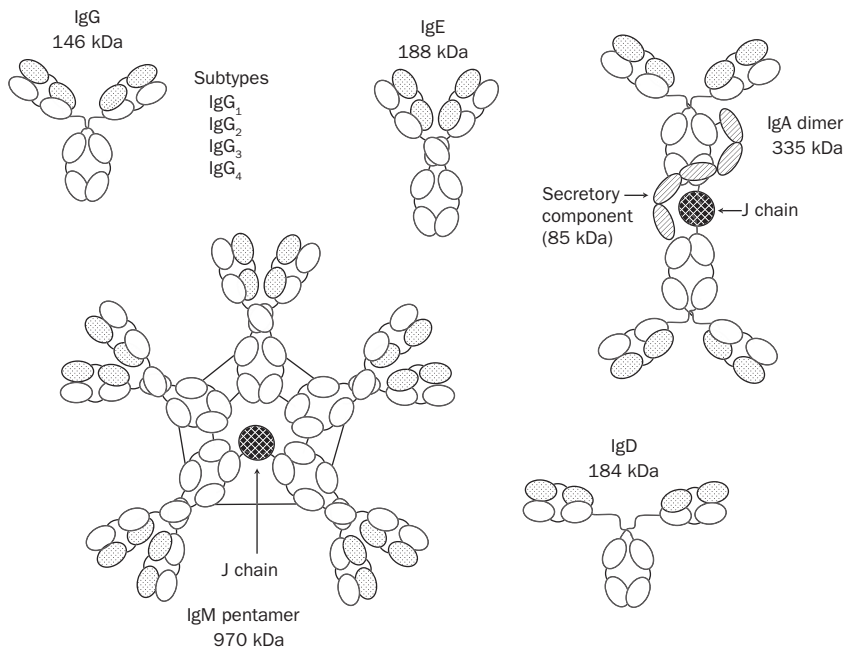


Figure 9.1 Human Ig classes

Shown are drawings of IgG, IgD, IgE, pentameric, J-chain-containing IgM, and dimeric, secreted IgA containing both a J-chain and a secretory component, the latter polypeptide obtained from plgR as part of the secretory process, as described in the text.

structure in which each of a pair of heavy chains, ca. 50–75 kDa in size and constructed of 4–5 Ig domains, is paired with a light chain of about 25 kDa and constructed of two Ig domains. The heavy chains α , δ , ϵ , γ , and μ comprise the IgA, IgD, IgE, IgG, and IgM classes, respectively. The IgA and IgG are further categorized into subclasses based on sequence and functionality. There are two subclasses for IgA (A1, A2) and four for IgG (G1–G4). The light chains, which are shared by all classes, are comprised of either κ or λ types. There are four subtypes in the λ family.

As described in an earlier chapter, Igs have two major moieties, the Fc and Fab, both of which are constructed of domains with β -pleated sheets. The Fc is comprised of domains 3–4 or 3–5 of the heavy chain, whereas Fabs are comprised of the two N-terminal domains of the heavy chain paired with the light chain. The V_L and V_H chains, which are the N-terminal Ig domains of both the heavy and light chains, comprise the antigen binding sequences of Igs (Chapters 3 and 5) and are derived from families of V and J (and in heavy chains, D) genes that are shared by all Ig classes. The other Ig domains in antibodies are referred to as C (constant) domains because

they are invariant among antibodies of the same heavy chain class or subclass and light chain type or subtype.

For the IgA, IgD, and IgG classes, a flexible, non-structured hinge separates the FAb and Fc moieties. The IgE and IgM classes possess an extra β -sheet constant domain that positionally replaces the hinge (see Figure 9.1), resulting in less flexible FAb domains. Igs of the IgD, IgE, and IgG classes are essentially monomeric, although dimers of certain IgGs can be found, as will be described later. IgAs can be found as both monomers in serum and dimers, which can occur both in serum and on mucosal surfaces. IgMs are multimeric with pentamers and hexamers being the predominant oligomers.

The IgA and IgM classes also possess two unique structural features not found in the other classes that confer additional functionalities. The first of these is an associated joining chain (J-chain), which confers the ability on IgAs and IgMs to bind to specific receptors such as the polymeric Ig receptor (pIgR) (Brandtzaeg and Prydz, 1984). The second unique feature is a C-terminal “tailpiece” which is an 18 amino acid non-structured sequence found in both IgAs and IgMs that confers the ability to multimerize through a critical cysteine residue (Sørensen et al., 1999). IgDs also have a short tailpiece that allows them to be anchored to the surface of B cells (Sun et al., 2005). All isotypes, however, can be synthesized with a transmembrane domain spanning sequence and can be expressed as antigen receptors on B cells.

Under natural conditions, antibodies bind targeted epitopes on foreign cells, viruses, or proteins with their variable regions. Particularly with foreign cells or viruses, the result is an antibody-coated particle that the immune system targets for removal from circulation. The binding of an antibody via its variable chains to its cognate target defines the primary characteristic of an antibody. As described in Chapters 7–8, the Fc domains of the antigen-bound antibodies then bind and cross-link a variety of receptors on immune effector cells, the result of which is usually removal of the antigen from circulation as well as stimulation of a cytokine response that ultimately further strengthens the immune response against the targeted antigen. Thus the functionality of the Fc of antigen-bound antibodies is a critical part of the overall humoral response to antigen stimulation. While the antigen-binding activity is critical for the function of the antibody, a given variable chain sequence will yield essentially the same intrinsic affinity whether it is incorporated into one Ig scaffold or another. On the other hand, each Ig class or subclass can exert a unique set of Ig-specific Fc-mediated immunological activities based on their sequence and structure. Additionally, the Ig class or subclass can dramatically affect the avidity of binding to the target as well as the degree of cross-linking. This chapter compares and contrasts the various Ig scaffolds that could be incorporated into a therapeutic antibody or FcFP.

9.2 Human IgG isotypes

9.2.1 Overview

There are four human IgG isotypes, IgG₁, IgG₂, IgG₃, and IgG₄, each of which possesses unique sequences and properties. All of these isotypes, except IgG₃, have been used to generate human therapeutic MABs. Phylogenetically, it appears that both intraspecies and interspecies IgG isotypes have evolved as a result of gene duplication, which occurred after the diversification of mammals (Vernersson et al., 2004; Hsu et al., 2006). This is consistent with the fact that humans, rats, and mice have four IgG isotypes, albeit with significant differences between species, whereas rabbits appear to possess only a single major IgG isotype (Knight and Winstead, 1997). Additionally, isotypes within the species arose after speciation, so there is no direct correlation, for example, between isotypes of rodent IgGs and their human counterparts (Hsu et al., 2006). Finally, recent evidence also suggests that avian IgY is a predecessor to mammalian IgG and IgE (Taylor et al., 2008).

The major properties of human IgG antibodies are provided in Table 9.1. Human IgG antibodies are the most prevalent antibodies in the serum, with an average total concentration of about 6–16 mg/mL. The intrinsic half-life for IgG₁, IgG₂, and IgG₄ isotypes is typically given as ~14–21 days. An analysis of >20 marketed and clinical candidate antibodies spanning these isotypes demonstrated that both the mean and median half-life values in humans were in the 12–13 day range (Strohl, 2009a). This practical value includes the impact of antigen load on clearance. IgG₃s have a shorter half-life of about 5–7.5 d (see Table 9.1).

IgGs are primarily described as serum antibodies in the textbooks, whereas IgA and IgM are well noted for their ability to translocate to the mucosal surfaces through their interaction with the pIg receptor, as discussed in Chapter 7. It might be surprising, then, to know that IgGs are found in many mucosal secretions, including female genital tracts, rectal secretions, and lung, where they function in combination with sIgA in host defense. In fact, it has been demonstrated that rectal secretions may contain as much as 700 µg/mL of IgG (Kozłowski et al., 1997). A testament to the importance of IgG in these secretions is that humans with IgG deficiency syndrome exhibit a substantially increased rate of mucosal and system infections, particularly in the respiratory tract (Spiekermann et al., 2002).

The sequences of the four human IgG isotypes are shown in Figure 9.2. The sequences involved in binding to FcγRs (Sonderman et al., 2001;

Table 9.1 Functional characteristics of human antibody IgG isotypes

Characteristic or function (human data only)	Subclass or isotype			
	IgG ₁	IgG ₂	IgG ₃	IgG ₄
Approximate molecular weight	146 kDa	146 kDa	165 kDa	146 kDa
Protein chains ^a	γ2L2	γ2L2	γ2L2	γ2L2
Ratio of κ/λ usage (Wingren et al., 2005)	2.4/1	1.1/1	1.4/1	8.0/1
Functional valency	2	2–4 ^b	2	1 ^c
N-linked glycosylation sites per Fc monomer	1	1	1	1
Hinge length (number of amino acids)	15	12	62	12
ADCC-NK cells	+	–	+	–
ADCP-macrophages	+	+	+	–
ADCC/ADCP PMNs	+	+	+	–
C1q binding	++	+/-	++	–
Complement-mediated cell lysis	++	+/-	++	–
FcRn binding	+	+	+/- ^d	+
Ability to cross the placenta	+++	+/- ^f	++	++
Nominal serum half-life (in days)	21 d	21 d	5–7.5d ^d	21 d
Number of allotypes ^e	4	1	13	0
Approximate mean serum concentration	9 mg/mL	3 mg/mL	1 mg/mL	0.5 mg/mL

Notes:

^a Protein chains: γ, heavy chain; L, light chain.

^b IgG molecules have two binding sites but IgG₂ can form covalent dimers leading to molecules with four binding sites (Salfeld, 2007).

^c Functionally unable to cross-link two antigens, and thus behaves as a monomer (van der Zee et al. 1986; Aalberse and Schuurman, 2002) and undergoes exchange of the heterodimeric heavy chain – light chain units with other IgG₄ molecules *in vivo* to create functional monovalency (Labrijn et al., 2009).

^d Based on data from Morell et al. (1970) and Kim et al. (1999). Binding of IgG₃ to FcRn is poorer than IgG₁, apparently due to the H435R substitution in the IgG₃ (Kim et al., 1999).

^e From Salfeld (2007).

^f Hashira et al. (2000).

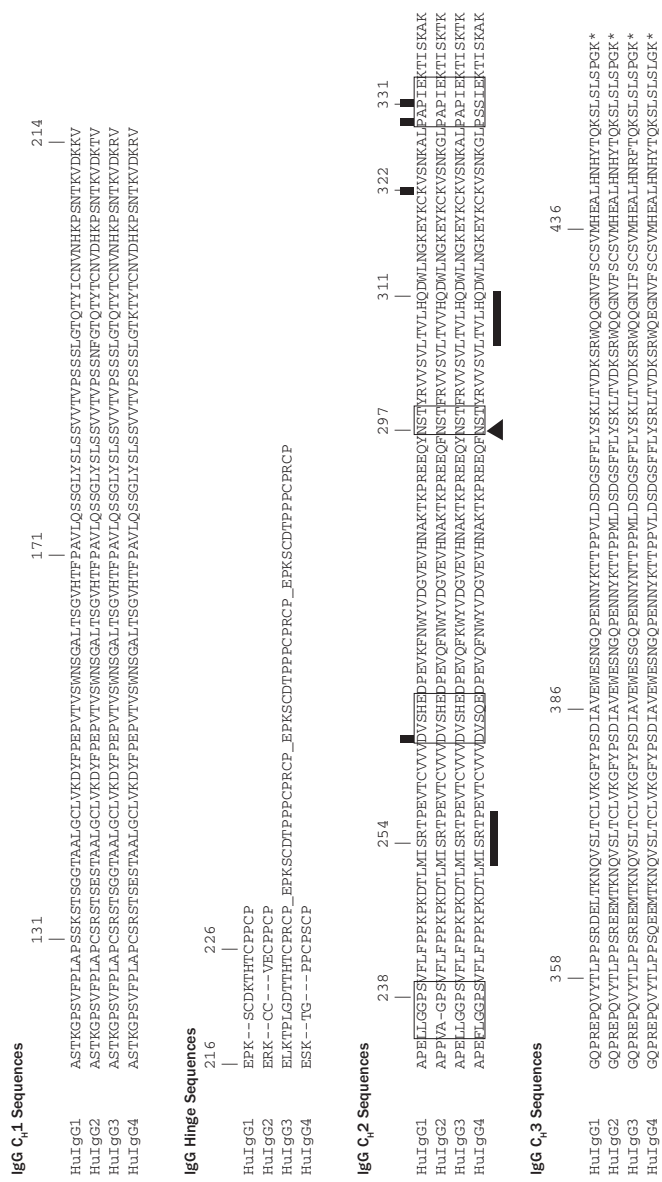


Figure 9.2 Sequences of human IgG₁, IgG₂, IgG₃, and IgG₄

The sequences of the human IgGs shown to bind human FcγRs, as described by Sonderrmann et al. (2001) and Radaev and Sun (2001), are boxed; residues known to bind to C1q (Idusogie et al., 2000; Thommesen et al., 2000) have a small rectangle over the sequence (note that for IgG₄, residues 330–331 are SS instead of AP, which eliminates C1q binding to IgG₄), the N-glycosylation site at N297 is depicted by a triangle under the sequences, and sequences involved in binding FcRn (Martin et al., 2001) are underlined with a thick line. Reference residues are numbered according to the EU numbering for IgG₁. Note that the lower hinge, as described in Figure 3.2, is included in the C_{1,2} domain in this figure.

Radaev and Sun, 2001), to complement component C1q (Idusogie et al., 2000; Thommesen et al., 2000), and to FcRn (Martin et al., 2001) are highlighted. Human IgGs have a single glycosylation site in the constant region at N297 (based on EU numbering) on each Fc chain. Additionally, 20–30% of naturally occurring IgG antibodies also have N-linked glycosylation in the variable chains (Jefferis, 2007).

Specific sequences of the antibody Fc regions have been demonstrated to be responsible for binding various receptors or complement components to effect these responses (see Figure 9.2). The human IgG isotypes each have different binding to Fc γ receptors due to the differences in their sequences, particularly in their hinge regions, and thus their three-dimensional structures (Jefferis, 2007). They also have sequence-based differential binding to the initial component of the complement cascade, C1q (Sontheimer et al., 2005). Finally, IgGs, but not other human Ig classes, bind FcRn (the neonatal receptor, also known as the Brambell receptor), which binds internalized IgG in acidic endosomes and, through a pH-dependent step, recycles the antibodies back into the serum, which gives IgGs their prolonged serum half-life (Ghetie and Ward, 2000) and also leads to trans-placental transfer of antibodies from mother to neonate. The fundamental structure of the Fc was described in Chapter 3. The following sections delve into the details of isotype, glycoform, and engineered Fc selection.

By our analysis, there are currently 75 MAbs, FAbs, and Fc-fusion proteins on the market or in advanced clinical trials (see Table 9.2). Of these, about 68% utilize a natural, normally glycosylated IgG₁ Fc. The remainder include about 9% using murine Fcs, 9% with engineered Fcs or significantly modified glycan structure, 4% IgG₂s, 4% antibody fragments (either FAbs or scFvs), and 4% IgG₄s. The surprising fact was that the “engineered Fc” group, which includes IgGs possessing modifications either to increase or decrease effector functionality, was larger than either the IgG₂ or IgG₄ isotypes in the advanced molecules, showing the critical importance placed on developing antibodies with optimized Fc functionality for the desired pharmacology.

9.2.2 Human IgG activities and binding to Fc γ Rs

As described in Chapter 7, myeloid cells such as monocytes, macrophages, neutrophils, and dendritic cells express a variety of Fc γ receptors, including at least one activating Fc γ R (Fc γ RI, Fc γ RIIa, Fc γ RIIIa/b), as well as the inhibitor Fc γ RIIb. Neutrophils express Fc γ RIIa and Fc γ RIIIb (but not Fc γ RIIIa), and also express Fc γ RI after activation with G-CSF (Desjarlais et al., 2007).

Table 9.2 IgG isotypes incorporated into marketed and late-stage clinical candidate therapeutic MABs and FcFPs^a

Isotype or form	Marketed	Late clinical phase	Totals	Percentage
Human IgG ₁	25	26	51	68%
Human IgG ₂	2 (Vectibix®, Prolia®)	1 (Tremelimumab)	3	4%
Human IgG ₄	2 (Mylotarg®, Tysabri®)	2 (Reslizumab, LY2439821)	4	5%
Engineered Fc ^b	4 (Soliris®, Orenicia®, Nplate®)	3 (Obinutuzumab, Tanezumab, Otelixizumab)	7	9%
IgG fragment (FAB)	3 (Reopro®, Lucentis®, Cimzia®)	0	3	4%
Murine isotype	4 (Removab®, Bexxar®, Zevalin®, Orthoclone OKT3®)	3 (Edrecolomab, Oregovomab, Pentumomab)	7	9%
Totals	40	35	75	

Notes:

^a These numbers include some antibodies that have either been discontinued or dropped from marketing.

^b Includes glycan engineering.

Binding of IgGs to FcγRs on monocytes, macrophages, and neutrophils can lead to phagocytosis of the target cells or particles (Clark, 1997; Ravetch and Bolland, 2001; Nakamura et al., 2005). Human natural killer cells express FcγRIIIa, as well as FcγRIIc, and binding of cell-bound IgGs to FcγRIIIa on NK cells leads to ADCC of the targeted cells (Ravetch and Bolland, 2001). Finally, IgG binding to FcγRI, FcγRIIa, and FcγRIIIa on these immune cells results in the release of inflammatory cytokines, as well as of reactive nitrogen and oxygen species that can induce apoptosis of target cells (Li et al., 2006b; Desjarlais et al., 2007).

Polymorphisms of IgG receptors such as FcγRIIa, FcγRIIIa, and FcγRIIIb can have important implications in susceptibility to certain diseases (van Sorge et al., 2003; Bournazos et al., 2009; Chapter 7) as well as on the efficacy of therapeutic IgGs binding those receptors (Cartron et al., 2002; van Sorge et al., 2003; Weng and Levy, 2003; Desjarlais et al., 2007; Zhang et al., 2007; Musolino et al., 2008). Bruhns et al. (2009) recently carried out a detailed analysis of the binding of human IgGs to FcγRs, and their data are presented in tabular form in Table 9.3 compared with measurements made by other investigators.

Table 9.3 Binding of IgGs to FcγRs

Human Fcγ receptor	K _D values for IgG isotype binding to FcγR (in nM)			
	Hu IgG ₁	Hu IgG ₂	Hu IgG ₃	Hu IgG ₄
FcγRI	15 ^a , 0.1 ^b , 8 ^c , 0.33 ^d	Nb ^a	15 ^a	30 ^a
FcγRIIa – H131	200 ^a , 850 ^b , 1000 ^d , 690 ^e	2000 ^a	1000 ^a	6000 ^a
FcγRIIa – R131	300 ^a , 910 ^b , 1000 ^d	10000 ^a	1000 ^a	5000 ^a
FcγRIIb and FcγRIIc ^h	8000 ^a , 2300 ^b , 1400 ^d , 1670 ^e	50000 ^a	6000 ^a	5000 ^a
FcγRIIIa – F158	800 ^a , 1000 ^b , 380 ^c , 2000 ^d , 1570 ^e , 710 ^f , 77 ^g	30000 ^a	130 ^a , 560 ^e , 51 ^f	5000 ^a
FcγRIIIa – V158	500 ^a , 280 ^b , 220 ^c	15000 ^a	100 ^a	4000 ^a
FcγRIIIb-NA1	5000 ^a	Nb ^a	900 ^a	Nb ^a
FcγRIIIb-NA2	5000 ^a	Nb ^a	1000 ^a	Nb ^a
FcγRIIIb-SH	5000 ^a	Nb ^a	900 ^a	Nb ^a

Abbreviation: Nb: no measureable binding with method used.

Data are from the following references and recalculated from K_A or K_D values, and rounded where necessary:

^a Bruhns et al. (2009).

^b Richards et al. (2008).

^c Oflazoglu et al. (2009) (cell-based data).

^d Horton et al. (2008).

^e Maenaka et al. (2001).

^f Monomeric IgG (Li et al., 2007b).

^g Multimeric IgG binding with ca. tenfold greater avidity than monomeric form (Li et al., 2007b).

^h Human FcγRIIb and FcγRIIc share identical exodomains (Bruhns et al., 2009), whereas FcγRIIb and FcγRIIc have ITIM (inhibitory) and ITAM (activating) cytoplasmic domains, respectively (Nimmerjahn and Ravetch, 2008) (see Chapter 7).

9.2.3 Human IgG₁

The IgG₁ isotype is not only the most abundant isotype found in human serum, but it is also the most well studied and most well-understood human antibody form. Thus, as biopharmaceutical companies initiated their early discovery and development on antibody therapeutics, it was an obvious choice to make the therapeutic candidates IgG₁ isotypes. Additionally, the IgG₁ activity profile reasonably suited many of the initial targets which were soluble or cell-surface with a goal of depletion (see below). Thus, of the first 14 chimeric, humanized, or human antibodies, or FcFPs, 13 incorporated an IgG₁ Fc. Additionally, 51 out of 75 (68%) of the

current marketed and late-stage clinical trial antibodies incorporate an unaltered IgG₁ isotype, making it by far the most popular isotype for therapeutic antibodies to date (see Table 9.2). Due to the vast amount of data available on the biologics, biophysical behavior, and expression of the human IgG₁ isotype, it is expected that IgG₁s will continue to be the favored isotype to make therapeutic MAb and Fc fusion proteins when suited to the target.

For therapeutic MABs that target proteins on the surface of cancer cells (e.g. trastuzumab, rituximab, cetuximab) or infectious disease agents (e.g. palivizumab), the ability of the MAB to bind FcγRs on immune cells to trigger cell-killing responses and/or to activate the complement cascade are typically viewed as important positive attributes. For this type of purpose, IgG₁s are typically the isotype of choice due to their ability to bind all of the FcγRs and activate complement. The IgG₁ isotype also has been used widely to block soluble proteins from binding their cognate receptors, including ustekinumab (anti-IL12/IL23p40), canakinumab (anti-IL-1β), omalizumab (anti-IgE), and bevacizumab (anti-VEGF). This approach appears to work well, having already resulted in several blockbuster monoclonal antibody products.

Human IgG₁s bind to every type of human FcγR (see Table 9.3) found on immune cells, leading to a variety of events including pro-inflammatory cytokine production, phagocytosis, ADCC, and complement activation, depending on the receptor or protein bound, the cell type with which the receptor is associated, and other immune factors present locally. Human IgG₁ antibodies can trigger phagocytosis through multiple receptors (e.g. FcγRI, FcγRIIa, FcγRIIIa) on monocytes and macrophages (Nimmerjahn and Ravetch, 2008), neutrophil-mediated phagocytosis or cell killing by binding FcγRIIa and FcγRIIIb (Jakus et al., 2008), and ADCC via binding to FcγRIIIa and FcγRIIc on NK cells (Ernst et al., 2002). IgG₁ binds to the high-affinity Fc receptor, FcγRI, with a K_D of 15 nM (see Table 9.3), which is tight enough to activate the receptor through a monomeric interaction (Bruhns et al., 2009). On the other hand, IgG₁ binding to the low-affinity receptors, FcγRIIa (K_D for IgG₁, 200–300 nM) and FcγRIIIa (K_D for IgG₁, 500–800 nM), requires the formation of immune complexes resulting in avidity affects to induce activity (Nimmerjahn and Ravetch, 2008; Bruhns et al., 2009). As mentioned previously, human IgG₁s also bind complement factor C1q with a K_D of about 48 nM (Moore et al., 2010) and is capable of activating the complement cascade via the classic route as well as the alternative route (Brüggemann et al., 1987; Seino et al., 1993).

9.2.4 Human IgG₂

Human IgG₂ antibodies are produced as part of the natural response to microbial pathogens possessing complex repeating epitope antigens such as capsules, complex carbohydrate antigens, or cell wall components. Thus human IgG₂s play an important role in the defense against infection by encapsulated microorganisms (Ferrante et al., 1990). Physiologically, human IgG₂s bind only to a single Fcγ receptor, FcγRIIa, which has two major polymorphic forms, as discussed in more detail in Chapter 7. Moreover, human IgG₂s bind to the H131 form of FcγRIIa with a fivefold greater affinity ($K_D \sim 2 \mu\text{M}$) than to the R131 isoform ($K_D \sim 10 \mu\text{M}$) (see Table 9.3). This fivefold difference in binding is physiologically very significant, as demonstrated by the greater susceptibility of individuals homozygous for the lower affinity R131 FcγRIIa receptor to infections by pathogenic microorganisms (Sanders et al., 1995). Even when binding to the H131 higher affinity form of FcγRIIa, human IgG₂s must form immune complexes in order to develop the avidity required to activate the receptor. Upon complex formation, IgG₂s can trigger complement-independent, macrophage, and PMN-mediated phagocytic activity, particularly against encapsulated bacteria such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Neisseria meningitidis* (Sanders et al., 1995; Yee et al., 2000). Human IgG₂s also possess a greater resistance as compared with the other human IgG isotypes to microbial proteases such as *Staphylococcus aureus* gluV8 and *Streptococcus pyogenes* IdeS (Brezski and Jordan, 2010), mostly due to the unique sequence of their lower hinge. Perhaps this affords an evolutionary advantage to IgG₂s in dealing with these kinds of encapsulated, protease-producing pathogens. Finally, human IgG₂s bind C1q very weakly, but it is believed that they typically activate complement only via the alternative pathway rather than by the classical pathway (Seino et al., 1993). In support of that possibility, Sensel et al. (1997) demonstrated that human IgG₂ bound complement factors C4b and C3b, but only at higher antigen density.

Human IgG₂ antibodies have pharmacokinetics very similar to those of human IgG₁ antibodies, in the range of 14–21 days, and bind FcRn similarly to IgG₁ antibodies. Nevertheless, IgG₂ antibodies apparently do not cross the placenta as readily as other human IgG isotypes (Hashira et al., 2000), including the IgG₃ isotype that has poorer binding to FcRn and a concomitantly shorter half-life (Kim et al., 1999). The reasons for these discrepancies are not known.

It has been historically difficult to demonstrate activity of human IgG₂s in standard cell-based *in vitro* assay systems due to their very poor binding to FcγRIIa and complement proteins. Complex assay environments such as

whole blood, which allow for immune complex formation, are required to evaluate the true effector functions of IgG₂ antibodies (Lai et al., 2005; Jefferis, 2007). When assayed in the presence of monocytes or with serum alone, several human IgG₂ anti-*Pseudomonas* antibodies failed to lyse the microorganisms, whereas when whole blood was used as the assay medium, the antibodies triggered substantial lysis of the pathogens (Lai et al., 2005).

Human IgG₂s can form covalent dimers through inter-protein disulfide formation by the cysteine residues in the hinge region (Yoo et al., 2003). It has been hypothesized that covalent dimerization may confer an avidity-based advantage to human IgG₂s in binding repeat polysaccharide antigens present on encapsulated bacteria (Yoo et al., 2003; Salfeld, 2007). While dimer formation has been ascribed particularly to human IgG₂s, they are not the only isotype that dimerize. Human IgG₁s also have been shown to form both non-covalent and covalent dimers, albeit via mechanisms different from those used by human IgG₂s (Yoo et al., 2003).

There currently are two marketed antibodies that incorporate the natural IgG₂ isotype, the anti-EGFR MAb, panitumumab (Vectibix®), and the anti-RANK-L MAb, denosumab (Prolia®, Xgeva®), both of which were developed and are manufactured by Amgen. Late-stage clinical candidates utilizing the natural IgG₂ isotype include the anti-CTLA4 MAb, tremelimumab, and the anti-IGF1-R MAb, figitumumab, both being developed by Pfizer.

Of the four human IgG isotypes, human IgG₂ antibodies have the lowest overall level of effector function, which makes them an attractive scaffold on which to design modified IgGs possessing little to no effector function (Dillon et al., 2008; An et al., 2009; Strohl, 2011; see also Chapter 10). With that in mind, three other notable MAbs incorporate at least part of the IgG₂ isotype. The first is eculizumab (Soliris®), an anti-C5 MAb that has an engineered IgG₂-IgG₄ hybrid isotype designed to reduce effector function (Rother et al., 2007). The other two are tanezumab, an anti-NGF MAb, and ponezumab, an anti-amyloid-β MAb, both of which are modified IgG₂ antibodies with mutations resulting in reduced effector function activity. These will be discussed in greater detail later in Chapter 10.

IgG₂s possess some characteristics that may make them less than ideal from a manufacturing perspective, where tight comparability between manufacturing lots, high levels of molecular stability, and product homogeneity are highly valued. With the development of the marketed IgG₂ MAbs, panitumumab and denosumab, as well as the late clinical development of other IgG₂ MAbs, the biophysical behavior of the IgG₂ isotype has come under more detailed scrutiny. Besides the ability of human IgG₂s to form intermolecular disulfide bonds resulting in covalent dimer formation, IgG₂ FAbs also have been shown to undergo disulfide bond isomerization,

resulting in heterogeneous isoforms that appear to retain biological functionality (Dillon et al., 2008; Guo et al., 2008; Wypych et al., 2008). Characterization of IgG₂ isoforms can add to the analytical efforts during development, but at present, isomerization does not appear to detract from incorporation of the IgG₂ isotype into therapeutic candidates (Dillon et al., 2008; Guo et al., 2008; Wypych et al., 2008). Additionally, as Salfeld (2007) pointed out, there are currently no pre-clinical or clinical data suggesting that human IgG₂s pose any unique safety issues. Nevertheless, additional analytical assays and follow-up protocols have been proposed for IgG₂ and other “novel” antibody isotypes or forms (Swann et al., 2008).

9.2.5 Human IgG₃

With its uniquely long hinge (Michaelson et al., 1992), comparably short half-life of 5–7.5 days (Morell et al., 1970; Kim et al., 1999), enhanced proteolytic susceptibility (Jefferis, 2007), and multiple natural forms with hinge length and constant domain polymorphisms (Jefferis, 2007), human IgG₃ is the most different of the human natural IgG isotypes. The short half-life is probably due to multiple mechanisms, including the proteolytic susceptibility which may reduce serum stability, and a H435R substitution as compared with the other human isotypes, which appears to influence the binding kinetics of the IgG₃ to FcRn (Kim et al., 1999).

Human IgG₃s bind all of the human FcγRs (Bruhns et al., 2009; see Table 9.3), mediating strong ADCC (Michaelson et al., 1992) and other immune-mediated cell killing mechanisms. Human IgG₃ antibodies also bind strongly to C1q and activate complement via both the classical route and alternative pathways (Seino et al., 1993). The hinge of human IgG₃ contains 62 amino acids and 11 disulfide bonds, and is encoded genomically by four exons. Interestingly, when the IgG₃ hinge was shortened to 15 amino acids, or when an IgG₄ hinge was substituted for the natural IgG₃ hinge, the resultant mutant IgG₃s still retained strong ADCC activity, suggesting that other regions contribute to this activity (Michaelson et al., 1992).

Human IgG₃ also is the only natural human IgG isotype not to be incorporated into therapeutic antibodies in the market or clinical development. There are only a few reports of investigators investigating the IgG₃ isotype for the design of potentially therapeutic molecules. Penichet and Morrison (2001) reported the construction of anti-HER2, IgG₃-based MAb-cytokine fusion proteins. They fused GM-CSF, IL-2, and IL-12 to an IgG₃ targeting HER2 in an effort to increase the potency of the anti-tumor antibody (Penichet and Morrison, 2001; De La Cruz et al., 2006). At this

time, however, there do not appear to be any significant efforts to make a therapeutic based on an intact human IgG₃.

On the other hand, Natsume et al. (2008) constructed several IgG₁/IgG₃ hybrid molecules in an effort to optimize effector function activity. They found that a variant comprised of an IgG₁ C_H1 and hinge and an IgG₃ C_H2 and C_H3 (called "1133") possessed the optimal set of characteristics, including the strongest complement-dependent cytotoxicity. Since hybrid 1133 did not bind Protein A, the Protein A-binding sequences from IgG₁ were grafted back into the hybrid 1133 molecule, resulting in a 113F molecule that was produced in a cell line which yielded antibodies lacking a fucose moiety. This 113F-minus fucose molecule retained its extraordinary C1q-binding activity and complement-mediated lysis activity, as well as strong FcγRIIIa binding (Natsumi et al., 2008), and demonstrated superior CDC and B cell depleting activity as compared with a wild-type IgG₁ (Natsumi et al., 2008). This hyper-potent, high CDC, high ADCC, modified MAb isotype has been dubbed the AccretaMAb™ (Kaneko and Niwa, 2011). Thus, hybrid IgG molecules containing IgG₃ sequences, similar to 113F, might be suitable for some future therapeutic MABs.

9.2.6 Human IgG₄

Human IgG₄, the least abundant IgG in human serum (see Table 9.1) is, along with IgE, part of the Th2 antibody response to antigens. Thus nematodes and pollen, which are known Th2 allergens, stimulate a class-switch response mediated by IL-4 to generate both IgE and IgG₄ (Aalberse et al., 2009; Nirula et al., 2011). Additionally, when humans are chronically exposed to protein antigens, including biopharmaceuticals, they typically undergo an isotype switch from IgG₁ to IgG₄ (Jefferis, 2007).

Marketed and late-stage clinical antibodies employing the human IgG₄ isotype include the anti-CD33 MAb gemtuzumab ozogamicin (Mylotarg®; recently withdrawn), the anti-VLA4 antibody natalizumab (Tysabri®), the Phase III candidate anti-CD22 antibody conjugate, inotuzumab ozogamicin, the Phase IIb/III anti-IL-5 candidate reslizumab, and the Phase II anti-IL-13 antibody tralokinumab.

For several years, it was thought that IgG₄ antibodies lacked any significant level of effector function, mostly due to their low affinity to FcγRIIa and FcγRIIIa, as well as the lack of binding to C1q, which, if true, would make them attractive as anti-cell surface target antibodies retaining a long half-life but lacking the ability to kill the targeted cells. The concept that human IgG₄ is a relatively silent isotype, however, was dispelled by

Reddy et al. (2000), who showed that an anti-CD4 IgG₄ antibody triggered cell depletion activity. Bruhns et al. (2009) recently demonstrated that human IgG₄ binds human FcγRI with high affinity (K_D 30 nM) and binds all isoforms of the low-affinity receptors, FcγRIIa, FcγRIIb, FcγRIIc, and FcγRIIIa, with affinities in the range of 4–6 μM (see Table 9.3). Moreover, antigen-complexed human IgG₄ has been shown to mediate ADCP and ADCC through those binding activities (Reddy et al., 2000; Ernst et al., 2002; Nielsen et al., 2007). The level of ADCC mediated by human IgG₄ antibodies is lower than by IgG₁, but it is still significant. For example, non-fucosylated IgG₄ mediated as strong ADCC as did the same antibody in a non-fucosylated IgG₃ isotype (Niwa et al., 2005). Since most existing commercial therapeutic antibody preparations do contain a low percentage (e.g. 1–5%) of non-fucosylated IgG, IgG₄ antibodies specific for cell-surface targets could lead to unwanted cell-depleting activities. Whether by this or another mechanism, an anti-CD52 IgG₄ containing the V-regions from CAMPATH mediated undesired first injection-related cytokine release syndrome (CRS), depleting lymphocytes in serum, and fostered injection site reactions (Isaacs et al., 1996). Additionally, IgG₄ purified from the serum of patients with Wegener's granulomatosis activated neutrophils by a mechanism requiring the engagement of FcγRIIa and/or FcγRIIIb. (Holland et al., 2004). As described in Chapter 10, several approaches have been used to reduce the Fc activity of human IgG₄s.

Human IgG₄ antibodies have been shown to have unique properties among the human IgG isotypes (Aalberse and Schuurman, 2002; Aalberse et al., 2009; Nirula et al., 2011). On the one hand, human IgG₄s are reportedly unable to cross-link antigens *in vivo*, making them functionally monovalent (van der Zee et al., 1986). This property actually could be beneficial in cases in which undesired cross-linking of receptors might result in agonism. On the other hand, human IgG₄ antibodies have been demonstrated to bind multiple different antigens in serum, functionally behaving like a bispecific molecule (Schuurman et al., 1999; Aalberse and Schuurman, 2002; van der Neut Kolfshoten et al., 2007). Both of these properties are linked to the ability of IgG₄s to dissociate in solution into two half IgG molecules, each containing one FAb arm (Angal et al., 1993; Aalberse and Schuurman, 2002; van der Neut Kolfshoten et al., 2007). This dissociation appears to be due to the flexibility of the human IgG₄ hinge region around the “Cys-Pro-Ser-Cys” sequence that favors intra-chain disulfide bond formation over inter-chain disulfide bonding, leading to an equilibrium of intra-chain and inter-chain disulfides that ultimately leads to dissociation (Schuurman et al., 2001; van der Neut Kolfshoten et al., 2007).

While reducing conditions activate this process, evidence also has shown that the human IgG₄ C_H3 domain is involved as well (van der Neut Kolfshoten et al., 2007; Labrijn et al., 2011). Residue Arg409 in the human IgG₄ C_H3 domain, as differentiated from Lys409 in human IgG₁, IgG₂, and IgG₃ isotypes, was recently found to be a key residue involved in FAb-arm exchange (Labrijn et al., 2011). Once dissociated, the half-IgG₄ molecules can pair with other half-IgG₄ molecules possessing the same or different specificity. This exchange has been demonstrated in the laboratory by mixing two IgG₄s with different specificities and showing that they “recombine” over time (Aalberse and Schuurman, 2002). More significantly, this principle was also demonstrated to occur in human serum in which resident IgG₄ molecules remix to form antibodies with heterogeneous binding specificities (Labrijn et al., 2009), resulting in natural bispecific antibodies *in vivo* (van der Neut Kolfshoten et al., 2007; Labrijn et al., 2009). Data indicate that the equilibrium between self-reassociation and heteromolecular association is driven by a purely stochastic process dictated by the concentration of each of the IgG₄s in solution (van der Neut Kolfshoten et al., 2007). Thus a therapeutic IgG₄ MAb at 20 µg/mL in a serum containing 500 µg/mL of endogenous IgG₄ molecules should have a high propensity to form heterodimeric IgG₄ molecules with a patient’s IgG₄ antibodies. Additionally, Rispens et al. (2011) have demonstrated that the local redox conditions play a significant role in the IgG₄ FAb arm exchange, and that the intrinsic barrier to half-antibody formation is the slow dissociation between the two C_H3 domains. Scientists at Celltech demonstrated nearly twenty years ago that an S228P (EU numbering based on IgG₁ sequence) mutation of the natural IgG₄ hinge reduced the level of half antibody species and improved the PK and tissue distribution properties (Angal et al., 1993). In a recent study, the S228P mutation was demonstrated to reduce half-antibody generation to background levels (van der Neut Kolfshoten et al., 2007). Thus most human IgG₄ molecules made today incorporate the S228P mutant to help limit the dissociation process. Nevertheless, in response to the potential safety risks associated with this phenomenon, scientists at Biogen-IDEC have developed detailed assays to measure the content of intact natalizumab (Tysabri®), as well as exchanged half-natalizumab (Shapiro et al., 2011).

Finally, IgG₄ antibodies have been shown to be pathogenic at high levels in human serum (Khosroshahi et al., 2010; Nirula et al., 2011; Zen and Nakanuma, 2011). For example, elevated IgG₄ levels have been associated with the onset of Wegener’s granulomatosis. Additionally, a new condition known as “IgG₄-related sclerosing disease” has been proposed to cover IgG₄-related clinical diseases of the pancreas, bile duct, gall bladder, and

other organs (Kamisawa and Okamoto, 2008). Many, but not all, of these sclerosing diseases appear to be associated with autoimmune pancreatitis (Kamisawa and Okamoto, 2008). IgG₄-related systemic diseases have recently been treated successfully by depleting B cell lymphocytes with 1 g doses of Rituxan® (Khosroshahi et al., 2010).

Despite the issues described above, treatment of multiple sclerosis patients with natalizumab (Tysabri®) appears to be manageable. However, considering the growing weight of issues, it is probable that the use of the natural IgG₄ as a scaffold for therapeutic MAb will diminish over time (Labrijn et al., 2008; Strohl, 2011). Modification of the natural scaffold including the hinge mutants noted above and the Fc-muted “IgG₄-ala-ala” (Reddy et al., 2000; also see Chapter 10 on Fc-silenced antibodies) may overcome these issues and lead to a new generation of IgG₄-based therapeutics.

9.2.7 Allotypes of human IgGs

The constant regions of human IgG antibodies can have different sequences based on genetic variants that are inherited in a co-dominant allelic manner. This concept of allelic variants of IgGs, i.e. allotype, was first described by Grubb (1956), and evolved into a classification of Ig allotypes using serological approaches (Lefranc and Lefranc, 2011). Subsequent sequence analysis has shown additional genetic differences, as well, although those are defined as alleles rather than allotypes. Human IgG₁, IgG₂, IgG₃, and IgG₄ have 4, 1, 13 and zero serologically defined allotypes, respectively (see Table 9.1). Additionally, the κ -light chain has three serologically distinct allotypes, whereas the λ -light chain does not have any known allotypes (Jefferis and Lefranc, 2009). The amino acid sequence correlates with the serologically defined allotypes are listed in Table 9.4 (Lefranc and Lefranc, 2011).

Natural IgG₁ allotypes, as measured serologically, that are expressed are G1m3, G1m17,1, and G1m17,1,2 (Table 9.5), which could be paired with a Km1, Km1,2, or Km3. Thus an IgG₁ with the G1m3; Km1 allotype would be different from a G1m17,1,2; Km3 allotype by six amino acid residues in the constant region (see Table 9.5) (Jefferis and Lefranc, 2009; Lefranc and Lefranc, 2011). It has been proposed that allotypic differences might contribute to anti-antibody responses, i.e. therapeutic antibody immunogenicity (Jefferis and Lefranc, 2009). For example, an individual possessing a homozygous G1m3; Km1 allotype might be expected to generate anti-allotypic responses against a therapeutic MAb such as rituximab or adalimumab, which differ by five residues from their endogenous IgG₁s (see Table 9.5). Nevertheless, recent studies on the

Table 9.4 Human IgG₁ and IgG₂ allotypes and their correlated amino acid sequence changes

IgG allotype	Alleles found in:	Correlated amino acid substitution	Antibodies used to determine allele
G1m1 (G1m(a))	<i>IGHG1*01, IGHG1*02</i>	Asp356, Leu358	HP6184, MG102-A2
nG1m1 (nG1m(a)) ^a	<i>IGHG1*03</i>	Glu356, Met358	
G1m2 (G1m(x))	<i>IGHG1*04</i>	Gly431	
G1m3 (G1m(f))	<i>IGHG1*03</i>	Arg214; Glu356, Met358	HP6027, %A1
G1m17 (G1m(z))	<i>IGHG1*01, IGHG1*02</i>	Lys214	HP6189, 5F10
G2m (G2m(n ⁻)) ^a	<i>IGHG2*01</i>	Pro189, Val282	–
G2m23 (G2m(n))	<i>IGHG2*02</i>	Thr189, Met282	SH21, 12D9
Km1		Val153, Leu191	
Km1,2		Ala153, Leu191	
Km3		Ala153, Val191	

^a Since no antibody reagent exists for this form, it is not a serological allotype.

Table 9.5 Examples of allotypes of some marketed MAb^s*

Molecule	Allotype	IgG ₁ heavy chain				Kappa chain	
		Position 214 (C _H 1)	Position 356 (C _H 3)	Position 358 (C _H 3)	Position 431 (C _H 3)	Position 153	Position 191
Expressed IgG allotype	G1m3; Km1	Arg	Glu	Met	Ala	Val	Leu
Expressed IgG allotype	G1m17,1; Km1,2	Lys	Asp	Leu	Ala	Ala	Leu
Expressed IgG allotype	G1m17,1,2; Km3	Lys	Asp	Leu	Gly	Ala	Val
Rituximab, Daclizumab, Alemtuzumab, Adalimumab	G1m17,1; Km3	Lys	Asp	Leu	Ala	Ala	Val
Trastuzumab, Omalizumab	G1m17; Km3	Lys	Glu	Met	Ala	Ala	Val
Basiliximab, Palivizumab, Cetuximab	G1m3,1; Km3,1	Arg	Asp	Leu	Ala	Ala	Val
Etanercept	–	–	Glu	Met	Ala	–	–

* All of the residues mentioned are based on EU numbering.

potential allotypic immunogenicity of infliximab (Remicade®) (Magdelaine-Beuzelin et al., 2009) and adalimumab (Humira®) (Bartelds et al., 2011) showed no association of anti-therapeutic MAb response with the allotype of the IgGs of the patients studied.

9.3 IgM

IgM is an ancient immunoglobulin found in all jawed vertebrates, from sharks to mammals. IgMs are large polyvalent antibodies derived from the μ gene locus, and are the first antibodies to be generated in response to an antigen *in situ*. As described in Chapter 5, B cell-bound IgMs play a significant role in B cell maturation in response to antigen stimulation. IgMs also make up the largest component of what is termed “natural antibodies” (also called non-immune antibodies), low-affinity, germline-encoded polyreactive Igs that are produced by long-lived B1 cells in the absence of antigen stimulation (Boes, 2000; Vollmers and Brändlein, 2006; Baxendale et al., 2007; Holodick et al., 2010). In mice, these B1 cells are characteristically CD5⁺ (Boes, 2000; Vollmers and Brändlein, 2006; Baxendale et al., 2007; Holodick et al., 2010), whereas in humans, it has recently been demonstrated that B1 cells, which are fully competent for “natural” IgM secretion, efficient T cell stimulation, and tonic intracellular signaling (i.e. ability to phosphorylate phospholipase C- γ 2 and spleen tyrosine kinase), are comprised of a subpopulation of CD20⁺ CD27⁺ CD43⁺ CD70⁻ cells (Griffen et al., 2011). Importantly, the “traditional” CD5⁺ marker, based on mouse B1 cell studies, did not accurately predict this population of natural IgM-secreting human B1 cells (Griffen et al., 2011).

These natural antibodies are largely localized in the pleural and peritoneal compartments where they provide a first-line defense against pathogens (Boes, 2000; Holodick et al., 2010). About 80% of the natural IgMs are derived from the V_H3 germline family, primarily from the DP47 and DP49 genes (Vollmers and Brändlein, 2006). There is significant interest in the use of natural IgM populations as sources of antibodies targeting infectious viruses such as HIV (Mouquet et al., 2010) and bacteria (Baxendale et al., 2007).

IgM monomeric units of about 190 kDa are comprised of heavy (C μ 1, C μ 2, C μ 3, C μ 4, V_H) and light chains (C κ -V κ or C λ -V λ). These monomeric units are typically polymerized into either a pentameric or hexameric format, although other multimers are known to exist as well. The ability to polymerize is conferred by the presence of an 18-amino acid sequence (PTLYNVSLVMSDTAGTCY) known as the tailpiece, which extends from the C-terminus of the heavy chain (Sørensen et al., 1999).

In its pentameric form, IgM also typically contains an additional 20 kDa subunit known as the joining chain, or J-chain. Approximately 95% of serum IgM is J-chain containing pentamer, with hexameric IgM lacking J-chains making up most of the remaining 5% of circulating IgM (Johansen et al., 2000). J-chain deficient pentameric IgM also has been described, and due to variations in disulfide bonds, several different oligomeric states of IgMs are produced, making “IgM” a collection of heterogenous molecules dominated by the pentamer (Collins et al., 2002). When produced in cell culture, the IgM fraction typically is represented by a ladder of species with different oligomeric states. Monomeric IgM, which also can be secreted by B cells, is usually associated with inflammatory conditions (Xu et al., 1994).

Both J-chain containing pentameric and J-chain lacking hexameric IgMs have a half-life in the range of about 5 d. Their clearance rates appear to be similar, so it has been hypothesized that dominance of the pentameric form in serum results from a higher secretion rate (Hughey et al., 1998).

Table 9.6 shows the major characteristics of pentameric and hexameric IgMs. The presence of the J-chain in the pentameric form confers on it the ability to bind the polymeric Ig receptor (pIgR), which conducts the transcytosis of pentameric IgMs from the vasculature to mucosal surfaces (Johansen et al., 2000). Pentameric IgM appears to have a non-symmetrical barrel-shape, or “mushroom shape” (Czajkowsky and Shao, 2009), possessing an antigen-binding face and a non-antigen binding face, which suggests that the ten V-regions may only result in a valency of five for the pentameric form (see Table 9.6) (Arnold et al., 2005). In this model, the J-chain would occupy the back side of the barrel and would physically impede antigen binding to the V-regions on that face (Arnold et al., 2005). The hexameric form, on the other hand, appears to be a planar molecule in which all of the V-regions are available for binding antigen, suggesting a functional valency of 12 (Arnold et al., 2005).

IgM is known to activate the complement pathway and carry out complement-mediated cell lysis. What often isn't appreciated, however, is that most of this activity is associated with the minority hexameric form. The hexameric form of IgM has three to twelvefold greater complement-activating activity via the classical pathway than the J-chain containing pentameric form (Collins et al., 2002; Arnold et al., 2005; Strohl, 2011). Pentameric IgM can also activate complement via the alternative pathway. Hexameric IgMs have been demonstrated to be involved in IgM-mediated autoimmune syndrome, possibly due to their strong activation of complement (Hughey et al., 1998).

Human IgM antibodies interact at high affinity with Fc α μ R (ca. 1 nM K_D; Ghumra et al., 2009), Fc μ R (ca. 10 nM K_D; Kubagawa et al., 2009),

Table 9.6 Characteristics of human IgM, IgA, and IgE antibody classes

Characteristic or function (human data only)	Class or isotype					
	Pentameric IgM	Hexameric IgM	Serum IgA	Dimeric sIgA ₁ ^a	Dimeric sIgA ₂ ^a	IgE
Approximate molecular weight	970 kDa	1140 kDa	160 kDa	445 kDa	445 kDa	188 kDa
Protein chains ^b	(μ_2L_2) ₅ J	(μ_2L_2) ₆	α_2L_2	(α_2L_2) ₂ , J, SC	(α_2L_2) ₂ , J, SC	ϵ_2L_2
Functional valency	5 ^c	12	2	4	4	2
No. of Fc N-linked glycosylation sites (per monomer) ^d	5	5	2	3	3–5	7
Primary location(s)	Serum; B cell surface	Serum; B cell surface	Serum ^e	External secretions ^f	External secretions ^f	Mast cell, basophil surfaces
Hinge region	–	–	+	+	+	–
ADCC-NK cells	–	–	?	–	–	–
ADCC-eosinophils	–	–	–	–	–	+
ADCC/ADCP PMNs	–	–	+	–	–	–
ADCP-macrophages	–	–	?	+	+	–
FcRn binding	–	–	–	–	–	–
Nominal serum half-life (in days)	6d	6d	5d	5d	5d	2 d
Approximate mean serum concentration	1.4 mg/mL (95% of IgM)	0.1 mg/mL (5% of IgM)	3.5 mg/mL ^e	–	–	300 ng/mL
Placental transfer	–	–	–	–	–	–
Produced by fetus during gestation in response to infection	+	+	–	–	–	–
Hinge length (number of amino acids)	C _H 2 domain replaces hinge	C _H 2 domain replaces hinge	Na	19	6	Domain replaces hinge
J-chain	+ (usually) ^g	–	–	+	+	–
Tailpiece	+	+	+	+	+	+/–
Secretory component (SC) ^a	–	–	–	+	+	–
pIgR-binding	+	–	–	+	+	–
Secreted into body fluids ^f	–	–	–	+	+	–
C1q binding	+/–	+++	–	–	–	–
Complement-dependent cell lysis	+/–	+++	–	+ ^h	+ ^h	–

Notes:

^a Secretory component (SC) of secretory IgA (sIgA) is derived from pIg receptor (pIgR) during the process of secretion.

^b Protein chains: L, light chain; J, joining chain; SC, secretory component.

^c While having ten V-regions available for binding, the barrel-like structure appears to be limited to five binding sites (Arnold et al., 2005).

^d Not including J-chain or secretory component.

^e Figure given is for all serum IgA, which is primarily monomer, but also contains minor dimeric and multimeric forms.

^f For example, saliva, urine, mucous, sweat, gastric fluids, tears.

^g J-chain-deficient pentameric IgM has also been described (Collins et al., 2002).

^h Through MBL mediated mechanism.

and pIgR (ca. 37 nM K_D ; Norderhaug et al., 1999). Binding of IgM to pIgR (Braathen et al., 2002) and Fc α/μ R (Ghumra et al., 2009) is J-chain dependent and thus does not occur with hexameric IgM. As mentioned in Chapter 7, pIgR is found on mucosal epithelial cells and is the mechanism by which IgAs and pentameric IgMs are translocated from circulation to mucosal surfaces (Kaetzel, 2005). Fc α/μ R and Fc μ R are found on B cells, suggesting roles in B cell regulation (Ghumra et al., 2009; Shima et al., 2010). Thus pentavalent and hexavalent IgM have substantially different activity profiles. These differences could have a significant impact on production, purification, and formulation of therapeutic pentameric IgMs, in which the hexameric forms might be present as contaminating species.

Several of the very early MAb therapeutic candidates were of the IgM isotype but no IgM has yet been commercialized. In part this was because in the early years following the development of hybridoma technology by Köhler and Milstein (1975), many of the antibodies produced by this technology were IgMs. Four such IgM candidates targeted microbial infections (Reichert and Dewitz, 2004), including edobacomab (XOMEN-55), a murine anti-lipid A IgM derived from mouse spleen cells, and nebucumab (Centoxin), a human anti-lipid A IgM derived from human spleen cells. Both of these antibodies reached advanced clinical trials in the early 1990s, but were discontinued due to lack of efficacy (Reichert and Dewitz, 2004). Several early anti-tumor antibodies were also pentameric IgM MAbs. As natural IgMs derived from cancer patients, they had low affinity and specificity (Vollmers and Brändlein, 2002). Additional limitations for use of IgMs as anti-tumor MAbs include poor tissue penetration and the inability of IgMs to cross the endothelial barrier, making it difficult for them to access some tumors (Vollmers and Brändlein, 2002).

Even with these caveats, several IgM antibodies have been, or are being, developed as anti-tumor MAbs, many of them targeting glycotopes unique to tumor cells. Patrys Ltd (Melbourne, Australia; <http://www.patrys.com/>) is the most active company pursuing the discovery and development of IgM-based therapeutics, with four anti-tumor IgM MAbs in early development. The most advanced is PAT-SC1, which is in Phase I/IIa trials to treat gastric cancer. SC-1 targets a specific glycoform on CD55 (denoted CD55^{PAT-SC1}) which is overexpressed on the surface of 74% of gastric cancer tissues (Patrys, <http://www.patrys.com/>, 2011). This particular epitope of CD55 has not been found to be expressed on normal tissues (Vollmers and Brändlein, 2006). Data from *in vitro* and *in vivo* models indicate that SC-1 functions by inducing apoptosis of the targeted cancer cells (Vollmers and Brändlein, 2006). Complement activation, which as noted above is not a property of pentameric IgM, and ADCC were ruled

out as mechanisms of action for SC-1 (Beutner et al., 2008). Thus a pentameric IgM which does not induce either CDC or ADCC appears to be a reasonable approach for this target. SM6, the second Patrys IgM MAb, targets GRP78, a multi-functional glucose-regulated protein apparently only present on tumor cells, and is in Phase I/IIa clinical trials for the treatment of melanoma. Patrys also has an IgM in late pre-clinical studies. LM-1, which targets the protein non-POU domain-containing octomer-binding protein (NONO), is reported to be expressed on nearly 100% of the cancer cells screened (Patrys, <http://www.patrys.com/>).

Several other IgM MAbs have been reported in either pre-clinical or early clinical development in recent years for a variety of indications. Morphotek (subsidiary of Esai) is developing MorAb-028, a fully human IgM that targets G-28 glycolipid on melanoma cells. It is in Phase I clinical trials for the treatment of metastatic melanoma (Clinical Trials # NCT01123304). Argenes has been developing ARG098, an anti-Fas (aka APO-1, CD95) agonist chimeric IgM for the treatment of rheumatoid arthritis. ARG098 has been demonstrated to function in the joint by the induction of apoptosis in Fas-expressing synoviocytes and infiltrating lymphocytes, reversing RA-associated synovial hyperplasia (Odani-Kawabata et al., 2010). ARG098 is formulated for injection directly into the synovium and is in Phase I/II clinical trials in Europe. In the neurosciences area, Acorda Therapeutics is developing a remyelination IgM that was discovered at the Mayo Clinic. The recombinant human IgM, rHIgM22, targets myelin on the surface of oligodendrites and appears to promote remyelination as determined by MRI analysis (Pirko et al., 2004). Doses as low as 500 ng appeared to promote spinal chord remyelination in murine models. The half-life of rHIgM22 in mice was 15 h, and in humans very little residual rHIgM22 was detected after 48 h suggesting a short half-life clinically as well (Warrington et al., 2007). MAb rHIgM22 is in pre-clinical studies at Acorda Therapeutics, who have contracted with Biotest for GMP manufacturing of the antibody.

In another example, MAb L612 was an anti-GM3 IgM derived at Chugai from Epstein-Barr virus (EBV)-transformed human B cells taken from a patient with melanoma (Hoon et al., 1993). GM3 is a glycotope found on the surface of melanoma cells, and L612 was found to kill GM3-expressing melanoma cells in a complement-dependent manner (Hoon et al., 1993). When L612 was advanced into Phase I clinical trials, it showed no significant adverse side effects (Irie et al., 2004) but lacked the desired potency. The L612 hybridoma cell line produced ca. 20% hexameric IgM, 74% pentameric IgM, and 6% of other minor IgM fractions, but most of the activity was complement-mediated cytotoxicity (CMC)

conferred by the hexameric fraction (Azuma et al., 2007). Thus a recombinant hexameric form of L612, CA19, was generated by production in CHO cells in the absence of J-chain (Azuma et al., 2007). CA19 produced about 80% hexameric species and yielded significantly stronger CDC on the melanoma cells than did the original L612 IgM (Azuma et al., 2007), demonstrating the possible manipulation of IgMs to optimize effector function to meet the needs of the therapeutic. Since CA19 does not appear in Chugai's 2011 published clinical pipeline, development may have been halted on this molecule.

Finally, HMMC-1, an IgM binding an O-glycan found specifically on the surface of uterine cancer cells, was shown to bind ca. 55% of uterine endometrial adenocarcinoma, 77% of uterine cervical adenocarcinoma, and 75% of epithelial ovarian cancer tissues but not normal ovarian tissues (Nozawa et al., 2004). At one point this antibody was being considered for treatment of ovarian cancer but there is no report of its development.

In summary, the IgM platform is not validated with a successful commercial product. Several questions need to be answered both clinically and from a CMC perspective before these molecules will be commercially viable. The first consideration is the consistency of the manufactured therapeutic antibody. Control of the ratio of pentameric to hexameric IgM forms produced in cell culture and the comparability of the product batch to batch are two issues to be addressed. Significant progress has been made in recent years. Several pentameric, J-chain-containing IgMs have been produced in Crucell's PER.C6 human cell line in a protein-free medium, with yields up to 2 g/L and cell productivities of ca. 20 pg/cell/day (Tchoudakova et al., 2009). Patrys (<http://www.patrys.com/>) also now claims to have production titers of over 4 g/L, suggesting that upstream production of IgMs has been adequately industrialized. Additionally, new hydroxyapatite and cation exchange chromatography methods for purification of IgMs based on their charge have been developed, as have conditions for their solubilization and stabilization (Gagnon et al., 2008).

9.4 IgA

Human IgA antibodies are the most abundant antibody isotype in mammals, most of which is produced locally in tissues and secreted across mucosal membranes to immerse the mucosal surfaces, including respiratory, digestive, and genitourinary tracts, to provide a non-inflammatory protection against microorganisms (Woof and Kerr, 2006) and maintain gut homeostasis (Cerutti and Rescigno, 2008). Human IgA monomers, which are ~160 kDa

in size, are comprised of heavy ($C_{\alpha 1}$, $C_{\alpha 2}$, $C_{\alpha 3}$, hinge region, V_H) and light chains ($C_{\kappa}-V_{\kappa}$ or $C_{\lambda}-V_{\lambda}$) in a structure similar to an IgG. Two isotypes of IgA, IgA₁, and IgA₂, are encoded by the $\alpha 1$ and $\alpha 2$ gene loci, respectively (Woof and Kerr, 2006). IgA antibodies are more heavily glycosylated than IgGs, with 2–5 N-linked glycosylation sites in the C_{α} chains, and 3–6 O-linked glycosylation sites in the hinge region (Woof and Kerr, 2006).

There are multiple differences between IgA₁ and IgA₂ isotypes, the most significant of which is the hinge. The hinge regions of IgA₁ antibodies are ~16–18 amino acids in length as compared with the 5 residue length in IgA₂ (Senior and Woof, 2005; Bonner et al., 2009). The longer hinge of the IgA₁ may allow for greater avidity, but at the cost of being considerably more sensitive to proteolytic degradation (Senior and Woof, 2005). Furthermore, the secretory form of IgA₁ (sIgA₁) has a nearly planar three-dimensional structure, whereas secretory IgA₂ (sIgA₂) is “bent.” It has been suggested that these structural differences may yield differential binding to IgA receptors which could result in different levels of IgA-dependent effector cell functionality between the isotypes (Bonner et al., 2009). Finally, the IgA₂ isotype has two allotypes, IgA₂m(1) and IgA₂m(2), which differ by both amino acid substitutions as well as the number of N-linked glycosylation sites (Woof and Kerr, 2006).

Serum IgA, which has a steady-state concentration of about ~3–5 mg/mL, is ~90% monomeric IgA₁ and is derived from bone marrow. Unlike IgG, serum IgA does not bind FcRn, and consequently the half-life for serum IgA is only about five days. Serum IgA that has aberrant galactosylation has been hypothesized to trigger IgA nephropathy (IgAN) (Lin et al., 2009).

Similar to IgM, the C-terminus of the IgA heavy chain contains a unique 18-amino acid tailpiece sequence (PTH-I/V-NVSVVMAEADGTCY) that directs dimerization through the penultimate cysteine residue (Sørensen et al., 1999). IgA dimers also possess a 15 kDa J-chain that is required for binding pIgR and Fc α μ R, similar to IgMs (Braathen et al., 2002; Ghumra et al., 2009). When IgA is translocated from serum to the mucosal surface by pIgR, it associates with an 80 kDa fragment from pIgR called the “secretory component” (SC) (Woof and Kerr, 2006). Thus the secreted form of IgA (sIgA) is dimeric and possesses both a J-chain and the SC chain. While the IgA-bound SC protects sIgA from proteolysis, it also interferes with the ability of sIgA to bind Fc α RI, leading some to label sIgA a “non-inflammatory immunoglobulin” (Monteiro and van de Winkel, 2003).

Mucosal microorganisms such as the group A and B streptococci, neisseriae, and *Haemophilus* spp. utilize a variety of mechanisms to neutralize sIgAs, including the production of sIgA binding proteins and

sIgA proteases, indicating the importance of sIgA in the homeostasis of gut microflora (Senior and Woof, 2005; Woof and Kerr, 2006; Kazeeva and Shevelev, 2009). SIgA proteases produced by the mucosal pathogens recognize a short sequence in the hinge shared by both sIgA₁ and sIgA₂ isotypes, although the longer hinge of sIgA₁ makes it more susceptible to microbial proteases than sIgA₂ (Senior and Woof, 2005).

As described in Chapter 7, human IgA antibodies bind Fc α RI (CD89), found primarily on neutrophils and monocytes, Fc α / μ R, found on pre-germinal B cells and follicular dendritic cells in humans, and pIgR, found primarily on epithelial cells, liver, lung, and small intestine (Takai, 2002; Monteiro and van de Winkel, 2003; Nakamura et al., 2005; Wines and Hogarth, 2006; Woof and Kerr, 2006). Additionally, human IgAs can bind the asialoglycoprotein receptor in the liver and the transferrin receptor in the kidney, which appear to mediate clearance of IgA₂ and IgA₁, respectively (Monteiro and van de Winkel, 2003).

Several attempts have been made to develop therapeutic sIgAs over the past decade or so. It appears that the first sIgA to enter clinical trials was CaroRxTM, a chimeric IgG-sIgA antibody that targeted plaque-forming *Streptococcus mutans* to prevent recolonization (Ma et al., 1998; Wycoff, 2005). CaroRxTM, which was constructed using FAbs from a mouse IgG₁ known as “Guy’s 13”, a mouse J-chain, and a rabbit SC, was produced in tobacco plants with a yield of 200–500 mg/kg of plant material (Larrick et al., 2001). CaroRxTM, which was delivered directly into the oral cavity, reached Phase II clinical trials in 2004 and was reportedly successful in preventing the recolonization of *S. mutans* over a four-month period without the generation of anti-antibody responses or adverse events (Ma et al., 1998; Wycoff, 2005). Nevertheless, it appears that the development of this antibody was eventually dropped, probably due to the economics of its intended use.

Other efforts have been made to produce sIgA in plants, but product quantity and quality, as well as potential regulatory hurdles for production, have limited the interest in using plants to produce sIgAs, especially if they are to be administered systemically (Stoger et al., 2002). Cell culture methods to produce sIgA have been developed by cloning IgA sequences, J-chain, and SC into facile cell lines (Morrison and Chintalacharuvu, 2001; Beyer et al., 2009), and downstream processing of sIgA has been facilitated by using either IgA-binding protein (IgAbp) or the IgA-binding lectin, Jacalin, as an affinity ligand for purification (Pack, 2000).

The questions for developing an IgA as a therapeutic include the format to be used (monomeric versus dimeric, and with or without J-chain or SC), the intended route of delivery (systemic or direct delivery to a mucosal

surface such as the oral cavity, gut, or intestine), and the intended Fc activity. Also, delivery of an sIgA directly to the mucosa may present a significant technical challenge. Finally, while the delivery of a J-chain-containing IgA dimer systemically might result in binding pIgR and translocation to the mucosal surface, the size of that total compartment ($\sim 400 \text{ M}^2$ of mucosal surfaces; Woof and Kerr, 2006) might require very high systemic doses to reach therapeutic levels at the desired mucosal surface.

Several reports demonstrate the potential for targeting cell depletion through the activity of Fc α RI, particularly through the activity of neutrophils, which comprise the largest fraction of white blood cells in circulation ($\sim 2.5\text{--}7.5 \times 10^6/\text{mL}$). In one example, Dechant et al. (2007) used the V-regions of MAbs 225, the precursor of cetuximab (targeting epidermal growth factor), to make monomeric IgA₁, IgA₂, and IgG₁ species for a direct comparison of their Fc activities. Both IgA₁ and IgA₂ constructs mediated ADCC of EGFR-bearing A431 cells using polymorphonuclear (PMN) cells but not mononuclear cells as effector cells (Dechant et al., 2007). IgA₂ was more effective in killing EGFR-bearing A431 cells in whole blood than either IgA₁ or IgG₁ (Dechant et al., 2007). Whether or not this enhanced IgA-directed activity would translate *in vivo* in humans is not known, but is intriguing.

On a related note, there are several examples of bispecific IgG-based antibodies in which one V-region bound HER2, CD20, or a fungal antigen while the second V-region bound Fc α RI (CD89), resulting in very active depletion of the targeted cells (Stockmeyer et al., 2000). This use of the neutrophil-centric, IgA receptor-driven cytotoxic approach toward targeted cell depletion may be suitable for some oncology or infectious disease targets, but must be balanced with safety concerns about the potential for lung, kidney, or other organ damage by highly activated neutrophils.

Antibody Fc engineering for optimal antibody performance

Abstract: The interactions of human IgGs with FcγRs and complement components dictate the extent to which the antibody utilizes the immune system as part of its mechanism of action (MOA). FcγRIIa and FcγRIIIa possess polymorphisms that alter the binding of natural IgGs to those receptors. These polymorphisms, which are found in portions of the population, have been demonstrated to be correlated with diseases and disease outcomes after antibody treatment, especially in oncology indications. On the other hand, there are circumstances in which IgGs are targeted toward receptors on immune or metabolic cells for which depletion is not desired as part of the MOA. Thus efforts have been made to engineer the Fc of various IgG isotypes to increase or decrease FcγR mediated activities and/or complement activation to tune the Fc activities to fit the desired outcomes. Finally, modifications have been made to IgG Fcs to modulate the interaction with FcRn, thereby changing the half-life characteristics of those modified antibodies. These Fc modifications result in engineered antibodies with more desirable characteristics for the indications for which they are intended, hence they can be called “fit-for-purpose” antibodies.

Keywords: IgG, isotypes, Fc, Fcγ receptors, FcγR polymorphisms, modified Fc, Fc engineering, effector function, fit-for-purpose antibodies, antibody optimization, silenced Fc, tissue targeting

10.1 Antibody engineering for decreased or increased effector function

The idea of designing antibodies that go beyond what nature provides (e.g. IgG₁, IgG₂, IgG₃, or IgG₄ natural isotypes) has been around for more than a quarter of a century (cf. Neuberger et al., 1984). Thus the concept of designing and developing an entire antibody, including the Fc, to best fit the biology required of it (i.e. “fit-for-purpose” MAbs) is nothing new.

Nevertheless, as shown in Chapters 2 and 9, most of the MAbs and FcFPs on the market or in late-stage clinical trials are built using a standard IgG₁ constant region. In the past few years, however, there has been a growing effort to design and make antibodies with modified Fc regions to engineer in or out desired Fc-mediated functionalities. Interestingly, the number of Fc-modified antibodies on the market or in late-stage clinical trials is equal to the number of IgG₂ or IgG₄ isotypes combined at the same stages (Table 9.2), indicating that Fc-engineered antibodies are as accepted as alternative isotypes. The ultimate goal of these efforts is to design and develop novel therapeutic antibodies with Fc functions that are most appropriate for the biology required, i.e. development of “fit-for-purpose” antibodies (Stockwin and Holmes, 2003; Laffly and Sodoyer, 2005; Chowdhury and Wu, 2005; Carter, 2006; Presta, 2006, 2008; Jefferis, 2007, 2009a, 2009b, 2009c; Dimitrov and Marks, 2008; Kubota et al., 2009; Strohl, 2009a, 2009b; Chan and Carter, 2010; Strohl, 2011; Desjarlais and Lazar, 2011).

Clinical efficacy and safety, the two most critical features of a therapeutic MAb or Fc fusion product, rely on several design features, including the biology of the target (signaling pathways, tissue distribution, turnover, interacting proteins, and so forth), potency, dosing, and selection of most pertinent patient population. These and additional factors must be taken into consideration when initiating a therapeutic MAb or FcFP project. Nevertheless, several additional design factors may be engineered into antibodies and FcFPs that can help to differentiate molecules against the same target from one another. What are the technologies that might differentiate one clinical candidate from others? While the answer for this question ultimately must come from clinical studies, a few high-level considerations are listed below:

- Affinity – including design of K_{on} or K_{off} where appropriate for a given target.
- Epitope – for many targets, both those on cell surfaces and soluble targets, epitope differences can lead to significant differences in functionality and therefore efficacy.
- Tuning Fc functionality to match the desired biology via modification of the interaction of the Fc with FcγRs and complement factors and the biology they confer.
- Overall potency – this is usually based on a combination of affinity, epitope, Fc functionality, and target biology.
- Dosing frequency and area under the curve (AUC) may be altered by modification of the MAb or FcFP half-life.
- Molecules that yield a larger efficacy to toxicity window or greater safety margin.

- Delivery – route of administration (e.g. subcutaneous, IV, inhaled, etc.).
- Tissue penetration and distribution.
- Size, shape, and flexibility of the molecule can affect biodistribution, tissue penetration, half-life and other key parameters.
- Physiochemical behavior of the molecule – solubility (especially at high concentrations), aggregation characteristics, biochemical stability, efficient folding (Honegger, 2008), spurious glycosylation sites, and so forth.
- Multispecificity, which may allow for targeting multiple proteins with a single molecule, in a format that can be stabilized and manufactured.
- Mixed-modality – the use of antibodies as half-life extension carriers or targeting agents for peptides, siRNA, protein toxins, cytotoxic natural products, or small molecules.

10.2 Current marketed MAbs and clinical candidates with modified Fc

As mentioned earlier in this book, antibodies have essentially two active parts, the antigen binding regions of the FAb (Chapters 3 and 5) and the Fc domain, which interacts with a series of Fc receptors and blood proteins (Chapters 7–9). Since the first antibody, Orthoclone OKT3[®], was developed as a therapeutic to treat patients for rejection of organ transplants, antibodies have been improved in a variety of ways. Some of these improvements include the humanization of murine antibodies to reduce immunogenicity (review by Almagro and Strohl, 2009), high concentration formulation of Enbrel[®], Xolair[®], and Humira[®] to allow for subcutaneous dosing, a preferred route of administration for both patients and caregivers, conjugation with either cytotoxic chemicals (e.g. generic name, Mylotarg[®]) or radionuclides (Bexxar[®]) for targeting tumors, the development of a peptibody (romiplostim, NPlate[®]) by Amgen as a thrombopoietin receptor agonist, and alternative formats such as Cimzia[®], which is a FAb PEGylated in a site-specific manner. While these improvements have generated significantly better, safer, and more efficacious molecules, they were largely limited to modification of the variable chains, fusion partner, or conjugation partner. Generation of a truly fit-for-purpose MAb or FcFP may include modification of the Fc to alter its interaction with either Fc receptors or blood factors such as C1q. Several companies have already spent years designing and developing Fc-modified MAbs or FcFPs to make them more “fit for purpose,” as indicated by the presence of several marketed products and advanced clinical candidates in which the Fc has been genetically engineered (Desjarlais and Lazar, 2011) (see Table 10.1 and Figure 10.1).

Table 10.1 Key Fc-engineered clinical candidates

Name	Company	Current status	Molecular target	Major indication	Protein format
Orencia® (Abatacept)	BMS	Approved 12/23/2005	CD80/CD86 T-cell co-stimulatory	RA	CTLA4-FcFP-modified Fc to reduce effector function
Soliris® (Eculizumab)	Alexion Pharma	Approved 03/16/2007	Complement C5	PNH (reduce hemolysis)	Humanized IgG ₂ / ₄ hybrid; modified Fc
Removab® (Catumaxomab)	Fresenius/Trion	Approved in EU 4/23/2009	EpCAM and CD3	Malignant ascites; cancer	Rat IgG _{2b} -mouse IgG _{2a} hybrid
Nulojix™ (Belatacept; LEA-29Y)	Bristol-Myers Squibb	Approved 6/16/11	CD80/CD86	Renal transplantation	CTLA-4 Fc fusion LEA (higher affinity than abatacept) – modified Fc to reduce effector function
Teplizumab; HOKT3γ1 (Ala-Ala)	Macrogenics/ Eli Lilly	Phase III	CD3	Diabetes	Humanized modified Fc – IgG ₁ -ala-ala to reduce FcγR binding
Tanezumab (RI-624)	Pfizer/Rinat	Phase III	NGF	Pain; osteoarthritis	Humanized IgG ₂ ; mutated to reduce effector function
Ertumaxomab (Rexomun)	Fresenius/Trion	Phase II/III	HER2/neu and CD3	Malignant ascites; cancer	Rat-murine hybrid; modified Fc; trifunctional bispecific
LY2469298 (AME-133v)	Eli Lilly	Phase II	CD20	NHL	IgG with modified Fc for increased ADCC
Ponezumab PF-4260365	Pfizer	Phase II	Amyloid-β	Alzheimer's	IgG ₂ κ – AP330/331SS mutations to reduce Fc functionality
PRO-131921	Genentech	Phase I/II – may have been dropped in 2010	CD20 (third generation)	Oncology	IgG with modified Fc sequence for increased ADCC
TRU-015	Trubion (now Emergent Bio-solutions)	Phase I	CD20	Oncology, RA	Fc sequence modified to reduce C1q binding and complement activation, with retention of normal ADCC functionality
Mor208 Xmab-5574	Morphosys/ Xencor	Phase I	CD19	NHL	IgG – modified Fc to increase ADCC, ADCP
MEDI-557	AstraZeneca	Phase I	F-protein on RSV	RSV	YTE mutant – longer half-life MAb

Abbreviations: NHL: non-Hodgkin's lymphoma; RA: rheumatoid arthritis; RSV: respiratory syncytial virus.

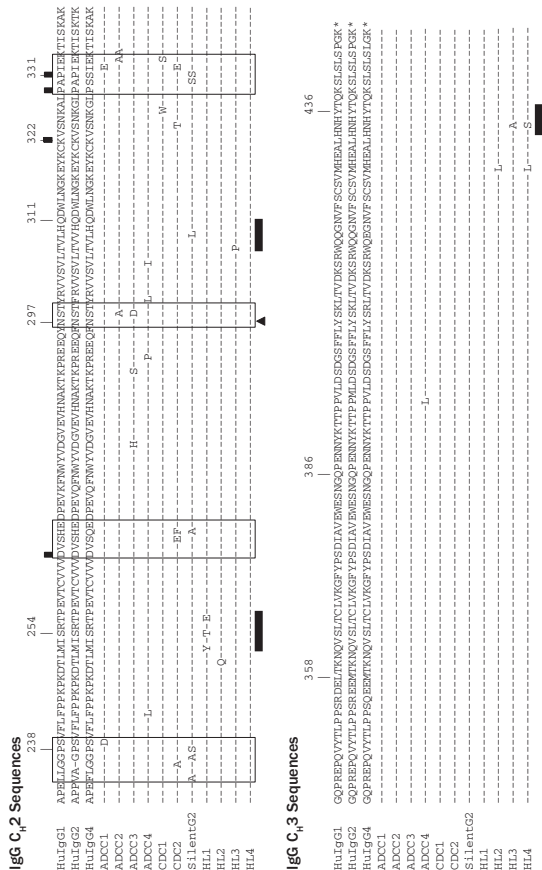


Figure 10.1 Examples of IgG effector function and FcRn sequence modifications

Shown are the aligned sequences of human IgG₁, IgG₂, and IgG₄, with examples of mutations that have been made to the C_H2 and C_H3 domains of IgGs to increase or decrease effector function or extend half-life. Note that the binding sites for FcγRs (boxed), C1q (black rectangles over sequence) and FcRn (thick bar below sequence) are depicted as in Figure 9.2. Dashes indicate that the sequences are identical to the parental IgG (all IgG₁ except for the “SilentG2,” which is based on IgG₂). Numbering is according to the EU numbering scheme.

Key: ADCC1 (increased effector function, typically measured as higher ADCC) – IgG₁ S239D, I332E (Lazar et al., 2006); ADCC2 – IgG₁ S298A, E333A, K334A (Shields et al., 2001); ADCC3 – IgG₁ D280H, K290S, S298D (Watkins and Allan, 2004); ADCC4 – IgG₁ F243L, R292P, Y300L, V305I, P396L (mutant 18 of Stavenhagen et al., 2007); CDC1 (increased CDC) – IgG₁ K326W, E333S (Idusogie et al., 2001); CDC2 – IgG₁ G236A, S267E, H268F, S324T, I332E (Moore et al., 2010); SilentG2 (completely silenced Fc function) – IgG₂ V234A, G237A, P238S, H268A, V309L, A330S, P331S (Vafa et al. submitted); HL1 (half-life extension 1) – IgG₁ M252Y, S254T, T256E (Dall’Aqua et al., 2002); HL2 – IgG₁ T250Q, M428L (Hinton et al., 2006); HL3 – IgG₁ T308P, N434A (Yeung et al., 2010); HL4 – IgG₁ M428L, N434S (Zalevsky et al., 2009).

Note that MAbs and FcFPs in which the glycosylation has been modified to increase or decrease effector function also fit into the fit-for-purpose category but are handled separately in the next chapter.

The marketed and clinical candidate MAbs and FcFPs shown in Table 10.1 exemplify a wide range of “fit-for-purpose” engineered molecules. They include antibodies in which the Fc has been modified to reduce Fc γ R interactions, antibodies modified to increase Fc functionality, and antibodies modified to increase half-life through modification of interaction with FcRn. There are currently two marketed therapeutic antibodies and FcFPs that possess genetically engineered Fc regions for the purpose of modulating Fc functionality, including ecolizumab (Soliris[®]) (Rother et al., 2007) and abatacept (Orencia[®]). Additionally, catumaxomab (Removab[®]), which has been approved in Europe (Seimetz et al., 2010), has been engineered by creating a non-natural mouse-rat hybrid Fc that yields both strong Fc functionality as well as two different binding capabilities (CD3 by one FAB arm and EpCAM by the other) (Zeidler et al., 1999; Hess et al., 2012). Another 11 current or recent clinical candidates have been developed that contain modified Fc domains (see Table 10.1), although a few of these have either been halted for business reasons, placed on clinical hold, or dropped for lack of efficacy.

There are at least three antibody candidates in recent clinical trials that have genetically engineered Fc regions to increase Fc immune functionality, including anti-CD19 MOR208 (Xmab-5574) (Zalevsky et al., 2009), anti-CD20 PRO-13921, and anti-CD20 LY2469298 (AME-133v; increased affinity to Fc γ RIIIa; Weiner et al., 2005) (Bello and Sotomayor, 2007; Alduaij and Illidge, 2011). Note that it appears as though PRO-13921 may have been dropped from clinical trials in 2010, as it no longer appears in the public Genentech/Roche pipeline, perhaps in favor of GA101, a low fucose anti-CD20 with increased ADCC. Other examples of MAbs in later-stage clinical trials that possess modified Fc domains for tuned functionality include teplizumab (humanized anti-CD3 OKT3- γ 1-ala-ala; Li et al., 2006b), visilizumab (anti-CD3 MAb with substantially reduced Fc γ R binding), AME-133v (anti-CD20 with increased affinity to Fc γ RIIIa; Weiner et al., 2005), and rhuMab V114 (anti-CD20 with increased affinity to Fc γ RIIIa). Additionally, there are several additional MAbs in the clinic produced by recombinant cell lines engineered to either reduce or eliminate the fucose residue on the glycan attached to N297 in the Fc to increase ADCC – these will be covered separately in Chapter 11.

The CTLA4 FcFPs abatacept and belatacept, the anti-C5 MAb ecolizumab, the anti-CD3 MAb teplizumab, the anti-NGF tanezumab, and the

anti-amyloid- β MAb ponezumab are all examples of Fc engineering to reduce Fc effector functionality (see Table 10.1). Additionally, the anti-CD3 MAb oteelixumab has a mutation of N297 to remove the Fc glycosylation, resulting in reduced Fc functionality as well (this will be covered in more detail later in this chapter).

An Fc-modified, third-generation anti-RSV MAb, MEDI-557, is in Phase I clinical trials. MEDI-557 is a modified version of Numax with the “YTE” mutation in the Fc that confers an elongated half-life on the antibody (Dall’Acqua et al., 2006). This is the first clinical example of an antibody specifically engineered for half-life modification.

In all of the cases mentioned above, modification of the human Fc sequence could potentially yield neo-epitopes that could invoke a potential immune response in some patients. This has not yet been a significant issue, although this approach is still relatively new and immunogenicity problems may be observed when more patients are dosed with Fc-modified therapeutic MABs. In the best known case thus far, eculizumab (Soliris®), which has a hybrid Fc comprised of IgG₂ and IgG₄ sequences, has very low immunogenicity (Soliris® prescribing information, 2011). Similarly, abatacept (Orencia®), which has a modified Fc region to reduce Fc effector functionality, also is reported to have low immunogenicity rates (Orencia® prescribing information, 2011).

Additionally, two unique clinical candidate antibodies, catumaxomab and ertumaxomab, have been constructed through a novel cell fusion process resulting in production cell lines known as quadromas (Zeidler et al., 1999). These antibodies are mouse IgG_{2b}-rat IgG_{2a} hybrids, with one half of the antibody targeting CD3 on T cells and the other half targeting tumor cell antigens EpCAM (catumaxomab) and HER2/neu (ertumaxomab). The hybrid mouse IgG_{2b}-rat IgG_{2a} Fc has strong Fc functionality in humans (Zeidler et al., 1999; Hess et al., 2012). Both of these antibodies should be expected to provoke a significant immune response due to the fact that they are non-human sequences, but that has been taken into account in the dosing regimens (Zeidler et al., 1999).

As mentioned previously, the field of Fc engineering is highly competitive, with significant activity in all phases, including research, early development, late development, and marketed candidates. A few key papers and reviews are cited here for further reference: Shields et al. (2001), Lazar et al. (2006), Presta (2006, 2008), Jefferis (2007, 2009a, 2009b, 2009c), Desjarlais et al. (2007), Richards et al. (2008), Strohl (2009a, 2009b, 2011), Moore et al. (2010), Chan and Carter (2010), Jiang et al. (2011), Desjarlais and Lazar (2011), Kaneko and Niwa (2011).

10.3 The effect of human Fc polymorphisms on disease and therapeutic index

Antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell phagocytosis (ADCP) are mediated by IgGs binding to FcγRs on immune cells to mediate cellular responses. Complement-dependent cytotoxicity (CDC) through the classical pathway is initiated by the binding of an IgG to complement C1q. Generally, if a cell-depleting activity is desired, as in the case of cancer-cell target antibody therapy (Cartron et al., 2002; Weng and Levy, 2003; Strome et al., 2007; Musolino et al., 2008; Bibeau et al., 2009), then either an IgG₁ or a modified IgG₁ with increased affinities to FcγRIIa and FcγRIIIa, in particular, may be desired.

In theory, these higher affinity antibodies should drive effector functions such as ADCC, ADCP, and/or CDC even when the patient has the low-affinity homozygous forms (i.e. FcγRIIa-131RR; FcγRIIIa-158FF) of those receptors (Desjarlais et al., 2007; Strohl, 2009a, 2009b; Strohl, 2011). But is there validation of this hypothesis? There are at least four lines of evidence that support the hypothesis that affinity of an IgG to either FcγRs or complement components can increase the potency of the antibody to kill targeted cells. The first level is *in vitro*, in which many researchers have demonstrated increased antibody-dependent killing of targeted cells in buffers, in serum, and in whole blood (Dall'Ozzo et al., 2004; Taylor et al., 2009). The second level of evidence comes from animal models in which antibodies with enhanced Fc activities have demonstrated superior activities (Steplewski et al., 1988; Oflazoglu et al., 2007; McEarchern et al., 2007); in some cases, these models include dosing in cynomolgus monkeys which have an immune system more representative of, albeit not identical (see Chapter 17) to, the human immune system (Shearer et al., 1999; Scinicariello et al., 2004). The third level is a genetic correlation of either resistance to a disease or increase in disease susceptibility with polymorphisms of antibody receptors. In these correlations, the assumption is made that the affinity of endogenous antibodies binding to those receptors influences disease outcomes. As described in detail in Chapter 7, polymorphisms of both FcγRIIa (high-affinity H131 and low-affinity R131 alleles, respectively) and FcγRIIIa (high-affinity V138 and low-affinity F138 alleles, respectively) have been shown to have significant impact on the etiology of certain inflammatory diseases (e.g. van Sorge et al., 2003; Brownlie et al., 2008) as well as on the susceptibility to infectious diseases (e.g. Sanders et al., 1995; Yee et al., 2000; Rhein et al., 2008). FcγR polymorphisms, on the other hand, were not correlated with increased risk of incurring breast cancer in Japanese and Brazilian populations (Iwasaki et al., 2011).

The final and most convincing level of evidence comes from studies on clinically relevant antibodies indicating that the efficacy of the antibody can be correlated in a statistically significant manner with the polymorphism of Fc γ Rs of the populations being tested (see Table 10.2). Specifically, significantly improved objective responses of patients with follicular NHL treated with rituximab (Rituxan[®]) were correlated with the high-affinity form (V158) of Fc γ RIIIa (Cartron et al., 2002; Weng and Levy, 2003; Zhuang et al., 2010) and the high-affinity H131 polymorphism of Fc γ RIIa (Weng and Levy, 2003) (see Table 10.2). Kim et al. (2006) also showed that the overall response rate to treatment of diffuse large B cell lymphoma (DLBCL) with rituximab plus chemotherapy was correlated with the high-affinity Fc γ RIIIa polymorphism, but not with that of Fc γ RIIa (see Table 10.2). Additionally, overall response rates of patients with Waldenstrom's macroglobulinemia to treatment with rituximab also were shown to correlate with the higher-affinity 158VV polymorphism of Fc γ RIIIa (Treon et al., 2005). That study also suggested that a second mutation of Fc γ RIIIa, position 48 (LL, LR, LH), that may impact response to rituximab treatment (Treon et al., 2005). In contrast, neither the high-affinity Fc γ RIIa nor high affinity Fc γ RIIIa polymorphism played a role in the outcomes of patients treated with rituximab for chronic lymphocytic leukemia (CLL; Farag et al., 2004; Zhuang et al., 2010; see Table 10.2). One caveat important to all of these data (see Table 10.2) is that concurrent or previous chemotherapeutic treatment may have led to leukopenia which would have decreased Fc γ receptor-based activity that could complicate the interpretations of Fc γ R polymorphisms on MAb efficacy.

Musolino et al. (2008) demonstrated a significant difference in response rates for metastatic breast cancer patients with the high-affinity form of Fc γ RIIIa treated with trastuzumab (Herceptin[®]) plus a taxane versus the low-affinity forms of that receptor, whereas the high-affinity Fc γ RIIa-131HH form did not correlate with complete and partial responses. Nevertheless, both Fc γ RIIa and Fc γ RIIIa high-affinity forms did correlate independently with progression-free survival, and when combined (i.e. Fc γ RIIIa-158VV and/or Fc γ RIIa-131HH), they correlated with total response rates (Musolino et al., 2008) (see Table 10.2). Tamura et al. (2010) also demonstrated that the high-affinity form of Fc γ RIIa, but not Fc γ RIIIa, significantly correlated with complete and partial response rates to treatment of metastatic breast cancer with Herceptin[®].

Finally, two separate studies demonstrated that progression-free survival of patients with metastatic colorectal cancer treated with cetuximab with or without chemotherapy was significantly correlated with the combination of the high-affinity forms of Fc γ RIIa and Fc γ RIIIa (Zhang et al., 2007;

Table 10.2 Examples of Fc γ R polymorphisms as a correlation of clinical response to antibody treatment for various forms of cancer

Antibody and study	Target	Indication	Patients (#)	End-points	Response rates and significance			
					Fc γ R11a (position 158)	Fc γ R11a (position 131)		
					Percent response*	P value	Percent response*	P value
Rituximab ^a	CD20	Follicular NHL	49	12 mo objective response	VV = 90% VF, FF = 51%	0.03	Nd	nd
Rituximab ^b	CD20	Follicular NHL	87	12 mo objective response	VV = 75% VF, FF = 26%	0.002	HH = 55% HR, RR = 26%	0.027
Rituximab ^c	CD20	Follicular NHL	92	Time to progression	VV = 534d VF, FF = 170d	0.023	HH = 434d HR, RR = 140d	0.005
Rituximab ^d	CD20	CLL	30	2 mo Partial response	VV = 33% VF = 42% FF = 50%	>0.2	HH = 78% HR = 31% RR = 65%	0.7
Rituximab ^e plus chemotherapy	CD20	DLBCL	113	Overall response rate	VV = 98% VF = 90% FF = 50%	<0.001	HH = 78% HR = 31% RR = 65%	0.7
Trastuzumab plus taxane ^f	HER2	MBC	54	Complete plus partial response	VV = 82% VF = 42% FF = 35%	0.03	HH = 70% HR = 44% RR = 40%	0.17
Trastuzumab plus taxane ^f	HER2	MBC	17	Complete plus partial response	Combined Fc γ R data: VV and/or HH = 71% VF, FF and HR, RR = 29%			0.04

Trastuzumab plus taxane ^f	HR2	MBC	54	Progression-free survival	VV = not reached VF = 15 mo FF = 11.1 mo	0.008 (VV vs VF)	HH = 29.5mo HR = 16.8mo RR = 10.0mo	0.04 (HH vs RR)
Trastuzumab ^g	HER2	MBC	35	Complete plus partial response	VV = 40% VF = 6% FF = 33%	0.053	HH = 40% HR = 12% RR = 0%	0.043
Cetuximab ^h	EGFR	MCRC	39	Progression-free survival	VV = 1.1mo VF = 2.4mo FF = 2.3mo	0.055	HH = 2.4mo HR = 3.7mo RR = 1.1mo	0.037
Cetuximab ^h	EGFR	MCRC	35	Progression-free survival	Combined FcγR data: HH,HR plus VF,FF = 3.7mo RR plus VV = 1.1mo			0.004
Cetuximab ⁱ plus irinotecan	EGFR	MCRC	61	Progression-free survival	Combined FcγR data: VV and/or HH = 5.5 mo VF,FF and HR,RR = 3.0 mo			0.005

Abbreviations:

CLL: chronic lymphocytic leukemia; DLBCL: diffuse large B cell lymphoma; MBC: metastatic breast cancer; MCRC: metastatic colorectal cancer; NHL: non-Hodgkin's lymphoma.

Notes:

- ^a Carton et al. (2002).
- ^b Weng and Levy (2003).
- ^c Desjarlais et al. (2007).
- ^d Farag et al. (2004).
- ^e Kim et al. (2006).
- ^f Musolino et al. (2008).
- ^g Tamura et al. (2010).
- ^h Zhang et al. (2007).
- ⁱ Bibeau et al. (2009).

* Or time, if end-point is based on disease progression.

Bibeau et al., 2009). On the other hand, Paez et al. (2010) recently reported a lack of correlation between the high-affinity forms of Fc γ RIIa and Fc γ RIIIa and progression-free survival of patients with metastatic colorectal cancer treated with anti-EGFR MABs.

The combined data from these studies using three different therapeutic MABs suggest a strong case for the role of therapeutic antibody interaction with Fc γ RIIa and Fc γ RIIIa as part of the anti-tumor response, particularly when combined. There are cases in which the high affinity form of one receptor or the other is not correlated independently with response rates or progression-free survival, but in each case where the data were combined, statistical significance was achieved. Much focus has been placed on the role of Fc γ RIIIa affinity on NK cells driving ADCC activity. Considerably less emphasis has been placed on the role of phagocytosis by monocytes, macrophages, and/or neutrophils in the treatment of different cancers with antibodies. Since Fc γ RIIa is not found on NK cells and is thus not involved in ADCC, but both Fc γ RIIa and Fc γ RIIIa can be implicated in ADCP, the data in Table 10.2 beg the question of the significance of ADCP vs ADCC in the treatment of tumors with therapeutic MABs. Additionally, the combined data from a variety of separate studies shown in Table 10.2 form a reasonable implication, albeit not yet concrete proof, that patient outcomes can be improved by increasing the affinity of the Fc of therapeutic MABs to both Fc γ RIIa and Fc γ RIIIa. The data, while still rather sparse, also suggest that MABs with increased affinities to Fc γ RIIa and Fc γ RIIIa may not work for all types of cancer, so it will require years of empirical studies to demonstrate where these modified antibodies will work best, if at all, in the treatment of cancer.

As demonstrated by the engineering approaches described in this chapter and in Chapter 11, many researchers in the biopharmaceutical industry are betting significant resources that those correlations will turn into improved antibody-based therapeutics. In the end, as with today's therapeutic antibodies, the key will be to strike a balance between therapeutic efficacy and the potential toxicities that additional Fc functionality may bring with it.

10.4 Fc engineering of IgGs to increase effector function

As discussed above, clinical data have demonstrated a correlation between clinical outcomes in some forms of cancer and Fc receptor polymorphisms. In short, individuals with low-affinity forms of Fc γ RIIa and Fc γ RIIIa may have poorer prognoses than those patients with the higher-affinity forms of those receptors when treated with biologics such as Rituxan[®],

Herceptin®, and Erbitux® for a variety of cancer types. The corollary to this is that increasing the affinity of an antibody to the activating Fcγ receptors might improve outcomes both for those patients who already possess high-affinity receptors as well as for those patients with low-affinity Fcγ receptor polymorphisms, who then would have IgG-Fcγ receptor interactions similar to those now observed with high-affinity Fcγ receptor patients treated with standard IgG₁ antibodies. Moreover, Preithner et al. (2006) demonstrated that serum IgGs acted as a “buffer” against the ADCC activity of potential therapeutic IgGs, requiring higher concentrations of the therapeutic IgG to compensate for the excess endogenous serum IgG. Thus several groups have invested in the construction and characterization of IgGs containing Fc mutations to increase various Fc functionalities such as ADCC, ADCP, and CDC, which hopefully will result in therapeutic candidates with enhanced effector function activities and therapeutic efficacies. It is expected that many of these mutant Fc sequences (see Table 10.3) will be applied to therapeutic candidates over the next several years, and that additional Fc-modified antibodies currently in the discovery stage or in pre-clinical development will soon enter clinical development.

As mentioned previously in Chapters 7 and 9, antibodies of different isotypes interact differentially with Fc receptors and complement factors based on their sequences and structure. Several academic and industrial researchers have worked since the late 1980s to figure out what sequences were important to what function and how changes in those sequences might alter functionality. The earliest efforts included domain swapping between the various IgG isotypes, followed by analyses of C1q binding, complement activation and/or complement-mediated cell lysis (Tao et al., 1991; Greenwood et al., 1993; Sensel et al., 1997; Natsume et al., 2008), FcγR binding and/or mediated functionality (Canfield and Morrison, 1991; Chappel et al., 1991; Greenwood et al., 1993), and half-life in mice (Zuckier et al., 1998). These low-resolution approaches subsequently led to the identification of key residues that were mutated using site-specific approaches to gain additional key information on the interaction of IgGs with receptors and complement (e.g. Canfield and Morrison, 1991; Chappel et al., 1991; Chappel et al., 1993; Brekke et al., 1994; Armour et al., 1999; Idusogie et al., 2000; Xu et al., 2000; Thommesen et al., 2000; Shields et al., 2001; Armour et al., 2003).

Table 10.3, which is modified from Strohl (2009a, 2009b) and Carter (2006), shows several examples of Fc mutations that have been demonstrated to result in increased ADCC, ADCP and/or CDC. It is important to note that several factors other than Fc structure may be included in the actual level of ADCC observed with an antibody, including affinity of the antibody

Table 10.3 Examples of Fc sequence engineering for increased effector functionality*

Mutations or changes	Desired function**	References
IgG ₁ -S298A, E333A, K334A	Increased ADCC, ADCP	Shields et al. (2001)
IgG ₁ -S239D, I332E	Increased ADCC, ADCP	Lazar et al. (2006); Horton et al. (2008); Zalevsky et al. (2009)
IgG ₁ -S239D, A330L, I332E	Increased ADCC, ADCP; ablated CDC	Lazar et al. (2006); Bruckheimer et al. (2009)
IgG ₁ -G236A, S239D, I332E	Increased ADCP	Richards et al. (2008)
IgG ₁ -P247I, A339D or Q	Increased ADCC, ADCP	Allan et al. (2006)
IgG ₁ -D280H, K290S with/ without S298D or V	Increased ADCC, ADCP	Watkins and Allan (2004)
Mutant 12: IgG ₁ -F243L, R292P, Y300L Mutant 16: IgG ₁ -F243L, R292P, Y300L, P396L Mutant 18: IgG ₁ -F243L, R292P, Y300L, V305I, P396L	Increased ADCC, ADCP	Stavenhagen et al. (2007)
IgG ₁ -K290E (or N), S298G, T299A, and/or K326E	Increased binding of aglycosylated IgG to FcγRs over wild-type	Sazinsky et al. (2008)
IgG ₁ -E382V, M428I produced in <i>E. coli</i>	Selective binding to FcγRI of non-glycosylated IgG mutant	Jung et al. (2010)
IgG ₁ -K326A, E333A	Slightly increased CDC while maintaining ADCC	Idusogie et al. (2001)
IgG ₁ -K326W, E333S and IgG ₂ - E333S	Increased CDC, abolished ADCC	Idusogie et al. (2001)
IgG ₁ /IgG ₃ chimeras "1133" and "113F"	Increased CDC while maintaining ADCC	Natsume et al. (2008); Kaneko and Niwa (2011)
IgG ₁ -S267E, H268F, S324T	Substantially increased CDC (also increased ADCC, ADCP)	Moore et al. (2010)
IgG ₁ -S267E, H268F, S324T, G236A, I332E	Substantially increased CDC (normal ADCC)	Moore et al. (2010)
IgG ₁ -F243L	Increased binding to FcγRIIIa; decreased fucose content in glycan	Stewart et al. (2011)

Abbreviations: ADCC: antibody-dependent cellular cytotoxicity; ADCP: antibody-dependent cellular phagocytosis; CDC: complement-dependent cytotoxicity; FcγR: Fc-gamma receptor.

* Plus description of several other single and multiple mutations.

** Note that many of the original publications on these mutations did not measure ADCP, per se, and the notation here for increase in ADCP is based on expectation of that result from the biology of the mutants listed.

to the cell surface target (Tang et al., 2007), target density on the cell surface (Patel et al., 2010), epitope on the target to which the antibody binds (Alduaij and Illidge, 2011; Niederfellner et al., 2011), internalization rate, and perhaps other factors. Shields et al. (2001) carried out a landmark study in which many of the key residues of the Fc were mutated individually, or sometimes in pairs or triplets, to alanine, followed by assays to determine binding to FcγRs, C1q, and FcRn. One mutant (IgG₁ S298A/E333A/K334A; see Table 10.3) possessed significantly increased binding to FcγRIIIa as well as a concomitant increase in ADCC activity (Shields et al., 2001). This “alanine scanning” effort by Shields et al. (2001) also helped to define the functionalities of a wide variety of residues in the Fc and pointed the way for identification of subsequent specific substitutions that resulted in additional modified Fcs with modified functionality (see Table 10.3). One of these second-generation mutants, IgG₁ S239D/I332E, was shown to significantly enhance FcγR binding and increase ADCC and ADCP (Lazar et al., 2006). This S239D/I332E mutation was incorporated into a humanized anti-CD19 MAb, XmAb5574, giving the engineered Fc antibody an 8- to ~140-fold higher affinity to various FcγRs than the wild-type IgG₁, depending on the receptor (see Table 10.3). Presta (2008) pointed out that the increased binding of the IgG₁-S239D/I332E and IgG₁-S239D/A330L/I332E mutants to FcγRIII ranged from 30- to 170-fold, at least in part target-dependent, and the increase in ADCC ranged from 10- to 100-fold. The greatest enhancement in affinity with the anti-CD19 IgG₁ S239D/I332E mutant was against the lower affinity human FcγRIIIa-F158 variant (Horton et al., 2008; see Table 10.3). Lazar et al (2006) also showed that addition of an A330L mutation to the IgG₁ S239D/I332E mutant eliminated CDC activity, providing the potential option to exclude complement activation if that functionality were not desired (Lazar et al., 2006). Since it has been demonstrated that CDC may be linked with injection site reactions (Van der Kolk et al., 2001; Tawara et al., 2008), there may be cases in which the A330L (or similar mutation) to eliminate CDC, while retaining strong ADCC/ADCP, might be advantageous.

In some cases, enhanced CDC might be desired. As mentioned in Chapter 9, Natsumi et al. (2008) constructed an IgG₁/IgG₃ hybrid molecule, 113F plus/minus fucose, that exhibited increased C1q-binding activity and complement-mediated lysis activity, as well as normal ADCC (plus fucose) or increased ADCC (minus fucose). Moore et al. (2010) showed that 113F had a sevenfold increased binding affinity to C1q (6.9 nM K_D as compared with wild-type, which was 48 nM K_D). Additionally, they described a series of mutants with enhanced C1q binding and concomitant CDC activity, and showed that there was a direct correlation between C1q binding affinity

and CDC activity. Of the single mutations obtained, mutant S267E yielded the strongest CDC activity. They demonstrated that one triple-residue mutant, IgG₁-S267E/H268F/S324T, had both the highest affinity C1q binding (1 nM K_D) and highest CDC activity (6.9-fold over wild-type IgG₁) (Moore et al., 2010). This mutant also possessed increased ADCC and ADCP activity, but if a pair of additional mutations (G236A/I332E) were also added, the resultant penta-mutant IgG₁ resulted in a 23-fold enhanced CDC activity over wild-type IgG₁ with normal level of ADCC activity (Moore et al., 2010).

While most N297A-mutant non-glycosylated IgGs have been shown to have substantially decreased binding to FcγRs (e.g. Bolt et al., 1993), Sazinsky et al. (2008) found that a non-glycosylated S298G,T299A mutant of IgG₁ bound both FcγRI and FcγRIIa, and activated FcγRIIa *in vitro*. Moreover, variants of IgG₁-S298G,T299A containing the additional mutations of K290E (or N) and/or K326E also bound FcγRIIIa better than wild-type IgG₁ (Sazinsky et al., 2008). Additionally, a non-glycosylated trastuzumab IgG₁ with two mutations in the Fc, E382V/M428I, demonstrated substantial and specific binding to FcγRI (Jung et al., 2010). Thus these investigators were able to demonstrate gain in function mutations to the non-glycosylated form of IgG. This approach offers a potentially separate path to obtain antibodies with increased or selective Fc functionality.

There are other approaches being tested to increase the ADCC of existing anti-tumor therapeutic MABs. For example, the small molecule lenalidomide (Revlimid™, a derivative of thalidomide approved for the treatment of certain myelodysplastic syndromes), which has been found to enhance the ADCC of therapeutic MABs (van der Veer et al., 2011; Wu et al., 2011), is currently being studied in Phase II clinical trials as an adjuvant to rituximab therapy as an approach to potentially increase ADCC activity of the therapeutic MAB. In recent studies of follicular lymphoma patients reported by Celgene (http://ir.celgene.com/phoenix.zhtml?c=111960&p=irol-newsArticle_print&ID=1570835&highlight=), 85% (38/44) of patients achieved a complete response, providing encouragement for this type of approach.

10.5 Fc engineering for silenced effector function

Approximately two-thirds of all marketed and clinical candidate antibodies are directed against receptor or cell-surface proteins (Strohl, 2009a, 2009b; see Table 8.1). As noted in the previous section, when these cell surface targets are cancer targets, the presence of ADCC, ADCP, CDC, or direct cell-killing

activities may be desired and even enhanced. On the other hand, if these cell-surface targets are for inflammatory, metabolic, or biological indications other than cancer, the desired product profile will often require non-depletion of the cells while blocking the activity of the receptor (Labrijn et al., 2008). Examples of this type of profile include targeting of CD3 for autoimmune disorders, IL-4R on immune cells for asthma, IL-6R for inflammatory diseases, or any of the co-stimulatory ligand/receptor interaction proteins (e.g. CD80, CD86, PD-1, PD-L1, CTLA4). For these cell-surface targets for which cell depletion via ADCC, ADCP, and/or CDC would be detrimental and potentially pose a safety risk, it is important to silence Fc functionality.

Another risk associated with the interaction of Fc γ Rs and IgG₁ antibodies used for therapy in oncology has also been highlighted. Immune complexes formed by the interaction of anti-tumor IgGs with their targets have been shown to promote angiogenesis and matrix tissue degeneration, which can promote tumor invasion and metastasis (Barbera-Guillem et al., 1999). Similarly, natural IgG antibodies interacting with Fc γ Rs in the tumor stromal environment have recently been shown to play a role in establishing chronic inflammation that helps to promote tumor growth (Andreu et al., 2010). Finally, even bevacizumab (Avastin[®]), which is a human IgG₂ MAb, has been demonstrated to form immune complexes with stromal, platelet-bound VEGF, most likely via its low-affinity but high-avidity interaction with Fc γ RIIa on platelets (Meyer et al., 2009), increasing the risk of thromboembolism in patients twofold (Scappaticci et al., 2007; Meyer et al., 2009). Thus an Fc-silenced MAb may be appropriate to improve the safety index for certain oncology indications as well.

Four marketed MAb/Fc fusions, eculizumab (Soliris[®]), abatacept (Orencia[®]), belatacept (Nulojix[™]), and romiplostim (Nplate[®]), already employ this principle. The Fc effector function of eculizumab has been significantly reduced by the construction of an IgG₂-IgG₄ fusion design (Rother et al., 2007; see Table 10.4). Abatacept and belatacept, which are CTLA4-Fc fusion proteins that bind CD80 and CD86 on T and B cells, utilize an IgG₁ Fc with a modified hinge region in which the cys residues at positions 220, 226, and 229 and the pro residue at position 238 in the Fc domain are modified to ser residues, significantly reducing ADCC and CDC effector functions (Davis et al., 2007; see Table 10.4). Romiplostim, which is a fusion of a TPO-mimetic peptide on the C-terminus of an IgG₁ Fc, is produced in *E. coli* and thus is non-glycosylated, giving it reduced Fc effector function (Kumagai et al., 2007).

Alternatively, scFv fragments, Fab fragments, or F(ab')₂ fragments have been used as benign blockers, although their pharmacokinetics are very poor when compared to full IgGs. These antibody fragments could be coupled to polyethylene glycol (PEG) or other half-life extension modules to increase

Table 10.4 Examples of Fc sequence engineering for silenced (or immunosuppressive) effector functionality

Mutations or changes	Function	References
IgG ₁ -N297A	Decreased ADCC, ADCC in anti-CD3 MAb (ChAglyCD3; TRX4; otelexizumab)	Bolt et al. (1993)
IgG ₁ -L234A, L235A	Decreased ADCC, ADCC, cytokine release in humanized OKT3-ala,ala variant	Alegre et al. (1994); Xu et al. (2000)
IgG ₄ -F234A, L235A	Decreased ADCC, ADCC in GLP-1-FcFP LY2189265	Glaesner et al. (2010)
IgG ₂ -V234A, G237A	Decreased ADCC, ADCC, cytokine release in visilizumab (M291-ala,ala)	Cole et al. (1999)
IgG ₄ -F234A, G237A, E318A	Decreased ADCC, ADCC in Campath IgG ₄ variant	Hutchins et al. (1995)
IgG ₂ /IgG ₄ hybrid Fc (C _H 1 and hinge from IgG ₂ , C _H 2 and C _H 3 from IgG ₄)	Decreased Fc functionality	Mueller et al. 1997
IgG ₄ -S228P,* L236E**	Decreased ADCC, ADCC in anti-CD4 MAb clenoliximab	Reddy et al. (2000)
IgG ₁ -E233P, L234V, L235A, G236Δ, A327G, A330S, P331S	Decreased ADCC, ADCC	Ghevaert et al. (2008)
IgG ₂ -H268Q, V309L, A330S, A331S	Decreased ADCC, ADCC, CDC; ("IgG _{2m4} ") in anti-ADDLs and anti-IL-13R MAbs	An et al. (2010); Strohl (2010)
IgG ₁ -C220S, C226S, C229S, P238S	Decreased ADCC, ADCC in abatacept	Davis et al. (2007)
IgG ₁ -C226S, C229S, E233P, L234V, L235A	Decreased ADCC, ADCC, CDC in anti-CD70	McEarchern et al. (2007)
Produced in <i>E. coli</i> , so not glycosylated	Decreased ADCC, ADCC in romiplostim	Kumagai et al. (2007)
IgG ₁ -L234F, L235E, P331S	Decreased binding to FcγRs	Oganesyan et al. (2008)
IgG ₁ -L234F, L235E, P331S	Decreased ADCC, ADCC, CDC	Sazinsky et al. (2008)
IgG ₂ -V234A, G237A, P238S, H268A, V309L, A330S, P331S	IgG _{2σ} ; Completely silenced Fc with fully eliminated FcγR and C1q interactions in anti-CD20 and anti-HER2 MAbs	Vafa et al. (submitted)
IgG ₁ -S267E, L328F	Selective binding to inhibitory FcγRIIb receptor in anti-CD19 MAb	Chu et al. (2008)

Abbreviations: ADCC: antibody-dependent cellular cytotoxicity; ADCC: antibody-dependent cellular phagocytosis; CDC: complement-dependent cytotoxicity; FcγR: Fc-gamma receptor.

* S228P (EU numbering) is the IgG₄ stabilization mutation to help prevent half-antibody formation (Angal et al., 1993).

** Numbering as defined by the authors.

half-life, as discussed further in Chapter 12. Certolizumab pegol (Cimzia®) is a good example of this approach, which has resulted in the development and marketing of a novel TNF- α -blocking antibody with a half-life of about 14 days. While PEGylation is still the method of choice for increasing the half-life of small proteins and antibody fragments (Werle and Bernkop-Schnürch, 2006; see Chapter 12), it has not yet been widely translated into antibody therapeutics. Thus the need for an IgG benign blocker still exists.

Since the very beginning of therapeutic antibody development, strategies have been employed to generate IgGs with reduced or silenced Fc-mediated cellular responses and complement activation (Labrijn et al., 2008; see Table 10.4). The first antibody marketed as a therapeutic, the anti-CD3 MAb, Muromonab OKT3® (Orthoclone OKT3), is a murine IgG_{2a} antibody targeting the ϵ -chain of T-cell receptors and has been used since 1986 to treat graft vs host disease (GVHD). OKT3 was demonstrated to induce both a substantial anti-antibody immune response in patients, as well as induction of pro-inflammatory cytokines, known as a “cytokine release syndrome” (CRS) or “cytokine storm” (Chatenoud et al., 1990; Xu et al., 2000). The cytokine storm was shown to be due to the interaction of the mouse IgG_{2a} Fc with human activating Fc γ Rs (Alegre et al., 1994; Xu et al., 2000). As efforts were made to improve OKT3, the molecule was humanized to reduce the anti-drug response and provide a human Fc. Additionally, a series of mutations were made in the lower hinge region of the human Fc regions of OKT3 to abrogate the Fc γ R interactions (Alegre et al., 1994; Xu et al., 2000). The resulting antibody candidates, including humanized OKT3 IgG₁ (L234A, L235A) (-Ala-Ala), demonstrated reduced binding to Fc γ Rs and a concomitantly reduced induction of pro-inflammatory cytokines (Bolt et al., 1993; Alegre et al., 1994; Cole et al., 1999; Xu et al., 2000; see Table 10.4). Visilizumab, another anti-CD3 MAb developed separately, also was humanized and built into a modified IgG₂ isotype (IgG₂-V234A, G237A) to reduced Fc γ R interaction and CRS (Cole et al., 1999).

One of the most significant cases of documented CRS occurred with the anti-CD52 MAb, CAMPATH (Wing et al., 1996). As previously mentioned, lymphocyte depletion, injection site reactions, and CRS were all observed with an IgG₄ version of CAMPATH (Isaacs et al., 1996). A triple-mutant (L235A, G237A, E318A) of human IgG₄ containing the variable chains from CAMPATH was constructed that reduced Fc functionality, cell-depletion activity, and CRS (Hutchins et al., 1995). A similar approach to modify the lower hinge of IgG₄ (IgG₄-S228P/L236E) was also employed to reduce binding of the anti-CD4 MAb clenoliximab to Fc γ receptors as well as to stabilize the two heavy chains of the MAb (Reddy et al., 2000) (see Table 10.4). Walker et al. (2011b) recently documented that many

commercial MAbs have the ability to confer antibody-dependent cytokine release, indicating the potential seriousness of this issue in developing new MAbs, especially against cell receptor targets.

The crystal structure of an Fc γ R and CDC-negative IgG₁ with the mutations L234F/L235E/P331S (called “TM”; see Table 10.4) suggested that the absence of binding was likely due to small variations in the local Fc γ R and C1q binding sites, because the antibody lacked any significant structural changes (Oganessian et al., 2008).

Labrijn et al. (2008) point out that many investigators have utilized IgG₂ or IgG₄ as silenced isotypes, but with untoward side effects caused by residual Fc γ R activities. Recently, several modifications of IgG₂ were demonstrated to reduce Fc γ R and CDC activity (Armour et al., 2006; Ghevaert et al., 2008; An et al., 2009; Strohl, 2010). A new version of these, IgG_{2 σ} , was recently developed at Centocor R&D (now Janssen R&D) that was demonstrated to be the first IgG completely devoid of Fc γ R activity (see Table 10.5) (Vafa et al., submitted). This antibody has been demonstrated to be not only completely silent with respect to Fc mediation functions, but also to retain complete FcRn functionality, normal half-life, high solubility, lack of aggregation, and reasonable expression levels (unpublished data). The absence of these activities also allowed us to probe the role of IgG₂ isotype antibodies in non-Fc function-dependent direct cell-killing mechanisms (Vafa et al., submitted).

As mentioned above, there is evidence pointing to the possibility that activation of the complement cascade by a therapeutic antibody may result in injection site reactions (Van der Kolk et al., 2001; Tawara et al., 2008). To eliminate the possibility of complement activation-mediated injection site reactions, TRU-015, an anti-CD20 small immunomodulatory pharmaceutical (SMIP), was mutated in the hinge region so that it eliminated C1q binding while retaining Fc γ R binding and normal ADCC activity (Hayden-Ledbetter et al., 2009).

10.6 Fc γ RIIb-dependent suppression of immune response

Three different strategies have been used to address the inhibitory receptor Fc γ RIIb as a potential therapeutic target (Smith and Clatworthy, 2010). In one approach, a highly specific anti-Fc γ RIIb antibody that does not bind to the highly homologous activating receptor, Fc γ RIIa (Veri et al., 2007), was generated and demonstrated to block the ability of immune complexes to bind to the inhibitory receptor. In theory, this antibody may be used therapeutically to block inhibitory responses resulting in an improvement of the activity of therapeutic antibodies that rely on ADCP or ADCC for

Table 10.5 Data comparing FcγR binding and functionality of several IgGs modified to reduce effector function*

Isotype/variant	Biacore assays (μM)			Bead-based alpha-screen avidity assays			Cell-based functionality assays			TNF-α cytokine release
	FcγRI K _D	FcγRIIIa K _D	FcγRIIIa K _D	FcγRI avidity	FcγRIIIa avidity	FcγRIIIa avidity	ADCC	ADCP	CDC	
IgG ₁	0.013*	1.7	0.16*	+++++	++++	+++	+++++	+++++	+++++	+++++
Aglycosyl-IgG ₁ (N297A)	3.7	>170	>95	+++	–	–	–	+++	–	++
IgG ₄ S>P, ala/ala**	15	150	55	+++	++	–	–	++	–	++
IgG ₂	120	1	41	–	+++	–	–	++	–	+++
IgG _{2m4}	210	2.7	95	–	+++	–	–	++	–	++
IgG _{2σ}	>210	>170	>95	–	–	–	–	–	–	–

* Data from Vafa et al. (submitted).

** IgG₄ S>P, ala ala is a human IgG₄ with S228P mutation to stabilize the IgG₄ and F234A, L235A to reduce effector function.

their efficacy (Veri et al., 2007). This could be applicable to certain cancers such as melanoma, which was recently shown to express FcγRIIb (Cassard et al., 2008). The tumor-expressed FcγRIIb was demonstrated to prevent NK cell-mediated ADCC of human metastatic melanoma cells *in vitro* as well as to protect B16F0 melanoma tumors from IgG-induced ADCC *in vivo* (Cassard et al., 2008). Since the ITIM motif of FcγRIIb was not required for this protection from ADCC, the authors proposed that FcγRIIb might be acting in this case as a decoy receptor to soak up anti-tumor IgG so that immune complexes could not be formed to trigger NK cell-mediated ADCC (Cassard et al., 2008).

In another approach for targeting FcγRIIb, an anti-CD19 antibody with a modified Fc (IgG₁-S267E/L328F) was generated that resulted in a 420-fold improved binding of the IgG to FcγRIIb, no substantial increase in binding to the activating FcγRs I and IIa, and a diminishment of FcγRIIIa binding (Chu et al., 2008; see Table 10.4). The co-engagement of CD19 and FcγRIIb by this antibody blocked B cell receptor (BCR) complex-mediated signaling, resulting in inhibition of B cell maturation and antibody generation (Chu et al., 2008; Horton et al., 2011). More significant, perhaps, is that co-engagement of CD19 and FcγRIIb did not result in B cell depletion as has been observed with other anti-CD19 and anti-CD20 antibodies (Horton et al., 2011).

A third potential approach to modulate the activity of FcγRIIb is through α-2,6-linked sialylation of IgGs, which was demonstrated in mice to indirectly stimulate the expression of FcγRII on mouse macrophages to provide an immunosuppressive effect without affecting FcγRII on B cells (Anthony et al., 2008a, 2008b). This is described in more detail in Chapter 11.

10.7 Antibody engineering for modulation of pharmacokinetics

As described in Chapter 7, section 7.3, IgG antibodies bind to the neonatal receptor, FcRn, in a pH-dependent manner which results in their recycling which contributes significantly to their long half-life of ~14–21 d (Martin et al., 2001; Dall'Acqua et al., 2002, 2006; Petkova et al., 2006). Details of the interactions between the Fc of human IgGs and FcRn (e.g. Martin et al., 2001) have led to several different efforts to modify IgG sequences to enhance FcRn binding and therefore elongate the half-life of potential therapeutic IgGs in human serum, which could result in less frequent dosing (Hinton et al., 2004, 2006; Dall'Acqua et al., 2006; Petkova et al., 2006; Datta-Mannan et al., 2007a, 2007b; Zalevsky et al., 2009; Yeung et al., 2010; Igawa et al., 2010). Table 10.6 shows several examples of IgG mutants that have been reported to confer either longer or shorter

Table 10.6 Examples of Fc sequence engineering for modification of half-life

Mutations or changes	Effect on half-life	Reference
IgG ₁ -M252Y, S254T, T256E	Longer	Dall'Aqua et al. (2002); Dall'Aqua et al. (2006); Oganesyan et al. (2009)
IgG ₁ -H433K, N434Y	Longer	Ward (2006)
IgG ₁ -T307A, E380A, N434A	Longer	Petkova et al. (2006)
IgG ₁ -T250Q, M428L	Longer*	Hinton et al. (2006); Datta-Mannan et al. (2007a, 2007b)
IgG ₁ -M428L, N434S	Longer	Zalevsky et al. (2009)
IgG ₁ -T307Q, N434A (or N434S)	Longer	Yeung et al. (2010)
IgG ₁ -T308P, N434A	Longer	Yeung et al. (2010)
IgG ₁ -N434H	Longer	Yeung et al. (2010)
IgG ₁ -N434A; V _H Y27H, S31H; V _L R53H, H55L	Longer	Igawa et al. (2010)
IgG ₁ -M252Y, S254T, T256E, H433K, N434F, 436H	Shorter	Ward (2006)
IgG ₁ -I253A	Shorter	Petkova et al. (2006)
IgG ₁ -P257I, N434H or D376V, N434H	Shorter	Datta-Mannan et al. (2007a, 2007b)

* The T250Q,M428L mutant may enable half-life extension for only some antibodies.

pharmacokinetics on IgGs in non-human primates. The most well known half-life mutant is “YTE” (IgG₁ with mutations in M252Y/S254T/T256E), which conferred a ca. fourfold longer half-life in non-human primates (NHPs) than the wild-type IgG₁ of the same antibody (Dall’Acqua et al., 2006). MEDI-557, which is a YTE-mutant version of motavizumab, has completed Phase I clinical trials, so information on the half-life of this mutant may be available soon. MEDI-557 is currently in a clinical trial (NCT01562938) in adults in which dosing at 12-month intervals is being tested, strongly suggesting that the extended half-life YTE mutant functions very well in humans, similar to the results obtained with NHPs.

Very few changes were observed in the structure of the YTE mutant Fc as compared with wild-type IgG₁-Fc, suggesting that the eight- to tenfold improvement in affinity for FcRn at pH 6.0 for that mutant was due to direct, local Fc–FcRn interactions (Oganesyan et al., 2009). On the other hand, there is mounting evidence that other factors are also involved in

both the affinity to FcRn and overall half-life of an IgG *in vivo* (Igawa et al., 2010; Suzuki et al., 2010). While Suzuki et al. (2010) demonstrated a general correlation between the affinity of IgG binding to FcRn, the pH-dependent binding characteristics alone may not be enough to prolong the half-life of an IgG. As mentioned in Chapter 7, the overall charge of the V-region can have a profound impact on half-life (Igawa et al., 2010). Moreover, since it is clear that long-range interactions between the Fv of an antibody and FcRn can affect its binding to FcRn and half-life *in vivo* (Igawa et al., 2010), what may work for one antibody may not for another. For example, an IgG₁-T250Q/M428L mutant (Hinton et al., 2004, 2006) was able to extend the half-life of an anti-hepatitis B virus antibody (Hinton et al., 2006), but did not appear to increase the half-life of an anti-TNF- α antibody (Datta-Mannan et al., 2007a, 2007b). Finally, Yeung et al. (2009) reported that an N434W mutation in IgG₁ resulted in a 40-fold improvement in affinity to FcRn without a concomitant improvement in pharmacokinetics, suggesting other factors are involved beyond direct FcRn binding affinity.

10.8 Tissue targeting

Perhaps the ultimate engineering of an antibody or related protein would be the ability to target the protein to desired human tissues. In one sense, most MABs and FcFPs that are designed to bind to cell surfaces in effect are targeting those cells, which may be specific to certain tissues or compartments. Thus anti-CD3 and anti-CD4 antibodies target T cells, anti-CD19 and anti-CD20 MABs target B cells, the anti-HER2 MAB trastuzumab targets HER2-positive breast cancer cells, and so forth. A slightly more sophisticated version of tissue targeting is the use of bispecificity to target tumor cells with one binding specificity and to use the second binding specificity to enhance the engagement of T cells. Antibodies using this targeting approach include catumaxomab (Removab®), which targets EpCAM on the surface of tumor cells with one FAB arm and CD3 on T cells with the other FAB arm (Lindhofer et al., 1995; Zeidler et al., 1999). Similarly, the BiTE therapeutics from Micromet (now Amgen) utilize a bispecific pair of scFvs, one of which targets a tumor antigen such as CD19 (blinatumomab, MT103), EpCAM (MT110), or CEA (MT111, MEDI565) (Wolf et al., 2005; Baeuerle et al., 2008), while the other linked scFv targets CD3 on T cells. More on multi-target specificity can be found in Chapter 13.

There are a few additional examples of efforts to target antibodies and FcFPs to specific tissues, but for the most part, this is still a relatively nascent area of effort. The most widely publicized effort is to target the brain by

“Trojan Horse” types of antibodies that bind to internalizing receptors on the surface of the blood–brain barrier (BBB) to confer transcytosis across the BBB. Under normal physiological conditions, approximately 0.1% of antibodies get across the BBB into the brain (Reiber and Felgenhauer, 1987). Thus special methods are required to get antibodies across the BBB to interact with the multiple potential targets behind the BBB (Pardridge, 2010; Neuwelt et al., 2011). The most widely utilized BBB surface receptors for this technology are the transferrin receptor (Pardridge et al., 1991; Lee et al., 2000; Boado et al., 2009; Yu et al., 2011), the insulin receptor (Pardridge et al., 1995; Boado et al., 2007), and low-density lipoprotein receptor (LDLR; Spencer and Verma, 2007). While many publications have been written on the use of this technology to cross the BBB, most of the publications have come up short of the high level of proof required to demonstrate unequivocally that the targeting agent actually traversed the BBB, entered the parenchyma, and yielded a pharmacological effect within the brain. The greatest concern to date has been the proof that the antibodies have indeed crossed into the parenchyma rather than remaining in association with the brain endothelium. Scientists at Genentech, however, recently generated a knobs-into-holes bispecific MAb (Ridgeway et al., 1996; Merchant et al., 1998) targeting transferrin receptor (TfR) with one half of the antibody and the other half of the MAb targeting β -secretase (BACE-1) (Atwal et al., 2011; Yu et al., 2011). They obtained clear, unambiguous penetration of the central nervous system with their bispecific antibody, along with statistically significant lowering of amyloid- β levels in both the brain and the plasma (Yu et al., 2011). Additionally, they demonstrated that the combination of a high dose and a lower affinity antibody against the TfR resulted in greater BBB transcytosis with entry into the parenchyma and anti-BACE-1 activity (Yu et al., 2011). One question that will need to be addressed if success is achieved in getting IgGs across the BBB at any physiologically relevant level is the role of Fc γ Rs on microglia, astrocytes, oligodendrocytes, and neurons (Okun et al., 2010), and how such IgGs would interact with those receptors.

Besides targeting the CNS, other efforts have been made to target specific tissues with modified MABs. ENB-0040 is a tissue non-specific alkaline phosphatase (TNALP)-FcFP for treatment of the bone disorder hypophosphatasia. This enzyme-FcFP is targeted to bone tissue with a 10-mer peptide fused to the C-terminus of the Fc (Millán et al., 2008), so that the enzyme will primarily function at the desired site of activity. In a different example, AS1409 is an anti-tumor antibody targeting BC1, a fibronectin splice variant of ED-B, that is fused with the pro-inflammatory cytokine IL-12 (Lo et al., 2007). In this case, the BC1 targeting is used to carry the IL-12 to the desired site of activity (Lo et al., 2007).

IgG glycans and glyco-engineering

Abstract: Human IgG antibodies are glycosylated at asparagine-297 (N297) in the C_H2 domain. This glycosylation occurs in multiple different forms, the most prevalent of which are bi-antennary structures known as G0F (N297-GlcNAc-GlcNAc-Man-(Man-GlcNAc)₂ with fucose attached to the first GlcNAc), G1F (one added galactose), and G2F (two galactose residues). Many minor glycoforms also typically are found. Thus purified antibodies are not a single chemical entity, but instead are a mixture of highly related compounds. IgGs lacking glycosylation at N297 have slightly lower stability as well as diminished binding to FcγRs, but with no apparent change in binding to FcRn or half-life. Antibodies lacking the fucose residue, or having very low fucosylation content, have been demonstrated to have significantly higher affinity binding to FcγRIIIa, resulting in stronger NK cell-mediated, antibody-dependent cellular cytotoxicity (ADCC). On the other hand, IgGs containing terminally sialylated oligoglycans appear to have an immunosuppressive effect at high concentrations.

Keywords: glycosylation, glyco-engineering, CHO and NS0 cell lines, non-glycosylated IgG, non-fucosylated IgG, low fucose, antibody-dependent cellular cytotoxicity (ADCC), sialylated IgGs

11.1 Introduction to Fc glycosylation

Human IgGs have a single conserved N-glycosylation site at N297 in the C_H2 domain of their constant region (Sethuraman and Stadheim, 2006; Jefferis, 2007; Raju, 2008; Jefferis et al., 2009a, 2009b), as shown in Figure 10.2. The N-glycans attached to N297 of antibody Fc domains are usually very heterogeneous, which results in an IgG being a “collection of closely related structures” often referred as microheterogeneity (see Chapter 16 for additional details on heterogeneity of IgGs) (see Figure 11.1).

A significant feature of the glycans attached to N297 of C_H2 is that they are buried between the two Fc chains and are not as accessible to various

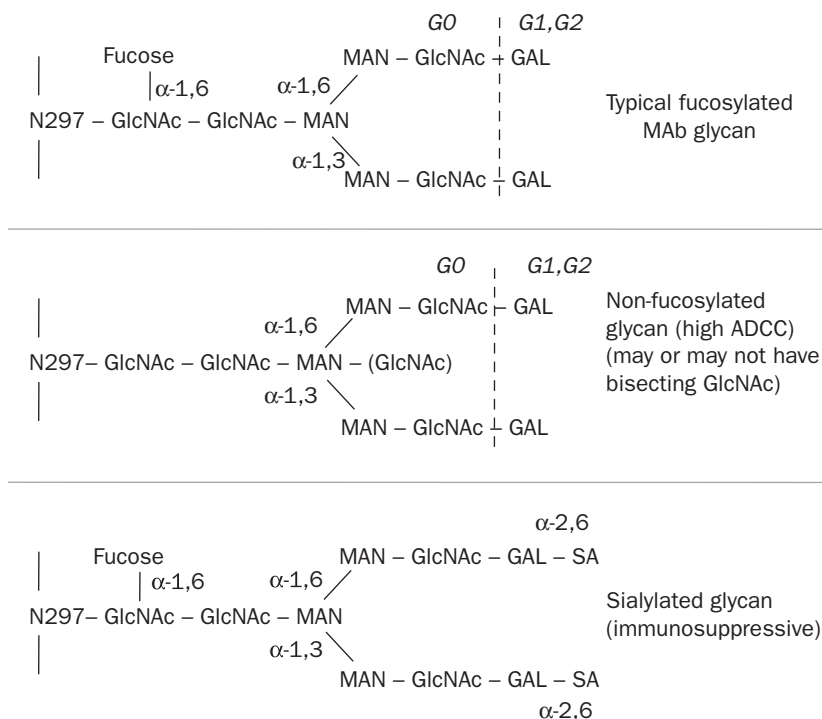


Figure 11.1 MAb and Fc fusion protein N-glycosylation patterns

The major species of N-glycosides from N297 in the C_H2 domain of a human IgG is typically bi-antennary, fucosylated G0, G1, and G2 (*top*), with minor non-fucosylated (*middle*) and sialylated (*bottom*) species as described in the text. Generation of mostly or completely non-fucosylated IgG, with or without bisecting *N*-acetyl-glucosamine (GlcNAc; *middle*), results in significantly higher $Fc\gamma RIIIa$ binding and higher ADCC, as described in the text. Generation of highly α -2,6-sialylated oligoglycans (*bottom*) results in immuno-suppressive effects at high IgG concentrations, as described in the text. *Abbreviations*: GAL: galactose; GlcNAc: *N*-acetyl-glucosamine; MAN: mannose; SA sialic acid.

glycan-modifying enzymes as most protein glycans (Sinha et al., 2008). Thus the Fc glycans tend to be simpler bi-antennary forms with relatively low levels of terminal sialylation (Sinha et al., 2008; Raju, 2008; Jefferis, 2009a). Additionally, Fc glycans can be either symmetrical (both with the same oligosaccharides) or asymmetrical (Sinha et al., 2008).

The core IgG oligosaccharide consists of a bi-antennary heptasaccharide structure containing four GlcNAc and three Man residues in the following sequence of: N297-GlcNAc-GlcNAc-Man-(Man-GlcNAc)₂, where N, GlcNAc and Man represent asparagine, *N*-acetyl-glucosamine, and mannose, respectively. Fucose is often attached to the GlcNAc that is core-linked to N and the fucose linkage in mammals is in an α 1,6-configuration (Sinha et al., 2008) (see Figure 11.1). A shorthand nomenclature has been established for

antibody Fc glycans, in which the afucosyl, agalactosyl core structure is called G0, with the terms G1 and G2 referring to core structures to which one or two galactosyl residues have been added, respectively (Jefferis, 2009a). The fucosylated version of these would be G0F, G1F, and G2F, respectively. The letter B is used to designate a bisecting *N*-acetylglucosamine, e.g. G0B, G1B, G2B. Finally, the letter S designates sialic acid residues, so G2S1 and G2S2 would refer to singly and doubly sialylated bi-antennary structures, respectively. Glycans containing the bisecting GlcNAc are typically not fucosylated, since the substrate for the fucosyltransferase is a non-bisected glycan (Umana et al., 1999; Shinkawa et al., 2003).

It has been calculated that each heavy chain of an IgG may possess up to 32 unique glycoforms, and since the chains may possess different glycans, a given IgG may have up to 128 different glycovariants in a single culture broth (Jefferis, 2009a). Flynn et al. (2010) analyzed naturally occurring glycans on IgG₁ and IgG₂ antibodies isolated from human serum and found that the IgG₁ pool had 21 different glycan forms, of which 11 were neutral, five were complex charged glycans (containing sialic acid) and seven were hybrid glycoforms. Similarly, for naturally isolated human IgG₂, 18 different glycoforms were found that largely overlapped with those found on IgG₁ (Flynn et al., 2010). G1F (35–42%), G0F (12–25%), and G2F (16–21%) dominated for both natural IgG isotypes (Flynn et al., 2010).

Therapeutic antibodies are made in cell culture, which these days is usually accomplished using Chinese hamster ovary (CHO) cells (see Chapter 18). In an unmodified CHO cell culture process, more than 90% of the glycans contains the core fucose residue, and less than ~5–10% of the glycans may contain terminal sialic acid residues (Raju, 2008) (see Figure 11.1). The parental CHO cell lines used for production of most MAbs to date do not express $\beta(1,4)$ -*N*-acetylglucosaminyl-transferase III (GnTIII), the enzyme that mediates the addition of bisecting GlcNAc, so they are mostly or completely devoid of that glycoform (Jefferis, 2009b). Thus an IgG produced by a typical CHO cell culture process may contain ~35% G0F, ~35% G1F (two isomers), and ~16% G2F glycans as the major glycoforms, with minor glycoforms comprising the remaining ~15% of the oligosaccharides (Arnold et al., 2006). These ratios of major glycoforms compare very favorably with the major glycoforms found on IgGs from human serum as noted above (Flynn et al., 2010). Most of the sialylated glycans produced by CHO cells are α -2,3-sialylated, because CHO cells do not typically express the α -2,6-sialyltransferase enzyme and they account for ~5–10% (Son et al., 2011). In any case, it has been well documented that neither the presence nor absence of glycan attached to N297 substantially affects the clearance of MAbs (Chen et al., 2009).

Mouse myeloma cell lines such as NS/0, SP2/0, and P3x63Ag8.653 (“Ag653”), on the other hand, produce only about 70–90% fucosylated glycans, so the percentage of non-fucosyl oligosaccharides is much higher (Naso et al., 2010). Additionally, mouse cells typically produce up to 10% of the oligosaccharides with a bisecting GlcNAc residue (Naso et al., 2010). The final significant difference between CHO and mouse myeloma cell lines is that the mouse lines also produce more glycans with the α -1,3-galactose linkage (Jefferis, 2009b). Chung et al. (2008) reported that cetuximab, which is produced in SP2/0 cells, produced an anaphylactic reaction in some patients due to the presence of anti-galactose- α -1,3-galactose IgE antibodies in the serum of those patients. However, this has been attributed to the α -1,3-galactose residues of N-glycans of FAb domain and not due to the α -1,3-galactose residues of Fc glycans. Cetuximab contains a conserved N-glycosylation site in the Fc and another N-glycosylation site in the variable region of the FAb domains (Chung et al, 2008).

The following sections describe the biological effects exerted by different glycan forms attached to N297 of an IgG Fc, and how engineering of those glycans (antibody glyco-engineering) can be used to obtain therapeutic MAb candidates with widely different biological properties. Table 11.1 summarizes several examples of MAb clinical candidates in which glyco-engineering has been used to modulate the biological activities of those candidates.

11.2 Non-glycosylated IgGs for lowered effector function

Other than the Fc γ R-binding non-glycosylated mutants described by Sazinsky et al. (2008), IgGs rendered non-glycosylated by N297Q/A mutations have long been known to exhibit reduced binding to Fc γ receptors and C1q (Tao and Morrison, 1989; Walker et al., 1989; Bolt et al., 1993). It is known that non-glycosylated IgG₁ retains some residual minor binding to Fc γ RI (Nesspor et al., 2012). Although this has been speculated not to result in effector function activity (Davis et al., 2007), the data shown in Table 10.5 in Chapter 10 suggest that ADCP activity might be retained. Several groups have mutated N297 to either A or Q to generate antibodies or FcFPs with reduced effector functions (Tao and Morrison, 1989; Walker et al., 1989; Bolt et al., 1993). As shown in Table 10.5 (see p. 245), a non-glycosylated IgG₁ N297A mutant still was capable of binding Fc γ RI at a moderate affinity, resulting in *in vitro* cell-based Fc-mediated activities such as ADCP and induction of TNF- α release by PBMCs (Table 10.5; Vafa et al., submitted; Nesspor et al., 2012).

Table 11.1 Examples of glyco-engineered MABs and FcFPs in clinical trials

Compound name	Company	Current status (US)	Molecular target	Major indication	Protein format
Nplate® (Romiplostim, AMG-531)	Amgen	Approved 08/22/08	TPO-R	Thrombocytopenia	Fc-peptide fusion ("peptibody") produced in <i>E. coli</i> – non-glycosylated
Orelizumab (ChAglyCD3; TRX4)	GSK/Tolerx	Recently in Phase III	CD3	Type 1 diabetes	Non-glycosylated, humanized IgG ₁
Obinutuzumab (GA-101) RG7159	Genentech/ Biogen/IDEC	Phase II	CD20	Front-line CLL and other cancers	IgG ₁ κ humanized; non-fucosylated form generated using Glycart technology
BMS-945429 (ALD518)	Alder Bio-pharmaceuticals/ BMS	Phase II	IL-6	Non-small cell lung cancer-related fatigue and cachexia	Non-glycosylated, humanized IgG produced in yeast
A-623	Anthera	Phase II	BLys	RA	Anti-BLys peptibody – produced in <i>E. coli</i> – non-glycosylated
Benralizumab MEDI-563 (BIW-8405)	AstraZeneca	Phase II	IL-5R	SLE	Non-fucosylated IgG ₁ κ by BioWa's Potelligent technology
Poteligeo (mogamulizumab; AMG-761; KW-0761)	Amgen/Kyowa Hakko	Phase I/II; approved in Japan	CCR4	Inflammation and oncology	Humanized IgG ₁ ; non-fucosylated by BioWa's Potelligent technology
MDX-1411	Medarex	Phase I	CD70	RCC	Human IgG; non-fucosylated by BioWa's Potelligent technology
MDX-1401	Medarex	Phase I	CD30 (backup to MDX-060)	Oncology (lymphoma)	Human IgG ₁ ; non-fucosylated by BioWa's Potelligent technology

Table 11.1 Examples of glyco-engineered MABs and FcFPs in clinical trials (*cont'd*)

Compound name	Company	Current status (US)	Molecular target	Major indication	Protein format
LFB-R603	LFB; French Biopharmaceutical Group LFB	Phase II	CD20	Lymphoid leukemia	Low fucose-chimeric IgG ₁ (EMABling™) – rat myeloma YB 2/0 produces low fucose glycans
MDX-1342	Medarex/BMS	Phase I	CD19	CLL, RA	Human IgG ₁ ; non-fucosylated by BioWa's Potelligent technology
BLIB-022	Biogen/IDEC	Phase I	IGF-1R	Solid tumors	IgG ₄ -non-glycosylated; human IgG from Dyax library
BIW-8962	Kyowa Hakko	Phase I	GM2	Blood tumors	IgG ₁ ; non-fucosylated by BioWa's Potelligent technology
GT-MAB 2.5-GEX™	Glycotope GmbH	Phase II	Tumor-specific carbohydrate-protein epitope	Oncology; solid tumors	Glycosyl-engineered MAb for greater ADCC, ADCP (GlycoExpress™)
GT-MAB 5.2-GEX™	Glycotope GmbH	Phase I	Tumor-specific carbohydrate-protein epitope	Oncology; solid tumors	Glycosyl-engineered MAb for greater ADCC, ADCP (GlycoExpress™)
LFB-R593 Anti-D MAb ADNC	French Bio-pharmaceutical Group LFB	Phase I	RhD factor	Rhesus incompatibility between mother and fetus	Low fucose-chimeric IgG ₁ (EMABling™); rat myeloma YB 2/0 produces low fucose glycans

Abbreviations: ADCC: antibody dependent cellular cytotoxicity; ADCP: antibody dependent cellular phagocytosis; CLL: chronic lymphocytic leukemia; RA: rheumatoid arthritis; RCC: renal cell carcinoma; RhD: rhesus factor D; SLE: systemic lupus erythematosus.

Non-glycosylated IgG or Fc platforms have been incorporated into a limited number of marketed and clinical candidate antibodies and FcFPs to minimize effector function activities. The most advanced is romiplostim (Nplate®, also known as AMG 531; Nichol, 2006), a thrombopoietin (TPO) receptor peptibody agonist approved by the FDA in 2008. This peptibody, which contains a TPO mimetic peptide genetically fused to the C-terminus of an Fc moiety, is produced in *E. coli* (Kumagai et al., 2007), which lacks the ability to N-glycosylate proteins. Similarly, A-623, an anti-BLys peptibody, is also produced in *E. coli* and thus is non-glycosylated.

Otelixizumab (also known as ChAglyCD3; TRX4), a non-glycosylated anti-CD3 IgG₁, was recently in Phase III clinical development by Tolerx and GlaxoSmithKline for the treatment of Type I diabetes. While Otelixizumab was produced in mammalian cell culture, it has an N297A mutation to make it a non-glycosylated product, resulting in reduced effector function (Bolt et al., 1993). Additionally, BMS-945429 (ALD518) is a non-glycosylated anti-IL-6 MAb produced in *Pichia pastoris*, and BIIB-022 is an anti-IGF-1R non-glycosylated IgG₄ MAb with reduced effector functions. Particularly for BIIB-022, the choice of low effector functions was made to reduce potential adverse events from IGF-1R on non-cancer cells. Jefferis (2007) also has suggested that use of a non-glycosylated IgG₂ might work best to reduce effector functions in IgG antibodies.

Non-glycosylated IgGs have been shown to have decreased thermostability (Ghirlando et al., 1999) and appear to be more susceptible to proteolysis than glycosylated IgGs (Raju and Scallan, 2006), but these are considered to be manageable differences. Importantly, non-glycosylated MAbs retain the FcRn interactions and long serum half-life of a wild-type IgG (Tao and Morrison, 1989). The crystal structure of a non-glycosylated mouse IgG was recently obtained, revealing differences from wild-type glycosylated IgG (Feige et al., 2009). The non-glycosylation IgG adopted a “superclosed” conformation that is not conducive to FcγR binding, and the structure of the C_H2 domain of the Fc was modified with some destabilization of the C'E loop, leading to the overall lower stability of the non-glycosylated IgG (Feige et al., 2009).

11.3. Low- or non-fucosylated oligosaccharides result in higher ADCC

As noted above, CHO cells do not express GnTIII and therefore lack bisecting GlcNAc in their glycoforms. Umana et al. (1999) engineered a CHO cell line with recombinant GnTIII resulting in the cell line producing MAbs with the majority of N-glycans containing bisected, non-fucosylated

oligosaccharides possessing terminal galactose residues. These antibodies also exhibited the ability for significantly higher ADCC than those produced by the control cell line. Later, it was shown that it was the lack of fucosylation rather than the presence of the bisected GlcNAc that was responsible for triggering the higher level of ADCC (Shinkawa et al., 2003). In the meantime, Shields et al. (2002) demonstrated that human IgG₁s possessing non-fucosylated glycans were able to bind FcγRIIIa with higher affinity than normal IgG₁s, the result of which was the higher ADCC. Studies have now demonstrated that either low- or non-fucosylated IgGs result in up to 50-fold higher affinity binding for FcγRIIIa (Okazaki et al., 2004; Ferrara et al., 2006). Okazaki et al. (2004) suggested that the increased binding was due to an increase in the K_{on} rate, while Ferrara et al. (2006) demonstrated that non-fucosylation of the oligosaccharide on IgG₁ yielded a stronger interaction of the IgG glycan with a glycan attached to Asn162 of FcγRIIIa. They noted that only FcγRIIIa and FcγRIIIb possess a glycan in this position, which may help to explain why low- or no-fucosyl IgGs have increased binding to FcγRIIIa/b and not the other human Fcγ receptors (Ferrara et al., 2006).

Importantly, the lack of fucosylation results in tighter binding by all four human IgG isotypes to FcγRIIIa, which demonstrates the ability of all four isotypes to show increased ADCC *in vitro* when non-fucosylated (Niwa et al., 2005). Scallon et al. (2007a) demonstrated that IgGs possessing about 40% fucosylated glycans had substantially increased ADCC over those with 90% fucosylated glycans. Moreover, those IgGs with 10% fucosyl glycans had about the same ADCC as those with 40% fucosylated glycans (Scallon et al., 2007a), indicating that the level of non-fucosylated glycans does not need to be as low as originally thought in order to increase ADCC.

Recently it was demonstrated that a non-fucosylated anti-CD20 IgG₁ also possessed enhanced binding to FcγRIIIb, resulting in greater neutrophil-mediated phagocytosis than a standard IgG₁ (Shibata-Koyama et al., 2009). This result shows even greater effects by non-fucosylated antibodies than previously understood (Shibata-Koyama et al., 2009). Similarly, Iida et al. (2009) showed that a non-fucosylated anti-CD20 IgG₁ could outcompete fucosylated IgG for binding to its antigen. They also showed that the non-fucosylated anti-CD20 IgG₁ could outcompete serum antibodies for binding to FcγRIIIa (Iida et al., 2009), which is also critical since serum antibodies are capable of dampening ADCC by a wild-type IgG₁ (Preithner et al., 2006). These studies combined suggest that non-fucosylation (or low fucosylation) is a physiologically significant modification that can confer significant advantages to the antibody *in vivo*.

There are now a number of methods described in the literature to generate low or non-fucosylated antibodies. As described above, cloning of GnTIII into a CHO or other production cell line results in the formation of bisecting glycans that typically lack core fucose (Umana et al., 1999; Shinkawa et al., 2003). Similarly, the gene *FUT8*, encoding fucosyltransferase, can be deleted from CHO or other host production cell lines (Yamane-Ohnuki et al., 2004; Kanda et al., 2006b; Malphettes et al., 2010) to generate completely non-fucosylated IgGs. Another recent method for generating a CHO cell line producing IgGs lacking fucose includes the heterologous expression of the prokaryotic enzyme GDP-6-deoxy-D-lyxo-4-hexulose reductase within the cytosol, which was able to deflect the fucose *de novo* pathway, resulting in production of non-fucosylated IgGs with enhanced ADCC (von Horston et al., 2010). Finally, glycosylation enzyme inhibitors such as kifunensine can be added to cell cultures, resulting in non-fucosylated oligomannose-containing IgG antibodies (Zhou et al., 2008).

Alternatively, a different type of cell line might be used that naturally produces low- or non-fucosylated IgGs. One such cell line is the rat myeloma cell line YB2/0, which naturally has low *FUT8* expression, the gene encoding fucosyltransferase. The YB2/0 cells produce IgGs with significantly lower fucose content that have been demonstrated to possess higher ADCC (Sibéril et al., 2006; Kanda et al., 2006b; Ishiguro et al., 2010). Further modification of the rat myeloma cell line YB2/0 by disruption of the GDP-fucose transporter gene GFT resulted in even lower and more consistent fucose-free IgG (Ishiguro et al., 2010). Another alternative host approach is the use of *Pichia pastoris*, which has been glyco-engineered to generate IgGs with human-like glycans that are non-fucosylated since the fucosyltransferase was never present in the first place (Li et al., 2006a).

As shown in Table 11.1, there are at least nine non- or low-fucosylated IgG₁-based antibodies now in clinical development. These include, for example, MEDI-563 (formerly BIW-8405), an anti-IL-5 receptor antibody developed using BioWa's Potelligent™ technology (Shinkawa et al., 2003), GA-101 (Robak, 2009), a third generation anti-CD20 antibody from Roche/Genentech using the GlycArt technology, and Poteligeo® (mogamulizumab; AMG-761; KW-0761), an anti-CCR4 MAb (Yamamoto et al., 2010).

Within the next few years, additional results should become available to demonstrate whether the *in vitro* and non-clinical *in vivo* data obtained with low- or non-fucosylated IgGs translates into efficacy, dose-sparing, expansion of patient pool (e.g. those patients with lower affinity polymorphisms of FcγRIIa and FcγRIIIa), or other medically-related outcomes in the clinic. Ishiguro et al. (2010) recently demonstrated that a non-fucosylated anti-HM1.24 MAb produced by a rat YB2/0 cell line killed

cancer cells, taken from a multiple myeloma patient, much better than normally fucosylated IgG. Additionally, the anti-CCR4 MAb Poteligeo® (mogamulizumab; AMG-761; KW-0761) was recently demonstrated to have potential efficacy in Phase I clinical trials in patients exhibiting relapsed CCR4-positive adult T-cell leukemia-lymphoma (ATL) or peripheral T-cell lymphoma (PTCL) (Yamamoto et al., 2010). This led to the recent approval of Poteligeo® in Japan for the treatment of PTCL.

11.4 Non-sialylated IgG glycans result in increased ADCC

As mentioned above, the oligosaccharides attached to N297 of an IgG are very heterogeneous, with as much as 5–10% terminating in sialic acid residues. As discussed throughout this chapter, this level of glycan microheterogeneity yields a mixed biological response. Naso et al. (2010) engineered a CHO-K1 cell line by expression of the catalytic domain of sialidase A from *Arthobacter ureafaciens* to produce IgGs bearing oligosaccharides completely devoid of sialic acid capping residues. IgG₁s completely lacking sialic acid residues had the same pharmacokinetics and antigen-binding characteristics as identical IgG₁s with the normal 5–10% sialic acid, but exhibited about twice the level of ADCC activity (Naso et al., 2010).

11.5 Sialylated IgG glycans may result in immunosuppressive effects

Intravenous immunoglobulin (IVIG) has been used to treat a variety of autoimmune diseases (see Chapter 13) due to its general immunosuppressive activity (Nimmerjahn and Ravetch, 2007). Samuelsson et al. (2001) demonstrated a potential link in mouse models between the immunosuppressive effects of IVIG and the inhibitory Fc receptor, FcγRII, by showing that IVIG failed to be immunosuppressive in FcγRII knock-out mice or in mice treated with an anti-mouse FcγRII-blocking antibody. Over the past few years, it has become apparent that the IgG molecules containing α-2,6-linked sialylated glycans (Figure 11.1) are present in IVIG. Such sialylated IgG molecules may be responsible for the anti-inflammatory action of high-dose IVIG (Kaneko et al., 2006; Nimmerjahn and Ravetch, 2007; Anthony et al., 2008a; Anthony and Ravetch, 2010). Approximately 10% of IgGs in IVIG are sialylated, 7–9% of which are monosialylated and 1–3% disialylated (Anthony and Ravetch, 2010). Anthony et al. (2008b)

demonstrated a potential mechanism of action for this effect by showing that sialylated murine IgGs had reduced binding to activating FcγRs but increased binding to the mouse lectin, SIGN-R1, which resulted in the upregulation of the inhibitory receptor FcγRII on mouse immune cells, contributing to an anti-inflammatory response. Sialylated mouse IgG also was shown to bind DC-SIGN, the human ortholog of SIGN-R1 in *in vitro* experiments (Anthony et al., 2008b). Scallon et al. (2007b) also showed that highly sialylated human IgGs had reduced affinity for both human FcγRIIIa and to their immobilized cell-surface targets, resulting in decreased ADCC. These results suggest that the anti-inflammatory properties of highly sialylated IgGs may be equally applicable to both mouse models (Kaneko et al., 2006; Nimmerjahn and Ravetch, 2007; Anthony et al., 2008a, 2008b) and humans. It is apparent, however, that very high concentrations (e.g. ~30–100 mg/kg doses) of sialylated IgGs are required for this effect both *in vitro* and *in vivo* (Kaneko et al., 2006; Anthony and Ravetch, 2010), so whether this can be translated clinically into a therapeutic approach is debatable. Recently, it was demonstrated that administration of 2,6-sialylated IgG to transgenic mice expressing humanized DC-SIGN resulted in the production of the T_H2 cytokine, IL-33 (Anthony et al., 2011). The increased IL-33 expression led to expansion of basophils and IL-4 secretion, which led to the increased expression of FcγRII on monocytes and macrophages, completing the anti-inflammatory pathway (Anthony et al., 2011). Mice deficient for IL-4, or mice treated with antibody blocking the IL-33 receptor, were unable to mount the sialylated IgG-induced anti-inflammatory response, thus confirming the linkages in the pathway (Anthony et al., 2011). An alternative mechanism of action also has been proposed recently for the immunosuppressive effects of IVIG. Sialylated, but not asialylated, IgG was found to bind the B cell surface receptor CD22 and down-regulated the activation, proliferation, and function of B cells, thereby yielding an immunosuppressive effect (Séité et al., 2010). It is possible that a combination of these mechanisms of action may contribute to the well-documented immunosuppressive effects of sialylated IgG in IVIG.

11.6 High-mannose glycoforms

Kanda et al. (2006b) explored the effect of various ramifications of non-fucosylated, high manose glycoforms attached to N297 as compared with more complex glycoforms. Three significant messages can be obtained from their data. First, high mannose forms led to lower C1q binding and lower activation of the complement system. Second, the non-fucosylated high

mannose oligosaccharides displayed higher ADCC than normally fucosylated antibodies (Kanda et al., 2006b). With respect to the higher ADCC observed with high mannose-containing IgGs, it is important to note that all man-3, man-4, man-5, man-6, man-7, man-8, and man-9 oligosaccharides on IgGs are non-fucosylated (Chen et al., 2009) and therefore will bind FcγRIIIa better and demonstrate higher ADCC than fucosylated IgGs (Kanda et al., 2006b). When non-fucosylated, high-mannose IgGs were compared side by side with non-fucosylated IgGs with complex glycans, the non-fucosylated, high-mannose IgGs had slightly lower ADCC. Thus the ADCC observed with the high-mannose IgGs was due to lack of fucosylation rather than to high-mannose content per se (Kanda et al., 2006b).

Finally, Kanda et al. (2006b) showed that high-mannose IgGs had a shorter half-life in mouse models than IgGs with complex oligosaccharide content, probably due to clearance via mannose-related receptors, since the binding to FcRn was essentially equivalent to all of the glycoforms tested (Kanda et al., 2006b). Similarly, Liu et al. (2011b) showed that IgGs containing high mannose-containing glycans produced in wild-type *Pichia pastoris* were cleared more quickly in human FcRn transgenic mice than were IgGs containing normal glycans produced by “humanized” *Pichia*. On the other hand, Zhou et al. (2008) did not observe a difference in pharmacokinetics with a pool of high-mannose IgGs versus a pool of antibodies with more complex oligoglycans. In humans, the data are more intriguing. Chen et al. (2009) showed data suggesting that IgGs containing high mannose content were not cleared more quickly in humans than IgGs containing more complex oligosaccharides. They demonstrated that man-8 and man-9 oligoglycans were cleaved to man-5 glycans in serum and proposed that this was what other investigators were visualizing as the more rapid clearance of IgGs with high mannose glycan. More recently, however, the same group very clearly demonstrated, using more sophisticated measurements, that natural IgGs containing high mannose (e.g. man-5) oligosaccharides were cleared more quickly in humans than antibodies with more complex glycans (Goetze et al., 2011). Thus, while this story has taken a while to gain clarity, it does in fact appear that high-mannose IgGs are cleared more rapidly in humans than are IgGs containing more complex glycans (Goetze et al., 2011).

11.7 FAb glycosylation

Approximately 20–30% of all human serum IgG molecules are N-glycosylated in the FAb domains (Holland et al., 2006; Mimura et al., 2007;

Jefferis, 2009a, 2009b). These glycosylation sites, identified by motifs of NxT/S, in which x is not a proline residue, are usually found in the V-regions as a result of somatic hypermutation during antibody maturation. The fact that glycosylated V-regions are selected during antibody maturation in B cells suggests that they may have a significant role in antigen binding and thus host defense, perhaps binding to antigens that would be difficult to bind with amino acid residues alone.

Most of the V-region glycans are complex structures that are highly galactosylated and sialylated, very different from the oligosaccharides present at the N297 position in the C_H2 domain of the constant region (Holland et al., 2006; Mimura et al., 2007; Jefferis, 2009a, 2009b). These V-region-encoded glycosylation sites may have a positive, neutral, or negative impact on the binding of certain antigens, and typically enhance the solubility of the antibody (Jefferis, 2009a).

The only commercial antibody to possess V-region glycosylation is cetuximab (Erbix[®]), which contains an N-linked oligosaccharide occupying N₈₈ of the V_H chain (Qian et al. 2007). In engineering MAbs as therapeutics, most groups shy away from selecting lead antibodies that have potential V-region glycosylation sites. Moreover, potential NxS/T sites in V-regions are often removed, if possible without damaging the activity or solubility characteristics, as part of the optimization process to improve the overall developmental characteristics of the antibody (see Chapter 16).

Antibody fragments as therapeutics

Abstract: A wide variety of antibody fragments has been developed as alternative platforms to IgGs. The most significant advantages to antibody fragments include size, manufacturing, tissue penetration, and ability to concatenate to generate multi-specificity. Thus far, only FAb fragments have been marketed, although several clinical and pre-clinical candidates have been generated using scFv, human V_H or V_L domains, humanized camelid V_{HH} domains, and Ig_{NAR} single domains. A new antibody fragment scaffold has recently also been constructed from the C_{H2} domain of a human IgG. Several strategies have been used to improve the naturally short half-life of antibody fragments, including PEGylation, the use of repeating peptide sequences, polysialylation, albumin or IgG binding or fusions, and other approaches.

Keywords: antibody fragments, FAb, scFv, ReoPro®, V_{HH} , domain antibodies, camelids, nanobodies, sharks, Ig_{NAR}, nanoantibodies, C_{H2} domains, half-life extension, PEGylation, albumin-binding

12.1 Introduction to antibody fragments and alternative formats

An “antibody” is defined by the variable chains it contains, irrespective of the constant regions or lack thereof. In natural antibody development, the variable chains go through a series of somatic hypermutations and affinity maturation processes as the B cell population producing them is further selected and expanded. At certain points in that development process, the B cells undergo chain switch recombination (CSR), allowing for an expansion of the biology associated with the variable chains through the introduction of different constant chains (see Chapter 5). But what if the antibody activity required only the activity of the variable chains themselves? The natural process does not account for this, but with genetic

engineering, we can produce in the laboratory virtually any form of the variable chains needed. Thus smaller recombinant antibody fragments, such as FAb (“fragment, antibody”), scFv (single chain variable chain fragments), and domain antibodies (dAbs) are credible alternatives to traditional IgG-based MAbs for certain functions. These fragments retain the targeting specificity of IgG-based MAbs but also possess other characteristics, such as smaller size, monovalency, ease of engineering and manufacture, improved tissue penetration, and broader biodistribution, as well as lack of potentially deleterious Fc effector function, that may be desired for certain applications.

Strategic reasons for selecting an antibody fragment as the drug candidate, instead of an intact IgG, for a particular application may include:

- desire or requirement for a short circulating half-life in serum, as was important for the first recombinant antibody ever made, Reopro® (Knight et al., 1995; Valgimigli et al., 2008);
- a molecule lacking an Fc effector functionality to eliminate both cellular responses against the target and potential for dimerization of receptors due to bivalency (see Chapter 8);
- a smaller biologic that would have broader tissue distribution or the ability to penetrate tumors (Schmidt and Wittrup, 2009);
- a molecule that can be manufactured in either yeast or *E. coli* to potentially reduce cost of goods or increase scale of manufacturing (Andersen and Reilly, 2004);
- a multi-specific antibody fragment (for more on multi-specificity, see Chapter 13), such as has been demonstrated by BiTEs (bispecific T cell engagers; Wolf et al., 2005; Baeuerle et al., 2008), diabodies (Holliger et al., 1993), and most recently, DARTs (Johnson et al., 2010); or
- a monovalent binding molecule that cannot activate receptors by dimerization, such as might be required for receptors such as c-met (Jin et al., 2008).

Often times, it is a combinations of these strategies that drive the decision to use antibody fragments. This chapter looks at the use of antibody fragments as potential biologic drug candidates and weighs the pros and cons of fragments versus full IgG constructs. An excellent review on antibody fragments has been written by Holliger and Hudson (2005).

In this chapter, we will cover many of the antibody fragments that have been, or are now being, developed as platforms for biologic drugs. Figure 12.1



Figure 12.1 Models of a FAb, scFv, and a nanobody domain Ab

Comparison of size and structure of a FAb (PDBid, 3g6a) (Teplyakov et al., 2009), scFv (PDBid, 1dzb) (Ay et al., 2000), and nanobody domain Ab (PDBid, 1xip) (Van Den Abbeele et al., 2010).

shows the basic comparison of a FAb fragment (ca. 48 kDa), a single chain Fv (scFv; ca. 24 kDa), and a domain antibody (dAb, ca. 12 kDa). Table 12.1 shows some of the fundamental differences exhibited by antibody fragments versus intact IgGs. The development and use of antibody fragments face the same general issues as faced by the non-antibody scaffolds such as DARPins (designed ankyrin repeat proteins), anticalins, and similar such scaffolds. As such, information on the development and use of non-antibody binding scaffolds, as reviewed by Strohl and O'Neil (2012), may be useful.

There are now at least 17 known antibody fragments either approved for marketing or in clinical trials for indications ranging from rheumatoid arthritis to bone disorders (Table 12.2). Three antibody fragments have been approved for marketing in the US, abciximab (ReoPro®), ranibizumab (Lucentis®), and certolizumab pegol (Cimzia®). Six of these 17 known antibody fragments target TNF- α , which is also the target of three marketed IgGs (Remicade®, Humira®, Simponi®) and a highly potent FcFP (Enbrel®). Additionally, two of these (blinatumomab and MT-110) are known bispecific T-cell engagers (BiTEs), which will be described in more detail in Chapter 13.

Table 12.1 Comparison of general properties of IgGs with antibody fragments

Property	IgGs	Antibody fragments
Size	150 kDa	~50 kDa (FABs); ~25 kDa (scFvs); ~12 kDa (domain antibodies); ~3 kDa (V _H FR2)
Proven commodity	+++ (>20 marketed)	+++ (several marketed)
Marketed examples	24 marketed IgG MAbs: Infliximab (Remicade®), Rituximab (Rituxan®), Ustekinumab (Stelara®), etc.	Three marketed FAb fragments: abciximab (ReoPro®), ranibizumab (Lucentis®), certolizumab pegol (Cimzia®)
High affinity	+	+
High selectivity	+	+
Manufacturing	CHO cells	CHO, <i>E. coli</i> , yeasts
Stability (e.g. pH, temperature, oxidation, shear stress)	+	++ (especially for domain antibodies)
Ease of purification	+++; standard platform; affinity with Protein A or Protein G	+/-
Potential for ambient temperature storage formulation	+/- (some antibodies may have potential for ambient storage)	+/-
Alternative routes of administration (e.g. intranasal, inhalation)	-	+
Tissue penetration	+	+++ (especially smaller forms)
Cavity binding	+	++
Manufacturable bi-, tri-, and multi-specificity	+	++
Effector functionality (e.g. ADCC, ADPC, CDC)	+++	-
Natural half-life (without half-life extension technology)	10–30 d (av. 14–17 d)	< 1 h
Ability to extend half-life	+++; via manipulation of FcRn binding characteristics	+++ (up to ~10–14 d with PEGylation)*
Disulfide bonds	+	+
Potential for intracellular targeting	-	+ (scFvs or domain Abs)
Human origin	+ or -	+ or -
Ease of manipulation	+/-	+
Ability to concatenate	-	+ (scFvs and domain Abs)

*Based on extension of Cimzia® half-life by PEGylation.

Abbreviations: ADCC: antibody dependent cellular cytotoxicity; ADPC: antibody dependent cellular phagocytosis; CDC: complement dependent cytotoxicity; CHO: Chinese hamster ovary; FcRn: neonatal Fc receptor; scFv: single chain Fv.

Table 12.2 Examples of antibody fragments on the market or in clinical trials*

Name	Company	Current status (US)	Molecular target	Major indication	Protein format
ReoPro® (Abciximab)	Centocor (now Janssen Biotech, Inc.)/ Eli Lilly	FDA approved 12/22/1994	gPIIb/IIIa on platelets	CVD	Chimeric FAb produced by enzymatic cleavage of recombinant IgG
Lucentis® (Ranibizumab)	Genentech/ Novartis	FDA approved 06/30/2006	VEGF-A	Wet AMD	Humanized IgG ₁ k FAb fragment produced in <i>E. coli</i>
Cimzia® (Certolizumab pegol)	UCB/Schwartz	FDA approved 05/14/2009	TNF- α	RA	Single-site PEGylated humanized FAb produced in <i>E. coli</i> ; purified, then PEGylated
Afelimomab AZD9773 (MAK195F) CytoFab™	AstraZeneca	Phase IIb	TNF- α	Severe sepsis	Mouse FAb for sepsis
CEP-37247 (formerly known as ART-621)	Cephalon	Phase II	TNF- α	Stable plaque psoriasis, RA	dAb fused to human Fc
KB-001	Kalobios/ Sanofi-Pasteur	Phase II	<i>Pseudomonas aeruginosa</i>	Infection	PEGylated FAb targeting the Type III secretion system
ALX-0081 (IV) and ALX-0681 (SC)	Ablynx	Phase II	von Willebrand Factor (vWF) + T _{1/2} extender	thrombosis	28 kDa camelid bivalent Nanobody®
PF-05230896 (ATN-103)	Ablynx (returned from Pfizer/Wyeth)	Phase II	TNF- α	Chronic inflammatory conditions	Nanobody® (Camelid dAb)
SBI-087 PF-05230895	Trubion (now Emergent)	Phase II	CD20 – B-cell deplete	RA	Humanized SMIP

Table 12.2 Examples of antibody fragments on the market or in clinical trials* (*cont'd*)

Name	Company	Current status (US)	Molecular target	Major indication	Protein format
Blinatumomab (MT103/MEDI-538)	Micromet (now Amgen)	Phase II	CD19, CD3	ALL, NHL, CLL	CD19, CD3 bispecific scFv-based BiTE
Alacizumab pegol (CDP-791)	UCB-Celltech/ ImClone	Phase II	VEGF-R2 (inhibits activation by VEGF-A, C, D)	Lung cancer	Humanized F(ab') ₂ -PEG conjugate
Topical ESBA105	Esbatech	Phase II	TNF- α	Uveitis; dry eye; wet AMD (for topical administration)	Single chain Ab
ALX-0061	Ablynx	Phase I /II	IL-6R (receptor)	Autoimmune disorders	Domain antibody
PF-05230905	Ablynx (returned from Pfizer)	Phase I	TNF- α	Chronic inflammation	Nanobody®
MT110	Micromet (now Amgen)	Phase I	CD3, EpCAM (CD326)	Gastrointestinal cancer	CD3, EpCAM bispecific scFv-based BiTE
Anti-VEGF scFv	ESBATEch (now Alcon)	Phase I	VEGF	Ophthalmology indications	ScFv
ALX-0141	Ablynx	Phase I	RANKL	Bone disorders	Nanobody®
ALX-0171	Ablynx	Preclinical	RSV	RSV	Nanobody®

*Data as of July 2, 2011, obtained from company websites, Prous Science Integrity, and <http://www.clinicaltrials.gov>.

Abbreviations: ALL: acute lymphoblastic leukemia; AMD: age-related macular degeneration; BiTE: bispecific T cell engager; CVD: cardiovascular disease; dAb: domain antibody; FDA: Food and Drug Administration; NHL: non-Hodgkin's lymphoma; RA: rheumatoid arthritis; RANKL: receptor activator of nuclear factor kappa-B ligand; RSV: respiratory syncytial virus; TNF- α : tumor necrosis factor-alpha; VEGF: vascular endothelial growth factor.

12.2 FAb and scFv antibody fragments

As described in Chapter 3, FAb fragments, $F(ab')_2$ fragments, Fv fragments, and Fd fragments can be obtained from an IgG via proteolytic degradation and other manipulations. On the other hand, genetic engineering technologies have provided a basis to produce antibody fragments more readily and in more stabilized formats, as described in the sections below.

12.2.1 FAb fragments

A FAb fragment or region consists of the Fd (C_H1 , V_H domains) and the light chain (C_L and V_L domains, which could be either λ or κ) linked through a disulfide bond. Naked FAb fragments are about 47–48 kDa in size and contain approximately 440–450 amino acids. Three FAb fragments have been approved for marketing in the US, abciximab (ReoPro®), ranibizumab (Lucentis®), and certolizumab pegol (Cimzia®) (Tables 2.7, 12.1, 12.2). ReoPro®, which was the first recombinant antibody of any format to be developed and approved for marketing, is a FAb fragment that binds gp IIb/IIIa ($\alpha IIb/\beta 3$) on platelets to inhibit their aggregation (Knight et al., 1995; Reopro® prescribing information, 2005). Ranibizumab (Lucentis®) is an anti-VEGF FAb antibody administered by intravitreal injection for the treatment of neovascular (wet) age-related macular degeneration. Certolizumab pegol (Cimzia®) is a PEGylated FAb fragment administered subcutaneously for the treatment of rheumatoid arthritis. At the time of writing, there do not appear to be any other FAb fragments in late-stage clinical development (Table 12.2), so it may be several years before a fourth FAb is approved for marketing.

A FAb can be generated and manufactured in multiple ways (Knight et al., 1995; Andersen and Reilly, 2004; Zhao et al., 2009). Abciximab, for example, is produced as a full, chimeric IgG and then cleaved by papain to produce the free FAb fragments as well as the Fc (Knight et al., 1995; Reopro prescribing information, 2005). The Fc is purified away and the FAb fragments are then purified and polished (Reopro prescribing information, 2005). Ranibizumab (Lucentis®) and certolizumab pegol (Cimzia®), on the other hand, are produced as recombinant FAb fragments in *E. coli* (Andersen and Reilly, 2004). Alternatively, FAb fragments might be produced as recombinant proteins in mammalian cells, either transiently for research or in stable cell lines for manufacturing (Zhao et al., 2009).

An alternative version of a FAb fragment is the $F(ab')_2$ fragment, which possesses two FAb fragments bound together by a disulfide bond from the hinge

region, maintaining the bivalency of a full antibody. $F(ab')_2$ fragments are obtained by cleavage of an IgG with pepsin, which cleaves below the core hinge between two Leu residues, resulting in two FAb fragments bound together by disulfide bonds (Bennet et al., 1997; Smith, 2007; see also Chapter 3). It is unlikely that $F(ab')_2$ fragments would make good drug candidates, however, because the new exposed C-terminal end derived from the hinge region, which is found in naturally cleaved antibodies in serum (Gearing et al., 2002; Brezski et al., 2009), is highly immunogenic (Lutz and Fumia, 2008; Fumia et al., 2008). The $F(ab')_2$ fragment can be treated with a reductant such as dithiothreitol (DTT) to yield two identical $F(ab)'$ fragments, each nine amino acid residues longer at the C-terminus than a FAb fragment. Other variations on the FAb fragment include the single chain FAb (scFAb) fragment, which would allow a FAb fragment to be produced from a single gene (Hust et al., 2007), and the “scAb” fragment, a single chain which contains the C κ domain in combination with the V_H and V_L chains (McGregor et al., 1994; Harvey et al., 2004).

Without additional half-life extension engineering, naked FAb fragments have a β half-life measured in minutes to about an hour. Abciximab, for example, has an α -half-life of about 10 minutes and a terminal (β) half-life in humans of about 30 minutes (Reopro[®] prescribing information, 2005; Valgimigli et al., 2008). Elimination of FAbs should likely be via the kidney because they are under ~70 kDa, the generally accepted size for cutoff for elimination by kidney glomerular filtration (Caliceti and Veronese, 2003). To achieve a longer half-life in circulation, polymers such as polyethylene glycol can be conjugated to give the antibody fragments a large Stokes radius and therefore reduce filtration by the kidney. Certolizumab pegol (Cimzia[®]) has also been engineered to include a single heavy-chain C-terminal Cys residue to which the 40 kDa polyethylene glycol (PEG) is site-specifically attached using maleimide chemistry (Chapman et al., 1999; Cimzia[®] prescribing information, 2011). This increases the hydrodynamic volume of certolizumab pegol by three to fourfold, which results in a terminal half-life of about 14 days (Chapman et al., 1999; Weir et al., 2006; Cimzia[®] prescribing information, 2011) and a volume of distribution that approximates the total blood volume, similar to that of an IgG antibody.

12.2.2 Single chain fragments (scFv)

For several years in the 1980s, efforts were made to stabilize the Fv after proteolytic cleavage, but with limited success (Hochman et al., 1973;

Sen and Beychok, 1986; Glockshuber et al., 1990). In 1988, two groups (Bird et al., 1988; Huston et al., 1988) independently developed stabilized Fvs by linking the two components V_H and V_L using short peptide linkers with the assumption that the linker did not restrict the folding of the variable domains to reconstitute the native Fv. Bird et al. (1988) constructed single chain Fvs (scFvs) of three different antibodies in the format V_L -(18 amino acid residues linker)- V_H , in which the most successful linker they tested was “KESGSVSSEQLAQFRSLD”. In one of their constructs tested, the scFv form of the antibody retained an affinity about fourfold lower than the FAb. Huston et al. (1988), on the other hand, built an scFv with the format V_H -(15 amino acid residues linker)- V_L , in which the linker used was (GGGGS)₃. In this case, the affinity of the scFv for its target was about sixfold lower than the FAb fragment bearing the same V-regions. These two studies also resulted in two lines of intellectual property for scFv construction, one owned by Micromet and the other owned by Enzon. In 2002, Micromet and Enzon cross-licensed their linker technology, creating a single source from which to license the technology for therapeutic antibodies. The (G₄S)_n linker is highly flexible and does not impact the structure of the domains and has become the favored linker strategy not only for scFvs but across the industry.

The order of the V_H and V_L in an scFv can make a considerable difference in expression, secretion into the *E. coli* periplasm, folding, and multimerization (Tsumoto et al., 1994; Huston et al., 1995; Dolezal et al., 2000; Kim et al., 2008b). In some cases the V_H - V_L orientation (V_H being the N-terminal domain) was best for expression and folding (Huston et al., 1995; Kim et al., 2008b), while in others, the V_L - V_H orientation was favored (Tsumoto et al., 1994; Huston et al., 1995; Dolezal et al., 2000). In at least one case, the V_L - V_H orientation resulted in stable monomers, whereas the V_H - V_L orientation of the same antibody and linker system resulted in multimer formation (Dolezal et al., 2000). Huston et al. (1995) showed that V_H - V_L orientation also affected spacing of the domains, whereby the V_L - V_H bridging distance was always ~5–10 Å greater than with scFvs containing identical linkers in the V_H - V_L orientation. Since it is not predictable which orientation might work best for a particular construct, both should be tested empirically.

ScFvs have been used for a variety of purposes, most notably as fragments fused into the P3 protein of M13 filamentous phage for display purposes (Vaughan et al., 1996; Glanville et al., 2009) and in the construction of *in vitro* display antibody libraries (Lipovsek and Plückthun, 2004; Rothe et al., 2006). ScFvs have also been developed as possible drug candidates in their own right, as well as components or domains of drug candidates. The most significant limitation with scFvs is their propensity to aggregate after they have been purified, most likely due to exposed hydrophobic regions and

the tendency to unfold (Wörn and Plückthun, 2001; Pedroso et al., 2002; Demarest and Glaser, 2008; Miller et al., 2010). Because scFvs are normally characterized by poor stability, low expression yields and aggregation, various efforts have been made to stabilize scFv structure (Reiter et al., 1996; Wörn and Plückthun, 2001; Jordan et al., 2009; Miller et al., 2010; Zhao et al., 2011; Lowe et al., 2011a). Stabilization can be achieved either through internal disulfide bonding (e.g. positions V_L S43C and V_H Q105C (Reiter et al., 1996), positions V_L G/Q100C and V_H G44C (Orcutt et al., 2010; Zhao et al., 2011)) or via stabilization through non-disulfide-based modifications (Jordan et al., 2009; Miller et al., 2010; Dong et al., 2011a).

Despite the potential issues with stability, aggregation, and unfolding, there are several recent and current clinical candidates that either are scFvs or incorporate a scFv into their structure. Efungumab (Mycograb™) and Aurograb™ were two scFv antimicrobial clinical candidates that, until recently, were being developed by Neutec Pharma and Novartis for the treatment of candidiasis and methicillin-resistant *Staphylococcus aureus*, respectively. Clinical development of both molecules has been discontinued, but both reached relatively advanced clinical trials before being halted. Additionally, the BiTE (bispecific T cell engager) technology pioneered and developed by Micromet utilizes the linkage of two single chains together to form a bispecific, scFv-based antibody that binds CD3 on T cells and a cancer cell surface target with the other binding chain (see Chapter 13). Finally, the small modular immunopharmaceutical product (SMIP) technology developed by Trubion (now part of Emergent BioSolutions) fuses scFvs to the N-terminus of IgG Fc domains (see Chapter 14). TRU-015 (recently dropped from Phase II clinical trials) and SBI-087 (in Phase II clinical trials), both anti-CD20 SMIPs, and TRU-016 (Phase I clinical trials), an anti-CD37 SMIP, all incorporate scFvs into their structure.

12.3 Domain antibodies, including nanobodies, Ig_{NAR}s, and nanoantibodies

In their early work on the cloning of antibody genes and construction of human antibody libraries, Greg Winter and his colleagues also found that single domains comprised of either V_H or V_L derived from human antibodies could also be stabilized as stand-alone, antibody fragments (Ward et al., 1989) (Figure 12.2). Similarly, two additional families of antibodies were discovered in camelids (Hamers-Casterman et al., 1993) and nurse sharks (Greenberg et al., 1995; Roux et al., 1998) that consisted of a lone V_H domain (Figure 12.2), lacking a paired V_L , attached to a constant region (Figure 12.3).

More recently, a new type of “domain antibody” was constructed using the C_H2 domain of an IgG as the base scaffold into which CDR loops were grafted (Gong et al., 2009; Xiao et al., 2009). Taken together, these novel antibody constructs comprise the “single domain” antibodies, as described below. These antibodies with unpaired antigen binding domains, and the single domain versions derived from them, have unique properties and have already shown promise as potential therapeutic antibodies (Wesolowski et al., 2009). In recent years, domain antibodies have become very popular antibody fragment alternatives (Dumoulin et al., 2002; Harmsen and De Haard, 2007), as evidenced by the recent acquisition of Domantis by GlaxoSmithKline. The primary advantages of domain antibodies as compared with scFvs are generally better folding and stability characteristics, the absence of the linker, and size. Table 12.3 compares some of the high-level similarities and differences of FAb fragments, scFvs, and domain antibodies. The different domain antibody formats are described in the following sections.

12.3.1 Human domain antibodies

Human domain antibodies (dAbs) are the smallest functional binding units of an antibody (12 to 15 kDa), and can be constructed using either the V_H or V_L domain as the base scaffold with randomized complementarity-determining regions (CDRs). The first isolated domain antibodies were V_H domains derived from immunized mice which demonstrated moderate affinity (~20 nM) to the antigens used for immunization (Ward et al., 1989). These murine V_H domain antibodies, however, both aggregated and were poorly expressed (Ward et al., 1989; Tanha et al., 2006), leaving some unanswered questions about the ability to stabilize and solubilize potential therapeutic candidates made with this format. The most significant issue was that the hydrophobic face of the murine or human V_H domain antibodies that was buried in the V_H-V_L interface of a normal Fv was now exposed, which led to aggregation (Holt et al., 2003; Jespers et al., 2004b). Camelid V_{HH} antibodies were first described in 1993 (Hamers-Casterman et al., 1993), which reinforced the premise that single domain antibodies might be designed to enhance both stability and solubility characteristics (Holliger and Hudson, 2005). The camelid V_{HH} antibodies have the ability to reversibly unfold and refold when heated, a property which gives them their characteristically high solubility and stability (Ewert et al., 2002). Several highly conserved, mostly hydrophilic mutations in the β -sheet scaffold (e.g. Tyr/Phe37 replacing Val37; Glu/Gln44 replacing Gly44; Arg45

Table 12.3 Comparison of general properties of different forms of antibody fragments

Property	FAb fragments	ScFvs	Domain Abs**
Size	~50 kDa (FAbs)	~25 kDa	~12 kDa
Proven commodity	+++ (several marketed)	+/- (late phase clinical trials)	+/- (early phase clinical trials)
High affinity	+	+/-	+/- (depends on technology used)
Manufacturing	CHO, <i>E. coli</i> , yeasts	<i>E. coli</i> , yeasts	<i>E. coli</i>
Stability (e.g. pH, temperature, oxidation, shear stress)	+	+/-	++
Ease of purification	+/-	+/-	+/-
Potential for ambient temperature storage formulation	+/-	–	+
Alternative routes of administration (e.g. intranasal, inhalation)	–	+/-	+
Potential for blocking enzyme active sites	+/-	+/-	+ (particularly nanobodies and Ig _{NAR} S)
Tissue penetration	++	+++	++++
Cavity binding	–	–	++
Manufacturable bi-, tri-, and multi-specificity	–	+/-	++
Effector functionality (e.g. ADCC, ADPC, CDC)	–	–	–
Serum half-life (without half-life extension technology)	< 1 h	< 1 h	< 1 h***
Ability to extend half-life	+++ (up to ~10–12 d with PEGylation)*	+++ (up to ~10–12 d with PEGylation)*	+++ (up to ~10–12 d with PEGylation)*,***
Potential for intracellular targeting	–	+/-	+ (especially if disulfide bonds are removed)
Ease of manipulation	+	+/- (some aggregation issues)	+
Ability to dimerize or concatenate	–	+	++

* See section 12.5 of this chapter.

** General principles covering both human domain antibodies, camelid nanobodies, and Ig_{NAR} shark antibodies.

*** Ability to make albumin binding domains that can be fused to domain antibodies, yielding significantly better half-life with only modest increase in size (Holt et al., 2008; Tijink et al., 2008).

replacing Leu45; Gly/Ser/Leu/Phe47 replacing Trp47; Muyldermans et al., 1994; Holt et al., 2003) (see Figure 12.2) have been proposed to contribute to this reversible unfolding characteristic (Ewert et al., 2002; Jespers et al., 2004b). These four residues (37, 44, 45, 47) have been called the V_{HH} tetrad (Holt et al., 2003) (see Figure 12.2).

Several different approaches have been taken to increase the solubility of human V_H domain antibodies, including “camelization,” which involved changing residues Gly44, Leu45, and Trp47 to their camelid counterparts (Davies and Reichmann, 1995, 1996; Tanha et al., 2001, 2006), although this approach still resulted in camelized V_H dAbs that had a tendency to aggregate and express poorly, as well as being less thermodynamically stable than native V_{HH} antibodies (Holt et al., 2003; Tanha et al., 2006). Jespers et al. (2004a, 2004b) demonstrated that a V_H antibody called HEL4 possessed desirable solubility and stability characteristics without being camelized. Instead, the framework regions of HEL4 were germline DP47 (V_H 3-23), but HEL4 possessed changes in CDRs that allowed for the improved properties (Jespers et al., 2004a). A key observation was that Gly35 in CDR-1 formed a cavity into which the sidechain of Trp47 was inserted, thereby increasing the hydrophilicity of the face that normally would be paired with a V_L (Jespers et al., 2004b; Barthelemy et al., 2008). Using that knowledge, Jespers et al. (2004a) developed a heat denaturation process as part of the V_H library panning to select aggregation-resistant dAbs. Additionally, the length of CDR-3 appears to play a potentially large role in helping to solubilize V_{HH} and Ig_{NAR} V-domain (also called V_{NAR})

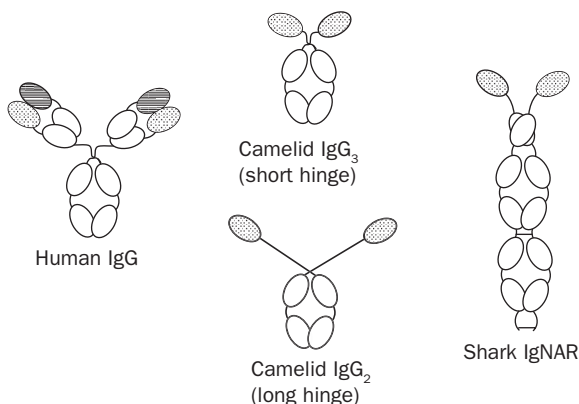


Figure 12.3 Comparison of human IgG with camelid heavy-chain IgGs and IgNAR structures

Human IgG (left), camelid IgG₂ (long hinge) (Hamers-Casterman et al., 1993), camelid IgG₃ (short hinge) (Hamers-Casterman et al., 1993), and IgNAR (Simmons et al., 2006) structures.

antibodies (Wesolowski et al., 2009). With the incorporation of longer CDR-3 loops into human dAb libraries, some of the residues can cover the hydrophobic V_H/V_L face, resulting in increased solubility of the dAbs in the library (Ewert et al., 2002; Wesolowski et al., 2009). A library consisting of the V_H3 germline also has been demonstrated to possess more improved solubility, aggregation, and expression characteristics than libraries made from other germline families (Holt et al., 2003). Van den Beucken et al. (2001) developed a non-biased dAb library based on V_L chains and selected several antibodies out of that library against CD80 (B7.1) and CD86 (B7.2). They found that their selected dAbs possessed high solubility, good expression in *E. coli*, and lack of aggregation (Van den Beucken et al., 2001). Bond et al. (2003) demonstrated that CDR-3 residues could pack against the framework of the exposed V_H/V_L interface and play a significant role in the stabilization and solubilization of human dAbs. Tanha et al. (2006) reported the construction of a partially camelized human dAb library BT32/A6.L1 from which several reasonable binders were obtained. A key to this library is that several residues different from the V_{HH} tetrad were also found to help stabilize the V_{HH} domains in library format.

Barthelemy et al. (2008) carried out a thorough analysis of a V_H dAb derived from antibody 4D5. Among their findings were the discovery that two changes, Q39R/L45E, not found in camelid V_{HH} domains, could significantly stabilize and solubilize the dAb structure, suggesting that several alternative pathways might exist to achieve the same end (Barthelemy et al., 2008).

Domantis, a biotechnology company founded in 2000 to develop fully human dAbs, has built several phage display libraries of human V_H and V_L dAbs with diversities of $>10^{10}$ unique members. Domantis was acquired in 2006 by GlaxoSmithKline (GSK) and as of February 2011, GSK were reporting at least one domain antibody in their biologics pipeline, GSK 2401502, a multi-specific domain antibody for use in oncology. Cephalon CEP-37247 (formerly known as ART-621), an anti-TNF- α domain antibody isolated at Domantis fused to a normal human IgG Fc, is the most clinically advanced domain antibody fragment (Doyle et al., 2007; Gay et al., 2010). CEP-37247 was in Phase II clinical trials for the treatment of rheumatoid arthritis (Clinical Trial #NCT00854685). Other companies currently generating libraries of human dAbs include X-Body Biosciences (libraries of human dAbs, analyzed by deep sequencing approaches) and Harbour Antibodies BV (transgene mice producing human single chain domain antibodies coupled to an Fc domain).

12.3.2 Camelid-derived nanobodies

Along with a normal IgG₁ repertoire, camelids also have a second type of γ -chain antibody in which each of paired chains are comprised of a single V_{HH} domain linked through a hinge to two constant domains, C_H2 and C_H3 (Hamers-Casterman et al., 1993; Conrath et al., 2003). These heavy chain antibodies (HCAbs) make up as much as 50% of the camelid IgG-like repertoire (Hamers-Casterman et al., 1993; Conrath et al., 2003; De Genst et al., 2006). As compared with a standard IgG, the C_H1, C_L, and V_L domains are absent from the camelid HCAbs. Two major versions of the camelid HCAbs have been described, an IgG₂ generally containing a longer hinge of ca. 30 amino acid residues that can bind Protein A but not Protein G, and an IgG₃ containing a short hinge of ca. 15 amino acid residues and the ability to bind to both Protein A and Protein G (Hamers-Casterman et al., 1993; Conrath et al., 2003; De Genst et al., 2006). Pairing of two of these chains forms a normal Fc and two antigen-binding non-associated V_{HH} domains with a molecular weight of about 95 kDa (Figure 12.3). Both old world (dromedary and bactrian camels) and new world (llamas) camelids possess these novel HCAbs (Hamers-Casterman et al., 1993; De Genst et al., 2006).

Based on X-ray crystal structure analysis, the camelid V_{HH} fragment has a relatively conserved Ig fold including three CDR loops and a conserved intra-domain disulfide bridge (Spinelli et al., 1996; Desmyter et al., 1996). Several features, however, appear to be unique to the camelid V_{HH} structure. As mentioned above in section 12.3.1, several of the V_{HH} surface residues in the framework 2 region (Phe37, Glu44, Arg45, Gly47, the “V_{HH} tetrad”) which lie in positions that would normally make contact with the V_L in an Fv contain residues that make the surface naturally more hydrophilic (Muyldermans et al., 1994). Camelid IgG₁s, on the other hand, generally have conserved IgG residues (Val37, Gly44, Leu45, Trp47) in those positions (Conrath et al., 2003), consistent with their H₂L₂ structure similar to human IgGs. Note that, due to a difference in the starting position, positions 37, 44, 45, and 47 are also numbered in the nanobody field as 42, 49, 50, and 52, respectively (De Genst et al., 2006). The V_{HH} tetrad has been suggested to give camelid V_{HH} dAbs the ability to reversibly unfold and refold into a functional form when heated (Ewert et al., 2002), resulting in their unique high solubility and stability (Pérez et al., 2001). The unfolding and refolding process is a two-state reversible process that is unique among antibody structures (Pérez et al., 2001). The CDR-3 hypervariable loop averages ~17 amino acid residues in length (range, ~10–24 residues), longer than the CDR-H3 loops of traditional antibodies (Muyldermans et al., 1994; Ewert et al., 2002). This long loop can potentially protrude into deep

depressions on a protein surface such as the active site of an enzyme or indented clefts of receptors, which may allow for an expanded array of targets as compared with normal human IgGs. Moreover, this long CDR-3 loop partially buries residues that normally would be in contact with V_L , helping the V_{HH} domain antibody to retain its solubility and stability (Muyldermans et al., 1994; Ewert et al., 2002). To help stabilize this long CDR-3 hypervariable loop, an additional disulfide bridge is sometimes formed between Cys residues of the CDR-3 loop and either the CDR-1 loop or in the framework region between CDR-1 and CDR-2; this type of inter-loop disulfide bridge is not typically found in other antibody structures (Muyldermans et al., 1994). Finally, the conformations of the CDR-1 and CDR-2 hypervariable loops of the V_{HH} domains often appear to fall outside of the traditional canonical structures for these loops (Decanniere et al., 2000). Saelens et al. (2008) investigated the possibility of incorporating an extra disulfide bond in a nanobody to increase stability even further. They found that one type of nanobody had a unique natural disulfide bond between positions 54 and 78. When they compared the effects of incorporation of that “natural” disulfide bond with those of a rationally designed disulfide bond between residues 39 and 87, they found that only the “natural bond” between residues 54 and 78 was tolerated, and that the 54/78 disulfide bond could stabilize a variety of different nanobodies (Saelens et al., 2008).

Ablynx is a company that was founded in 2001 in Ghent, Belgium, to commercialize the V_{HH} domains of camelid antibodies as domain-like antibodies with unique properties. Ablynx obtains V_{HH} domain antibodies (“Nanobody™”) using their “Nanoclone™” screening technology which recovers single-chain antibody genes from lymphocytes derived from immunized llamas. Due to certain restrictions, Ablynx currently does not utilize phage display approaches to either isolate or optimize nanobodies, which does present some limitations to the use of their technology. Ablynx currently has six nanobodies in clinical trials, including the anti-von Willebrand factor (vWF) ALX-0081, anti-TNF- α nanobodies ATN-103 and PF-05230905, anti-IL-6 receptor ALX-0061, anti-RANKL ALX-0141, and most recently, the anti-CXCR4 (GPCR) ALX-0651. Additionally, nanobodies have been made against a number of other targets including RSV, several cancer targets (e.g. EGFR, CEA), as well as several bacterial targets (Harmsen and De Haard, 2007; Monegal et al., 2009).

Other groups besides Ablynx have also begun to take advantage of the camelid system to generate tools, reagents, and potential therapeutic candidates. Several groups have generated libraries of camelid antibodies, either using a semisynthetic approach (Goldman et al., 2006) or as naïve libraries obtained from llama lymphocytes (Monegal et al., 2009).

Additionally, efforts have been made to render camelid antibodies more human-like to reduce the potential for immunogenicity (Vincke et al., 2009). These small, novel, highly stable antibodies exhibit significant promise in the engineering of next-generation antibodies.

12.3.3 Shark-derived Ig_{NAR}s

Another unusual antibody called immunoglobulin new antigen receptors, or Ig_{NAR}s (Greenberg et al., 1995; Roux et al., 1998), was discovered to be produced by nurse sharks (*Ginglymostoma cirratum*) and wobbegong sharks (*Orectolobus maculatus*). An Ig_{NAR} consists of a V_H domain fused to a short (five amino acid residues) flexible hinge region and five constant domains (Figure 12.3) (Greenberg et al., 1995; Dooley and Flajnik, 2006). The Ig_{NAR} V_H domains (V_{NAR}s), which are about 13 kDa in size, have an immunoglobulin fold that contains eight β -strands instead of the ten β -strands found in the V_H domain antibody (Stanfield et al., 2004). As compared with a normal V_H, the C' and C'' strands are absent, resulting in the absence of the CDR-2 loop (Stanfield et al., 2004; Figure 12.2). Moreover, the CDR-1 of an V_{NAR} is short and CDR-3 is typically long, ranging from 7 to 34 residues in length (Dooley and Flajnik, 2005). Additionally, the CDR-3 loop in V_{NAR}s represents one of the greatest combinations of loop size variability, sequence diversity, and loop conformations found in any antibody form (Stanfield et al., 2004; Streltsov et al., 2004; Shao et al., 2007). The absence of the CDR-2 loop and the long CDR-3 loop in V_{NAR}s results in a highly diversified functional binding site (solvent accessible surface; SAS) that surprisingly is quite large, ranging from ~540 to 830 Å², a figure not all that different from human Fv paratopes using similar antibody-antigen interactions (Stanfield et al., 2004; see Chapter 3, Table 3.2). While V_{NAR}s are clearly phylogenically isolated, they do share certain structural features with human T cell receptor V α - and IgG V κ chains (Greenberg et al., 1995; Fennell et al., 2010).

Based on the location of non-canonical Cys residues, three different subgroups of Ig_{NAR} V_H domains (V_{NAR}s) have been identified. Type I V_{NAR}s have four key non-canonical Cys residues, two in the framework regions 2 and 4 and two in CDR-3. To date, it appears that Type I V_{NAR}s are limited to the nurse shark (Streltsov et al., 2004). Type II V_{NAR}s, on the other hand, have non-canonical Cys residues in CDR-1 and CDR-3 (Roux et al., 1998). Type III V_{NAR}s, which are very closely related to Type II V_{NAR}s, are found primarily in embryonic sharks (Diaz et al., 2002). Structural analyses of Type I and II Ig_{NAR}s alone or bound with antigen have shown that the

unusually long CDR-3 plays the major role in antigen binding (Stanfield et al., 2004; Streltsov et al., 2004). The different disulfide bond patterns of Type I and II Ig_{NAR}s confer different conformations of CDR-3. In the Type I Ig_{NAR}s, CDR-3 folds over due to a disulfide bridge between CDR-1 and CDR-3, whereas the CDR-3 in Type II Ig_{NAR}s is upright (Roux et al., 1998). Type I (Stanfield et al., 2004) and Type II (Stanfield et al., 2007) V_{NAR}s from naïve libraries have been found that bind hen egg-white lysozyme in the nM range, suggesting a broad potential for manipulation of the V_{NAR} scaffolds to find high-affinity binders to a wide variety of targets.

V_{NAR}s have been manipulated by genetic engineering methods to generate libraries for panning antigens (Nuttall et al., 2001; Dooley et al., 2003; Shao et al., 2007), for affinity optimization (Stanfield et al., 2007; Fennell et al., 2010), and for the construction of dimeric formats (Simmons et al., 2006). The key to library construction is maintenance of the unusually long CDR-3 structure. The biotechnology company Haptogen, which was founded in 2002 to commercialize the Ig_{NAR} platform, was acquired in 2007 by Wyeth (now Pfizer).

12.3.4 Micro-antibodies (partial domains)

As mentioned in Chapter 3, the immunoglobulin fold consists of β -pleated sheets that run in anti-parallel fashion, linked by a loop at each end. If the domain is constructed and stabilized so that it does not bind indiscriminately with other domains, one can build an antibody-like molecule from virtually any Ig fold. Pessi et al. (1993), who were the first to build such a molecule, utilized a 61 amino acid sequence from a V_H domain containing CDRs 1 and 2, to build a ~7 kDa metal-binding “minibody.” While the original minibody was poorly soluble, modifications were made to increase its solubility (Bianchi et al., 1994) and its ability to be expressed in *E. coli* as a phage displayed protein (Tramontano et al., 1994). A small library was made and utilized to pan proteins such as IL-6 (Martin et al., 1994b; Martin et al., 1996), resulting in ca. 220 nM binders that inhibited IL-6 from binding to its receptor.

Qiu et al. (2007) also developed a micro-antibody by connecting CDR-H1 and CDR-L3 using the V_H framework region 2 (V_H-FR2) to yield a small (28 amino acid residues; ~3 kDa) mimetic that retained the antigen recognition of the parent IgG molecule. This molecule was fused with bacterial toxin colicin Ia to make what the authors termed a “pheromonicin” which was able to target a tumor antigen using the recognition sequences of the micro-antibody and stop tumor growth in mice with the activity of the colicin (Qiu et al., 2007).

12.3.5 C_H2 -domain-derived human nanoantibodies

Recently, Dimiter Dimitrov and his colleagues at the National Institutes of Health have constructed a novel domain antibody structure, dubbed “nanoantibody,” that has some intriguing potential properties. They have made libraries consisting of the ~110 amino acid residue C_H2 domain of an IgG₁ into which CDR loops were grafted, resulting in a domain antibody of about 14.7 kDa (Xiao et al., 2009; Dimitrov, 2009). Libraries of mutant residues were grafted into BC and FG loops of C_H2 (Xiao et al., 2009). They determined that a single human IgG₁ C_H2 domain had a T_m of 54.1°C and that while this was more stable than a mouse IgG₁ C_H2 domain, it still lacked the thermal stability desired in a library platform. Thus they inserted a disulfide bridge between N-terminal (A strand) and C-terminal (G strand) strands which resulted in a highly stabilized C_H2 domain with a T_m of 74–83°C (Gong et al., 2009).

One rather intriguing possibility that could differentiate the C_H2 domain-based libraries from other antibody fragments and scaffolds is pharmacokinetics. Kim et al. (1994) demonstrated that the C_H2 domain of a murine antibody retained a ~25 h half-life in mice, which is significantly longer than would be expected for a protein the size of ~12–15 kDa. If, in fact, the C_H2 domain retains enough binding capacity to FcRn to give it even a modest boost in terminal half-life, this could be a significant differentiator for this type of molecule, as compared with scFvs and other dAbs. This, however, remains to be rigorously tested at this point.

12.3.6 Antibodies with only a single FAb arm

It is well known that dimerization of some receptors by normal IgGs containing two FAb arms can cause activation of those receptors. Perhaps the classic example is c-met, the receptor for hepatocyte growth factor (HGF). It has been known for years that the targeting of c-met with IgGs results in substantial agonism (Prat et al., 1998). In fact, one such anti-c-met agonist antibody is in development for the protection of cardiac cells from apoptosis (Pietronave et al., 2010). On the other hand, for the treatment of c-met-positive tumors, in which the desire is to induce apoptosis and block proliferation, antagonism of c-met has been of great interest. The lead antibody in this field is an anti-c-met MAb, onartuzumab, also called “MetMAB,” a one-armed antibody in Phase III development by Genentech for a variety of oncology indications (Martens et al., 2006; Jin et al., 2008).

MetMab has been shown to bind to the SEMA domain of c-met which is required for HGF-mediated dimerization of c-met, to inhibit HGF-induced c-met phosphorylation, and to decrease tumor cell proliferation in pre-clinical models (Martens et al., 2006; Jin et al., 2008). While the actual strategy for construction of the one-armed MetMab has not been published, it appears that the antibody is derived from an anti-c-met scFv named 5D5 (Nguyen et al., 2003) and built into a modified IgG in which one half contains a Fab arm with the 5D5 V-region sequences and the other half is devoid of a Fab arm. The two halves are apparently constructed using the knobs-into-holes technology (Ridgeway et al., 1996; Merchant et al., 1998) for the generation of bispecifics (<http://www.gene.com/gene/research/sci-profiles/anteng/carter/profile.html>).

Genmab's UniBody® represents another univalent binding antibody fragment (<http://www.genmab.com/docs/technology/unibody-fact-sheet.pdf>). The UniBody® is derived from the concept of IgG₄ Fab-arm exchange (Schuurman et al., 1999; Aalberse and Schuurman, 2002; van der Neut Kolfshoten et al., 2007), which was described in more detail in Chapter 9. By removing the core and upper hinge of the IgG₄, the resultant molecules are unable to dimerize, resulting in stable "half-antibodies" of about 72 kDa. Proof-of-concept data have been presented at meetings in which a UniBody® targeting c-met protein tyrosine kinase was demonstrated to have the pertinent *in vitro* activity. The UniBody® retains a moderately long half-life since it can still interact with FcRn, albeit in a monomeric manner.

12.4 Antibody size and tissue distribution

Antibody size, along with other factors such as affinity, half-life, Stokes/hydrodynamic radius, location of target (soluble versus cell bound), target turnover rate, and hydrostatic pressure of the tissue, can alter biodistribution, in particular with respect to tumor penetration (Yokota et al., 1992; Thurber and Wittrup, 2008; Zhang et al., 2009). Intact IgGs generally have limited tissue distribution and their Vss, volume of distribution, is generally approximately the same as serum volume.

Thus, if deep tumor penetration is required for efficacy of a particular antibody molecule, or if deep tissue penetration is required to access a particular target, the size of the antibody may make a significant difference. Thurber and Wittrup (2009) and Schmidt and Wittrup (2009) demonstrated that the optimal size and affinity profile for cancer tissue penetration was a molecule smaller than ~12 kDa and very high affinity, or alternatively a

molecule larger than ~75 kDa, with no particular need for high affinity (i.e. low nM affinity was enough; Schmidt and Wittrup, 2009). Additionally, they showed that molecules falling into the size ranges of an scFv or FAb, i.e. 25–50 kDa, were potentially the worst possible candidates for deep tumor penetration, no matter the affinity of the molecules (Thurber and Wittrup, 2009; Schmidt and Wittrup, 2009).

12.5 Strategies for half-life extension of antibody fragments

12.5.1 Introduction

Elimination of proteins from the serum occurs by several different tissue and receptor-mediated mechanisms, the most predominant of which are membrane transport-mediated clearance and endocytosis by liver hepatocytes and filtration by the kidney glomerulae (Caliceti and Veronese, 2003). While the precise mechanisms for exclusion from kidney filtration are still not fully understood, it has been demonstrated that size, shape, charge, and hydrodynamic radius all play significant roles (Caliceti and Veronese, 2003). As a general rule, proteins with molecular masses ≥ 70 kDa are less likely to be eliminated by kidney filtration than are smaller proteins and peptides (Caliceti and Veronese, 2003; see Figure 7.2). Additionally, negatively charged smaller proteins also may be excluded from filtration due to repulsion by the negatively charged basement membrane of the kidney glomerulae (Caliceti and Veronese, 2003).

In some cases, the short half-life of an antibody fragment is desirable and therefore no method of half-life elongation is desired. Abciximab (ReoPro®) binds to glycoprotein (gp) IIb/IIIa on platelets and is indicated as an adjunct to percutaneous coronary intervention to prevent complications from acute cardiac ischemia. In this particular case, the short half-life exhibited by Abciximab is a beneficial property of the drug, as an elongated half-life for a platelet blocker might lead to excess bleeding. In a different example, antibodies that are delivered to special compartments, e.g. the vitreous of the eye, may not benefit from the half-life extension offered by FcRn-mediated recycling. Ranibizumab, for example, has a ~2.9 d half-life in the vitreous humor of the rabbit eye, but is cleared rapidly once it gets into the serum (Bakri et al., 2007). In this case, the antibody fragment functions just as well as a full IgG in the specialized compartment.

One of the significant limitations of developing antibody fragments such as FAbs (~50 kDa), scFvs (~25 kDa), or domain antibodies (~12–15 kDa) is their inherent short half-life in serum, driven in large part by their sizes,

which are below the lower limit for exclusion from the glomerular filtration in the kidney (Caliceti and Veronese, 2003), as noted above. Secondly, because these antibody fragments are devoid of the Fc, they are not subject to recycling via FcRn as are IgGs, so there is no active recycle mechanism to lengthen their half-lives. Thus virtually everyone trying to develop antibody fragments has investigated alternative mechanisms to lengthen the half-life of their molecules.

The fundamental trade-off that needs to be considered, however, is that while a key value of antibody fragments is their small size that may yield biodistribution patterns that allow them to access tissues and spaces not available to the larger IgGs (Schmidt and Wittrup, 2009; Montgomery et al., 2009; Sabin et al., 2010), this comes at the price of a very short half-life. A fascinating example of this paradox is in the discovery of fusion inhibitors for HIV. The anti-gp41 MAb D5, which was the first MAb to be targeted to the hydrophobic pocket in gp41 (Miller et al., 2005), was affinity matured and tested in both *in vitro* and cell-based assays for its ability to inhibit HIV entry into cells (Montgomery et al., 2009). While IgG versions of affinity-matured D5 were more potent than FAb or scFv formats of the same antibody in direct binding assays, the scFv formatted MAb was the most potent in neutralization assays, which take into account the interaction of the virus and cell surface (Montgomery et al., 2009). The results indicated that a steric blockage of the larger IgGs by the small space between the virus and cell during the entry process was at least in part responsible for the loss in activity of the full IgG. More recently, Sabin et al. (2010) observed a similar phenomenon with anti-HIV MAb HK20 in which the scFv form of HK20 was more potent than the full IgG format of the same antibody. These are examples of why it is critically important to understand the biology of the system for which therapeutic MAbs are being developed, and why, in cases like these, size does matter.

Application of the half-life extension technologies described in this section almost uniformly add bulk to and increase the hydrodynamic radius of the antibody fragments to which they are applied, which may neutralize the very advantage they have in the first place.

To get around these shortcomings, several strategies for half-life extension of antibody fragments have been, or may be, used (see Table 12.4), including:

- extension of half-life by increasing the hydrodynamic radius of the Ab fragment through chemical conjugation to repeat-structure moieties, e.g. conjugation to polyethylene glycol (PEG; PEGylation); hydroxyl-ethyl starch (HES; HESylation), or polysialylation; or

Table 12.4 Half-life extension strategies for antibody fragments

Strategy	Construct
PEGylation	Chemical conjugation of linear or branched polyethylene-glycol (PEG) to Ab fragment via one of several known chemistries
HESylation	Chemical conjugation of hydroxy-ethyl starch (Fresenius Biotech) to Ab fragment would increase hydrodynamic radius similar to the mechanism of PEGylation
Polysialylation	Conjugation of a polysialyl moiety to the Ab fragment to increase half-life and possibly improve tumor uptake
ELPylation	Genetic fusion to elastin-like peptide repeat sequence (PhaseBio) can extend half-life
XTENylation ("recombinant PEG" or "rPEG")	Genetic fusion of non-exact repeat peptide sequence (Versartis, Amunix) to therapeutic peptide
PASylation	Genetic fusion of polypeptide sequences composed of Pro-Ala-Ser (XL-protein GmbH) form uncharged random coil structures with large hydrodynamic volume.
CTP (carboxy-terminal peptide)	Genetic fusion of the CTP peptide from human chorionic gonadotropin β -subunit to Ab fragment (Prolor Biotech)
Fusion to serum albumin	Genetic fusion to human serum albumin (HSA) which has ~19 d half-life (see Figure 7.2)
Binding to serum albumin	Extend half-life by binding HSA with second domain Ab (e.g. AlbuDAb for Domantis version; Ablynx has similar strategy)
Fc fusions	Genetic fusion of scFv to N-terminus of human IgG Fc (e.g. Trubion SMIPs); see also Chapter 14
Binding to serum IgG	Bind IgG with second domain

- increase half-life by genetic fusion of Ab fragment to the inert polypeptides, e.g. XTEN (also known as recombinant PEG or "rPEG"), HAPylation (for homo-amino acid polymer), PASylation (for proline-alanine-serine polymer), or ELPylation (for elastin like peptide); or
- genetic fusion of Ab fragment to a highly sialylated peptide (e.g. C-terminal peptide (CTP)), known to extend the half-life of natural proteins such as human chorionic gonadotropin β -subunit, to the biological drug candidate; or

- genetic fusion to a naturally long-half-life protein or protein domain (e.g. Fc-fusion, human serum albumin (HSA) fusion); or
- extension of half-life by binding at moderate to low affinity to normally long half-life proteins such as HSA, human IgG, or human transferrin.

12.5.2 PEGylation

The most widely used strategy for extending the half-life of small proteins, peptides, and antibody fragments is conjugation of the proteins to either linear or branched-chain monomethoxy polyethylene-glycol (PEG) (Caliceti and Veronese, 2003; Kontermann, 2009; Kang et al., 2009; Jevševar et al., 2010). PEG is a neutral, highly flexible, very hydrophilic molecule that is not metabolized *in vivo* (Caliceti and Veronese, 2003; Jevševar et al., 2010). The general structure of PEG is a random coil that absorbs water (approximately three water molecules per monomeric unit; Caliceti and Veronese, 2003), which swells it and creates a hydrodynamic radius larger than that of a protein of the same molecular weight (Caliceti and Veronese, 2003; Jevševar et al., 2010). Several different chemistries are available to attach PEG to proteins (Roberts et al., 2002; Constantinou et al., 2010).

PEG can be generated in both linear and branched forms, and has been used in many formats ranging from linear short chain PEG to long chain linear PEG, to moderately and even highly branch-chain PEG (Caliceti and Veronese, 2003). The size and structure of protein-conjugated PEG are generally correlated with serum half-life (Jevševar et al., 2010), so proteins conjugated with a shorter PEG (e.g. < 20kDa) are still likely to be eliminated via renal filtration, whereas those conjugated to PEG moieties of >30 kDa are more likely to be eliminated by the hepatic route, resulting in a longer half-life (Jevševar et al., 2010). This is because the pore size in the glomerular basement membrane is ca. 3–5 nm (Nakaoka et al., 1997), while PEG moieties of 12, 20, and 30 kDa have hydrodynamic radii of 5, 7, and 8 nm, respectively. Since PEG is highly flexible and deformable, however, even larger PEGs can still enter the pores, albeit at slower rates the larger they are (Nakaoka et al., 1997; Caliceti and Veronese, 2003). The correlation of the size of a PEG-conjugate to serum half-life, however, is not linear, with a very steep curve in the 20–30 kDa PEG size range, followed by a gradual increase in half-life with PEG size over 40–50 kDa (Nakaoka et al., 1997). Thus conjugation of an scFv (~25 kDa protein) or Fab (~50 kDa protein) to a >30kDa PEG would result in a hydrodynamic radius of the protein PEG conjugate to a level in which it is no longer

actively filtered through the glomerulae of the kidney for elimination (Nakaoka et al., 1997; Jevševar et al., 2010).

One interesting feature is that conjugation of PEG to a protein often decreases the activities of the protein *in vitro* (Kubetzko et al., 2005), while *in vivo* pharmacological activity of conjugated proteins is often enhanced (Bailon et al., 2001). Moreover, the lowering of *in vitro* activity of PEG-conjugated proteins has been directly correlated with the size of the PEG, but those reductions in *in vitro* activities do not necessarily correlate with *in vivo* efficacy (Jevševar et al., 2010).

PEG conjugation has been reported to reduce immunogenicity (Abuchowski et al., 1977a; Sehon, 1991), to alter the biodistribution of the protein, and to protect the protein from proteolytic degradation (Kontermann, 2009). PEG has been approved by the US FDA as a “GRAS” (generally recognized as safe) molecule (Pasut and Veronese, 2007). Nevertheless, PEG has been associated with vacuolization of renal cortical tubular epithelium cells (Bendele et al., 1998), so its safety needs to be carefully evaluated with each molecule developed. Additionally, many people harbor anti-PEG antibodies in their serum, which may limit the use of PEG in those patients.

Adagen®, a PEGylated conjugate of adenosine deaminase used for the treatment of severe combined immunodeficiency disease, was the first protein to be approved for marketing, whereas the first and thus far only PEG-conjugated antibody to be approved for marketing is Cimzia® (certolizumab pegol), a site-specifically PEGylated anti-TNF- α FAb fragment, for the treatment of rheumatoid arthritis.

12.5.3 HESylation

One alternative approach for increasing the hydrodynamic radius of an scFv or FAb is via conjugation to hydroxyl-ethyl starch (HES) (i.e. “HESylation”) (see Table 12.4). HES is a highly branched form of starch containing both α -1,4- and α -1,6-linked glucose residues. It is derived from amylopectin by limited acid hydrolysis to achieve the desired polymer size, followed by ethylation of the free 2, 3, and 6 hydroxyl positions of the glucose residues. Fresenius Kabi has developed the HESylation platform for half-life improvement of peptides and proteins, demonstrating that the AUC of HESylated G-CSF was similar to that of the PEGylated G-CSF, Neulasta® (<http://www.fresenius-kabi.com/Introduction.htm>). Thus far, there are no reports in the literature of any HESylated antibody fragments.

12.5.4 Polysialylation

The basement membrane of kidney glomerulae is negatively charged, and it has been demonstrated that the elimination of negatively charged proteins such as polysialylated proteins via filtration by the glomerulae is significantly reduced as compared with neutral proteins of the same size (Caliceti and Veronese, 2003). Gregoriadis et al. (1993) first demonstrated that polysialylation could elongate the half-life of peptides and proteins in serum. More recently, Constantinou et al. (2008) have demonstrated that the half-life of FAb could be extended significantly by polysialylation.

Polysialic acid (PSA) contains N-acetylneuraminic acid residues linked in an α -(2 $\rightarrow$ 8) glycosidic linkage (Gregoriadis et al., 2005). PSAs, which have a molecular weight of ~11 kDa, ~22kDa, or ~39 kDa (Jain et al., 2003), are typically conjugated to the ϵ -amino groups of lysines via the periodate-oxidized non-reducing end of the PSA. Sialylation of several proteins (e.g. L-asparaginase, catalase, interferons, insulin) has now been demonstrated to confer on those proteins a consistent improvement in half-life (Gregoriadis et al., 2005), likely both through increase in size and negative charge. Polysialylation also may decrease the potential immunogenicity of a protein, similar to PEG (Gregoriadis et al., 2005).

An anti-carcinoembryonic antigen (CEA) scFv containing an extra C-terminal free Cys residue was site-specifically polysialylated with an 11 kDa (average ~35 sialic acid residues) PSA moiety using maleimide chemistry to that Cys residue (Constantinou et al., 2009). The serum half-life of the polysialylated scFv was improved 3.6-fold, while tumor accumulation of the PSA-scFv increased 30-fold (Constantinou et al 2009).

12.5.5 Recombinant polypeptide chains

Several new polypeptide-based strategies have recently emerged as alternatives to chemical conjugation to PEG or other non-biologic polymers to improve half-life. All of these new technologies have in common the genetic fusion of inert peptide repeat polymers to the recombinant protein.

To investigate the ability of an amino acid repeating polymer to extend the half-life of various versabodies (small, highly folded peptide toxins) and other small proteins, a library of sequences containing the amino acid residues A, E, G, P, S, and T was constructed (by Amunix) and then analyzed for expression, genetic stability, solubility, lack of aggregation, and heat stability (Schellenberger et al., 2009). Several largely unstructured

polypeptides, which were named “XTEN” sequences, were tested for their ability to lengthen the half-life of the peptides, exenatide and glucagon, and proteins, green fluorescent protein (GFP) and human growth hormone (HGH). In several animal models, XTEN lengths of 288 (~32 kDa) to 1,008 (111 kDa) residues resulted in half-life improvements of 50–125-fold for the peptides and up to ~12-fold for GFP (Schellenberger et al., 2009). No immune response was observed against the XTEN sequences in the animal models. Recently, several different XTEN peptides were fused to glucagon and tested for their pharmacodynamics in fasted dogs, in which they were shown to prevent hypoglycemia without inducing temporary hyperglycemia (Geething et al., 2010). The best glucagon-XTEN polypeptide contained 144 amino acid residues fused to the C-terminus of glucagon (Geething et al., 2010). In the past year, Versartis, Inc., who are developing the XTEN platform, have placed two “bio-better” XTEN-ylated molecules into Phase I clinical trials, VRS-859, an exenatide-XTEN molecule, for glycemic control of patients with type 2 diabetes mellitus, and VRS-317, an XTEN-fused, once monthly recombinant human growth hormone.

Elastin-like polypeptides (ELPs) are polypeptide units similar to elastin that contain repeats of V-P-G-x-G, where x is any amino acid except proline (Floss et al., 2010). “ELPylation” is the fusion of these elastin-like peptide repeat sequences to a target protein to enhance a thermal phase transition (Floss et al., 2010; MacKay et al., 2010). At higher temperatures ELPs aggregate and fall out of solution, but when the temperature is decreased below the transition point, they become fully soluble (Floss et al., 2010; MacKay et al., 2010). The chemistry of the residue in the “x” position (i.e. polar, charged, hydrophobic) affects the transition state of the polymer. Moreover, the fusion of ELP sequences to small proteins enhances the half-life of those proteins by increasing their hydrodynamic radius, resulting in decreased elimination by the kidney (Conrad et al., 2010). ELPylation of a ~12 kDa anti-TNF- α V_{HH} domain antibody recently was demonstrated to substantially increase the half-life of that antibody in rodent models (Conrad et al., 2010). PhaseBio, Inc., has developed ELPylation for both protein purification and as a half-life extension module, and recently began Phase I/IIa clinical testing on Glymera™, an ELPylated version of GLP-1 with extended steady-state levels in pre-clinical models.

Another approach for polypeptide-directed half-life extension is the use of a homo-amino acid polymer (HAP) of primarily glycine residues fused to the protein of interest. While inert repeat sequences such as (Gly₄Ser)_n have been used for thirty years as linker sequences, Schlapschly et al. (2007) tested the hypothesis that longer Gly-Ser polypeptides might increase the hydrodynamic radius and thus extend the half-life of the anti-HER2 FAb, 4D5. They

demonstrated that a repetitive sequence $(\text{Gly}_4\text{Ser})_n$ in which $n = 200$ fused to the light chain of 4D5 increased its hydrodynamic volume more than double that of the FAb alone, and moderately increased the half-life of the molecule.

A fourth approach for using polypeptide repeat sequences to extend the half-life of antibody fragments would be to use a polymer consisting of three repeating amino acids, such as proline, alanine, and serine (PAS). Fusion of PAS polymers of 100–200 repeats in length improved the half-life of small proteins by 3.5–6-fold over native proteins in mouse models, and PASylation of interferon increased its half-life by a reported 60-fold over native interferon (Skerra, 2009). A new company, XL-Protein GmbH (<http://www.xl-protein.com>), has been formed to commercialize the use of PASylation to improve the half-life of peptides and small proteins (Skerra, 2009).

These polypeptide-based genetic fusion strategies have four advantages over PEGylation:

- They eliminate the cost of the PEG moiety, as well as the time, process cost, and yield losses for the chemistry to couple it to the protein.
- The constructs result in single molecular entities that can be expressed in *E. coli* or other hosts with no post-translational modification.
- Since no chemistry is performed, only a single round of purification is required as compared with PEGylation, which requires purification, conjugation, and then repurification.
- The peptides are biodegradable and should not be toxic *in vivo*.

The primary potential disadvantages include potential immunogenicity and lack of clinical proof-of-concept for all but the XTEN platform.

12.5.6 CTP fusion

Human chorionic gonadotropin (HCG), follicle stimulating hormone (FSH), luteinizing hormone (LH), and thyroid stimulating hormone (TSH) are heterodimeric glycohormones consisting of a common α -subunit, but unique β -subunits that confer on them different activities (Matzuk et al., 1990). The half-life of HCG is longer than FSH, LH, and TSH; moreover, FSH, LH, and TSH are largely eliminated through the kidney, whereas a much smaller fraction of HCG is cleared renally. The HCG β -subunit (HCG- β) possesses a ~25 amino acid residue long C-terminal peptide (CTP) with the sequence of: ... S*KAPPPS*LSPS*RLPGPS*DTPILPQ (where * denotes a glycosylation site) which possesses four O-glycosylation sites,

each of which terminates with sialic acid. CTP has been shown to naturally extend the half-life of HCG in human serum, most likely because the negatively charged sialylated CTP impairs renal clearance (Fares et al., 1992; Caliceti and Veronese, 2003).

Fusion of the β -CTP to FSH was shown to result in a twofold improvement in its half-life in humans as compared with recombinant FSH (Duijkers et al., 2002), no matter whether it was dosed subcutaneously or intravenously (Klein et al., 2002; Duijkers et al. 2002). As a result, FSH-CTP was developed as a long-acting FSH (Klein et al., 2002; Duijkers et al., 2002), and was approved in 2010 by the European EMEA as a long-acting fertility drug under the name of ELONVA® (corifollitropin alfa).

CTP also has been fused to other hormones, including erythropoietin (EPO) (Fares et al., 2007) and human growth hormone (HGH) (Fares et al., 2010), resulting in extension of their half-lives and increases in the area under the curve (AUC). Prolor Biotech, Inc., which has commercialized the CTP half-life extension technology, has several molecules in development including interferon- β -CTP, which they claimed to have 55-fold greater overall exposure over IFN- β alone, Factor VII-CTP, Factor IX-CTP, and a CTP fusion with a naturally occurring anti-obesity peptide. Overall, it appears that fusion of CTP to a protein will extend its half-life by two to fourfold, whereas fusion to peptides may lengthen their half-life even more. An important limitation of this technology is that the recombinant CTP fusion proteins must be produced in CHO or other mammalian cell systems to obtain the requisite O-glycosylation of the CTP extension module. Whether CTP fusion to an antibody fragment will prove to significantly enhance their half-life is yet to be determined.

12.5.7 Binding to human serum albumin (HSA) or HulgG

Another important strategy for extension of the half-life of antibody fragments is to bind them non-covalently to proteins that have a long half-life in human serum. Three such proteins are human IgG (see Chapter 7), human serum albumin (HSA; ~19 d half-life; Peters, 1985), and transferrin (10–12 d half-life; Kontermann, 2009). A significant advantage of this approach is that small binding domains can be used to bind to these “carrier proteins,” resulting in a final molecule that retains much of the small size inherent in the fragments. Albumin or “serum protein” binding, which requires a balance between protein binding and dissociation of the small molecule to carry out its function, has been used for years as a method to improve the functional half-life of small molecules (Bertucci and Domenici, 2002).

Dennis et al. (2002) first described a strategy for albumin binding of proteins as a method for extending their half-life. They found that the short peptide SA21 (Ac-RLIEDICLPWGCLWEDD-NH₂), isolated from peptide phage display on various albumins, could bind human, rabbit, and rat albumin at 467, 320, and 266 nM, respectively. They showed that the core consensus sequence required for optimal albumin binding was “DICLPWGCLW”. Fusion of SA21 to a FAb improved its half-life 26-fold and 40-fold in mouse and rabbit, respectively (Dennis et al., 2002). Nguyen et al (2006) found that higher-affinity binding to albumin correlated with longer half-life and reduced clearance in rat models. They also demonstrated by allometric scaling that a small protein with an affinity of ~500 nM for albumin would have a half-life in humans of about 4 d and a clearance rate of 76 mL/h (Nguyen et al., 2006). Finally, a recent study has shown that the albumin-binding domain of streptococcal protein G could be used to bind HSA and “hitch a ride” through the FcRn recycle mechanism, yielding a longer half-life for those molecules to which it was fused (Andersen et al., 2011). This same principle has been applied extensively in the past half-dozen years using scFvs, V_{HH} nanobodies, domain antibodies, and non-antibody binding proteins. Both domain antibodies (dAbs), dubbed “AlbudAbs” (Holt et al., 2008), and nanobodies (Coppieters et al., 2006; Tijink et al., 2008) have been developed that bind HSA for half-life extension.

AlbudAbs were found to possess a half-life in mice (24 h) and rats (43–53 h), similar to the half-life values of the mouse serum albumin (35 h) and rat serum albumin (53 h), indicating that the AlbudAbs should be able to extend the half-life of dAbs to near that of the SA to which they are attached (Holt et al., 2008). Since HSA has a half-life of ~19 days (Peters, 1985), the expectation is that AlbudAbs might extend the half-life of dAbs to the two week-plus range. In another example, an anti-albumin V_{HH} domain was demonstrated by Coppieters et al. (2006) to extend the half life of anti-TNF- α bivalent V_{HH} molecules 54–58-fold from ~50 min to ca. 2 days in mice, approximately the same half-life as mouse albumin (Coppieters et al., 2006). The expectation is that these 36 kDa, trivalent, bispecific anti-TNF- α /anti-albumin V_{HH} constructs would have a half-life similar to HSA (Coppieters et al., 2006; Tijink et al., 2008), as mentioned above. Additionally a very small (5 kDa) naturally occurring albumin-binding three-helix bundle was engineered to bind albumin with sub-picomolar affinities (Jonsson et al., 2008). This approach has been commercialized as Affibody's Albumod™ platform for half-life extension of biopharmaceuticals.

Alternatively, a fragment might be used to bind hIgG instead of HSA. The anti-IL-6 avimer non-antibody binding protein that was taken into

Phase I clinical trials had one avimer domain that bound IgG, giving the avimer trimer a half-life in non-human primates of 89 h (Silverman et al., 2005), as compared with about 1 hour for a non-IgG-binding avimer.

These types of approaches would yield the desired half-life improvement without a substantial increase in size and bulk. This could be particularly useful for very small (~10–12 kDa) high-affinity, anti-tumor antibody fragments or binding proteins, where the difference in penetrability between ca. 10 kDa and 25 kDa has been shown to be very significant (Schmidt and Wittrup, 2009).

12.5.8 Albumin fusion

Similar to huIgGs, HSA (66.5 kDa protein) has a long average half-life of about 19 d (Peters, 1985; Strohl and O'Neil, 2012) (see also Figure 7.2). Moreover, HSA is the most abundant protein in human serum (~50 mg/mL; ~600 μ M), where it functions to help maintain plasma pH, metabolite and fatty acid transport, and consistent blood pressure. HSA is strongly anionic and is at the upper limit of size for filtration via kidney glomerulae, a combination of features that help to retard its filtration via the kidney (Caliceti and Veronese, 2003). HSA also accumulates in tumors and at sites of inflammation, so using HSA as a carrier might help to target antibody fragments to those sites (Kratz, 2008). Finally, HSA binds FcRn in a pH-dependent manner similar to antibodies, albeit at a different site and via a different mechanism to IgG binding (Chaudhury et al., 2003; Chaudhury et al., 2006), and is efficiently recycled in a manner similar to that of IgGs resulting in an extended half-life (Chaudhury et al., 2003, 2006; Roopenian and Akilesh, 2007).

Chemical conjugation of a FAb to albumin Cys34 was demonstrated to increase the AUC by 17-fold over that of the the FAb-Cys alone (Smith et al., 2001) which, along with the work of Dennis et al. (2002), helped to lay the foundation for coupling small proteins to albumin to increase their half-life.

Human Genome Sciences (HGS) have built a pipeline of peptide and small proteins genetically fused to HSA to extend the half-life of those molecules (Subramanian et al., 2007). An HSA-interferon- α -2b fusion (Albugon; GSK 716155), has been taken into Phase III clinical trials, validating this approach. HGS is also developing albiglutide (SyncriaTM), an HSA-fused glucagon-like peptide-1 (GLP-1) peptide in Phase III clinical trials for the treatment of type 2 diabetes mellitus, as well as an HSA-interferon α 2b fusion (ZALBINTM) for the treatment of chronic hepatitis C,

an HSA-granulocyte colony-stimulating factor (G-CSF) (AlbugraninTM) for neutropenia, an HSA-insulin fusion (AlbulinTM) for diabetes, and an HSA- β -type natriuretic peptide fusion (CardevaTM) for chronic heart failure, further demonstrating the potential breadth of this approach to half-life extension of peptides or small proteins. Merrimack Pharmaceuticals, Inc., has developed a bispecific HSA-fusion antibody, MM-111, which is comprised of a modified HSA fused with an anti-ErbB3 scFv at one end and an anti-ErbB2 scFv at the other. MM-111 is currently in Phase II clinical trials for the treatment of breast cancer. Similar to Fc fusions, an HSA-antibody fragment fusion would result in a relatively large protein (~80–100 kDa), which would likely limit its tissue distribution.

12.5.9 Fc fusion

The use of Fc fusions with antibody fragments is both well known and has been widely utilized. In any case, an scFv, domain antibody (or Nanobody[®] or Ig_{NAR}), or a micro-antibody can be fused to either the N-terminal sequence or C-terminal sequence (or both) of a human IgG Fc. Trubion's small modular immunopharmaceuticals (SMIPs) (Hayden-Ledbetter et al., 2009) are typical examples. Further details about these types of Fc fusion proteins (FcFPs) in general can be found in Chapter 14.

Multiple antibody and multi-specificity approaches

Abstract: One strategy to potentially improve the therapeutic index of antibodies is to engage multiple targets simultaneously, thereby using polypharmacology as a mechanism to better treat a disease. The first antibody treatment ever used, serum therapy, used this approach in a crude manner. Intravenous immunoglobulin (IVIG), which uses purified serum IgGs from donors, is an improvement to the serum therapy approach in wide use today. Additionally, there are now at least nine clinical candidates in which the therapeutic consists of multiple IgGs. Several antibodies have been demonstrated to engage multiple targets using a single combining site, perhaps the most interesting of which is ustekinumab (Stelara®), which targets the p40 subunit shared by IL-12 and IL-23. Finally, while a wide variety of bispecific IgGs or antibody fragments containing multiple combining sites have been tested since the early 1980s, only recently have the bispecific technologies been good enough to result in clinical candidates that have a significant chance of being marketed. The most advanced of these is blinatumomab (AMG-103) (CD19 x CD3 BiTE), which is currently in Phase II clinical trials for the treatment of acute lymphoblastic leukemia (ALL).

Keywords: intravenous immunoglobulin (IVIG), multi-antibody approaches, bispecific IgGs, bivalent, tetravalent, polypharmacology, bispecific antibody fragments, diabodies, TandAbs, DARTs

13.1 Introduction

Using multiple therapeutics simultaneously has been of interest to the pharmaceutical industry and healthcare professionals for several years (Zimmermann et al., 2007). HAART (highly active antiretroviral therapy) treatment, defined as the combination of three or more different drugs (e.g. two nucleoside reverse transcriptase inhibitors (NRTIs) and a protease

inhibitor (PI), two NRTIs and a non-nucleoside reverse transcriptase inhibitor (NNRTI), etc.), of AIDS, for example, is now the standard of care. A variety of small molecule medications are formulated into single vials, including as a few examples: Vytorin[®], a combination of the cholesterol-lowering drug simvastatin and the cholesterol uptake inhibitor ezetimibe; Lotrel[®], an antihypertension drug consisting of the calcium channel antagonist amlodipine and the ACE inhibitor benazepril; and Advair[®], a combination of the glucocorticoid receptor agonist fluticasone and the β_2 -adrenergic drug salmeterol (Zimmermann et al., 2007). Similar interest extends to the use of antibodies as therapeutics, in which there is significant interest in developing multiple antibodies together in a single drug and in designing multi-targeting antibodies.

In this chapter, we will explore the concept of using multiple antibodies or multiple combining sites on a single antibody to address multiple epitopes, whether they reside on a single antigen or represent multiple antigens. As mentioned in Chapter 1, the field of therapeutic antibodies originated with the use of serum therapy in the late stages of the nineteenth century to treat bacterial diseases (Behring and Kitasato, 1890). This approach also was the first use of multiple antibodies, i.e. serum antibodies, to treat bacterial diseases. The second form of multi-epitope targeting to treat human diseases came in the form of human-to-human serum therapy, also known as “IVIG,” for intravenous immunoglobulin, therapy. There are a few antibody combinations in early clinical trials in which specific antibodies derived against different antigens have been combined to make a single product. Finally, most recently, companies have begun to explore the concept of deriving specific, engineered antibodies to multiple targets or multiple epitopes on a single target and producing those engineered antibodies in a single bioreactor (Rasmussen et al., 2007). These approaches all have the same common theme of targeting multiple epitopes or targets using multiple individual antibodies, ranging from the very crude, unpurified forms in which no specific components are known definitely to highly purified, recombinant forms in which every component is known and characterized. An alternative way to achieve the same goal is to include multiple combining sites into a single antibody or antibody-like molecule, a strategy that has been envisioned and worked on with limited success over the last ~30 years. We are now finally to the point where bona fide bispecific antibodies are poised to make a difference clinically and on the market (Peipp and Valerius, 2002; Kufer et al., 2004; Marvin and Zhu, 2005; Morrison, 2007; Cuesta et al., 2010; Chan and Carter, 2010; Kontermann, 2012).

13.2 Serum therapy

The original polyclonal therapeutic approach was serum therapy, which was first developed by Emil von Behring in the late nineteenth century for the treatment of anthrax and diphtheria (Behring and Kitasato, 1890; Strohl, 2009a). The serum used in the early stages of serum therapy was derived from immunized, non-human sources (e.g. horse, rabbits), and was not purified. Thus the sera used to dose patients contained not only the therapeutic antibodies but also many foreign proteins. This led to “serum sickness,” an immune-mediated response to the foreign proteins, in essentially 100% of patients treated with serum therapy (Lawley et al., 1984; Gronski et al., 1991). Serum therapy was used for a wide variety of diseases (diphtheria, streptococcal pneumonia, meningitis, tularemia, shigella dysentery, brucellosis, gas gangrene, tetanus, botulism, anthrax, whooping cough, measles, poliomyelitis, mumps, and chickenpox; Casadevall and Scharff, 1995) until just prior to World War II. Of those, serum therapy was reasonably successful in the treatment of diphtheria, meningitis, and pneumonia. Before the outbreak of World War II and the commercialization of penicillin and other antibiotics, about 86% of patients at Boston City Hospital diagnosed with type I streptococcal pneumonia were treated using type-specific serum therapy (Casadevall and Scharff, 1995). Serum therapy, however, was demonstrated not to be successful for the treatment of anthrax, whooping cough, or shigella-related dysentery (Casadevall and Scharff, 1995). The most important limitation of serum therapy was the serum toxicity, and many attempts by Behring and others were made to separate the “antitoxins” from other serum contaminants (Weil et al., 1938; Gronski et al., 1991). While delivering some successes, serum therapy never truly got past its most significant problematic issues, including not only serum sickness, but also batch-to-batch variation, variable pharmacokinetics, and intravenous administration (Casadevall, 1996).

13.3 IVIG

The most obvious potential improvements for serum therapy are the use of human serum, rather than heterologous serum derived from livestock animals, and fractionation of the human serum into an IgG enriched fraction to limit serum sickness. The first example of this approach occurred in 1952, when a patient with primary immunodeficiency was given intramuscular (IM) injections of purified human IgG (Bruton, 1952).

Unfortunately, the IM route of delivery limited the doses, resulting in suboptimal IgG replenishment and marginal clinical benefit.

Improvement in this approach was brought about by the development of methods for the delivery of fractionated IgG via intravenous administration (“IVIG,” also known as IGIV in the US). Early attempts at IVIG therapy were plagued with immunological reactions thought to be due to complement activation (Weiler, 2004). Improved purification methods ultimately removed the cause of this immune reaction, although it still took until the 1980s for widespread use of IVIG to treat primary antibody deficiencies (Mouthon and Lortholary, 2003).

IVIG comes in two forms, “normal immunoglobulins” and “specific immunoglobulins.” Normal immunoglobulin, also known as IVIG or “gamma globulin” treatment, is a term used for IgG obtained from a large pool of random donors. IVIG treatment was initially used as a replacement therapy for patients unable to generate their own immunoglobulins (Farrugia and Poulis, 2001; Orange et al., 2006). IVIG has also been shown to provide up to six weeks of protection against pathogens such as hepatitis A, measles, mumps, and other viral diseases that are relatively widespread in populations (Llewelyn et al., 1992).

In an ironic twist, although IVIG historically comes directly out of the use of serum therapy for infectious diseases, the most significant use for IVIG today is for humoral immunodeficiency (i.e. low IgG levels) rather than for infectious diseases. As of 2010, a total of 13 products have been approved by the FDA, including 12 for primary or humoral immunodeficiency, eight for idiopathic thrombocytopenic purpura (ITP), three for Kawasaki syndrome, and one each for infections associated with hypogammaglobulinemia or B-cell chronic lymphocytic leukemia, pediatric HIV infection, and hematopoietic stem cell (bone marrow) transplantation (Weiler, 2004; Orange et al., 2006) (see Table 13.1). IVIG also has been used off-label for a variety of conditions including aplastic anemia, pure red cell aplasia, autoimmune hemolytic anemia, immune-mediated neutropenia, and multiple sclerosis, as well as many other immune-mediated disorders. Additionally, several uses for IVIG have been proposed, including re-expanding the use of IVIG for infectious diseases (Wallington, 2004; Casadevall and Scharff, 2005), protection from potential biological warfare agents (Casadevall, 2002), prevention and therapy of Alzheimer’s disease (Dodel et al., 2010), and treatment of neurological disorders (Orange et al., 2006).

The preparations of IVIG noted in Table 13.1 differ in several ways, including the presence of IgA in some preparations, the buffers used, and the content of sugar. Thus, while the IgG component may be similar, different formulations may be better for some individuals than others.

Table 13.1 Approved IVIG products and indications in the United States

FDA approved indication	IVIG products approved for indication*
Primary humoral immunodeficiency	Gammagard liquid (BHC); Iivegam E/N (BHC); Polygam S/D (BHC); Carimmune NF (CSL); Gammar-P.I.V. (CSL); Panglobin NF (CSL); Privigen (CSL); Flebogamma DIF 5% (GBI); Venoglobulin-S (GBI); Octagam (OP); Gamimune N (TBI); Gamunex (TBI); Gamunex-C (TBI)
Immune mediated thrombocytopenia purpura	Gammagard S/D (BHC); Polygam S/D (BHC); Carimmune NF (CSL); Panglobin NF (CSL); Privigen (CSL); Venoglobulin-S (GBI); Gamimune N (TBI); Gamunex (TBI)
Kawasaki syndrome	Gammagard S/D (BHC); Iivegam E/N (BHC); Venoglobulin-S (GBI)
Prevention of bacterial infections associated with hypogammaglobulinemia and/or B-cell chronic lymphocytic leukemia	Gammagard S/D (BHC)
Hematopoietic stem cell transplantation	Gamimune N (TBI)
Pediatric HIV	Gamimune N (TBI)
Chronic inflammatory demyelinating polyneuropathy (CIDP)	Gamunex (TBI)

* *Guide to companies:* BHC: Baxter Healthcare Corporation; CSL: CSL Behring AG; GBI: Grifols Biologics, Inc.; OP: Octapharma; TBI: Talecris Biotherapeutics, Inc.

Over the past half-dozen years, there has been significant discussion concerning the mechanism of action for IVIG therapeutics (Samuelsson et al., 2001; Kaneko et al., 2006; Nimmerjahn and Ravetch, 2007). Ravetch, Nimmerjahn and their colleagues have recently demonstrated that the presence of terminal α -2,6-linked sialic acid on the oligoglycan attached to N297 in the Fc of IgG was responsible for the anti-inflammatory activity of high-dose IVIG (Nimmerjahn and Ravetch, 2007; Anthony et al., 2008a, 2008b; Kaneko et al., 2009; Anthony and Ravetch, 2010). In support of that hypothesis, Scallan et al. (2007b) have shown that increased sialylation of three different antibodies resulted in decreased ADCC by reducing the affinity of the antibodies to both Fc γ RIIIa and their target. As described in Chapter 11, it appears, based on a variety of model systems, that the high-dose sialylated IgG binds to the receptor

DC-SIGN, which in turn stimulates the production of IL-33, IL-4-producing basophils, and upregulation of the inhibitory Fcγ receptor FcγRIIb, resulting in the immunosuppressive effect (Anthony et al., 2011). Just recently, an alternative mechanism of action has been proposed for the immunosuppressive effects of IVIG. It was demonstrated that sialylated, but not non-sialylated, IgG bound to CD22, a B cell surface receptor, and downregulated the activation, proliferation, and function of B cells, thereby yielding an immunosuppressive effect (Séité et al., 2010). Which of these mechanisms linked with the immunosuppressive effects of IVIG treatment pre-clinically is actually functional in humans has not yet been fully established, although the immunosuppressive effect of IVIG itself has been demonstrated broadly.

IVIG doses tend to be very high, typically with a loading dose of 2 g/kg given in a 4–5 day period, followed by 600 mg/kg monthly (Orange et al., 2006; <http://www.cidpusa.org/ivig.html>). Thus a six-month regimen of IVIG treatment for a 70 kg individual may require as much as ~400 g of IVIG at a going cost of \$48–60/g, giving a six-month therapy cost of ~\$20,000–\$24,000. Including office visit costs, the cost of a single IVIG infusion can reach \$3,000 for a child and \$10,000 for adults. The high cost of IVIG is related to how it is produced. IVIG is obtained from paid plasma donors; it is then pooled, processed, purified, and filtered to remove viruses, and then freeze dried (<http://www.cidpusa.org/ivig.html>). Also, because IVIG is obtained from healthy donors, donation rate can vary, leading to periods in which there are shortages of qualified IVIG products available for patients. For example, there was a severe shortage of IVIG products in the 1997–98 timeframe, which resulted in additional regulation of the industry (Farrugia and Poulis, 2001).

Efforts have been made over the past five years to move to subcutaneous self-administration of Ig by patients with primary immunodeficiency diseases as a way to increase convenience and decrease doctor's office visits (Ochs et al., 2006). Considerable progress has been made recently in this approach. In July 2011, the FDA approved subcutaneous dosing of Baxter's GAMMAGUARD LIQUID 10% for treatment of primary immunodeficiency with the goal of once-weekly dosing by patient self-administration. Additionally, Baxter has set up a collaboration with the formulation and delivery company, Halozyme, to deliver IVIG subcutaneously in conjunction with human hyaluronidase, an enzyme that promotes adsorption and dispersion of protein therapeutics. They currently have a Phase III clinical trial studying the efficacy in primary immunodeficiency of the investigational IVIG treatment called HyQ, a mixture of IVIG and hyaluronidase delivered subcutaneously.

As differentiated from “normal immunoglobulin” or IVIG, “specific immunoglobulins” are obtained from vaccinated volunteers or from convalescent donors. One example of a specific immunoglobulin is the

passive immunization of human anti-rabies IgG to naïve patients bitten by animals suspected of or confirmed as being carriers of rabies. The anti-rabies IgG is generated by immunized human donors and provided to bite victims to provide virus-neutralizing antibodies until the patient can produce their own antibodies in response to concomitant vaccination. As of 2010, two anti-rabies IgG formulations (Imogam® Rabies-HT (Sanofi-Pasteur) and HyperRab™ S/D (Talecris Biotherapeutics)) are licensed for use in the United States. Another example of a specific immunoglobulin is anti-rhesus D-specific Ig, which is obtained from healthy volunteers specifically vaccinated with the RhD antigen (Llewelyn et al., 1992).

IVIG therapy led directly to the development of the anti-RSV monoclonal antibody, palivizumab (Synagis®). The general concept of a specific IVIG also has led to the development of a multi-antibody strategy, which is similar in principle to specific immunoglobulin, but significantly more refined and sophisticated. This approach, discussed in more detail in the next section, has been promoted by Symphogen, a biotechnology company in Denmark.

13.4 Multi-antibody approaches

The efficacy of specific IVIG therapy depends on a pool of antibodies derived from the serum from vaccinated individuals that possess the desired functional characteristics. The next step towards refinement of this approach would be to develop a specific pool of highly characterized monoclonal antibodies that would, as a pool, possess the desired characteristics. In theory, this pool could contain as few as two MABs of different structure and specificity to as many as dozens of MABs, each with different structure and specificity. This general approach of developing multiple antibodies for a single therapeutic indication is now gaining a significant following. There are now at least nine separate examples of multiple MABs being developed for single indications as noted below. Many of these focus on infectious disease targets, which seems to be a reasonable first step for this type of approach based on the natural job of the immune system.

The most sophisticated company in this space appears to be Symphogen, which has developed technologies for cell culture of multiple monoclonal antibodies in a single cell culture system (Rasmussen et al., 2007). They have isolated the genes encoding anti-infective or other antibodies directly from human patients or subjects, determined their specificities, potencies and other characteristics, and then developed master cell banks for each individual MAB. Individual cell banks are then used to inoculate a cell culture containing the producing cell lines for many individual monoclonal

antibodies, e.g. a form of polyclonal-monoclonal production strategy (Rasmussen et al., 2007). This concept simulates the natural mechanism the body uses to fight foreign antigens or invading pathogens, while lacking the huge volume of non-specific antibodies that would be present in an IVIG preparation. This approach also should result in low doses based on the potential synergies obtained with MABs hitting multiple epitopes on a single antigen (Nowakowski et al., 2002). Symphogen currently has developed an anti-RhD product candidate containing 25 different MABs that have been through Phase I clinical trials (Wiberg et al., 2006). Excelimmune is a second biotechnology company taking this approach of combining multiple monoclonal antibodies into a polyclonal product candidate.

Symphogen also has taken a more conservative approach to the multi-antibody strategy. They have developed Sym004, which consists of a pair of recombinant MABs targeting non-overlapping epitopes on EGFR. Sym004 is currently in Phase I/IIa clinical trials for treatment of cancer patients with EGFR-positive solid tumors. Pre-clinical data have suggested that Sym004 has superior anti-cancer efficacy over marketed single anti-EGFR antibodies *in vivo* in several tumor xenograft models. The mechanism of action, which includes very rapid internalization and degradation of the receptor, appears to be distinct from that of the marketed MABs. Similarly, Merrimack Pharmaceuticals announced recently that they were initiating Phase I clinical trials with a combination product, MM-151, consisting of three MABs binding non-overlapping epitopes on EGFR, discovered for them by Adimab.

Rabies is a severe disease that results in 55,000 deaths annually, about half of which are in the Far East (Bakker et al., 2008). Currently, the only post-exposure therapeutic approaches to rabies are administration of either equine immunoglobulin or human IVIG, but the availability, efficacy, and non-specificity of these approaches make them poor therapeutic approaches. Crucell currently has a pair of anti-rabies virus MABs in Phase II clinical trials that could replace the IVIG therapeutic approaches with more defined, better controlled, and more widely available MABs. These MABs were originally obtained from phage display libraries constructed from peripheral lymphocytes from individuals immunized with the Merieux Imovax rabies vaccine (Kramer et al., 2005). A large panel of potent, neutralizing antibodies was obtained from the library, from which pairs of non-competing anti-rabies virus glycoprotein MABs, CR57 (human IgG₁λ) and CR4098 (human IgG₁κ) (Bakker et al., 2008) were picked for clinical commercial development as the combination product CL184 (Goudsmit et al., 2006).

Similarly, Medarex generated a pair of fully human antibodies using their HuMAB mice against each of the A and B toxins produced by *Clostridium difficile* (Lowy et al., 2010). This MAB combination is

comprised of MDX-066 (also known as MBL CDA-1) and MDX-1388 (also known as MBL CDB-1), which target *C. difficile* toxins A and B, respectively. In Phase II clinical trials, IV administration of the MDX-066–MDX-1388 MAb pair reduced the rate of recurrence of the infection by 70%. Merck is developing these antibodies clinically in collaboration with Medarex. In a similar approach, Thallion and LFB are developing a pair of antibodies against *E. coli* shigatoxins 1 (Stx1) and 2 (Stx2) (Bitzan et al., 2009); these antibodies are currently in Phase II clinical trials.

In another example of multiple MAbs in a single product, IQ-DAA, a combination of two human MAbs, is currently in Phase I clinical trials for the treatment of inhalation anthrax. IQ-DAA, which stands for IQ Therapeutics' "dual antibody approach," combines MAb IQNPA, which targets the protective antigen part of *Bacillus anthracis* toxin, while IGNLF targets the lethal factor. Similarly, Xoma is developing the drug, XOMA 3AB, which is a combination of three IgG antibodies (affinity-matured versions of S25, C25, and 3D12; Levy et al., 2007) in a single product for prevention and treatment of botulism poisoning caused by exposure to *Clostridium botulinum* neurotoxin Type A. XOMA 3AB is under development in collaboration with the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

Finally, a clinical candidate called "Combotox" containing two separate ricin-conjugated murine IgG antibodies targeting CD22 (RFB4) and CD19 (HD37) has been studied in Phase I clinical trials for treatment of refractory B cell lymphoma (Messmann et al., 2000) and acute lymphoblastic leukemia (Schindler et al., 2011). These toxin conjugates target B cells with both members of the combination.

While most of these examples of multiple MAbs comprising a single "drug" are targeted towards infectious disease agents, they set a precedent that may one day be followed by targeting multiple MAbs against human targets. Examples where this already has been seen include the cancer treatment candidates Sym004 and MM-151 which target EGFR, and Combotox, which targets B cells. Alternatively, we also may eventually see additional MAbs in a single vial that target different molecules, e.g. MAbs targeting EGFR and HER2. As one example of this, Tang and Zhao (2011) have recently hypothesized that the treatment of ovarian cancer may in fact require multiple MAbs targeting different receptors to achieve the potency needed to obtain efficacy. In a second example, it was recently demonstrated that despite HAART therapy, HIV can continue to replicate and spread cell to cell, with the daunting possibility that multiple infections of a recipient cell could promote resistance of the HIV particles within that cell (Sigal et al., 2011). Coincidentally, Walker et al. (2011a) demonstrated that the use of multiple, newly isolated, highly potent anti-HIV

antibodies could provide neutralization against a broad series of HIV strains, which might be used in conjunction with HAART therapy to kill viruses being spread from cell to cell, perhaps decreasing resistance to HAART therapy.

13.5 Bispecific antibodies based on IgGs

13.5.1 Introduction

An alternative to multiple antibodies in a single product is the generation of a single molecule containing multiple, independent binding activities, hence the term “bispecific antibodies.” The concept of bispecific antibodies has been around for nearly 30 years (Milstein and Cuello, 1983), and has taken on many different forms and formats. Milstein and his colleagues figured out how to make bispecific IgGs using hybrid-hybridoma technology, which they first used to simplify immunoassays (Milstein and Cuello, 1983; Suresh et al. 1986). The first wave of significant bispecific antibodies, mostly derived using the hybrid hybridoma approach, that were constructed with therapeutic mechanisms in mind were T cell (or other immune cell) engagement antibodies, in which one FAb arm targeted CD3 an Fc γ R, or other immune cell components, and the other arm bound to an epitope on the target cell (Clark and Waldmann, 1987; Gilliland et al., 1988). The concept was to bring cytotoxic T cells, or other immune cells such as NK cells, into contact with target cells such as cancer cells to promote lysis and elimination of the target cells (Clark and Waldmann, 1987). One of the first recombinant methods for producing bispecific antibodies involved the use of retroviral-derived shuttle vectors to generate a recombinant hybridoma cell line producing two MAbs (De Monte et al., 1990). In all of these early approaches, the key issue was the need to purify the desired bispecific antibody, containing the right light chain specificities, from all of the other products including the parental antibodies and mixed forms containing the wrong light chain combinations (Milstein and Cuello, 1983; Suresh et al., 1986; De Monte et al., 1990).

In terms of therapeutic use, bispecific antibodies have not really yet found their niche, although this seems to be changing quickly with several biopharmaceutical companies investing heavily in bispecific antibody technology. It is expected that bispecific antibodies will play a significant therapeutic role in the next decade or so. In late 2011 and early 2012, we are already starting to see a significant trend toward multi-specific antibodies, with at least a half-dozen new bispecific antibodies entering clinical trials over the past year. Additionally, there have been several new and exciting approaches for making multi-specific antibodies (Schaefer et al., 2011; Gu and Ghayur, 2012; Hess et al., 2012; Kontermann, 2012; Strop et al., 2012).

One significant issue that all drugs face is the window that separates minimum effective dose (MED) and maximum tolerated dose (MTD). When two small molecule drugs are incorporated into a single pill, or when two combining regions are included in the same antibody protein, the window between MED and MTD becomes dictated by two mechanisms of action instead of one, and potentially shrinks in size (Figure 13.1). With the generation and testing of new antibodies with multi-specificity and multi-functionality, more sophisticated approaches for understanding the polypharmacology they bring with them will need to be developed.

In the following sections, we will describe some of the formats used to make bispecific-bivalent IgGs, bispecific-tetravalent IgGs, and a variety of bispecific antibody fragments. A PubMed search of the words “bispecific antibody” made in July 2012 turned up 1,771 references, indicating that this is a very active area of research. Several excellent reviews have been written on bispecific antibodies (see Peipp and Valerius, 2002; Kufer et al., 2004; Marvin and Zhu, 2005; Morrison, 2007; Cuesta et al., 2010; Chan and Carter, 2010; Gu and Ghayur, 2012; Hess et al., 2012; Kontermann, 2012). Chemical coupling approaches for making bispecific antibodies (e.g. Brennan et al., 1985; Garrido et al., 1990) will not be included in this discussion.

13.5.2 Bispecific activities with single combining (or binding) sites

While the concept of “bispecific antibody” typically conjures up the image of two separate and distinct combining sites, that is not always the case.

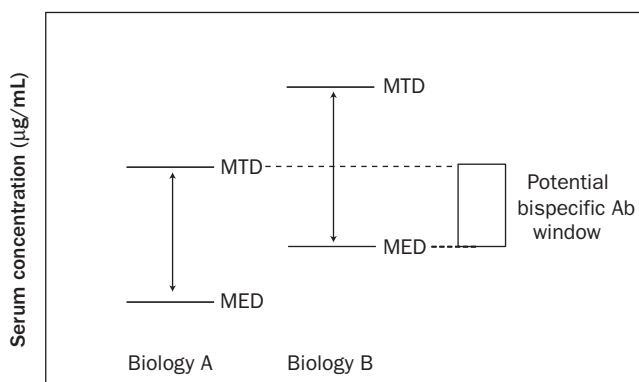


Figure 13.1 Minimum effective dose (MED) vs maximum tolerated dose (MTD) for bispecifics

Considering that a bispecific antibody has two targeted biologies, the combination of the MED and MTD for each targeted biology may shrink the window for effective dosing without toxicity.

In fact, interestingly enough, the first recombinant antibody ever to be approved for marketing, the FAb abciximab (ReoPro®), was also the first bispecific (FAb) antibody, since it binds to both the gpIIb/IIIa receptor on platelets and the vitronectin receptor on platelets and endothelial cells (Reininger et al., 2000). This bispecificity was not intended, however, since abciximab was developed prior to the discovery of the vitronectin receptor. Both the gpIIb/IIIa and vitronectin receptors are heterodimeric proteins and share a common subunit. Similarly, the recently approved antibody ustekinumab (Stelara®) binds to both IL-12 and IL-23 because it targets the p40 subunit that is shared by these two different cytokines (Benson et al., 2011). As described for abciximab, when ustekinumab was first discovered, neither IL-23 nor the p40 subunit of IL-12 were known, so the antibody was made to block IL-12 without knowledge of the epitope. Only later when IL-23 was discovered, and it was demonstrated that ustekinumab bound the p40 subunit shared by IL-12 and IL-23, did it become apparent that the antibody had bispecific properties (Benson et al., 2011). These are examples of cases in which multiple proteins with distinct physiological functions may share a subunit, and targeting of that subunit ultimately provides multi-specificity and some level of polypharmacology.

In a more directed effort, Dobson et al. (2009) screened an scFv phage human antibody library for antibodies that specifically cross-reacted to TRAIL-R1 and TRAIL-R2. They obtained antibodies specific to each of those related receptors, as well as a pool of 134 antibodies that cross reacted with both receptors, demonstrating that with proper screening, antibodies with single combining domains may be selected to bind to multiple, albeit related targets (Dobson et al., 2009). A second recent example of selecting antibodies with single combining sites recognizing multiple related proteins was demonstrated by Garcia-Rodriguez et al. (2011), who showed that antibodies 1B18 and 4E17 could recognize multiple related *Clostridium botulinum* neurotoxins both in their originally identified form, as well as after affinity optimization. MAb 1B18 recognized botulinum neurotoxins (BoNT) A and B, while 4E17 recognized BoNT A, B, E, and F, even after substantial affinity optimization (Garcia-Rodriguez et al., 2011).

A novel approach for the generation of a “natural IgG”-like bispecific antibody with a single combining site capable of binding both HER2 and VEGF was recently described by Bostrom et al. (2009). Noting that the binding of the anti-HER2 MAb, trastuzumab, to HER2 was primarily dictated by the V_H chain, they generated a library of LC variants that were screened for their ability to bind to VEGF while maintaining their ability to bind to HER2. The clone with the highest dual affinity, bH1, had binding affinities (K_Ds) of 26 nM and 300 nM for HER2 and VEGF, respectively (Bostrom

et al., 2009). Structures of each of the FAb-target complexes demonstrated that the paratopes overlapped and that CDR-L1 had two different conformations depending on which antigen was bound (Bostrom et al., 2009). The conformations of the other V_H and V_L CDRs were similar with either antigen. More recently, Schaefer et al. (2011a) have described using these same principles to generate an anti-HER3, anti-EGFR dual targeting antibody. This antibody, MEHD7945A, has recently entered Phase I clinical trials.

Additionally, two marketed Fc fusion proteins share the property of multi-specificity due to the binding proteins used in their construction. Due to the incorporation of p75 as the binding domain, etanercept (Enbrel®) binds to both TNF- α and TNF- β , a factor that differentiates it from infliximab (Remicade®), adalimumab (Humira®), golimumab (Simponi®), and certolizumab pegol (Cimzia®), all of which bind only TNF- α (Tracey et al., 2008). Abatacept (Orencia®), which is comprised of CTLA4 exodomain fused to the Fc of an altered IgG, also binds two targets, the receptors CD80 and CD86, and blocks their ability to interact with CD28 on T cells (Korhonen and Moilanen, 2009).

13.5.3 *Bispecific, bivalent IgG antibodies*

The first bispecific, bivalent IgG antibodies were made using the hybrid hybridoma approach or variations on that theme (Milstein and Cuello, 1983; Suresh et al. 1986; Clark and Waldmann, 1987; Gilliland et al., 1988; De Monte et al., 1990). Bispecific IgG antibodies can be made in various ways, including various manipulations or “recombinations” of two IgG molecules resulting in a series of IgGs, including the parental molecules, the desired bispecific and many heavy and light chain assortments in between. Suresh et al. (1986) pointed out that the generation of hybrid hybridomas resulted in ten possible different rearrangements from the pair of IgGs, only one of which would be the desired molecule. Thus this approach put a lot of emphasis on purification of the correct form, with significant waste of mismatched molecules in the process.

A variation on the hybrid hybridoma theme was used to generate the first rationally designed bispecific antibody to be approved for marketing, catumaxomab (Removab®), an anti-CD3-anti-EpCAM mouse IgG_{2a}-rat IgG_{2b} hybrid IgG (Lindhofer et al., 1995; Zeidler et al., 1999; Seimet et al., 2010; Hess et al., 2012) (see Figure 13.2 and Table 13.2).

To construct that MAB, a mouse-rat quadroma called BiUII was generated that produced the mouse-rat hybrid antibody as well as each of the parental antibodies. The use of MAbs originating from different species

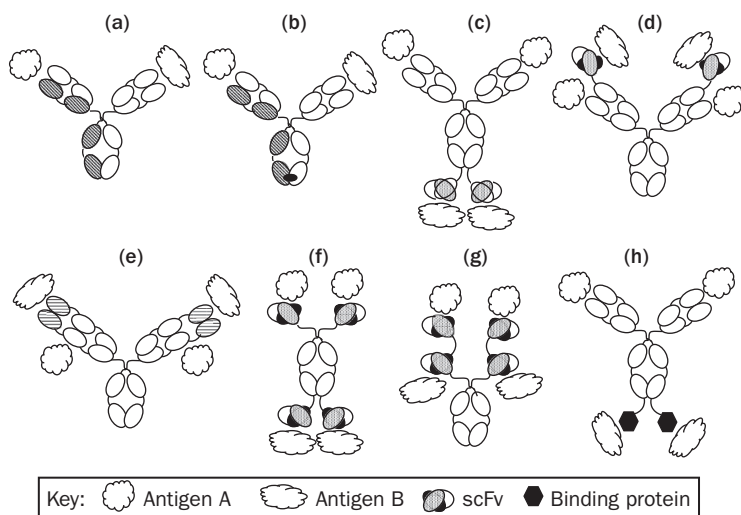


Figure 13.2 Examples of bispecific IgG-like molecules

(a) Bivalent, bispecific IgG based on hybrid hybridoma approach. In this case, the desired correct sequence must be isolated from a pool of ten different possible combinations. (b) Bivalent bispecific IgG based on the “knobs-into-holes” approach (Ridgeway et al., 1996; Merchant et al., 1988; Carter, 2001) or the equivalent molecule produced using an electrostatic steering approach (Gunasekaran et al., 2010). In either case, light chain pairing (or mismatching) results in multiple candidates from which the properly paired molecule must be purified. (c) and (d) Tetravalent, bispecific IgG-scFv fusion molecules with the scFv on the C-terminus of the heavy chain and N-terminus of the light chain, respectively (e.g. Jordan et al., 2009; Michaelson et al., 2009; Miller et al., 2010; Dong et al., 2011a, 2011b; Orcutt et al., 2010; Schantzer et al., 2011). (e) Bivalent, bispecific dual-variable domain (DVD)-Ig (Wu et al., 2007a; Gu and Ghayur, 2012). (f) Tetravalent, bispecific Scorpion construct, which is built as: N-scFv1-linker- C_H2-C_H3 -linker-scFv2 (http://www.emergentbiosolutions.com/?q=node/47&quicktabs_technologies=1#quicktabs-technologies). (g) Tetravalent, bispecific dual scFv construct, which is built as: N-scFv1-linker-scFv2-linker- C_H2-C_H3 . (h) Tetravalent, bispecific IgG-alternative scaffold fusion (e.g. Wittrup et al., 2011).

as the parental molecules resulted in species-restricted heavy/light chain pairing (Lindhofer et al., 1995; Zeidler et al., 1999; Hess et al., 2012). Thus the bispecific needed only to be purified from the parental mouse and rat antibodies, making both purification and subsequent analysis much simpler than the outcomes of standard hybrid hybridoma approaches (Lindhofer et al., 1995). The resultant rat-mouse hybrid IgG is called trifunctional, due to its retained Fc activities (Zeidler et al., 1999; Hess et al., 2012) as well as its demonstrated T cell-mediated tumor cell lysis activities (Schmitt et al., 2004). Hess et al. (2012) have suggested that the combination of T cell engagement, via binding CD3 with one arm and the targeted cell with the other, and potent Fc functionality can link the innate and adaptive immune systems in such a way as to make the most potent possible multi-functional antibodies. This potency, however, brings with it the potential for cytokine

Table 13.2 Examples of technologies for bispecific antibodies based on IgGs

Technology	Brief description	Format	Reference
Hybrid hybridoma	Fusion of two hybridomas; yields 10 possible variants from which desired bispecific must be purified	BS, BV, normal Fc	Milstein and Cuello (1983); Suresh et al. (1986)
Triomab	Rat IgG _{2b} -mouse IgG _{2a} hybrid antibody produced by quadroma	BS, BV, hybrid Fc	Lindhofer et al. (1995); Zeidler et al. (1999)
Overlapping paratopes	Mutation of the combining site of anti-HER2 MAb so that it also binds EGFR, mostly by residues contributed by the light chain	BS, BV, normal Fc	Bostrom et al. (2009)
Knobs-into-holes	Mutations in C _H 3 domains resulting in a knob on one side (larger amino acid residue) and a hole (smaller residue) on the other that generate a novel site favoring heterodimeric Fc formation; light chain mispairing is a potential issue	BS, BV, modified hybrid Fc	Ridgeway et al. (1996); Merchant et al. (1988); Carter (2001)
Cross-MAb	Exchange of C _H 1 and C _L domains of one half of the antibody to eliminate light chain mispairing; can be used with knobs-into-holes or electrostatic steering to eliminate light chain pairing issue	BS, BV, modified hybrid Fc and modified FAb	Schaefer et al. (2011b)
Electrostatic steering	Mutations in charged amino acid residues in C _H 3 domain resulting in preferential heterodimerization; light chain mispairing is a potential issue	BS, BV, modified hybrid Fc	Gunasekaran et al. (2010)
Strand-exchange engineered domain (SEED)	Incorporation of IgA sequences into IgG C _H 3 domain to generate asymmetric but complementary pairs, AG and GA, favoring heterodimeric Fc formation	BS, BV, modified hybrid Fc	Davis et al. (2010); Muda et al. (2011)
DVD-Ig	An extra Fv added to the N-terminus of the FAb with linkers to both heavy and light chains	BS, TV, normal Fc	Wu et al. (2007a); Gu and Ghayur (2012)
IgG fused with scFv	Stabilized scFvs targeting a second epitope or protein fused to the N-terminus of the heavy chain or C-terminus of the heavy or light chain	BS, TV, normal Fc	Jordan et al. (2009); Michaelson et al. (2009); Miller et al. (2010); Dong et al. (2011a, 2011b); Orcutt et al. (2010); Schantzer et al. (2011)

Table 13.2 Examples of technologies for bispecific antibodies based on IgGs (*cont'd*)

Technology	Brief description	Format	Reference
IgG fused with domain antibody	Single domain targeting a second epitope or protein fused with either the C- or N-terminus of an IgG	BS, TV, normal Fc	Shen et al. (2007)
Fc fused with diabody	Diabody recognizing two epitopes or proteins fused to Fc through natural hinge	BS, TV, normal Fc	Alt et al. (1999); Lu et al. (2005)
Surrobodyes	Incorporation of the pre-B pseudo light chain resulting in fusion capability to both the $\lambda 5$ and V_{preB} domains	BS, TV, normal Fc	Xu et al. (2008, 2010)
IgG-alternative scaffold fusion	Alternative scaffold such as FN3 domain, DARPin, or other non-antibody binding protein fused to IgG via a linker	BS, TV, normal Fc	Wittrup et al. (2011)
Scorpions™	scFvs linked to both the N- and C-terminus of each chain of a normal Fc	BS, TV, normal Fc	http://www.emergentbiosolutions.com/?q=node/47&quicktabs_technologies=1#quicktabs-technologies

Abbreviations: BS: bispecific; BV: bivalent; DVD: dual variable domain; FN3: fibronectin domain 3; TV: tetravalent.

release syndrome (CRS), so when used, this approach will have to be managed very carefully, perhaps through microdosing.

A variety of recombinant methods have also been used to restrict the number of variants obtained in bispecific, bivalent antibodies. The first and perhaps most well known approach was the “knobs-into-holes” approach developed by Paul Carter and his colleagues (Ridgeway et al., 1996; Merchant et al., 1998; Carter, 2001). In this design, changes were made to the C_H3 domain of two different antibodies, one (Y407T) which would form a hole due to the replacement of a large residue with a smaller one, and the other (T366'Y) which would form a knob that would fit into the hole (Ridgeway et al., 1996) (see Figure 13.2 and Table 13.2). This resulted in a high percentage of the resultant antibody being the correct bispecific. If two different light chains are used with this type of approach, however, light chain mispairing can still occur, creating the possibility for multiple different species and potentially low recovery (Carter, 2001). Thus, if the same light chain can be used for the two antibodies making up the bispecific antibody, the light chain pairing issue would be eliminated and the efficiency can be increased to virtually 100% (Merchant et al., 1998; Carter, 2001). The downside to utilization of the

common light chain design is that some antibodies simply may not function with the light chain required by the other half, potentially restricting the antibodies made by this approach. A potential improvement on the theme of the knobs-into-holes plus common light chain design is the domain crossover design, called “CrossMab,” recently described by Schaefer et al. (2011b). In this approach, antibodies designed with a knobs-into-holes Fc are also modified by switching out the C_H1 and C_L domains of one half of the antibody, resulting in one half with a normal heavy-light chain pairing, and the other half with the constructs of: V_H-C_L-hinge-C_H2-C_H3 and V_L-C_H1 (Schaefer et al., 2011b) (see Table 13.2). This construct prevents light chain mispairing because the C_H1 would not naturally pair with another C_H1 and thus prevents the need for identical light chains to be incorporated into each half of the pair. Antibodies constructed with this design behaved similarly to standard IgGs by all tests conducted (Schaefer et al., 2011b).

Several other variations on the knobs-into-holes theme were described by Carter (2001), including a disulfide approach (D399C/K392'C), a salt bridge approach (L368D, Y407F/T366'K), and knobs-into-holes combinations utilizing phage approaches. Jackman et al. (2010) recently used the knobs-and-holes design to develop an anti-FcεRI-FcγRIIb bispecific antibody that should completely inhibit IgE signaling through the high-affinity FcεRI receptor (Jackman et al., 2010). Jackman et al. (2010) used a two-step approach by using multiple light chains for initial screening followed by focusing on common light chains once candidates had been found. Another modification was that they carried out both the screening and production using *E. coli*, so the final bispecific product was non-glycosylated (Jackman et al., 2010).

Another example of an approach that somewhat mimics the knobs-into-holes design to achieve a bivalent, bispecific antibody is an electrostatic steering approach recently described by Gunasekaran et al. (2010). In their design, they modified one of the Fc halves in C_H3 by making a K409D mutation, and in the other Fc half a D339'K mutation, which resulted in electrostatic interactions favoring the hybrid molecule over either of the parental molecules (Gunasekaran et al., 2010) (see Table 13.2). To avoid the light chain problem, they fused scFvs binding to CD3 and TARTK, respectively, to the first (Fc) and second (Fc') mutated Fc halves (Gunasekaran et al., 2010). Another strategy to make bispecific, bivalent antibodies is the strand-exchange engineered domain (SEED) approach taken by scientists at EMD Serono Research Institute (Davis et al., 2010; Muda et al., 2011) (see Table 13.2). For the SEED approach, sequences from IgA were incorporated into the C_H3 domain of an IgG to generate asymmetric but complementary pairs, AG and GA, such that only the heterodimeric protein would bind and fold into an Fc (Davis et al., 2010; Muda et al., 2011).

The authors envision using this format to build bispecific antibodies, antibody-immunofusion pairs, and other bimodal types of binding proteins. An additional novel approach resulting in a bispecific, bivalent IgG is the new DuoBody™ approach used by GenMAB to make bispecific IgGs. While their technology appears to be derived from their previous research on FAb-arm exchange by IgG₄ molecules (Aalberse and Schuurman, 2002; van der Neut Kolshoten et al., 2007; Labrijn et al., 2009), the details are not yet available publicly (<http://www.genmab.com/research-and-technology/genmab-technology>). Finally, Strop et al. (2012) have recently described the generation of bispecific antibodies by modifying positions 368 and 409 in the C_H3 domain of either IgG₁ or IgG₂ isotypes.

13.5.4 Bispecific, tetravalent IgG antibodies

A different approach to making bispecific antibodies is to add another binding domain of some sort to an IgG to generate a bispecific, tetravalent modified antibody that is generally larger than a normal IgG. The first significant effort along these lines was the generation of an IgG-scFv fusion in which the scFvs were attached at the C-terminal end of each Fc chain (Coloma and Morrison, 1997). The positive side to this type of construct is that only one form is produced in recombinant cell culture, making purification easier than bispecifics from hybrid-hybridomas, as noted above. The negative side is that scFvs are notoriously unstable and tend to unfold (Wörn and Plückthun, 2001), leading to a product that could easily aggregate (via interchain interaction of the unfolded scFvs), degrade, and cause other developability problems (Jenkins et al., 2008; Voynov et al., 2009; Shire, 2009; Conley et al., 2011).

This type of approach for the generation of bispecific tetravalent antibodies has been used by a variety of other investigators with many variations on the general theme. In one set of examples, Mabry et al. (2010) designed a series of anti-IL-17A, anti-IL-23p19 bispecific antibodies using single chains fused to various parts of an IgG or Fc. One construct featured tandem scFvs fused to the N-terminus of an Fc (called TascFv-Fc fusion), another featured different scFv constructs fused to each of the N- and C-termini of an IgG Fc, and the third construct featured an scFv fused to the C-terminus of an IgG (Mabry et al., 2010). To reduce scFv-related issues, Mabry et al. (2010) screened for highly stable scFvs using the V_H 3-23 germline known for its stability (Ewert et al., 2003) to incorporate into their bispecific antibody formats.

Over the past few years, several efforts have been made to generate bispecific, tetravalent antibodies based on the fusion of scFvs to the two C-terminal domains of an IgG Fc (Demarest and Glaser, 2008; Jordan et al., 2009; Michaelson et al., 2009; Miller et al., 2010; Dong et al., 2011a, 2011b) or alternatively, to the C-terminal sequences of the light chain (Orcutt et al., 2010) (see Figure 13.2 and Table 13.2). As mentioned above, the key has been the stabilization of the scFv (Demarest and Glaser, 2008), either through the addition of a disulfide bond to keep it from unfolding (Orcutt et al., 2010) or via mutation of the scFv to achieve significantly higher stability scFvs (Jordan et al., 2009). Orcutt et al. (2010) stabilized the scFv by incorporating an internal disulfide bond (either V_L G100C – V_H G44C or V_L V43C – V_H Q105C) to link the V_H and V_L chains together followed by fusion of that construct to the C-terminus of the light chain. Their construct, which was ~200 kDa in size, behaved much like an IgG including stability of the IgG and pharmacokinetics in mice (Orcutt et al., 2010).

Schanzer et al. (2011) made a series of bispecific, tetravalent constructs against the HIV co-receptor CCR5, one to the second extracellular loop and the other to an N-terminal epitope. They tested placement of the scFv on either the C-terminal or N-terminal ends of both the heavy and light chains, $(Gly_4Ser)_n$ linker length, and the effect of disulfide stabilization (V_H G44C – V_L Q100C). They found that disulfide stabilization did not affect functional activity but decreased expression substantially, that linker length had little effect on expression but longer linkers reduced aggregation, and that all constructs possessed some level of aggregation issues but that the introduced disulfide bonds reduced those problems. They demonstrated that several of these constructs achieved more than a two to fivefold increase in potency over a monospecific antibody due to the strong avidity effect of binding to two epitopes on the same protein. The disulfide stabilized, heavy chain C-terminal fusion with a linker length of 30 residues was considered to be the optimal construct for further studies due to its lowered tendency to aggregate (Schanzer et al., 2011).

Jordan et al. (2009) and Miller et al. (2010) showed that substitutions V_H S16E, V55G, P101D, and V_L S46L resulted in a ~30°C improvement in T_m of an anti-lymphotoxin- β (LT β) receptor scFv, which they incorporated into their IgG-scFv bispecific tetravalent antibody targeting TRAIL-R2 (with the IgG portion) and LT β R with the scFv. This TRAIL-R2-LT β R bispecific antibody, as well as another form in which the anti-LT β R scFv was fused to the N-terminus of the heavy chain, demonstrated good pharmacokinetics, stability, and pre-clinical efficacy (Michaelson et al., 2009). Dong et al. (2011a) generated a series of anti-IGF-1R scFvs and constructed a panel of bispecific anti-IGF-1R antibodies based on both N- and C-terminal IgG-scFv

fusions targeting multiple epitopes on IGF-1R. One scFv, BIIB4, was stabilized to $>65^{\circ}\text{C}$ T_m by a single mutation (V_L I83E) whereas a second scFv BIIB5 required four mutations (V_H E6Q, S49A and V_L L50N, V83E, A84G) to stabilize the scFv. BIIB5 also was fused to an anti-EGFR IgG to make a stabilized anti-EGFR-IGF-1R bispecific, tetravalent antibody (Dong et al., 2011b). Instructive in these significant efforts by the Biogen researchers (Jordan et al., 2009; Michaelson et al., 2009; Miller et al., 2010; Dong et al., 2011a) is the fact each scFv required a different set of mutations for effective stabilization, meaning that there is no single solution for the stabilization of all scFvs. Thus this IgG-scFv approach will unfortunately require separate engineering methods to stabilize each novel scFv.

Another form of bispecific antibody that utilizes a standard Fc but not the FAb arms is a ScorpionTM bispecific antibody (<http://www.emergentbio solutions.com/?q=node/47>). This is similar to the SMIP construct described in Chapters 12 and 14 but with a pair of second scFvs fused through short linkers to the C-terminus of the C_{H3} domains of the Fc. Thus this construct consists of an Fc to which four scFvs targeting two different epitopes or proteins are attached (see Figure 13.2 and Table 13.2).

Scientists at Abbott labs recent developed a highly stable and structurally sound bispecific technology based on the IgG by fusion of the Fv of a second IgG to the N-terminus of an IgG to generate a tetravalent, dual-target-specific, dual-variable domain (DVD) antibody of approximately 200 kDa (Wu et al., 2007a; Gu and Ghayur, 2012). To enable the fusion, the V_{κ} of the second Fv was fused to the N-terminus of the IgG V_{κ} using a short linker of “TVAAP” residues, while the V_H chain of the second Fv was fused to the N-terminus of the IgG V_H using a short linker of “ASTKGP” residues (Wu et al., 2007a). An anti-IL-12, anti-IL-18 DVD Ig was generated using this technology that was demonstrated to be highly stable, possessed normal pharmacokinetic properties, and was bioactive in disease models (Wu et al., 2007a). Moreover, expression, purification, and solubility properties of the DVD Ig were similar to those of a normal IgG (Wu et al., 2007a; Gu and Ghayur, 2012). Two DVD bispecific antibodies, including the anti-TNF- α /anti-IL-17A dual targeting antibody, ABT122, and the anti-IL-1 α /IL-1 β dual targeting antibody, ABT-981, are now in Phase I clinical trials.

Alt et al. (1999) demonstrated the feasibility of fusing a diabody construct (see section 13.6.2) recognizing two separate epitopes to the N-terminus of an IgG Fc to generate a long half-life, diabody-based, bispecific antibody. Zhengping Zhu and his colleagues also replaced the FAbs of an IgG with a pair of diabodies recognizing IGF1-R and EGFR (Lu et al., 2005) (see Table 13.2). These di-diabody-Fc fusion proteins strongly inhibited the growth of two different tumor models in xenograft studies.

An alternative approach to generate a bispecific, tetravalent antibody using a domain antibody was the fusion of a domain antibody either to the N-terminus of the V_L chain of a normal antibody (Shen et al., 2006), or to the C-terminus of the C_{H3} domain (Shen et al., 2007) (see Table 13.2), generating a bispecific antibody in which the IgG portion recognized mouse VEGFR2 and the domain antibody recognized mouse PGFR- α .

Scientists at Sea Lane Biotechnology described a novel approach for the construction of bispecific antibodies by using the pre-BCR fragment as the light chain to make what they termed “Surrobody™.” The pre-BCR two subunit light chain surrogate chimeras containing the fused pseudo-light chain (Ψ LC) segments, $\lambda 5$ and V_{preB} , yield bispecificity due to an extra fragment hanging off the surrogate light chain components that can be used to engage the second target (Xu et al., 2008) (see Table 13.2). Xu et al. (2010) demonstrated that scFvs or cytokines could be fused to either the C-terminal end of the pre-BCR tails or to the N-terminal end of the $\lambda 5$ domain to generate a functional, bispecific, tetravalent antibody or antibody-cytokine fusion, respectively.

Since fusion of a linker and protein to the C-terminus of an IgG does not significantly impair its ability to engage $Fc\gamma$ Rs or $FcRn$, it is not unreasonable to expect that a variety of new bispecific MAb approaches are being, or will be, developed by fusing small, highly stable, non-antibody binding proteins (NABPs) such as DARPins, adnectins, or anticalins to the C-terminus of the heavy chain (or the light chain) of normal IgGs via short flexible linkers. Wittrup et al. (2011) have demonstrated the feasibility of this approach by fusing an Fn3 domain to the C-terminus of the heavy chain of an IgG to make a bioactive, bispecific, and trispecific antibody/NABP hybrid (see Figure 13.2 and Table 13.2).

13.5.5 Marketed and clinical candidate bispecific IgG antibodies

There are currently at least eight bispecific constructs based on IgGs on the market or in clinical trials, three of which use the Trion Triomab® technology. Catumaxomab (Removab®) is a trifunctional, bispecific, hybrid, mouse-rat, monoclonal antibody (Triomab® technology) against human EpCAM and human CD3 that was approved for marketing in Europe in April 2009 (Seimetz et al., 2010). Catumaxomab is produced in a quadroma cell line derived from the three-way fusion of a mouse B cell, a rat B cell, and a myeloma cell (Zeidler et al., 1999), which produces a hybrid Fc containing one half mouse IgG_{2a} and the other half rat IgG_{2b} Fc,

with one FAb arm binding CD3 on T cells and the other FAb arm binding the cancer target EpCAM (Zeidler et al., 1999). The hybrid mouse IgG_{2a}/rat IgG_{2b} Fc actively binds human FcγRs I and III to trigger ADCC (Zeidler et al., 1999; Shen and Zhu, 2008; Hess et al., 2012). The second Triomab® in clinical trials is ertumaxomab, a trifunctional, bispecific hybrid MAB with one arm binding human CD3 and the other arm binding human HER2/neu mouse. Similar to catumaxomab, ertumaxomab is also functional on FcγRs I and III, resulting in trifunctionality. The third antibody incorporating Triomab® technology is FBT-A05, and anti-CD3 x anti-CD20 antibody targeting T cells. All three of these antibodies suffer from the fact that they are highly immunogenic in humans, which limits their dosing significantly as compared with other anti-tumor antibodies on the market.

Additionally, several new human or humanized bispecific IgG-like molecules have entered clinical trials within the last year. Phase I clinical trials were recently initiated with the DVD Igs (Wu et al., 2007a; Gu and Ghayur, 2012), ABT-122 (TNF-α/IL-17A) and ABT-981 (TNF-1α/IL-1β), for treatment of inflammatory diseases. Similarly, Genentech has placed MEHD7945A, the bivalent, bispecific antibody targeting HER3 and EGFR (Schaefer et al., 2011a) into Phase I clinical trials for the treatment of breast cancer. Finally, SAR156597, a bispecific antibody from Sanofi and Regeneron targeting IL-4 and IL-13, has also recently entered Phase I clinical trials as a candidate to treat idiopathic pulmonary fibrosis.

13.6 Bispecific antibody fragments

Similar to the concept of bispecific IgG-like molecules, the concept of generating bispecifics using antibody fragments has been around since the mid-1980s with the first description of a bispecific antibody fragment construct that brought together an effector cell (e.g. T cell) and its target cell (Staerz and Bevan, 1986). While there are many forms of antibody fragment bispecifics in the literature, this chapter will focus on just a few of the key formats, namely domain antibody dimers, diabodies including tandem antibody dimers (TandAbs), bispecific T cell engagers (BiTEs), and dual affinity retargeting (DART) molecules (see Figure 13.3). There are many multi-specific antibody fragments based on these general building blocks. For more information on some of the more exotic bispecific antibody fragments, a recent review that focuses primarily on antibody fragment bispecifics covers them (Cuesta et al., 2010).

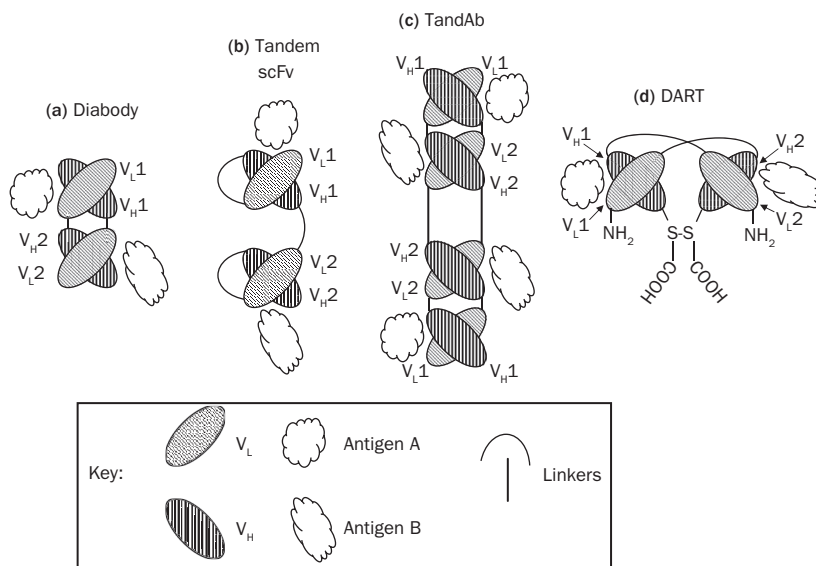


Figure 13.3 Examples of bispecific antibody fragments, shown with binding to two different antigens

(a) Bivalent, bispecific diabody, in which two different scFv constructs (V_{H1} - V_{L2} and V_{H2} - V_{L1}) interact with each other non-covalently due to the short linkers between each of the V_H and V_L chains to form a ~50 kDa bispecific molecule (Holliger et al., 1993). (b) Bivalent, bispecific tandem scFv, which are two scFvs linked with a relatively long linker to promote intramolecular (rather than intermolecular) interactions. This class would include the bispecific T cell engager (BiTE)-like molecules (Wolf et al., 2005; Baeuerle et al., 2008). (c) Tetraivalent, bispecific TandAb molecule, which consists of two chains, each containing four domains: V_{H1} - V_{L2} - V_{H2} - V_{L1} , that bind non-covalently in a head-to-tail orientation to generate a ~100 kDa molecule (Kipriyanov et al., 1999). (d) Bivalent, bispecific, dual-affinity re-targeting (DART) molecule (Johnson et al., 2010; Veri et al., 2010; Moore et al., 2011).

13.6.1 Domain antibody dimers

As described in Chapter 12, domain antibodies consist of a stabilized V_H , V_L , V_{HH} , or V_{NAR} domain and are about 12–15 kDa in size, and have been sourced from humans, mice, camelids, and nurse sharks. Perhaps the simplest bispecific antibody fragments that can be made are two domain antibodies fused together end to end, with or without a linker, resulting in a bispecific, bivalent protein of ca. 25 kDa. While considerable information has been presented at meetings on bispecific domain antibodies, very few papers actually describe such constructs. Conrath et al. (2001) demonstrated the ability to make bispecific camelid nanobodies using a 29-amino acid residue, proline-rich, linker derived from the upper hinge of the camel IgG_{2a}

antibody. They made nanobodies against β -lactamase and lysozyme and determined that the order did not affect the binding activity of the β -lactamase nanobody, but significantly impacted the binding activity of the anti-lysozyme nanobody. The anti-lysozyme nanobody had fourfold poorer binding when it was positioned second (C-terminal) in the pairing, indicating that a free N-terminus of that nanobody was more optimal for binding (Conrath et al., 2001). A set of single-chain monospecific, bivalent and bispecific, trivalent (with an albumin-binding half-life extension domain at the C-terminus) anti-EGFR nanobodies was constructed in which the nanobodies were separated by a 12-residue linker derived from the upper hinge of camelid IgG₃ (Roovers et al., 2007). In that study, it also was demonstrated that the nanobodies were active no matter what position in the chain they occupied (Roovers et al., 2007). Roovers et al. (2011) further demonstrated that a biparatopic nanobody targeting non-overlapping epitopes of EGFR were more potent both *in vitro* and *in vivo* than either a monovalent or monospecific bivalent nanobody, demonstrating the potential use of nanobody multimers. Hmila et al. (2010) developed a bispecific antibody targeting AaH1 and the more potent AaHII scorpion peptide toxins in which they coupled two anti-toxin nanobodies with a 16-residue hinge derived from camelid IgG_{2c} antibody. Their bispecific construct was very potent in neutralizing both toxins, showing promise as a potential for replacing horse serum derived antibodies as antitoxins (Hmila et al., 2010).

Simmons et al. (2006) demonstrated several methods for dimerization of shark-derived V_{NAR}s. They utilized different dimerization strategies including the incorporation of dimerized C1_{NAR} constant chain domains coupled with short linkers to the C-termini of the V_{NAR}s, as well as a helix-turn-helix dimerization domain and a disulfide-mediated dimerization. All of these approaches resulted in either poor expression, low activity, aggregated protein, or all of the above. When V_{NAR}s were dimerized by construction of a head to tail single domain, separated by a short 7-residue linker derived from the Ig_{NAR} hinge, only monomeric activity was observed. The authors subsequently determined that the second V_{NAR} in the single chain was inactive and that a free N-terminus was required for activity of that particular V_{NAR} (Simmons et al., 2006).

Domantis (a subsidiary of GlaxoSmithKline) has reported the ability to make DAb bispecific antibodies either by incorporation of V_HDAb and V_LDAb onto the N-terminus of the C_H1 and C_L domains, respectively, of a normal antibody (e.g. replacing V_H and V_L, respectively), or by generation of DAb single chain dimers (http://www.iptonline.com/articles/public/IPT_17_2005_p42_45.pdf). Also, where reported in the literature, it does not appear that a free N-terminus is required for all DABs. An example of this

is in the interferon- α 2b-AlbuDAb fusion that was made to extend the half-life of the interferon (Walker et al., 2010). In that work, the biologically active DAb domain was at the C-terminus of the construct, so its N-terminus was “covered” by the linker linking it to the N-terminal interferon (Walker et al., 2010). Finally, GSK has reported that their pipeline contains GSK 2401502, a multi-specific domain antibody for use in oncology.

Thus it appears that concatenation of nanobodies of DABs is achievable, robust, and attractive to make multi-epitope, multi-specific, and/or longer half-life domain antibodies. Whether this principle will also translated for V_{NARS} is not yet known. For additional information, Chames and Baty (2011) have recently reviewed the field of bispecific domain antibodies.

13.6.2 Diabodies

As described in Chapter 12, when scFvs were first designed by Bird et al. (1988) and Huston et al. (1988), they were built with linkers of 18 and 15 amino acid residues between the V_H and V_L chains, respectively. These linkers had enough length and flexibility that the two domains, V_H and V_L , could associate to form an Fv (Bird et al., 1988; Huston et al., 1988). Diabodies were first described in 1993 by Holliger et al. (1993), who demonstrated that if the linkers between the V_H and V_L domains of each of two different scFvs expressed as separate genes in *E. coli* were kept short enough, it would not allow pairing between the V_H and V_L of the same chain, resulting in an interchain pairing of $V_{HA}-V_{LB} - V_{HB}-V_{LA}$ (see Figure 13.3). They found that a $(\text{Gly}_4\text{Ser})_n$ linker of 15 amino acid residues, i.e. $n = 3$ (identical to that used by Huston et al., 1988), resulted in both intra-chain and inter-chain pairing, whereas linkers of five and ten residues ($n = 1$ and 2 , respectively) resulted in primarily inter-chain pairing and retained bioactivity of both scFvs (Holliger et al., 1993). They also demonstrated that a pair of heterologous V_H - V_L chains, each fused directly with no linker between them, also would result in inter-chain pairing and full bioactivity of both scFvs (Holliger et al., 1993). They called these inter-chain bispecific 50 kDa antibody fragments “diabodies.”

Over the years, diabodies have been used to make several different kinds of constructs, including:

- generation of tetravalent, bispecific di-diabodies using the Fc C_{H3} domain as the dimerization domain (Lu et al., 2003);
- construction of a humanized anti-EGFR, anti-CD3 T cell engagement diabody called hEx3 that was capable of binding both EGFR⁺ target

cells and T cells, and of prolonging the life of mice with EGFR⁺ colon carcinoma cell xenografts (Asano et al., 2006);

- engineering of a trifunctional diabody targeting CD3 on T cells and the cancer antigen CEA, fused with the streptococcal protein G albumin binding domain, providing both T cell engagement and lengthened half-life (Stork et al., 2007);
- construction of diabody-HSA fusions (Müller et al., 2007) and diabody albumin domain III fusions (Kenanova et al., 2010) for long half-life; and
- generation and analysis of an anti-CD30 diabody-drug toxin conjugate (Kim et al., 2008a).

In this last construct, the chemical cytotoxin and linker maleimidocaproyl-valine-citrulline-*p*-aminobenzyloxycarbonyl-monomethyl-auristatin E (mc-vcMMAE) was conjugated to an anti-CD30 diabody into which several Cys residues had been incorporated by mutagenesis. The diabody-vcMMAE conjugate, which averaged 3.7 drug molecules per diabody, demonstrated strong efficacy although it was slightly less potent than an IgG₄-vcMMAE control (Kim et al., 2008a). The strength of this effort is that the rapid clearance of the diabody-conjugate should also lower the toxicity. Müller and Kontermann (2011) have given a detailed accounting of all of the different kinds of diabody-like constructs that could possibly be made.

A variation of the bispecific, bivalent diabodies consisting of four domains (2 V_H, 2 V_L) is the construction of two diabody constructs that associate non-covalently to make a bispecific, tetrameric molecule of ~114 kDa (Kipriyanov et al., 1999). These molecules have been called TandAbs (Kipriyanov et al., 1999), and they have been developed into a commercial antibody platform by the biotechnology company, Affimed. The original TandAb had nine amino acid residue linkers separating each of the V_H-V_L pairs of “AKTTPALGG” residues which were short enough to force intermolecular interactions instead of intramolecular pairing, and a 12-residue linker of “RADAAASGGPGS” which allowed enough flexibility but still forced inter-chain molecular pairing (Kipriyanov et al., 1999). The design of the first TandAb was anti-CD3 V_H – anti-CD19 V_L – anti-CD19 V_H – anti-CD3 V_L which, when paired non-covalently with an anti-parallel fragment containing the same sequence (see Figure 13.3), resulted in a bispecific, tetravalent antibody fragment that contained Fvs with the order of anti-CD3 anti-CD3 – anti-CD19 – anti-CD19 – anti-CD3 (Kipriyanov et al., 1999). These TandAbs were found to be more potent than the equivalent diabodies in mediating T cell killing of target CD19⁺ B cells (Kipriyanov et al., 1999). Le Gall et al. (2004) investigated the effect of linker lengths and composition on the stability and activity of TandAbs,

and found that while a TandAb with linkers of six and ten residues joining the V_H - V_L and scFv moieties, respectively, yielded the best productivity in *E. coli*, the most active TandAb form was one which included ten amino acid residue linkers (SAKTPKLGG) between the V_H and V_L domains and a 12-residue linker (RADAAAAGGPGS) coupling the two halves (Le Gall et al., 2004). A CD19 x CD3 TandAb was demonstrated to have very strong cytotoxic T lymphocyte killing of CD19⁺ B cells from patients with B cell chronic lymphocytic leukemia (CLL), indicating the ability of this TandAb to recruit non-stimulated T cells into CTL killing activity (Reusch et al., 2004). One TandAb, AFM13, which is a CD30 x CD16 bispecific, tetrameric TandAb molecule of about 100 kDa, is currently reported to be in Phase I clinical trials (<http://www.affimed.com/tandab-antibodies>) for the treatment of Hodgkin's lymphoma.

13.6.3 Bispecific scFvs and BiTEs

As mentioned in Chapter 12 and noted above, scFv antibodies were first devised in 1988 by both Bird et al. (1988) and Huston et al. (1988). Shortly thereafter, investigators tried to devise different strategies for making bispecific single chain antibodies. Mallender and Voss (1994) developed a model bispecific, bivalent scFv containing a 24 amino acid residue linker connecting the individual scFvs. Both scFvs retained activity in the dimeric construct (Mallender and Voss, 1994). A variety of bispecific scFv constructs have been designed and tested since then, but none is perhaps as compelling as the BiTE platform (Wolf et al., 2005; Baeuerle et al., 2008) described in more detail in this section.

In the mid-1980s, it was demonstrated using conjugated "bispecific" IgG antibodies that one antibody targeting a cancer cell and another targeting a T cell could result in T cell-mediated killing of the targeted cell (Staerz et al., 1985; Perez et al. 1985). Thus it was understood very early in the development of recombinant antibodies that targeting T cells and target cells with the same molecule could redirect the T cells to kill the target cells. Once the concept of bispecific scFvs was envisioned (Holliger et al., 1993), the retargeting idea was applied to them immediately. Both Gruber et al. (1994) and Kurucz et al. (1995) devised bispecific scFvs for retargeting CTL to mediate tumor lysis. Gruber et al. (1994) designed a ~57 kDa bispecific, bivalent scFv construct that bound fluorescein with one scFv and a T cell receptor epitope with the other scFv. They then covalently bound fluorescein to the surface of model tumor cells and demonstrated that the

bispecific scFv could redirect the T cells to lyse the fluorescein-coated tumor cells (Gruber et al., 1994).

Kurucz et al. (1995) targeted CD3 on T cells with one scFv and human transferrin receptor with the other scFv in a bispecific scFv in which the two scFvs were linked together using a 14 amino acid residue linker. While this construct suffered from some instability, they were still able to demonstrate redirected CTL activity (Kurucz et al., 1995). Mack et al. (1995) also made a T cell engaging bispecific scFv antibody using an anti-CD3 to target T cells and anti-epithelial 17-1A antigen (EpCAM) found on tumor cells. They produced the bispecific scFv with either a $(G_4S)_1$ or $(G_4S)_3$ linker in CHO cells and demonstrated potency at nM levels using several cancer cell lines. Those studies were the forerunner of what is now known as the “bispecific T cell engager” (BiTE) technology that the biotechnology company Micromet (recently acquired by Amgen) has built into a commercial platform (Wolf et al., 2005; Baeuerle et al., 2008). BiTE antibodies are bispecific scFvs that engage CTLs by binding to CD3, and target cells by binding to a target cell surface protein (e.g. EpCAM, CD19). They direct the engagement of the T cells with target cells to kill the target cells via T cell-mediated lysis, hence the name “bispecific T cell engager.” The first BiTE-like antibody to be built for clinical trials was the anti-CD3 x anti-CD19 bispecific single chain antibody in which the two scFvs were linked with a five-residue (G_4S) linker $[(V_LCD19-V_HCD19)\text{-GGGGS-}(V_HCD3-V_LCD3)]$. Two key features of this bispecific antibody were its ability to function with T cells which had not been pre-activated and its very high potency (Löffler et al., 2000). Dreier et al. (2003) optimized the anti-CD3 x anti-CD19 BiTE antibody and demonstrated in *in vitro* experiments that the optimized form lysed target cells within a few hours with an ED_{50} value of 30 pg/mL (600 fM) and had a 100,000-fold greater potency than rituximab run in the same assays. This potency was remarkable given that the antibody bound CD3 on T cells with a K_D of only 26 μ M, as compared with the binding of the anti-CD19 scFv portion to B cells of low nM affinity (Dreier et al., 2003). Bargou et al. (2008) reported that in Phase I/II clinical trials of blinatumomab (MT-103; anti-CD3 x anti-CD19 BiTE) for treatment of non-Hodgkin’s lymphoma, the antibody led to partial and complete responses and clearance of malignant lymphoma cells from bone marrow and liver.

Schlereth et al. (2005) also reported the construction of an anti-EpCAM x anti-CD3 BiTE that demonstrated remarkable *in vivo* potency in animal tumor models, and Hammond et al. (2007) reported the construction of an anti-EphA2 x anti-CD3 BiTE antibody that also redirected T cells efficiently and demonstrated remarkable potency.

Mølhøj et al. (2007) reported that the anti-CD19 x anti-CD3 (MT103) BiTE construct was markedly more potent (700- to 8,000-fold depending on the experiment) than a quadroma version (anti-CD19 x anti-CD3 IgG in which one Fab arm targets CD19 and the other targets CD3) or a TandAb version of the same antibody. Unfortunately, as Little and Kipriyanov (2007) pointed out, the supposed TandAb construct was made improperly and therefore did not truly represent that platform. Thus there is an open question as to whether other T cell engagement platforms with anti-CD3 x anti-tumor target domains, such as bispecific IgGs, TandAbs, or even bispecific non-antibody-binding proteins such as DARPins, might have the potency of BiTEs.

Micromet (now Amgen) has advanced the BiTE platform to mid-stage clinical trials (Bargou et al., 2008). Blinatumomab (MT-103) (CD19 x CD3 BiTE) is currently in Phase II clinical trials (Bargou et al., 2008), and MT-110 (EpCAM x CD3 BiTE) and MT-111 (CEA x CD3 BiTE) are currently in Phase I clinical trials, all for oncology indications.

13.6.4 DARTs

A new bispecific antibody fragment platform called “Dual Affinity Re-Targeting” (DART) antibodies has been developed in recent years by the biotechnology company, MacroGenics. DARTs are scFv-like antibodies with an unusual structure (Johnson et al., 2010; Veri et al., 2010; Moore et al., 2011). DARTs are constructed in two chains with a total molecular weight of ca. 50 kDa. From N-terminus to C-terminus, the first chain contains the V_L chain of antibody A with the V_H chain of antibody B, coupled with a G_3SG_4 linker and followed with the short C-terminal sequence “FNRGEC” (i.e. $[V_LA]-G_3SG_4-[V_HB]-FNRGEC_{SH}$), leaving a C-terminal sulfhydryl moiety. From N-terminus to C-terminus, the second chain contains the V_L chain of antibody B with the V_H chain of antibody A, coupled with a G_3SG_4 linker and followed with the short C-terminal sequence “VEPKSC” (i.e. $[V_LB]-G_3SG_4-[V_HA]-VEPKSC_{SH}$), also leaving a C-terminal sulfhydryl moiety (Johnson et al., 2010). These antibody chains, which are produced in mammalian cells, form a disulfide bond between the two chains and fold into the heterobispecific DART structure (see Figure 13.3), with each domain finding its partner on the opposite chain (Johnson et al., 2010). The first model DARTs consisted of a CD16a x CD32b design which functioned to deplete CD32b⁺ B cells from PBMCs by redirecting CD16a⁺ NK and other effector immune cells (Johnson et al., 2010; Veri et al., 2010).

Subsequently, DARTs have been constructed using CD32b x CD79b for inhibition of B cell proliferation (Veri et al., 2010), and CD19 x CD3 for T cell-redirection B cell depletion (Moore et al., 2011). In this last study, a CD19 x CD3 DART was compared directly with the same V-regions constructed in BiTE format as well (Moore et al., 2011). Interestingly, the DART molecules appeared to be significantly more potent than their identical BiTE counterpart in several types of assays, including the ability to cross-link T and B cells (Moore et al., 2011), leading Rader (2011) to comment that DARTs may have the potential to (minimally) rival BiTEs in clinical trials.

13.6.5 Other bispecific antibody fragments in clinical trials

One other bispecific antibody to enter the clinic recently is Merrimack Pharmaceutical's M-111, an anti-HER2/HER3 human serum albumin (HSA)-antibody fusion protein for the treatment of breast cancer which is currently in Phase I clinical trials. According to Merrimack's website (<http://www.merrimackpharma.com/pipeline/mm-111/>), MM-111 is a bispecific scFv-HSA molecule in which one scFv arm binds to ErbB2 with high affinity to target the antibody to HER2-overexpressing tumors, and the other scFv arm binds to ErbB3 to carry out its functionality.

FcFPs and similar constructs using Fc

Abstract: Proteins that are constructed by fusing the Fc domain of an IgG to a receptor protein, cytokine, peptide, scFv, or domain antibody are called Fc fusion proteins (FcFPs). FcFPs have been used widely as alternatives to antibodies to bind targets for a variety of disease indications. The first and most widely known Fc fusion protein product is etanercept (Enbrel®), which targets TNF- α and TNF- β . Another five FcFPs have been approved for marketing, and many more are currently in clinical trials.

Keywords: Fc fusions, traps, etanercept (Enbrel®), receptor fusions, half-life extension, peptides

14.1 Introduction

As mentioned previously in Chapter 3, the Fc domain of an antibody is comprised of a pair of C_H2-C_H3 domains with an approximate molecular mass of about 50 kDa. The C_H3 domains pair tightly, whereas the C_H2 domains do not directly interact. The combination of the Fc domains and the hinge, which stabilizes the inter-chain binding through disulfide bonding (Chapter 3), is even more stable than the Fc itself, and provides a molecule that retains much of the half-life of a normal antibody, as well as possibly some of the effector functions, depending on how intact the hinge is. Additionally, the Fc region and hinge combination are structurally very stable, can be expressed at high titers in mammalian cell culture, and can be purified using Protein A affinity matrices in a manner similar to antibodies.

An Fc fusion protein (FcFP), which is comprised of a protein, peptide, or receptor exodomain genetically fused to the Fc of an antibody (typically at the amino terminal end, although some peptides have been fused to the C-terminal end of the Fc), possesses many of the salient characteristics of antibodies, but with the activity of the fused proteins or peptides replacing

the variable chain binding function found in an antibody. Why construct an FcFP? In most cases, FcFPs are constructed to extend the half-life of a protein, peptide, or potentially even a small molecule. Other attributes that may be key factors for some fusion proteins include the fact that two proteins are attached, one to each arm of the Fc, which may allow in some cases for dimerization of the fused partners. Finally, if Fc mediator functions are desired, the Fc of the fusion protein could be engineered as described in Chapter 10 to yield the level of Fc-mediated activity desired. Peptides, proteins, soluble receptors, antibody fragments lacking the Fc domain, and non-antibody binding proteins generally have short serum half-life pharmacokinetics, most often because they fall below the protein size (~70 kDa) required to be filtered by the kidney (Caliceti and Veronese, 2003). This process of elimination limits their exposure to the target tissue and consequently their pharmacodynamic effects. Many of the key attributes that have contributed to the success of therapeutic MAbs are provided by the Ig constant region or Fc region. The Fc region can significantly prolong the serum half-life of a protein or peptide fused to it, due both to its pH dependent binding to FcRn, which salvages the protein from being degraded in endosomes (see Chapter 7, section 7.3), as well as to the larger size due to the homo-dimeric nature of FcFPs which limits renal clearance.

This chapter provides an overview of FcFP constructs as well as examples of those FcFPs and peptides currently or recently in clinical trials. Huang (2009) has recently reviewed all forms of FcFPs in detail, and Beck and Reichert (2011) highlight the recent successes of FcFPs on the market and in late-stage clinical trials.

The concept of making a fusion protein between an intact IgG Fc and another protein was first laid out by Capon et al. (1989), who described the fusion of the exodomain of the T cell receptor CD4 with an Fc domain of an IgG₁, a construct they called “immunoadhesin” (Ashkenazi et al., 1993; Ashkenazi and Chamow, 1995, 1997). Since then, many varieties of FcFPs have been made and tested either pre-clinically or clinically.

There are three fundamentally different types of FcFPs, each of which has its own type of use. The first type, which is a receptor exodomain fused to the Fc of an IgG, is exemplified by etanercept. In this case, the fusion protein may function to soak up the ligand or block cell–cell interactions. Etanercept (Enbrel®) is the prototypical example of this type of FcFP. An Fc trap is a sub-type of Fc-receptor fusion protein in which heterologous receptors or receptor domains are sequentially fused to the Fc to trap the natural ligand (Economides et al., 2003). The second is the fusion of a normal or mutated cytokine or other blood factor with the Fc of an IgG,

resulting in a stabilized version of the soluble protein. Examples of this type of FcFP are Factor IX-Fc (Valentino, 2011) and the EPO mimetibody construct, CNTO530 (Bugelski et al., 2008). The final version of an FcFP is the fusion of an agonist natural peptide hormone to an Fc to give the peptide agonist extended pharmacokinetics. Fc-GLP-1 fusions (Glaesner et al., 2010) are an example of this type of FcFP. This chapter describes the different types of FcFPs (see Figure 14.1) and gives some examples of these proteins currently in clinical trials.

Currently, seven FcFPs have been approved for marketing in the United States (the most recent of which is EyleaTM), another five are in late-stage clinical trials, and more than a dozen known FcFPs are in earlier stage development (see Table 14.1). Etanercept (Enbrel[®]), an FcFP in which the TNFR2 p75 exodomain was fused to an Fc domain (Mohler et al., 1993), was approved by the FDA in 1998, making it the first FcFP to be approved for marketing. Enbrel[®] is indicated for the treatment of rheumatoid arthritis, psoriasis, and several other inflammation-related conditions. The second FcFP to be approved for marketing was alefacept (Amevive[®]), which is comprised of the CD2-binding domain of LFA-3 fused to the Fc of an IgG₁. Amevive[®] is indicated for psoriasis, although since its approval in 2003 by the FDA, it has not gained significant market share (see Chapter 2, Table 2.7).

14.2 Receptor-FcFPs

The ability of cells to produce soluble cytokine receptors was first discovered in the mid-1980s, with the finding that several human T cell leukemia virus 1 (HTLV1)-positive T cell cultures produced soluble interleukin-2 (IL-2) receptors in their culture supernatants as a result of viral infection (Rubin et al., 1985). Subsequently, a large variety of soluble receptors has been found both in cell line culture supernatants and in serum of both humans and other species (Aricescu and Jones, 2007; Ware, 2008). Many of these receptors belong to either the TNF or Ig superfamilies (Aricescu and Jones, 2007; Ware, 2008). Moreover, for virtually every known cytokine receptor, there is a soluble receptor form, generated either by “shedding,” i.e. proteolytic cleavage of the receptor from the membrane surface, or alternative splicing of the mRNA encoding the receptor resulting in receptors lacking the membrane spanning or anchor domains (Gearing et al., 1994; Bremer et al., 2009). It was discovered that these soluble cytokine receptors acted in a regulatory manner as “decoy” molecules to block the activity of the cytokine or soluble protein to which they naturally

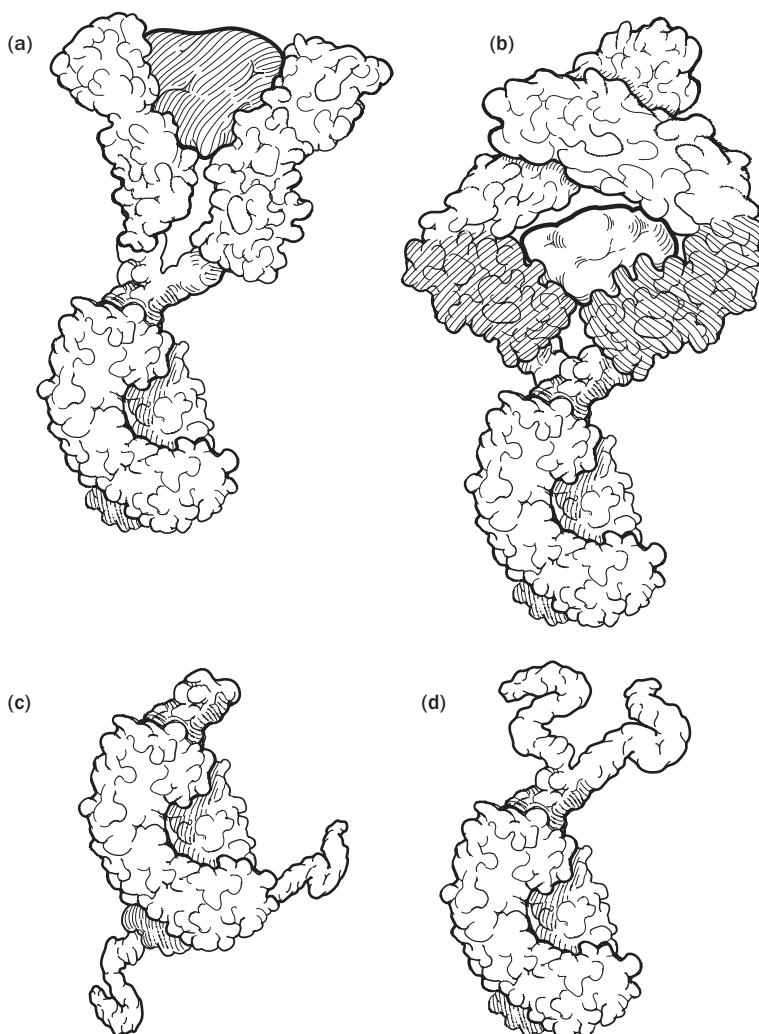


Figure 14.1 Four examples of Fc fusion molecules

(a) Typical receptor exodomain-Fc fusion protein in which the receptor exodomain is fused to the N-terminus of a human IgG₁ Fc using a short linker and/or the natural IgG₁ hinge. An example of this type of FcFP is etanercept (Enbrel®). (b) "Trap" FcFP in which two heterologous receptors or receptor domains (e.g. exodomain 2 of VEGFR1 fused to exodomain 3 of VEGFR2) are fused to huIgG₁ Fc domain (Economides et al., 2003). Aflibercept (Zaltrap®, approved by the US FDA on 8/3/12 for the treatment of metastatic colorectal cancer) is an example of this type of FcFP. (c) FcFP in which a peptide is fused via a linker to the C-terminus of a human IgG. Romiplostim (NPlate®) is an example of this type of FcFP. (d) FcFP in which a peptide is fused via a linker to the N-terminus of a human IgG Fc. Dulaglutide™ (LY2189265) (Glaesner et al., 2010), which consists of a GLP-1 mimetic peptide fused to huIgG₁ Fc domain, is an example of this.

Table 14.1 Examples of important FcFPs on the market or in clinical trials

Name	Company	Current status (US)	Molecular target	Major indication	Protein type and construct
Enbrel® (Etanercept)	Immunex (now Amgen)	Approved by FDA 11/02/1998	TNF- α and TNF- β	RA	FcFP; p75 exodomain of TNFR2 fused to hulG ₁ Fc domain
Amevive® (Alefacept)	Biogen	Approved by FDA 01/30/2003	CD2 – inhibits CD2-LFA-3 interaction on activated T cells	Psoriasis	FcFP; CD2-binding domain of LFA-3 fused to hulG ₁ Fc fusion domain
Orencia® (Abatacept)	BMS	Approved by FDA 12/23/2005	CD80/CD86	RA	FcFP; CTLA4 exodomain fused to sequence modified hulG ₁ -Fc domain
Arcalyst® (Rilonacept)	Regeneron	Approved by FDA 02/27/2008	Antagonizes IL-1 β , IL-1 α , IL-1RA	CAPS, Muckle Wells Syndrome	TRAP; IL-1R and IL-1 accessory protein fused in-line with hulG ₁ Fc domain
Nplate® (Romiplostim, AMG-531)	Amgen	Approved by FDA 08/22/2008	TPO-R	Thrombocytopenia	PFcFP; TPO mimetic fused to C-terminus of aglycosylated hulG ₁ Fc domain ("peptibody")
Nulojix™ (Belatacept; LEA-29Y)	Bristol-Myers Squibb	Approved by FDA 06/16/2011	CD80/CD86	Renal transplantation	FcFP; sequence modified higher affinity CTLA4 exodomain fused to sequence modified hulG ₁ -Fc domain
Eylea™ (VEGF Trap-Eye)	Bayer-Schering Pharma/ Regeneron	Approved by FDA 11/18/2011	VEGF	Wet AMD; central retinal vein occlusion	TRAP; exodomain 2 of VEGFR1 fused to exodomain 3 of VEGFR2 fused to hulG ₁ Fc domain
Atacicept (TACI-Ig)	Merck-Serono/ Zymogenetics	Phase III	BLyS, April antagonist	SLE (PII for MS, RA)	FcFP; exodomain of transmembrane activator and CAML interacting (TACI) fused to hulG ₁ Fc domain

Table 14.1 Examples of important FcFPs on the market or in clinical trials (*cont'd*)

Name	Company	Current status (US)	Molecular target	Major indication	Protein type and construct
Zaltrap® (Aflibercept)	Sanofi-Aventis/ Regeneron	Approved 8/3/12 as this book went to press	VEGF	NSCLC, colorectal, prostate	TRAP; exodomain 2 of VEGFR1 fused to exodomain 3 of VEGFR2 fused to hulG ₁ Fc domain
rFIXFc	Biogen Idec/ Biovitrum	Phase IIb/III	Factor substitute	Haemophilia	SPFcFP; Factor IX fused to the N-terminus of hulG ₁ Fc domain
Dulaglutide™ (LY2189265)	Eli Lilly	Phase II	GLP-1R	Type II diabetes	PFcFP; GLP-1 mimetic peptide fused to hulG ₁ Fc domain
Bamlanivcept alpha (LTβR-Ig)	Biogen Idec	Phase II	LTβR-Ig	RA	FcFP; exodomain of lymphotoxin-βR fused to hulG ₁ Fc domain
Blisibimod (A-623; AMG- 623)	Anthera (from Amgen)	Phase II	BLyS (BAFF)	RA	PFcFP; BAFF-specific binding protein fused to N-terminus of non-glycosylated hulG ₁ Fc domain ("peptibody") – produced in <i>E. coli</i>
Briobacept (aka BR3-Ig)	Biogen Idec/ Genentech	Phase II	BLyS	RA	FcFP; BR3 (aka BAFF-R) exodomain fused to hulG ₁ Fc domain
AMG-386	Amgen	Phase II	Angiopoietins 1 and 2	RCC and ovarian cancer	PFcFP; Peptide fused to C-terminus of aglycosylated hulG ₁ Fc domain ("peptibody")
ACE-031	Shire/ Accelleron	Phase II	Activins	Sarcopenia, cachexia; Duchenne muscular dystrophy	FcFP; ActRIIB exodomain fused to hulG ₁ Fc domain
ENB-0040	Enobia	Phase II	Inorganic pyrophosphate	Hypophosphatasia (bone disorder)	SPFcFP; TNSALP fused to hulG ₁ Fc domain, with 10-mer peptide on C-terminus of Fc to target to bone tissue

ACE-011	Acceleron/ Cellgene	Phase II	Activin	Chemotherapy- induced anemia	FcFP; activin receptor type IIA (ActRIIA) exodomain fused to hulG ₁ Fc domain
rFVIIIIFc	Biogen Idec/ Biovitrum	Phase I/IIa	Factor substitute	Hydrophilia	SPFcFP; FVIII fused to hulG ₁ Fc domain
ASP2408	Perseid (Maxygen)/ Astellas	Phase I	CD80/CD86	RA, inflammatory diseases	FcFP; sequence modified higher affinity CTLA4 exodomain fused to sequence modified hulG ₁ -Fc domain
ACE-041	Acceleron	Phase I	Activin	Oncology	FcFP; activin receptor-like kinase 1 (ALK1) exodomain fused to hulG ₁ Fc domain
FP-1039	Five Prime	Phase I	Multiple species of FGF	Oncology – solid tumors	FcFP; FGF-R exodomain fused to hulG ₁ Fc domain
APG101	Apogenix	Phase I	CD95L	Oncology (glioblastoma); GVHD	FcFP; CD95 exodomain fused to hulG ₁ Fc domain
AMG-745	Amgen	Phase I	Myostatin	Muscle loss	PFcFP; myostatin-binding peptide fused to hulG ₁ Fc domain ("peptibody")
AMP-224	Amplimmune/ GSK	Phase I	PD-1	Oncology	FcFP; PD-L2 (aka B7-DC) exodomain fused to hulG ₁ Fc domain
ALKS 6931	Alkermes/ Acceleron	Pre-clinical	TNF- α	RA	FcFP; TNFR exodomain fused to hulG ₁ Fc domain
TTP-4000	Pfizer	Pre-clinical	Amyloid- β	Alzheimer's	FcFP; receptor for advanced glycation end-products (RAGE) exodomain fused to hulG ₁ Fc domain

Constructs: FcFP: Fc fusion protein; PFcFP: peptide-FcFP; SPFcFP: soluble protein-FcFP; TRAP: trap-based FcFP as described in section 14.3.

Other abbreviations: BAFF: B cell activating factor; CAPS: cryopyrin-associated periodic syndromes; FGF: fibroblast growth factor; GLP-1: glucagon-like peptide-1; GVHD: graft-versus-host disease; LT β R: lymphotoxin- β receptor; RA: rheumatoid arthritis; RCC: renal cell carcinoma; TNF: tumor necrosis factor; TNSALP: tissue non-specific alkaline phosphatase.

bound. For example, the type II soluble IL-1 receptor (IL-1R2) binds IL-1 with high affinity but does not signal because it does not contain the membrane anchor or intracellular signaling domains (Colotta et al., 1993). Other soluble receptors of cytokines, e.g. IL-13R α 2 and soluble ST2, act in a similar manner (Mantovani et al., 2001). The discovery of these naturally acting soluble receptors led to the design of molecules in which the exodomain of a normal cytokine receptor was fused genetically to the Fc of an IgG, which resulted in a long half-life soluble receptor that blocked the activity of the cytokine to which it naturally bound (Capon et al., 1989). The first example of this was the construction of a protein containing the ligand binding domains, D1-D2, of CD4 to the N-terminus of the IgG₁ Fc domain (Capon et al., 1989). This FcFP, called an “immunoadhesin” protein because it was comprised of an IgG constant region and the ligand binding domain of the cell adhesion molecule CD4, specifically inhibited the natural ligand-mediated activities of CD4 (Byrn et al., 1990; Chamow et al., 1992; Ashkenazi et al., 1993).

Figure 14.1(a) shows an example of a typical receptor exodomain-FcFP in which either the ligand binding domain or the entire exodomain of a receptor is fused to the N-terminus of an Fc containing its hinge, either by fusion directly to the hinge or via a short linker. Similar to antibodies, the FcFP forms a homo-dimer through the strong interactions of the C_H3 domains and is stabilized by the inter-chain disulfide bonds of the hinge. Etanercept (Enbrel®) and alefacept (Amevive®) are examples of receptor exodomain Fc fusion molecules (see Table 14.1). Huang (2009) and Table 14.1 describe additional receptor exodomain Fc fusion molecules in greater detail.

14.3 Traps: multi-ligand binding domains of different receptor chains fused to Fc region

Whereas some receptors bind their cognate ligands with high affinity based on a single binding interaction, other receptors require two or more distinct binding regions, or even subunits from two different receptor chains, to achieve high-affinity binding to their ligands. Examples of this type of interaction include the high-affinity binding of IL-1 by the type I IL-1 receptor (IL-1R1) and IL-1R accessory protein (IL-1RacP), high-affinity binding of IL-4 by IL-4R α and IL-2R γ (common γ chain), high-affinity binding of IL-13 with IL-13R α 1 and IL-4R α , and high-affinity binding of IL-6 with IL-6R α and gp130. In most of these cases, the ligand binds one receptor chain at a low affinity and the complex then recruits the other receptor chain to form a high-affinity interaction (Miloux et al., 1997).

Regeneron Pharmaceuticals developed constructs in which two different ligand-binding domains from different receptor chains were sequentially fused together and the combination fused to the human IgG₁ Fc (Economides et al., 2003). They termed this type of construct a “cytokine trap” (Economides et al., 2003). These cytokine traps were found to have extraordinary potency with both binding affinities and cell-based assay IC₅₀s in the low picomolar range (Economides et al., 2003). A model of an example Fc-trap is provided in Figure 14.1(b).

One trap has been approved by the FDA and others are in late-stage clinical trials. Rilonacept (Arcalyst®) is a trap consisting of the C-terminus of the IL-1RAcP ligand-binding domain fused to the N-terminus of the IL-1RI exodomain, together fused to a human IgG₁ Fc domain. Rilonacept is a very potent, high-affinity IL-1R antagonist approved by the US FDA as an orphan drug for the treatment of patients with cryopyrin-associated periodic syndromes (CAPS) (Economides et al., 2003).

Aflibercept (approved by the US FDA on 8/3/2012 as Zaltrap® for the treatment of metastatic colorectal cancer) binds the cytokine vascular endothelial growth factor-A (VEGF-A) and placental growth factor (PLGF), both of which have been implicated in tumor angiogenesis. Aflibercept is comprised of the second domain of VEGFR1 fused to the third domain of VEGFR2 and the human IgG₁ Fc region (see Table 14.1). The original VEGF trap, which possessed the first three domains of VEGFR1 fused to the human IgG₁ Fc region, was highly potent but had a poor pharmacokinetic profile and non-specific binding interactions that made it unsuitable as a clinical candidate. Subsequent engineering resulted in the final molecule which possessed better PK, affinity, stability, and specificity (Holash et al., 2002). Several other traps, including the IL-4, IL-4/13, and IL-6 traps, have been developed to certain stages but ultimately all have been dropped from development for various reasons (Huang, 2009).

14.4 Soluble protein FcFPs

As described in Chapter 1, non-antibody-based biologics often are replacement proteins that are used as therapeutics to replace a protein or function missing in certain individuals, causing a disease state. Examples of these include the erythropoietins (EPOs), cytokines, enzymes such as alkaline phosphatase, and blood factors such as Factor XIII and Factor IX. In some cases, it is desired to stabilize or extend the half-life of recombinant factors such as these. This half-life extension can be achieved by PEGylation or other bulking approaches as described in Chapter 12, or by fusion to an

Fc of an IgG. Examples of these approaches include rFIXFc, an Fc fusion of Factor IX currently in Phase IIb/III clinical trials, and rFVIII_hFc, an Fc fusion of Factor VIII in Phase I/IIa trials, both for the treatment of hemophilia (see Table 14.1). Additionally, Jazayeri et al. (2007) have described the construction of a leukemia inhibitor factor (LIF) mutant (mucin)-Fc fusion protein as an antagonist of serum LIF and oncostatin M. Jazayeri and Carroll (2008) point out various approaches to stabilize cytokines or cytokine mutants via Fc fusion technologies. Huang (2009) describes additional soluble protein Fc fusions as well, many of which are in the pre-clinical phase of development.

A second type of soluble protein Fc fusion can be exemplified by the fusion of an enzyme to the Fc to extend the half-life of the therapeutic enzyme. An example of this is ENB-0040, which is a tissue non-specific alkaline phosphatase-FcFP, currently in Phase II clinical trials for treatment of the bone disorder hypophosphatasia (Millán et al., 2008). This construct is unique because it also incorporated a 10-mer peptide fused to the C-terminus of the IgG-Fc that helps to target the FcFP to bone tissue (Millán et al., 2008) (see also section 10.8).

14.5 Antibody fragment – Fc fusion proteins

As mentioned in Chapter 12, the Fc domain of an IgG has been used extensively to elongate the serum half-life of antibody fragments such as scFvs and domain antibodies, as well as a variety of different alternative scaffolds (Strohl and O'Neil, 2012). A typical structure would be a domain antibody, scFv, or even multiples of these fused to the N-terminus or C-terminus of the Fc, typically via a flexible linker.

Cephalon's CEP-37247 (formerly ART621), an anti-TNF- α domain antibody isolated at Domantis, is the most clinically advanced antibody fragment fused to an Fc domain (Doyle et al., 2007; Gay et al., 2010). CEP-37247, which is about 75 kDa in size, or half the size of a standard IgG, is currently in Phase II clinical trials for the treatment of rheumatoid arthritis (Clinical Trial #NCT00854685). Due to its smaller size, it is projected that CEP-37247 may provide greater tissue penetration and broader biodistribution than a normal IgG (Gay et al., 2010). Trubion (now Emergent Biosolutions) has developed its platform of small modular immunopharmaceutical (SMIPTM) proteins by fusing scFvs to the Fc of an IgG, resulting in proteins of about 100 kDa. The anti-CD20 SMIPs TRU-015 and SBI-087 have been in clinical trials for the treatment of

rheumatoid arthritis and systemic lupus erythematosus (Burge et al., 2008; Hayden-Ledbetter et al., 2009), although it now appears more recently that TRU-015 has been dropped from development. Similarly, Harbour Antibodies has fused V_{HH} domains to both the N- and C-termini of an IgG₁ Fc domain to generate a tetravalent antibody (Janssens et al., 2006). Trubion's ScorpionsTM have this same type of structure.

14.6 Fc peptide fusions as receptor agonist therapeutics

There are several native peptides less than 50 residues in length in human serum that have a variety of biologics functions by exerting agonist activity on receptors. These include GLP-1, GLP-2, PYY, CCK, pancreatic polypeptide, gastrin, oxyntomodulin, NPY, and similar such peptides (Chaudhri et al., 2006; Murphy et al., 2006). As naked peptides, their half-life is usually in the minutes to a few hours range, depending on the sequence and proteolytic sensitivity of the particular peptide. One widely accepted method to lengthen the half-life of these types of peptides for therapeutic indications is to conjugate them to PEG as described in Chapter 12 and by Strohl and O'Neil (2012). Often, a single, linear 40 kDa PEG can increase the hydrodynamic radius of a peptide to prevent kidney filtration, to protect the peptide from proteolysis, to reduce immunogenicity, and to improve stability (McGregor, 2008). A second strategy to elongate the half-life of these peptides has been to fuse them to human serum albumin, as described in Chapter 12. A third option is to fuse these peptides to the Fc domains of IgGs (Sutherland, 2004), which both makes them too large to be cleared by the renal filtration system (Chapter 12) and recycles them through interaction with FcRn (see Chapter 7, section 7.3).

The most advanced example of this type of molecule is Amgen's romiplostim (NPlate[®]) which was approved for the treatment of chronic immune thrombocytopenia purpura by the FDA in 2009 (see Table 14.1). Romiplostim is comprised of two copies of thrombopoietin mimetic peptides separated by a flexible linker which are fused to the C-terminus of an IgG₁ Fc (Kuter, 2007). Because romiplostim is manufactured by *E. coli* fermentations, the Fc is non-glycosylated, helping to reduce effector functionality (Kumagai et al., 2007). Amgen has produced several other peptibodies, including AMG 623, now licensed to Anthera as A623 (see Table 14.1), a B-cell inhibitor for treating systemic lupus erythematosus, AMG 386, an angiopoietin-2 inhibitor for treating various cancers (Herbst et al., 2009), and AMG-745, a myostatin mimetic peptide Fc fusion

peptibody (Han et al., 2009b). All of these FcFP-peptide fusions appear to be produced in *E. coli*.

Another advanced peptide-Fc fusion agonists is Eli Lilly's GLP-1-FcFP, DulaglutideTM (LY2189265), which is now in Phase II clinical trials for the treatment of type II diabetes (Glaesner et al., 2010). They constructed a dipeptidyl peptidase IV (DPP4)-protected version of GLP-1 (V8-GLP-1) and fused it to the Fc of an IgG₄ (F234A/L235A) through a linker moiety (Glaesner et al., 2010). They found that without the linker, the activity of the GLP-1 mimetic FcFP was minimal, exemplifying the importance of the proper "presentation" of the peptide to its receptor (Glaesner et al., 2010). A general model of LY2189265 as compared with romiplostim is provided in Figure 14.1(c) and (d).

Centocor R&D (now Janssen R&D) has developed a mimetibody platform for the fusion of agonist peptides to the Fc of an IgG (typically IgG₁ or IgG₄ in which EU positions 234 and 235 were mutated to ala residues; Picha et al., 2008; Bugelski et al., 2008; Huang, 2009). Several mimetibody proteins have been made and placed into development. CNTO 528, which was comprised of a 20 amino acid erythropoietin mimetic peptide 1 (EMP1) fused to the Fc of a human IgG, was taken into Phase I clinical trials (Huang, 2009). CNTO530 is a second-generation version of CNTO528 improved by multiple protein engineering efforts for both biological and biophysical properties (Bugelski et al., 2008). CNTO530 was licensed to Edison Pharma, who reports that it has recently entered Phase I clinical trials for the treatment of inherited respiratory chain diseases and hemoglobinopathies. CNTO736 is a GLP-1 receptor agonist mimetibody protein which incorporates a DPP-4-resistant GLP-1 peptide analogue fused to an IgG₄ ala-ala Fc moiety (Picha et al., 2008). CNTO3649, an improved version of CNTO736, was taken into Phase I clinical trials in 2009.

14.7 Other FcFP structures

As noted earlier, FcFPs are dimeric proteins containing two Fc halves brought together by the strong interactions of the C_H3 domains and stabilized by the disulfide bonding of the hinge regions. This structure provides a potential strategy to generate constructs in which each Fc arm is fused to a different protein comprising a heterodimeric pair. An example of this principle was the construction of a heterodimer FcFP comprised of the α subunit of follicle stimulating hormone (FSH) attached to one Fc arm and the β subunit fused to the other arm (Low et al., 2005). The heterodimeric FSH-FcFP exhibited the desired extended serum half-life in rodents and

non-human primates as well as good *in vivo* activity in rats (Low et al., 2005). Similar constructs might be envisioned for other a/b heterodimeric soluble proteins or the exodomains of heterodimeric receptors such as IL-4R α and IL-13R α 1.

Syntonix (now part of Biogen Idec) developed a proprietary technology for the generation of monomeric Fc fusion molecules, in which a therapeutic protein is attached to only one arm of a dimeric Fc (see Figure 14.1(e)). Using this technology, Syntonix have generated several monomeric FcFPs such as EpoFc, interferon β -Fc, interferon α -Fc, and Factor IX-Fc. These monomeric FcFPs exhibited higher systemic bioavailability after pulmonary delivery in non-human primates than similar dimeric Fc fusions (Dumont et al., 2006). The most advanced monomeric FcFP is human Factor IX-Fc, which is currently in Phase IIb/III clinical trials for the treatment of Hemophilia B (see Table 14.1). Current Factor IX therapy requires multiple infusions per week, so the goal of the Factor IX-Fc is to reduce the frequency of administrations. The Factor IX-FcFP was shown to have a half-life in FIX-deficient mice, normal rats, FIX-deficient dogs, and NHPs of 52, 39, 47, and 50 hours, respectively, giving hope for a less frequent dosing regimen (Huang, 2009).

14.8 Issues to consider with FcFPs

14.8.1 Choice of Fc

For FcFPs potentially binding cells involved in the immune, blood homeostasis, or metabolic systems, the Fc functionality of the fusion protein may be a potential safety risk due to ADCC, ADCP, or CDC activities, as described in Chapters 7–10. Thus, for these FcFPs, the same approaches as described in Chapter 10 to silence MAbs may need to be undertaken. In fact, some of these approaches have already been used to mute the Fc activity of marketed and late-stage clinical candidate FcFPs. Abatacept (Orencia[®]) and belatacept both contain a modified IgG₁ in which several residues in the hinge region are replaced with serine residues, rendering the Fc less active than the wild-type IgG₁ (Davis et al., 2007) (see also Chapter 10). Similarly, the peptibody romiplostim (NPlate[®]) is produced in *E. coli*, resulting in an aglycosylated Fc and reduced Fc-mediated functions (Kuter, 2007). Additionally, Dulaglutide[™] (LY2189265), a DPP-4-protected version of GLP-1, is fused to the Fc of an IgG₄ (F234A/L235A), giving it substantially reduced Fc effector function activity (Glaesner et al., 2010). Similarly, receptor agonist mimetibody proteins such as CNTO530 (Bugelski et al., 2008), an

EPO-R agonist, and CNTO736 (Picha et al., 2008), a GLP-1 receptor agonist, were constructed in either IgG₁ or IgG₄ isotypes in which positions 234 and 235 were changed to ala residues to reduce Fc effector function (Hutchins et al., 1995). Both IgG₁ and IgG₄ “ala-ala” mutants were demonstrated to possess at least a 100-fold reduction in FcγRI and FcγRIIa binding activities (Xu et al., 2000). Finally, the hinge region of the anti-CD20 SMIP, TRU-015, was modified to reduce CDC while retaining wild-type ADCC activity (Hayden-Ledbetter et al., 2009).

14.8.2 Pharmacokinetics

The primary use of an FcFP is the extension of half-life conferred on a peptide or protein fused to the Fc of a human IgG. IgGs are endowed with their long half-life of nominally 14–21 days due to their recycling by FcRn (Roopenian and Akilesh, 2007), as described in Chapter 7. While fusion to a human IgG Fc does indeed confer longer half-life on the fusion proteins, very seldom does it reach the values observed with typical IgG antibodies. Etanercept, for example, has a half-life in humans of about 100 hours, which has led to the need for weekly dosing (Enbrel® prescribing information, 2011). Similarly, AMG 386, a selective angiopoietin 1/2-neutralizing peptibody that inhibits angiogenesis by preventing interaction between angiopoietins and Tie2 receptors, was shown in Phase I clinical trials to possess a half-life of 80–99 hours (Mita et al., 2010). CNTO528, an EPO mimetibody, was found in Phase I clinical trials to have a half-life of about 7.6 days in humans (Bugelski et al., 2008). Alefacept, on the other hand, has a longer half-life of about 12 days in humans (Strober et al., 2007). Suzuki et al. (2010) recently showed that most FcFPs have lower affinity to FcRn and concomitantly shorter half-lives than their IgG counterparts. While the Fc domains clearly confer a longer circulating half-life on the fused peptides and proteins, it is also clear that other mechanisms are involved in their clearance. These may include different kinetics of binding to FcRn caused by the presence of the fusion peptide or protein on the Fc or proteolytic cleavage.

14.8.3 Stability

FcFPs are by their very nature unnatural constructs comprised of an antibody Fc fused with some other protein or peptide. Because these

constructs are not natural, there is a variety of potential issues that may arise once they are built. For example, the pI of the original VEGF Trap led to aggregation and instability, resulting in a molecule with poor PK and developability characteristics (Huang, 2009). Additionally, proteases may either trim the ends of the Fc fusion constructs or cleave the fusion proteins, leading to product heterogeneity, inconsistent pharmacokinetic and pharmacodynamic profiles, and poor stability *in vivo* (Huang, 2009). In discovering and developing Fc fusion constructs, these potential “developability” issues (see Chapter 16 for more details) need to be carefully analyzed and corrected before moving the molecules into GLP manufacturing.

Antibody-drug conjugates

Abstract: Various methods have been used to improve the ability of antibodies to kill cells, perhaps the most complex of which are antibody-drug conjugates (ADCs). ADCs require an antibody or targeting moiety, a linker, and a toxic chemical. The antibody can be an IgG, an antibody fragment, or even a non-antibody binding protein. The linker is typically either a synthetic chemical linker or a peptide linker, and the toxic chemical can be either a cytotoxic natural product derivative such as monomethyl auristatin, a peptide or protein toxin usually derived from bacteria or plants, or a radionuclide. While in the past, ADCs were not significant drugs, recent improvements have made the newer generation of ADCs much more likely to succeed as therapeutics. Radionuclide-based conjugates also can be used for a variety of imaging modalities as diagnostics.

Keywords: antibody-drug conjugates (ADCs), chemical warheads (payload), linker, natural products, doxorubicin, calicheamicin, maytansanoids, monomethyl auristatins, duocarmycins, bystander effect, immunotoxins, PE38, radioimmunoconjugates, PET, SPECT, imaging

15.1 Introduction to antibody-drug conjugates

Antibody-drug conjugates (ADCs) are defined as antibodies to which other molecules are bound through a chemical linker. The term “conjugate” differentiates these modified antibody forms from peptide fusions that may be genetically fused to N- or C-termini of either the light or heavy chains of the antibody. The general principle of ADCs is to use the antibody to target a particular cell population, thereby carrying the conjugated molecule to that targeted cell population so that it can exert most, if not all, of its pharmacological activity upon just that population. In the most common case of ADCs, this is exemplified by antibody cell targeting, internalization by the cells (generally cancerous), and payload release to expose the highly

potent cytotoxic drugs to the tumor for toxin-mediated tumor cell killing (Lambert, 2005; Ricart and Tolcher, 2007; Chari, 2008; Carter and Senter, 2008; Beck et al., 2010). Thus far, most anti-tumor drugs need to be dosed at, or very near, their maximum tolerated dose in order to achieve the desired therapeutic efficacy (Chari, 2008). The goal of the anti-tumor ADC approach is to increase the specificity of cell targeting, thereby in principle increasing the efficacy to toxicity window for the chosen therapy, as well as lowering the dosage needed. In broad terms, there are six possible types of conjugates that have been linked to an antibody:

1. small molecules, which are usually highly potent cytotoxins (e.g. maytansinoid, auristatin) to kill tumor cells;
2. protein toxin (e.g. *Pseudomonas* exotoxin, diphtheria toxin) to kill tumor cells;
3. a cytolytic immunomodulatory protein (e.g. Fas ligand) to kill targeted cells;
4. a biologically active peptide (e.g. GLP-1) to extend the pharmacological half-life of the natural peptide;
5. enzymes (e.g. urease) to modify the biochemistry of the targeted microenvironment;
6. radionuclides (e.g. ^{90}Y , ^{111}In) for either killing or imaging of tumor cells.

As noted, the concept of ADCs can be extended to antibodies that carry conjugated bioactive peptides, such as have been developed as a platform by CovX (now Pfizer) (Doppalapudi et al., 2007), as well as targeting small molecules to specialized compartments. An example of a non-oncology use of ADCs is the targeting of osteoclasts by an anti-RANK receptor MAb conjugated with the peptide calcitonin, which was demonstrated to inhibit osteoclast differentiation (Newa et al., 2011).

When constructing ADCs, the most important considerations include target selection, which includes tumor or tissue specificity and ability to internalize, internalization rate of the ADC, potency of the cytotoxic drug, ability to mute the cytotoxicity of the drug until it reaches its destination, stability of the linker between the antibody and the drug, drug-to-antibody ratio, and the effects of the linker-conjugated drug on the antibody properties (Carter and Senter, 2008). In many ways, an ADC is much like a sophisticated pro-drug delivery system that is intended to deliver the highly cytotoxic drug directly to the tumor or targeted tissue without collateral damage to normal tissues. Once the ADC arrives at the desired site of activity, its cytotoxin cargo needs to be released through a chemical or biochemical mechanism found primarily or only at the desired site of activity

(Ducry and Stump, 2010). The greatest challenge in constructing ADCs is for the ADC to retain chemical and biochemical integrity and lack of toxicity on its way to its intended location, followed by the ability to be activated once it reaches its intended target.

Table 15.1 shows a list of example ADCs that are currently (or formerly, in the case of Mylotarg®) on the market as well as many of those in or known to be close to entering clinical trials. There may be additional ADCs in clinical trials as well, but unknown to the authors. Of the listed conjugates, 27 are small molecule (natural product) toxins conjugated to antibodies against tumor antigens, two are protein toxins conjugated to an anti-tumor antibody, another is an enzyme conjugated to a tumor-targeting antibody, seven are radioconjugates targeted against tumor antigens, and two are antibody-peptide conjugates, designed to extend the half-life of the bioactive peptides for non-oncology indications (see Table 15.1).

15.2 Overview and anatomy of a typical ADC

A typical ADC consists of three parts (see Figure 15.1):

1. the antibody (or binding protein), which is the method for targeting the payload to its ultimate destination;
2. the linker, which must be stable during circulation and behave as it is designed to behave once the antibody has reached the targeted receptor; and
3. the toxin or “payload”, which in the case of most ADCs is a highly potent cytotoxin that would be difficult to administer on its own due to the incredibly high toxicity (Ducry and Stump, 2010; Beck et al., 2010). The toxin can be a cytotoxin small molecule, a radionuclide, or a peptide or protein toxin.

Whatever linkers and toxins are used, it is critical that the ADC possess certain behavioral characteristics. First, while circulating through the bloodstream, the ADC needs to behave like a normal antibody. It must remain intact and soluble. In particular, the linkage between antibody and drug must remain intact so that the toxin or other payload is not separated from the antibody targeting moiety. Another critical factor is that the process used for formation of the ADC needs to result in a product that is relatively consistent with respect to a number of conjugation sites per molecule, and molar ratio of antibody to drug.

Table 15.1 Examples of antibody drug conjugates in development

US trade name (generic name)	Company	Current status (US)	Molecular target	Major indication	Protein and conjugate
Mylotarg® (Gemtuzumab ozogamicin)	Wyeth	Approved 05/17/2000; withdrawn 06/21/2010	CD33	Leukemia	Humanized IgG ₄ κ- calicheamicin cytotoxin conjugate
Zevalin® (Ibritumomab tiuxetan)	Biogen Idec	Approved 02/19/2002	CD20 on B cells	NHL	Murine IgG ₁ κ radioconjugate, using either ⁹⁰ Y or ¹¹¹ In
Bexxar® (Tositumomab-1131)	Corixa	Approved 06/27/2003	CD20 on B cells	NHL	Murine IgG _{2a} λ – ¹³¹ I radioconjugate
Adcetris™ (brentuximab vedotin; SGN-35)	Seattle Genetics/ Takeda	Approved 08/19/2011	CD30	Hodgkin's lymphoma	Chimeric IgG- MMAE cytotoxin conjugate
Trastuzumab emtansine (trastuzumab-DM1)	Genentech/ Immunogen	Phase III (BLA filing expected in 2012)	HER2	Breast cancer	Humanized IgG ₁ DM1 cytotoxin conjugate
Inotuzumab ozogamicin (G5/44)	Wyeth	Phase III	CD22	NHL	Humanized IgG ₄ calicheamicin cytotoxin conjugate
Lorvotuzumab mertansine IMGN901 (formerly huN901-DM1)	Immunogen	Phase II	CD56	SCLC, MM	Humanized IgG- DM1 cytotoxin conjugate
Glembatumumab vedotin (CDX-011; CR011-vcMMAE)	Celldex (formerly CuraGen)	Phase II	GPNCMB	Unresectable Stage III or Stage IV melanoma	Human IgG ₁ – MMAE cytotoxin conjugate
CME-548	Wyeth	Phase II	5T4	Solid tumors	IgG-calicheamicin cytotoxin conjugate
IMGN242	Immunogen	Phase II	CanAg	Oncology	Humanized IgG-DM4 cytotoxin conjugate
VB4-845 (Vicinium™ and Proxinium™)	Viventia	Phase I/II	EpCAM	Oncology	scFv-fused <i>Pseudomonas</i> exotoxin

Pentunomab AS1402 (Theragyn™; huHMFG1)	Antisoma	Phase III	MUC1	Breast cancer	Humanized IgG – ⁹⁰ Y radioconjugate
Clivatuzumab tetraxetan (Y90-hPAM4)	Immunomedics	Phase II	MUC1	Oncology	Humanized IgG – ⁹⁰ Y radioconjugate
Neuradiab (131I anti-tenascin MAb 81c6)	Bradmer Pharmaceuticals	Phase II	Tenascin	Glioblastoma multiforme	IgG- ¹³¹ I radioconjugate
J591-177Lu	Millenium	Phase II	PSMA	Prostate cancer	Murine IgG- ¹⁷⁷ Lu radioconjugate
Epratuzumab tetraxetan	Immunomedics	Phase I/II	CD22	NHL, SLE	Humanized IgG- ⁹⁰ Y radioconjugate
Milatuzumab conjugate	Immunomedics	Phase I/II	CD74	Multiple myeloma	Humanized IgG-doxorubicin cytotoxin conjugate
AVE-9633 (huMy9-6)	Sanofi-Aventis/ Immunogen	Phase I	CD33	AML	Humanized IgG-DM4 cytotoxin conjugate
Anti-CD22-PE (CAT-8015)	AstraZeneca	Phase I	CD22	Oncology, CLL	ScFv <i>Pseudomonas</i> exotoxin fusion protein
PSMA ADC MAb	Progenics/ Seattle Genetics	Phase I	PSMA	Oncology, prostate	IgG – MMAE cytotoxin conjugate
CVX-096	Pfizer	Phase I	GLP-1R	Diabetes	GLP-1 peptide mimetic-IgG conjugate
SAR-3419	Sanofi-Aventis/ Immunogen	Phase I	CD19	NHL	IgG-DM4 cytotoxin conjugate
BLIB-015	Biogen Idec – Immunogen	Phase I	Cripto	Solid tumors	IgG-DM4 cytotoxin conjugate
ASG-5ME	Seattle Genetics	Phase I	SLC44A4 (aka AGS-5) – novel tumor antigen	Pancreatic, prostate oncology	IgG-MMAE cytotoxin conjugate

Table 15.1 Examples of antibody drug conjugates in development (*cont'd*)

US trade name (generic name)	Company	Current status (US)	Molecular target	Major indication	Protein and conjugate
IMGN388	Immunogen	Phase I	αV integrins	Oncology	Humanized IgG-DM4 cytotoxin conjugate
CVX-045	Pfizer	Phase I	Angiogenesis inhibitor	Oncology	IgG-thrombospondin-1 mimetic conjugate
G193	Wyeth	Phase I	Lewis Y antigen	Non-small cell lung cancer	IgG-calicheamicin cytotoxin conjugate
BT-062	Biotest AG	Phase I	CD138	Multiple myeloma	Humanized IgG-DM4 cytotoxin conjugate
SGN-75	Seattle Genetics	Phase I	CD70	Advanced/recurrent RCC and relapsed/ refractory NHL	IgG-MMAF cytotoxin conjugate
Anti-CA IX	Bayer-Schering AG	Phase I	CA IX	Oncology	IgG-MMAE cytotoxin conjugate
AGS-16M8F	Agensys (Astellis)	Phase I	ENPP3	Metastatic renal cell carcinoma	IgG-MMAF cytotoxin conjugate
ASG-22M6E (aka AGS-22ME)	Seattle Genetics/ Astellas	Phase I	Nectin-4	Oncology – breast, bladder, lung cancer	Human IgG-MMAE cytotoxin conjugate
SAR566658 (huDS6)	Immunogen/ Sanofi Aventis	Phase I	CA6	Ovarian, breast, cervical, lung, pancreatic cancers	Humanized IgG-DM4 cytotoxin conjugate
MDX-1203	Medarex	Phase I	CD70	RCC and NHL	IgG-small molecule cytotoxin conjugate (pro-drug of CC- 1065 analog [duocarmycin])

BAY-94-9343	Bayer/ Morphosys	Phase I	Mesothelin	Oncology	Human IgG ₁ -DM4 cytotoxin conjugate
L-DOS47	Helix BioPharma	Entering PI	CEACAM6	Oncology	Camelid dAb-urease conjugate
Labetuzumab-SN-38 (CEAcide™)	Immunomedics	PC	CEACAM5	Oncology	Murine IgG-SN-38 cytotoxin conjugate
MEDI-547	Medimmune/AZ	Pre-clinical (near IND)	EphA2- conjugate	Oncology	IgG-MMAF cytotoxin conjugate

Abbreviations: CA IX: carbonic anhydrase IX; DM1: (N^E-deacetyl-N^E-(3-mercaptop-1-oxopropyl)-maytansine); DM4: (N^E-deacetyl-N^E-(4-mercaptop-4-methyl-1-oxopentyl)-maytansine); ENPP3: ecto-nucleotide pyrophosphatase/phosphodiesterase 3; MMAE and MMAF: monomethyl-austatin E and F, respectively; NHL: non-Hodgkin's lymphoma; PSMA: prostate-specific membrane antigen; RCC: renal cell carcinoma; SLE: systemic lupus erythematosus.

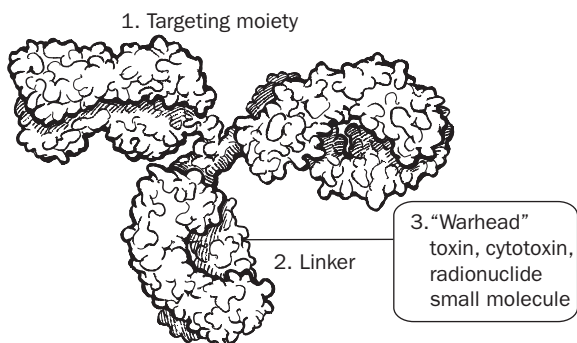


Figure 15.1 Antibody drug conjugate (ADC) model

Simple model of an IgG-based ADC showing the three critical components of the ADC that need to be optimized: (1) the antibody, or targeting moiety, which needs to bind specifically to the target and promote rapid internalization; (2) the linker, which needs to be stable in serum, but release the toxic molecule once it is in the endosome; and (3) the “warhead,” which can be a chemical (usually natural product) cytotoxin, a protein or peptide cytotoxin, radionuclide, or small molecule to be targeted to a specific cell or tissue type.

15.3 ADC antibodies and targets

15.3.1 Targets

Most MAbs used for ADC bind to a receptor or cell surface protein that is either found exclusively on tumor cells or is substantially overexpressed on tumor cells as compared with normal tissue. Examples of these kinds of targets include CD20, HER2/neu, CD22, CD33, CD30, CD40, CD56, CD70, CD74, and MUC1 (Teicher, 2009) (see also Table 15.1). Optimal ADC targets are those that are found primarily or at significantly higher concentrations on the cell or tissue type being targeted as compared with non-targeted tissues, and which can internalize when ligated with an antibody or other binding protein (Teicher, 2009). Teicher (2009) recently reviewed 34 different cell membrane receptors and proteins for ADC, highlighting their tissue distribution, structure, and efforts made to target them with ADCs. These included:

- carcinoma targets: EpCAM, GD2, EGFR, CD74, CD174, CD227, CD340, MUUC16, GD2, GPNMB, PSMA, Cripto, TMEFF2, EphB2, 5T4, mesothelin, TAG-72, and CA9;
- myeloid targets: CD33, CD64, CD40, CD56, and CD138;

- B cell proteins: CD19, CD20, CD21, CD22, CD40, CD70, CD79, and CD205;
- the T cell targets: CD25, CD30, CD40, CD70, and CD205 (note the overlap of B and T cell targets);
- an endothelial target: CD105;
- a stromal target: FAP; and
- a vascular target: ED-B.

(Teicher, 2009)

A potentially excellent target for the ADC approach may be CD30, which has a highly restricted expression on malignant cells of Hodgkin's disease, anaplastic large cell lymphoma, non-Hodgkin's lymphoma, multiple myeloma and cutaneous T cell lymphoma, and minimal expression on normal tissues (Doronina et al., 2003).

15.3.2 MAbs

Many types of MAb formats have been used as targeting agents, including IgGs (isotypes 1, 2, or 4), FAbs, F(ab')₂, scFvs, and TandAbs, to name a few (Wu and Senter, 2005). Two critical aspects of the different antibody formats described above are the roles of antibody half-life and avidity in optimal ADC-based therapy. In combination with affinity, these control the two significant variables tumor uptake and blood activity (Wu and Senter, 2005). Virtually all of the marketed and late-stage clinical candidate chemical ADCs to date are linked to IgGs that have reasonably long half-lives (see Table 15.1). While that fact alone does not prove that a long half-life of 14–21 days is the best approach for ADCs, there is concern about getting enough of the cytotoxic drug to the site of the tumor when antibody fragments, which have short half-lives, are used (Lash, 2010). To date, the use of short half-life antibody fragments has been largely limited to imaging efforts and protein toxin-ADCs, rather than a chemical ADC approach. In one example, however, anti-CD30 diabodies were tested against a similarly conjugated IgG as targeting agents for auristatins (Kim et al., 2008a). The auristatin-conjugated diabodies were slightly less potent than their IgG counterparts, even though both types of molecules contained approximately four toxins/antibody. The diabodies also demonstrated about three- to fourfold lower potency than the IgG equivalent *in vivo* as well, with clearance rates that were ~30-fold higher than the IgG (Kim et al., 2008a). These data minimally suggested further investigation of short half-life approaches might be worthwhile.

Antibodies used in ADCs must be naturally stable and non-aggregating and their ability to bind their targets must remain intact upon conjugation (Teicher, 2009). In the best case scenario, once the antibody engages the receptor or cell surface protein, it would be internalized into vesicles, thereby also carrying the toxin payload along with it. Thus, when selecting antibodies for this purpose, it is important to screen not only for binding characteristics, but also for high internalization rates, which can be critical to the success of the ADC approach (Becerril et al., 1999; Poul et al., 2000; Kuo et al., 2009; Poul, 2009; Zhou et al., 2010b).

There has been considerable discussion concerning the affinity of an antibody, its ability to be delivered to a tumor, its ability to penetrate tumor tissue, and its ability to internalize. A recent study using a series of MAbs targeting HER2 determined that delivery of the IgG to the tumor *in vivo* was inversely correlated with affinity, with the lower-affinity IgG having higher tumor penetration and broader biodistribution (Rudnick et al., 2011). That was balanced against the fact that higher affinity antibodies were internalized more rapidly (Rudnick et al., 2011). Thus, in the process of constructing a new ADC for a novel target, there might be a key balance to strike between internalization and tumor penetration. Factors affecting this balance would include target density on the tumor versus on surrounding tissues, tumor size, antibody affinity for the target, dose size and frequency, and potency of the cytotoxin.

15.4 ADC chemical “warheads”

Doxorubicin, calicheamicin, maytansines, auristatins, and other cytotoxic drugs have been the mainstays of chemical ADCs, some of them more potent than others (see Table 15.2). Over the past five to six years, several ADCs have emerged that have taken several of the lessons learned from earlier ADC attempts and made significant improvements, including more sophisticated linker technology, more potent cytotoxic drugs, and better choices of targets and epitopes. Most ADCs today utilize highly cytotoxic natural products derived from cyanobacteria, fungi, or actinomycetes. These typically have IC_{50} values in the range of 100–1,000 times lower than exhibited by cytotoxic agents that are given as “naked” chemotherapeutics (Ducry and Stump, 2010). Figure 15.2 shows four examples of ADCs, which include four different cytotoxins (doxorubicin, calicheamicin, the maytansinoid DM1, and the auristatin MMAE), and three different linker strategies (hydrazone, disulfide, and peptide).

Table 15.2 Common chemical cytotoxic molecules used for ADCs

Chemical or protein cytotoxin	Compound class and derivation	Pharmacological activity (MOA)	Approx. IC ₅₀ of free drug	Example where used	Reference
Doxorubicin	Anthracycline (NP from <i>Streptomyces peuceitius</i>)	DNA intercalator and stabilization of cleavable complex	200–300 nM range	BR-96	Strohl et al. (1997)
Calicheamicin γ 1	Enediene (NP from <i>Micromonospora echinospora</i>)	Generates double strand DNA breaks	Low pM range (~10–100 pM)	Mylotarg®	Hamann et al. (2002)
Maytansinoids DM1 and DM4	Ansamitocin (NP from <i>Actinosynnema pretiosum</i>)	Tubulin stabilizer	10–100 pM	Trastuzumab-DM1	Chari et al. (1992)
Monomethyl auristatins E and F (MMAE and MMAF, respectively)	Pseudopeptide toxin derivative (original NP from marine mollusk <i>Dolabella auricularia</i>)	Prevents microtubule polymerization; anti-mitotic	MMAE; ~10 pM–1 nM; MMAF, ~10–100 nM	Brentuximab vedotin (SGN-35)	Doronina et al. (2003, 2006); Senter (2009)
Duocarmycins A, SA and CC-1065 derivatives	Derivatives of NP from <i>Streptomyces zelensis</i>	Bind minor groove of DNA; adenine alkylation	5–10 pM (SA) and 50–100 pM (A)	MDX-1203	Boger and Johnson (1995)
Topotecan, Irinotecan, and derivatives (e.g. SN-38)	Pentacyclic alkaloids, camptothecins from Chinese tree <i>Camptotheca acuminata</i>	Topoisomerase I inhibitors	1–10 nM (for SN-38, potent metabolite of irinotecan)	Labetuzumab-SN-38	Li et al. (2006c); Burke et al. (2009)

Abbreviations: NP: natural product.

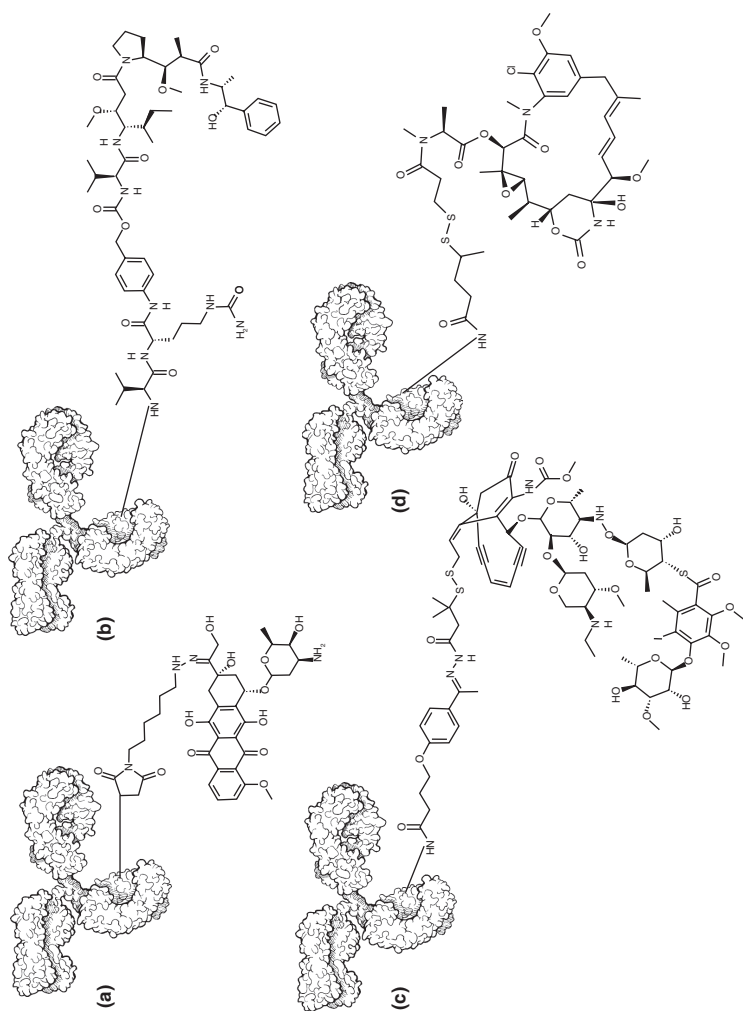


Figure 15.2 Examples of antibody drug conjugates (ADCs)

(a) Auristatin, linked via a peptide linker. (b) Doxorubicin, linked to the antibody via a hydrazone linker. (c) Calicheamicin, linked to the antibody via a hydrazone linker. (d) DM1 (maytansinoid), linked to the MAb via a disulfide linker.

Of the 27 ADCs listed in Table 15.1 that employ small molecule toxins, ten of them are built using the auristatins (either monomethyl-auristatin E or F [MMAE or MMAF]), another ten employ the maytansanoid cytotoxin (either DM1 or DM4), four utilize calicheamicin (one of which is gemtuzumab ozogamicin), one utilizes doxorubicin, one is an irinotecan derivative, and one utilizes duocarmycin (the toxin molecules are compared in Table 15.2). The number of ADC molecules per antibody depends on many factors including the method of coupling, the desired potency, and the linkers used (Beck et al., 2010; Ducry and Stump, 2010), but most are in the 3–4 molecules/drug range. For most ADCs developed today, the average appears to be about four toxin molecules per MAb.

Doxorubicin is an anthracycline anti-tumor natural product produced by *Streptomyces peucetius* and a variety of other actinomycetes (Strohl et al., 1997). Doxorubicin acts by intercalating into the double helix of DNA, whereby it stabilizes the topoisomerase II-DNA complex after initial strand cleavage, resulting in stabilization of what is known as the “cleavable complex,” leading to cell death (Strohl et al., 1997). The first significant ADC of note was called “BR-96,” an anti-Lewis Y antigen antibody linked to the intercalating anti-tumor drug, doxorubicin, via a 6-maleimidocaproyl-hydrazone linker (King et al., 2003). While BR-96 demonstrated significant pre-clinical efficacy, it failed to demonstrate efficacy in the clinic, likely due to the relatively low potency of doxorubicin (Ducry and Stump, 2010), even though the molar ratio of doxorubicin to MAb was high at 7.5–8.5/1 (King et al., 2003; Wu and Senter, 2005). This failure was actually a significant lesson for the field, as subsequent cytotoxic agents have been found that are significantly more potent (typically <1 nM IC₅₀ for killing of tumor cell lines) than doxorubicin (IC₅₀ for killing tumor cell lines in the 100–200 nM range) or other anthracyclines like it. Doxorubicin was the cytotoxic drug in BR-96, one of the first ADCs to undergo extensive clinical testing, as well as the current anti-CD74 ADC candidate, milatuzumab conjugate, which is in Phase I/II trials for the treatment of multiple myeloma. This effectively inhibits DNA replication and transcription, halting cell growth. Some consider that doxorubicin is not potent enough for broad use as the cytotoxic agent in ADCs (Ducry and Stump, 2010).

Calicheamicin is a member of the enediyne anti-tumor natural products produced by the actinomycete, *Micromonospora echinospora*. Calicheamicin γ 1 and a related enediyne, esperamicin, are potent cytotoxic agents with IC₅₀ values for cell killing in the 1–100 pM range. They function by binding to the minor groove of DNA and inducing diradical species that result in double-strand DNA cleavage (Hamann et al., 2002). The first chemical ADC to be approved by the FDA was gemtuzumab ozogamicin (Mylotarg®), which was approved for marketing in 2000 to treat acute myeloid leukemia, but was

subsequently removed from the market in 2010 (see Table 15.1). Gemtuzumab ozogamicin is an anti-CD33 IgG₄ humanized MAb to which N-acetyl- γ -calicheamicin is attached via a bifunctional hydrazone-based chemical linker (Hamann et al., 2002). The calicheamicin is released by hydrolysis of the hydrazone moiety of the linker when the gemtuzumab ozogamicin ADC is internalized into lysosomal vesicles (Ducry and Stump, 2010). For the period in which it was marketed, gemtuzumab ozogamicin was marginally effective and after recent studies demonstrated that the drug had more safety issues and even less efficacy than had previously been shown, it was voluntarily removed from the market by its manufacturer in June 2010.

Auristatins are pseudo-pentapeptide-based derivatives of dolastatin 10, a natural product toxin derived from the shell-less marine mollusk, *Dolabella auricularia* (also known as a “sea hare”) (Pettit et al., 1995; Doronina et al., 2003). These toxins function by inhibiting tubulin polymerization, which blocks mitosis and leads to apoptotic cell death of dividing tumor cells (Okeley et al., 2010). The two forms of auristatins used in clinical candidate ADCs today are monomethyl-auristatin E (MMAE) and monomethyl-auristatin F (MMAF), which have IC₅₀ values against a variety of cancer cell lines in the ranges of 10–100 pM and 10–100 nM, respectively (Senter, 2009). While MMAF potencies are typically much lower than MMAE potencies, an analog of MMAF, MMAF-O-methyl, was found that had sub-pM potency values. MMAF-O-methyl was described by the authors as the most potent auristatin they had ever found (Doronina et al., 2006). Currently there are ten auristatin-based ADCs in clinical trials, seven of which incorporate MMAE and three utilize MMAF (see Table 15.1). Both toxins are typically conjugated to the MAb via a peptide linker (Doronina et al., 2003, 2006). The lead auristatin ADC molecule is an anti-CD30 ADC developed by Seattle Genetics called Adcetris™ (brentuximab vedotin; SGN-35), which was approved for marketing in August 2011 for the treatment of Hodgkin’s lymphoma. Adcetris™, which has an average of about four MMAE molecules per MAb, is linked via a dipeptide linker and a cathepsin B cleavable bond (Okeley et al., 2010). This combination has been demonstrated to give Adcetris™ the ability to kill cells adjacent to CD30⁺ cells via the bystander effect (Ricart and Tolcher, 2007; Okeley et al., 2010). Additionally, cell-released cathepsin B may be present in the tumor micro-environment, which may allow for release of the toxin extracellularly, resulting in a potential extended bystander effect.

Maytansinoides, which are 19-membered macrocyclic lactams related to the ansamycins, are produced by plants and microorganisms, particularly the actinomycete *Actinosynnema pretiosum* (Yu et al., 2002). Ansamitocin P-3, the precursor of the maytansines DM1 and DM4, is a very potent semi-synthetic derivative of the maytansinoid natural product (Chari et al.,

1992; Yu et al., 2002). Microtubules are polar polymers of $\alpha\beta$ tubulin heterodimers built into long tubules that are dynamic in length, being constructed and deconstructed as required to support mitosis, cell division, and other cellular processes (Lopus et al., 2010; Lopus, 2011). Blocking the synthesis and degradation of tubulin, i.e. stabilizing the tubulin structure, interferes with the required normal dynamic processes, resulting in disruption of mitosis and the death of cells in active division. Maytansines act as tubulin stabilizers with IC_{50} values in the 10–40 pM range (Chari et al., 1992). Maytansines were tested as monotherapeutic cytotoxins for cancer chemotherapy in the 1970s but were too toxic as a systemic drug, so those efforts were halted (Tolcher et al., 2003). The two maytansines used in ADCs today, DM1 ($N^{2'}$ -deacetyl- $N^{2'}$ -(3-mercapto-1-oxopropyl)-maytansine) and DM4 ($N^{2'}$ -deacetyl- $N^{2'}$ -(4-mercapto-4-methyl-1-oxopentyl)-maytansine), have been developed by the biotech company Immunogen as one of the two most successful current ADC platforms in the business today. The lead compound in this class is trastuzumab-DM1 (T-DM1) which is currently in Phase III clinical trials for the treatment of breast cancer and other HER2-positive cancers (Lopus et al., 2010; Lopus, 2011). A total of two DM1 and eight DM4 conjugated ADCs are currently in clinical trials (see Table 15.1). New modifications of DM1 and DM4 include a new thio-trastuzumab-DM1 that appeared to possess greater potency than T-DM1 with a larger potency-to-toxicity window (greater therapeutic index; Junutula et al., 2010).

Duocarmycins and the related molecule known as CC-1065 (also called rachelmycin) are cytotoxins that bind the minor groove of DNA and alkylated adenine residues. These molecules, which are produced naturally by *Streptomyces zelensis*, recently have been developed as anti-tumor conjugates for ADCs (Boger and Johnson, 1995). One of the keys to these molecules is that they kill tumor cells at any point in their life cycle, rather than just killing dividing cells (Lash, 2010). This property could make them more toxic to non-tumor cells, emphasizing the need for optimized drug targeting. Synthron, which recently acquired Syntarga and their linker and ADC technology, is developing duocarmycin derivative ADCs as part of their platform (<http://www.syntarga.com/>), and Bristol-Myers Squibb, who recently acquired Medarax and their MAb platforms, are now developing a proprietary analog of CC-1065 as their ADC platform. MDX-1203, a Medarax ADC just entering Phase I clinical trials for the treatment of renal cell carcinoma and non-Hodgkin's lymphoma, targets CD70 with a pro-drug analog of CC-1065 linked to the targeting MAb by a cleavable peptide linker. The prodrug is activated at the tumor site by carboxylesterase II (http://www.ibclifesciences.com/upload/wysiwyg/drug_discovery_series/D10201/Passmore_Activation_of_Antibody.pdf).

Other recent examples of highly potent cytotoxic drugs being considered as ADC drugs are analogs of camptothecin, linked to their targeting antibodies via dipeptide or glucuronide-based linkers (Burke et al., 2009). Camptothecin is a pentacyclic alkaloid natural product produced by the Chinese tree *Camptotheca acuminata* (Wall et al., 1966). The camptothecin analogs, e.g. 7-butyl-10-aminocamptothecin and 7-butyl-9-amino-10,11-methylenedioxy-camptothecin, were found to be 10–1,000-fold more potent than the parental camptothecin molecule (Burke et al., 2009). Several derivatives of camptothecin have been made over the years to improve on the toxicity to efficacy ratio (Li et al., 2006b). Two of these, topotecan and irinotecan, have been approved for use as anti-tumor agents (Li et al., 2006b). Irinotecan has been shown to be metabolized into a 100–1,000-fold more potent derivative called SN-38 (Chabot, 1997). The biotechnology company, Immunomedics, has incorporated the irinotecan derivative, SN-38, into their anti-CEACAM5 ADC, labetuzumab-SN-38 (also known as hMN14 and CEAcide™) (Govindan et al., 2009).

In the best case scenario, the ADC should bind the target antigen through the activity of the antibody, be internalized by the receptor and be degraded by lysosomal activity to release the cytotoxic end-product to carry out its cell-killing mechanism of action before leaking out of the dead cell and killing adjacent cells (Ricart and Tolcher, 2007). This last part, where the released cytotoxin may kill adjacent cells, is called the bystander effect (Kovtun and Goldmacher, 2007; Ricart and Tolcher, 2007). If a tumor does not express the targeted surface antigen uniformly in the tumor cell population, an ADC that kills only the antigen-positive cells will not kill the entire tumor. ADCs, however, that possess the bystander effect will have a significantly better chance of killing both antigen-positive and antigen-negative cells (Kovtun and Goldmacher, 2007; Ricart and Tolcher, 2007). Adcetris™ (brentuximab vedotin, SGN-35), which was approved by the FDA in August 2011, was demonstrated to exert a significant bystander effect (Okeley et al. 2010), whereas trastuzumab-DM1, which has a non-cleavable linker terminating in a Lys residue, does not appear to exert a bystander effect on adjacent cells (Hughes, 2010). What is the difference between ADCs that exhibit the bystander effect and those that do not? Kovtun and Goldmacher (2007) make the case that hydrophobicity allows for neighbor cell penetration and promotes the bystander effect, whereas charged cytotoxic molecules cannot enter neighboring cells, and thus lack the bystander effect. They reviewed examples of both maytansinoids and auristatins in which this proposed differential mechanism held. If this is found to be widely true, then having a hydrophobic final cleavage product, after the antibody is degraded and the linker cleaved, may become one of the goals of making next-generation ADCs.

15.5 ADC linkers

The technology and approach used for the linker profoundly affects the toxicity, stability and half-life, and ultimately the efficacy of the ADC. Linkers fall into essentially two major categories, chemically or enzyme-labile linkers that are intended to be cleaved at the site of activity, and stable linkers that do not degrade, even at the intended site of action. In this case, degradation of the antibody itself may be the primary route of release for the cytotoxic drug (Ducry and Stump, 2010). One of the key shortcomings of early ADCs was the use of linkers that either degraded in the serum, exposing the patient to systemic release of the cytotoxic drug, or failure of the linker to degrade in the lysosome, reducing the potency of the drug (Beck et al., 2010). An additional complexity is the site of linker attachment – in this case, the linker carrying the cytotoxic drug may be attached randomly to the antibody, usually to exposed Lys residues (Beck et al., 2010; Ducry and Stump, 2010), or attached to specific residues that may have been modified (often to Cys residues) to be the specific attachment sites (Junutula et al., 2008, 2010; Beck et al., 2010; Ducry and Stump, 2010).

Chemically or biochemically labile linkers are linkers that are designed to release the active form of the potent cytotoxin upon cleavage, either by acidification (which occurs in the lysosome), or by a lower redox potential, or by some other mechanism such as enzymatic cleavage of an ester bond. The earliest cleavable linkers were acid-labile hydrazones that were intended to be cleaved once the ADC had been internalized and subjected to the acidic lysosomal environment (Wu and Senter, 2005; Ducry and Stump, 2010). One example of an ADC with this type of technology was BR-96, the anti-Lewis Y trisaccharide antigen MAb which utilized 6-maleimidocaproyl-hydrazone to link the targeting MAb to doxorubicin (King et al., 2003). BR-96, which contained eight doxorubicin molecules per MAb linked through Cys residues, was designed to release its cargo at pH 5, but was ultimately found to be unstable in serum and lacked the desired *in vivo* potency in clinical trials (Wu and Senter, 2005; Ducry and Stump, 2010). Gemtuzumab ozogamicin (Mylotarg®) also incorporated a hydrazone linker as well as a sterically hindered disulfide bond (Wu and Senter, 2005). The γ -calicheamicin was linked to the anti-CD33 gemtuzumab through exposed Lys residues at about 4–6 drug molecules per MAb and those were then mixed 50:50 with non-conjugated MAb to arrive at a final average of about 2–3 drug molecules per MAb total (Ducry and Stump, 2010). Gemtuzumab ozogamicin was withdrawn from the market in 2010 due to lack of an efficacy/toxicity window, at least in part caused by the instability of the hydrazone linker, which released some of the drug systemically (Ducry and Stump, 2010).

It was demonstrated more recently that chemically and enzymatically stable linkers tend to result in ADCs with higher efficacy to toxicity ratios due to their enhanced serum stability (Ducry and Stump, 2010). This general phenomenon appears to be the case with both the auristatins (developed by Seattle Genetics) and the maytansinoids (developed by Immunogen), the two major payloads used in ADCs today.

The first type of stable linker that has been used is a selectively cleavable peptide linker, which was first used to make a serum-stable but lysosomally cleavable BR96 MAb-peptide-conjugate (Dubowchik and Firestone, 1998; Wu and Senter, 2005). This peptide linker concept has been incorporated into the monomethyl auristatin product candidates developed at Seattle Genetics. Their approach has been to generate MMAE or MMAF ADCs containing 4–8 drug molecules per MAb linked to the antibody via exposed Lys residues (Wu and Senter, 2005; Doronina et al., 2006). These linkers are highly stable in serum, and are cleaved only when exposed to lysosomal acidic proteases such as cathepsin and plasmin (Wu and Senter, 2005; Ducry and Stump, 2010). The most advanced auristatin conjugate is brentuximab vedotin (SGN-35), which has the structure of cAC10 MAb-maleimidocaproyl-valine-citrulline-*p*-aminobenzoyloxycarbonyl-monomethylauristatin E, and contains four drug molecules/MAb, coupled through Cys residues (Doronina et al., 2006). Several different dipeptide linker designs have been tested for the cytotoxic drugs, monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF) (Doronina et al., 2003, 2006). It was demonstrated that the valine-citrulline dipeptide was more stable in serum than a similar phenylalanine-lysine dipeptide linker, and both were significantly more stable than a hydrazone-based linker (Doronina et al., 2003; Ducry and Stump, 2010).

Trastuzumab DM1 was constructed by conjugating the cytotoxin DM1 to lysine residues on trastuzumab via the uncleavable thioether linker, succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC; thus, MAb-SMCC-DM1). The antibody is completely degraded in the lysosome to yield the final stable toxin product, lysine-*N*^ε-SMCC-DM1, which has a potency (IC₅₀) on a variety of cell lines of about 8–16 nM, as compared with 30–90 pM for naked maytansine (Erickson, 2010). One potential drawback to this linker is that the final product has a terminal Lys residue, conferring a charge that appears to inhibit potential bystander activity on adjacent cells (Kovtun and Goldmacher, 2007). A twist on the concept of stable linker technology is the targeted antibody payload (TAP) technology developed by Immunogen. In this case, the antibody targeting moiety is connected to a maytansinoid cytotoxic drug, DM1 or DM4, via a sterically hindered disulfide linker (Erickson et al., 2010). The linker is stable in serum, where the

concentration of glutathione, the most important possible biological reductant, is in the μM range, as compared with cellular cytosol, which is an anoxic environment with glutathione concentrations reaching the mM range (Wu and Senter, 2005; Ducry and Stump, 2010). Moreover, the tumor microenvironment is anoxic, making delivery of the toxic maytansenoid payload, via cleavage of the disulfide bond, to the tumor both specific and potent. These linkers include the hindered disulfide *N*-succinimidyl-4-(2-pyridyldithio)pentanoate (SPP) linker coupled to an anti-CD56 MAb to make lorvotuzumab mertansine (MAb-SPP-DM1), and the sterically hindered disulfide *N*-succinimidyl 4-(2-pyridyldithio)butyrate (SPDB) linker included in combination with DM4 (MAb-SPDB-DM4). This latter linker-DM4 combination has been incorporated into multiple newer MABs, including SAR3419 (anti-CD19 MAb), IMGN388 (anti-pan αv MAb), BT-062 (anti-CD138 MAb), BIIB015 (anti-Cripto MAb), and SAR566658 (anti-CA6 MAb) (Erickson, 2010).

A study was recently carried out by Immunogen comparing their three different linker series, SMCC-DM1, SPP-DM1, and SPDB-DM4 (Erickson et al., 2010). They demonstrated that the same antibody, huC242, coupled with each of the three linkers, resulted in different degradation patterns and thus different end products. The sole metabolite of huC242-SMCC-DM1 was found to be lysine- N^ϵ -SMCC-DM1. On the other hand, the disulfide-linked conjugates huC242-SPP-DM1 and huC242-SPDB-DM4 were metabolized to lysine- N^ϵ -SPP-DM1 and lysine- N^ϵ -SPDB-DM4 metabolites, respectively. However, due to the disulfide bound in the linkers, they were further metabolized to the lipophilic thiol-maytansinoid metabolites that have been shown to possess bystander activity (Erickson et al., 2010). Additionally, the *S*-methyl-DM1 and *S*-methyl-DM4 metabolites possessed the activity of naked maytansines, whereas the lysyl- N^ϵ -DM compounds had significantly reduced potency, similar to that found with lysyl- N^ϵ -trastuzumab-DM1 (Erickson et al., 2010). Thus linker stability alone is not the most critical feature of stable linkers, but also the chemistry, potency, and lipophilicity of the final end products of lysosomal degradation may also be critical to success.

Typical attachment sites on antibodies are exposed Lys residues or some other non-specific approach toward labeling the antibodies with toxin conjugates. Junutula et al. (2008) recently designed a “THIOMAB” by mutating HC Ala114 (Kabat numbering) to a Cys residue, followed by >90% efficiency in coupling of auristatins to each Fab arm through the mutated residues. The resultant THIOMAB conjugates demonstrated superior potency and a larger efficacy-to-toxicity window (i.e. greater therapeutic index) than equivalent ADCs (Junutula et al., 2008). Junutula et al. (2010) extended this principle to the construction of thio-trastuzumab-DM1, and showed that the resultant ADC was both more potent and had a greater therapeutic index than normal trastuzumab-DM1.

15.6 Issues, limitations, and design of ADCs

Given all of the information above, as well as the lessons learned from the past decade of ADC design and trials, how can one make the best ADC possible? To determine this, the first question is what limitations do current ADCs have? First, calculation of the proper dose has been a difficult aspect of ADC technology because exposure is difficult to measure (Webb, 2011). Thus dose calculation for efficacy without dose-limiting toxicity becomes a delicate balance to achieve (Webb, 2011). Second, better targets still need to be found that have the optimal characteristics of a high ratio of tumor to normal cell expression, and high internalization rates when ligated with antibodies. Third, antibodies that possess ideal binding characteristics, limited immunogenicity (i.e. are humanized or fully human), and rapid internalization rates on the cell surfaces need to be engineered (Chari, 2008). Fourth, linker chemistries still need to be improved to achieve high stability in serum as well as degradation or cleavage to hydrophobic and highly toxic end products in cells (Kovtun and Goldmacher, 2007; Ducry and Stump, 2010). Fifth, the cytotoxic drugs used need to have high potency (i.e. IC_{50} s against cell lines of 10–100 pM) and a mechanism of action that supports the killing of cancer cells being targeted (e.g. tubulin stabilizers work against breast and ovarian cancers, but may have limitations with other types) (Chari, 2008). Sixth, cancer cell resistance is a concern that must be overcome for effective tumor efficacy.

One of the significant limitations of ADCs is the presence of ATP-binding cassette (ABC) transporters that export drugs and toxins out of the cells, rendering those cells multi-drug resistant (MDR) to the cytotoxic payloads (Kovtun and Goldmacher, 2007; Tang et al., 2009). Even if the drug is successfully targeted to the cell population of choice, if those cells have active ABC transporters, they can remove the cytotoxin before it accumulates to toxic levels. The human genome encodes 48 different ABC transporters, three of which are known to be expressed widely by cancer cells; these include P-glycoprotein (P-gp; also called “multi-drug resistance” MDR1), MDR associated-protein 1 (MRP1, also called ABCC1), and ABCG2 (also sometimes called MXR, BCRP) (Kovtun and Goldmacher, 2007). All three of these resistance proteins can pump out anthracyclines such as doxorubicin, MRP1 can export enedienes such as calicheamicin, and P-gp can export taxanes such as paclitaxel as well as maytansenoids (Kovtun and Goldmacher, 2007). In some cases, modification of the toxin or linker of the ADC can make them resistant to export-based resistance mechanisms (Kovtun and Goldmacher, 2007; Kovtun et al., 2010). Tang et al. (2009) demonstrated that at least one maytansine derivative, AVE933,

an anti-CD33-disulfide bond linked-DM4 ADC, was sensitive in cell culture to P-gp activity but not in cells from acute myeloid leukemia patients. Unfortunately, 40% of the cells from patients were resistant to AVE933 by another unknown mechanism. Kovtun et al. (2010) designed a polyethylene-glycol (PEG)-maleimide coupling to achieve significant reduction in MDR-related activity and kill normally DM1-resistant cell lines. These types of approaches bode well for the future improvement of ADCs that will not be subject to MDR-related resistance mechanisms.

15.7 Radioimmunoconjugates

Another approach to ADC is the use of radioimmunoconjugates (RICs) in which a radionuclide takes the place of the cytotoxic chemical. This section will briefly describe uses of RICs for both imaging for diagnostic purposes and therapeutic approaches. The different functions for RICs require different isotopes, so conjugation of the correct isotope to the carrier MAb is one of the key issues for RIC. For example, radioimmunoscintigraphy which utilizes γ -detecting cameras and single-photon emission computer tomography (SPECT) require γ -emitting isotopes such as ^{99m}Tc , ^{111}In or ^{123}I (see Table 15.3). Positron emission tomography (PET) imaging requires positron (β^+)-emitting isotopes such as ^{11}C , ^{18}F , ^{64}Cu , ^{68}Ga , ^{86}Y or ^{124}I (see Table 15.3). Finally, targeted delivery for therapeutic use requires either strong β -emitters such as ^{90}Y , ^{131}I , and ^{177}Lu , or α -particle emitters such as ^{211}At , ^{213}Bi , or ^{225}Ac (see Table 15.3) (Wu and Senter, 2005; Olafsen and Wu, 2010).

15.7.1 RICs for imaging

The targeting agent for RICs is usually a short half-life binding protein that can be administered for binding to the target and rapid elimination of circulating RIC to enhance the contrast between target-related signal and background radioactivity. Thus F(ab')_2 molecules, FAbs, scFvs, nanobodies, and non-antibody binding proteins (Olafsen and Wu, 2010; Vaneycken et al., 2011) have widely been used as the targeting agents for imaging purposes. Nevertheless, for longer studies, full IgGs and minibodies have also been used (Milenic et al., 2010; Olafsen and Wu, 2010). Bispecific MAbs that target two receptors on the cells or tissues of interest can enhance tissue selectivity and potentially yield better imaging results

Table 15.3 Examples of common radionuclides utilized in radioimmuno-conjugates (RICs)

Radionuclide	Type of emission	Isotopic half-life	Max. tissue penetration (mm)	Example use
¹¹ C (carbon)	β^+	20.4 min	NA	PET imaging
¹⁸ F (fluorine)	β^+	109.8 min	NA	PET imaging
⁶⁴ Cu (copper)	β^+ , β^- , EC	12.7 hrs	NA	PET imaging
⁶⁸ Ga (gallium)	β^+	68 min	NA	PET imaging
⁷⁶ Br (bromine)	β^+	16.2 hrs	NA	PET imaging
⁸⁶ Y (yttrium)	β^+	14.7 hrs	NA	PET imaging
⁹⁰ Y (yttrium)	strong β^- , γ	2.7 days	12 mm	Therapeutic (e.g. Zevalin®)
⁹⁴ Tc (technetium)	β^+	4.9 hrs	NA	PET imaging
^{99m} Tc (technetium)	β^+ , IT, γ	6 hrs	NA	SPECT imaging
¹¹¹ In (indium)	EC, γ	2.8 days	NA	SPECT imaging
¹²⁴ I (iodine)	β^+	4.18 days	NA	PET imaging
¹²⁵ I (iodine)	EC, Auger electrons, γ	59 days	0.001 mm	SPECT imaging and potential therapeutic
¹³¹ I (iodine)	strong β^-	8 days	2 mm	Therapeutic (e.g. Bexxar®)
¹⁷⁷ Lu (lutetium)	strong β^-	6.7 days	1.5 mm	Potential therapeutic
¹⁸⁶ Re (rhenium)	β^-	3.8 days	5 mm	Potential therapeutic
¹⁸⁸ Re (rhenium)	β^-	17 hrs	11 mm	Potential therapeutic
²¹¹ At (astatine)	α	7 hrs	0.05 mm	Potential therapeutic – localized killing
²¹² Bi (bismuth)	α	60 min	0.6–0.9 mm	Potential therapeutic – localized killing
²¹³ Bi (bismuth)	α	46 min	0.1 mm	Potential therapeutic – localized killing
²²⁵ Ac (actinium)	α	10 days	0.04 mm	Potential therapeutic – localized killing

Abbreviations: EC: electron capture; IT: isomeric transition; NA: not applicable for intended use; PET: positron emission tomography; SPECT: single-photon emission computer tomography.

(Sharkey et al., 2010). The three key differences between the different molecules (e.g. IgG, F(ab')₂, FAb, diabody, scFv) is their size, avidity, and half-life, and hence tissue penetration, distribution, target binding, and clearance (Yokota et al., 1992, 1993; Olafsen and Wu, 2010). Thus, if the desire in the imaging study is to obtain the highest tissue penetration, scFvs or domain antibodies such as nanobodies can be used (Vaneycken et al., 2011). One of the favorite isotopes used for imaging is technetium-99m (^{99m}Tc) (see Table 15.3), which combines a short half-life of six hours (compatible with short half-life antibody fragments), the ability to be produced in hospital nuclear medicine departments, and the ability to label through a Cys residue containing a free sulfhydryl moiety (Olafsen and Wu, 2010). RIC can be used for diagnostic purposes, can provide information on the presence and density of the target, and can potentially help to inform dosing (Olafsen and Wu, 2010). To this end, both of the current RIC B cell lymphoma therapeutics on the market, Bexxar and Zevalin, come as imaging reagents (¹³¹I-labeled Bexxar and ¹¹¹In-labeled Zevalin) (see Table 15.4) as well as therapeutics (¹³¹I-labeled Bexxar and ⁹⁰Y-labeled Zevalin) (Olafsen and Wu, 2010). It is clear from Table 15.4 that the field of MAb-conjugated radionuclides for imaging has had a checkered history. Of the 13 radionuclide-MAb conjugates that were approved at one point by either the EMEA or US FDA, only five remain on the market today. Nevertheless, with the increased pressure on personalized medicine and the need to better segment patient populations, there is a significant effort today in moving new radionuclide-MAb conjugates forward into clinical trials for potential marketing. Olafsen and Wu (2010) give an overview of the current situation in that field. One of the areas in which there is concerted effort is in the coupling of HER2 imaging (e.g. Milenic et al., 2010) with trastuzumab (Herceptin®) dosing in a proposed process called “image and treat” (Capala and Bouchelouche, 2010).

Two new forms of imaging by RIC are single-photon emission computer tomography (SPECT), typically using the γ -emitters ¹²⁵I, ¹¹¹In, ^{99m}Tc, and positron emission tomography (PET), which uses the β^+ (e.g. ⁶⁸Ga, ¹⁸F, ⁶⁴Cu, ⁸⁶Y, and ⁷⁶Br in order of increasing half-life) emitters. These methods both use RICs and provide visualization of physiological and biochemical changes coupled with the targeting potential of antibodies (McCarron et al., 2005; Olafsen and Wu, 2010). The criteria for choosing the nuclide for use in a particular study includes availability (oftentimes this requires a nearby cyclotron), cost, energy emitted, chemistry, and half-life match with biological half-life (McCarron et al., 2005; Olafsen and Wu, 2010).

A new and rapidly expanding field in imaging is the conjugation of non-radioactive quantum dots to targeting proteins to obtain three-dimensional

Table 15.4 Approved radioimmunoconjugate imaging agents

Name	Company	FDA approval	Target	Antibody format	Radiolabel	Imaging indication
OncoScint® CR/OV (satumomab pendetide)	Cytogen (now Eusa)	December 1992; withdrawn	Tumor associated glycoprotein-72 (TAG-72)	Mouse IgG ₁	¹¹¹ In	Colorectal and ovarian cancer
CEA-Scan (arctumomab)	Immunomedics	June 1996; withdrawn	CEA	FAB' from mouse IgG ₁	^{99m} Tc	Colorectal, breast, small lung cell carcinoma
Myoscint (imciromab pentetate)	Centocor	July 1996; withdrawn February 1999	Myosin	FAB' from mouse IgG _{2a}	¹¹¹ In	Myocardial necrosis
Verluma (nofetumomab merpentan)	Boehringer Ingelheim	August 1996; withdrawn	40 kDa glycoprotein	FAB' from mouse IgG _{2b}	^{99m} Tc	Small cell and non-small cell lung cancer
ProstaScint (capromab pendetide)	Cytogen (now Eusa)	October 1996	PSMA	Mouse IgG ₁	¹¹¹ In	Prostate cancer
Zevalin (ibritumomab tiuxetan)	Cell Therapeutics, Inc.	February 2002	CD20	Mouse IgG ₁	¹¹¹ In	NHL
Bexxar (tositumomab)	GSK	June 2003	CD20	Mouse IgG _{2a}	¹³¹ I	NHL

NeutroSpec (formerly LeuTech) (fanolesomab)	Palatin Technologies	July 2004; suspended December 2005	CD15	Mouse IgM	^{99m} Tc	Appendicitis
Lymphoscan (bectumomab)	Immunomedics	None – not marketed	CD22	FAb' from mouse IgG _{2a}	^{99m} Tc	NHL
HumaSPECT (votumumab)	Intracel	1998 (EU), withdrawn 2003	Altered cytokeratins	Human IgG ₃	^{99m} Tc	Colorectal, ovarian and breast cancer
Indimacis-125 (igovomab)	CIS Bio International	September 1996; withdrawn September 1999	CA-125	F(Ab') ₂ from mouse IgG ₁	¹¹¹ In	Ovarian cancer
LeukoScan (sulesomab)	Immunomedics	Approved in EU February 2007	NCA-90 on surface of granulocytes	FAB' from mouse IgG ₁	^{99m} Tc	Activated granulocytes in osteomyelitis, appendicitis, IBD
Scintimun (besitesomab)	CIS Bio International	Approved in EU January 2010	NCA-95 on surface of granulocytes	FAB	^{99m} Tc	Inflammatory lesions in osteomyelitis

Abbreviations: CEA: carcinoembryonic antigen; IBD: inflammatory bowel disease; NHL: non-Hodgkin's lymphoma; PSMA: prostate specific membrane antigen.

images of the targeted tumor, organ, or tissue (Byers and Hitchman, 2011). Quantum dots are photostable semiconductor nanocrystals typically comprised of a core of CdSe, CDS, or CdTe and a shell of ZnS that are manufactured into crystal form that can easily be conjugated to antibodies and other targeting proteins (Byers and Hitchman, 2011). Quantum dots possess wide excitation spectra and narrow, symmetrical emission spectra which allow for multi-spectral imaging using the various dots (Byers and Hitchman, 2011).

15.7.2 RIC therapeutics

As mentioned at the beginning of this chapter, there are seven clinical stage radionuclide ADCs (see Table 15.1). Two of these, ibritumomab tiutexan (Zevalin®) and tositumomab-I131 (Bexxar®) have been approved for marketing since 2002 and 2003, respectively. Ibritumomab tiutexan comes as either a ^{90}Y (therapeutic) or ^{111}In (imaging) radioconjugate, whereas tositumomab-I131 comes only as the ^{131}I radionuclide (used for both imaging and therapeutic purposes). Both of these marketed radionuclide ADCs target CD20 on B cells for the treatment of non-Hodgkin's lymphoma (NHL). Of the current clinical candidates, pemtumomab AS1402 (Theragyn™) and clivatuzumab tetraxetan both are in Phase II clinical trials targeting the MUC1 cancer antigen using a ^{90}Y immunoconjugate for treatment of breast and other solid-tissue carcinomas. Neuradiab is a ^{131}I radioconjugate in Phase II clinical trials targeting tenascin for the treatment of glioblastoma multiforme, J591-177Lu is a ^{177}Lu -radioimmunoconjugate in Phase II trials that targets PSMA for the treatment of prostate cancer, and epretuzumab tetraxetan is a ^{90}Y radioconjugate in Phase I/II clinical trials that targets CD22 for treatment of NHL (see Table 15.1).

The key to efficacy for therapeutic RICs is their ability to bind the target antigen on tumor cells and emit their energy into the surrounding cells and tissue. RICs depend on the “crossfire phenomenon,” which is that the path of emission from radioactive decay will interact both with cells that possess the target antigen as well as adjacent cells within the emission field. Thus cells in the proximity of the RIC binding can be irradiated from multiple directions simultaneously (Ricart and Tolcher, 2007). Due to the type of emission of a particular radioisotope used with RIC, the focus may be on killing at the molecular level, cellular level, and tissue level. For molecular level RIC, the isotopes most commonly used are ^{125}I and ^{165}Er , which have a very low emission strength of a few eV and a range of $\sim 1\text{ }\mu\text{m}$. For killing

at the cellular level, α -emitters such as ^{211}At , ^{212}Bi , and ^{213}Bi , which are very damaging with emission energies of $\sim 4\text{--}8$ MeV but have very low tissue penetration ($\sim 40\text{--}100$ μm) due to their size, are used. For tissue-level killing, which relies more on the crossfire phenomenon (Ricart and Tolcher, 2007), strong β emitters such as ^{131}I , ^{90}Y , and ^{177}Lu , which have an emission strength in the $1\text{--}3$ MeV range and deeper tissue penetration (up to ~ 1 cm), are often used (McCarron et al., 2005). The two RIC therapeutics that have been approved for marketing today are ^{131}I -labeled Bexxar and ^{90}Y -labeled Zevalin, both of which are strong β -emitters (see Table 15.3).

The most significant issue with the use of RIC for therapy is the generation of the short half-life radioisotopes in the proximity of the patient to be treated, followed by the necessity to conjugate it to the antibody at the site of therapy (i.e. hospital or hospital-related lab). Other issues include toxicity, clearance, and localized delivery (McCarron et al., 2005). These issues are significant enough to keep sales of both Bexxar (no data for 2010) and Zevalin (\$28 million in sales in 2011) to a minimum.

15.8 Protein immunotoxins

As an alternative to a chemical natural product toxin warhead, natural protein toxins have been used as immunotoxins for the treatment of various tumors. Several toxins have been tried including the plant toxins ricin (Schindler et al., 2011), abrin (Bora et al., 2010), gelonin (Pirie et al., 2011), bouganin (Cizeau et al., 2009), and pokeweed antiviral toxins (Teicher, 2009), and the bacterial toxins *Pseudomonas aeruginosa* exotoxin A (Weldon and Pastan, 2011; Pastan et al., 2011) and diphtheria toxin (Potala et al., 2008; Kuo et al., 2009). Note that while many of these are described in this chapter on “conjugates,” most of these biological toxin-MAb “conjugates” are actually genetically fused proteins.

The keys to the successful functionality of these immunotoxins include low immunogenicity, high targeted-cell internalization rates, proteolytic stability in the endosomal compartment, and endosomal escape into the cytoplasm for their activity (Kuo et al., 2009; Cizeau et al., 2009; Mathew and Verma, 2009; Pirie et al., 2011; Weldon and Pastan, 2011; Pastan et al., 2011). Additionally Pirie et al. (2011) recently demonstrated that for the plant toxin gelonin, a threshold of 5×10^6 internalized toxin molecules per cell were required for killing – this key finding may also apply to other similar toxins as well and help to design better immunotoxins in the future.

Of the protein toxins mentioned above, perhaps the most progress has been made with *Ps. aeruginosa* exotoxin A (ETA) (Weldon and Pastan, 2011).

Natural ETA has three domains: the LRP1 receptor targeting domain I, a second domain (II) that may be involved in intracellular trafficking, and the catalytically active domain III that inactivates mammalian elongation factor 2 (eEF2) by ADP-ribosylating it (Weldon and Pastan, 2011). Recombinant ETA-MAb conjugates require domain III and an additional small fragment to be fully functional as recombinant toxins; this construct containing ETA residues 253–364 fused to 381–613 is typically referred to as PE38. The two primary issues in developing ETA-MAb conjugates are the immunogenicity of the ETA fragment and the lysosomal proteolytic instability of ETA which reduces its potency (Weldon et al., 2009; Pastan et al., 2011). Weldon et al. (2009) recently generated a mutant PE38 that demonstrated complete stability in the lysosome and exhibited increased potency over normal PE38. Additionally, Pastan et al. (2011) recently highlighted other advances in PE38 engineering that have optimized it as a therapeutic toxin including the identification and removal of several B and T cell epitopes (resultant molecule called HA22-8X) and generation of the protease-resistant version. These advances may make future generations of ETA-MAb conjugates more potent and tolerable.

To date, perhaps the most advanced PE38 derivative is the anti-CD22 ETA-Fv fusion moxetumomab pasudotox (also known as CAT-8015 and HA-22), which is currently in Phase I/II clinical trials for the treatment of hematological malignancies (Pastan et al., 2011; Weldon and Pastan, 2011) (see Table 15.1). Other forms of ETA-MAb fusions are also in clinical trials, including several NCI and academic-sponsored (i.e. non-industry-sponsored) candidates such as:

- LMB-2, an anti-CD25 scFv-PE38 fusion protein currently in Phase II clinical trials for the treatment of adult T cell leukemia/lymphoma (ATL);
- SS1P, a mesothelin targeted scFv-PE38 fusion in Phase I clinical trials for the treatment of malignant mesothelioma;
- MR1-1, an anti-epidermal growth factor vIII (EGFR-vIII)-PE38 fusion in Phase I for the treatment of malignant brain tumors; and
- cintredekin besudotox, an IL-13 mutein (mutated form) fused to PE38 to target IL-13R (Rosada et al., 2010) in Phase I for the treatment of tumors (not a MAb-toxin immunotoxin) (Weldon and Pastan, 2011).

The second group of protein toxins that has sparked significant interest is the group of plant-derived mammalian ribosome-inactivating proteins (RIPs) that includes three subtypes: Type I RIPs include bryodin, gelonin, pokeweed antiviral protein, and bouganin; Type II RIPs include abrin and

ricin; and Type III RIPs, which are less studied (den Hartog et al., 2002). Of these, a deimmunized form of bouganin called VB6-845 has been fused to an anti-EpCAM FAb with a linker containing a furin cleavage site (Cizeau et al., 2009). The deimmunized, highly stable immunotoxin, VB6-845, is currently being studied in Phase I clinical trials for the treatment of cancer.

An interesting combination of two separate deglycosylated ricin-conjugated murine IgG antibodies targeting CD22 and CD19 have been studied in investigator-initiated clinical trials for the treatment of refractory B cell lymphoma (Messmann et al., 2000), and more recently, for acute lymphoblastic leukemia (Schindler et al., 2011). The deglycosylated ricin was conjugated to each of the IgGs through a heterobifunctional linker, yielding a conjugate that showed clinical activity for multiple indications in clinical trials (Messmann et al., 2000; Schindler et al., 2011).

One of the critical shortcomings of most plant and bacterial toxins is their natural immunogenicity when administered to humans. Thus a novel approach to the generation of immunotoxins has included the use of human proteins such as the pro-apoptotic molecules Fas-ligand and TRAIL, proteolytic granzymes, or RNAses as the toxins, and scFvs targeting tumor cell surface antigens as the targeting agents (Mathew and Verma, 2009). A few examples of these include an anti-EGFR scFv-sTRIAL fusion for the potential treatment of squamous cell carcinomas, an anti-CD7 scFv-FASL fusion for the potential treatment of leukemias, and an anti-HER2 scFv-granzyme B fusion for the potential treatment of epithelial cell tumors (Mathew and Verma, 2009).

15.9 ADEPT

For several years, scientists and companies alike have shown a keen interest in ADEPT (antibody-directed enzyme prodrug therapy) as a mechanism to target highly toxic molecules specifically to tumor tissues (Wu and Senter, 2005; Tietze and Krewer, 2009; Schellmann et al., 2010; Andrady et al., 2011). The success of the ADEPT approach is to maximize the tumor-to-blood ratio of the toxic therapeutic (Wu and Senter, 2005). Various ADEPT approaches have been tried since 1985 with relatively minor success and no true breakthroughs resulting in late-stage clinical candidates. The concept of ADEPT is to deliver an antibody conjugated enzyme “A,” specific for a particular chemistry, which would target the tumor based on the specificity of the antibody for a tumor antigen. Once the antibody-enzyme conjugate had localized to the tumor, a non-toxic pro-drug, equipped with a linker cleavable

only by enzyme “A,” would be delivered systemically (Bagshawe et al., 1994; Tietze and Krewer, 2009; Schellmann et al., 2010; Andrady et al., 2011). The most critical aspect of a successful ADEPT therapeutic is that the toxin “prodrug” must be non-toxic in circulation, and activated only at the tumor site by enzymatic cleavage by the targeted enzyme. The proposed advantage of ADEPT over standard ADCs is the amplification of the cytotoxin concentration in the local tumor environment and the ability of the toxin to kill not only the targeted cell but bystander cells as well (Tietze and Krewer, 2009; Schellmann et al., 2010; Andrady et al., 2011).

Each component of the ADEPT system is critical to its success. The issues with ADEPT are many, including continued serum circulation of antibody-enzyme conjugate (Bagshawe et al., 1994), lack of potency in the toxins used, tumor resistance to the toxin used (generally due to multi-drug resistance mechanisms), systemic toxicity due to linker instability in the serum, and immunogenicity of the enzymes used to cleave the linker (Bagshawe et al., 2004; Tietze and Krewer, 2009; Schellmann et al., 2010; Andrady et al., 2011). For targeting, scFvs, FAbs, F(ab')₂, or other antibody fragments with short half-lives have been used as the targeting agent. Enzymes not normally present in human serum, such as non-human-derived β -D-galactosidase, alkaline phosphatase, carboxypeptidases A and G2, β -lactamase, and β -glucuronidase, are example of enzymes that have been used as the pro-drug cleavage catalysts, but generally are immunogenic and have low catalytic rates (Bagshawe et al., 2004; McCarron et al., 2005; Schellmann et al., 2010; Chen et al., 2011a; Andrady et al., 2011). Finally, the cytotoxic drug needs to be a molecule that can be made in an enzyme-activatable, inactive pro-drug format that is 100% stable in serum (i.e. the activation enzyme activity must not be present in serum). Some of the most popular cytotoxic drugs for ADEPT have been doxorubicin, camptothecin, duocarmycin, and paclitaxel (Bagshawe et al., 2004; McCarron et al., 2005; Kratz et al., 2008; Andrady et al., 2011). To address some of these problems, one recent approach utilized a human, genetically enhanced β -glucuronidase fused with a humanized scFv targeting cancer-specific TAG-72 antigen (Chen et al., 2011a). With advances in genetic engineering and drug design, new approaches to ADEPT may finally make it a viable clinical approach to cancer therapy.

15.10 Other ADC-like approaches

Besides targeting tumor cells with either small or large toxic molecules, there are additional uses being considered for antibody conjugates. The first

of these is an interesting approach developed by CovX (acquired by Pfizer in 2008) which modifies a catalytic antibody with aldolase activity to generate a highly reactive lysine in the CDR-H3 of the V_H chain. This allows for highly specific ligation of a chemical linker to that site. This linker has been used to attach peptides and small molecules to extend their half-life and their pharmacological activity (Doppalapudi et al., 2007). Pfizer and CovX have two peptide conjugates in Phase I clinical trials, including CVX-096, a modified GLP-1 peptide conjugate for the treatment of diabetes, and CVX-045, a thrombospondin-1-mimetic peptide conjugate for the treatment of cancer (see Table 15.1).

A second, particularly interesting, approach was recently described by Helix BioPharma in which the enzyme urease was targeted to the tumor microenvironment using a tumor-specific camelid domain antibody against an epitope found on CEACAM6 predominantly associated with tumor cells of non-small cell lung cancer (NSCLC). Urease normally converts ammonia and CO₂ into urea, but in the therapeutic mode, the thermodynamics are reversed, and urea produced in the tumor microenvironment is converted to ammonia, hydroxyl ions, and CO₂. The concept is that the increase in pH in the normally acidic tumor microenvironment will be harmful to the cancer cells and ammonia taken up by those cells will upset their metabolism. The urease conjugate, L-DOS47, is currently entering Phase I clinical trials for the treatment of NSCLC (<http://www.helixbiopharma.com/ldos47>).

The final example of a non-oncology use of ADCs is the use of an anti-RANK receptor MAb conjugated with the peptide calcitonin to inhibit osteoclast differentiation (Newa et al., 2011). It is expected that as researchers understand how to utilize conjugates better to target non-toxic payloads to specific tissues, more examples like these will emerge.

Development issues: antibody stability, developability, immunogenicity, and comparability

Abstract: One of the key attributes of a therapeutic monoclonal antibody or Fc fusion protein candidate is that it must have the biochemical and biophysical attributes that will make it stable, soluble, and not prone to degradation or modification during manufacturing and storage. A wide variety of potential liabilities in the lead candidate(s) may be exposed that require correction, including the propensity to aggregate, poor solubility, propensity to fragment, deamidation, oxidation, cyclization, or other chemical modification of key residues, disulfide bond shuffling, glycation, and so forth. During antibody discovery and development, it is critical to use antibody modeling and engineering to optimize the antibodies for stability, solubility, and other biophysical characteristics. Two significant potential outcomes if antibodies are not properly behaved are immunogenicity and lack of batch-to-batch comparability.

Keywords: aggregation, solubility, self-interaction, hydrophobic residues, post-translational modifications, disulfide bonds, deamidation, fragmentation, oxidation, glycation, pyroglutamate, glycosylation, immunogenicity, biocomparability

16.1 Introduction

MAbs and FcFPs are complex, large molecule biologics produced in cell cultures, purified from those cultures, and formulated for use in some form of stabilization buffer. Thus variability is inherent to both the processes used and the molecules themselves (Wang et al., 2007; Manning et al., 2010). The key to the successful development for therapeutic use of complex biologics such as these is to understand the potential chemical

variations of the molecule, and find ways to minimize both the variations themselves and the impact they have on efficacy, safety, and immunogenicity of the molecule (Chirino and Mire-Sluis, 2004).

As mentioned in previous chapters, as antibodies and FcFPs are designed and engineered, careful consideration needs to be taken of affinity, potency, Fc functionality, and other attributes that specifically affect the efficacy of the biologic generated. It is just as important, however, that the molecules being constructed also have biophysical properties that lend themselves to being developed and manufactured without great difficulty. Thus molecules being developed need to possess chemical, biological, and physical stability so that as they are developed, they are not degraded, oxidized, chemically modified, or undergo other unwanted modifications significantly.

As shown in Figure 16.1, the potential unwanted degradation or modification of MAbs and FcFPs that can negatively affect the development of those molecules can occur in many ways (Wang et al., 2007; Manning et al., 2010), including:

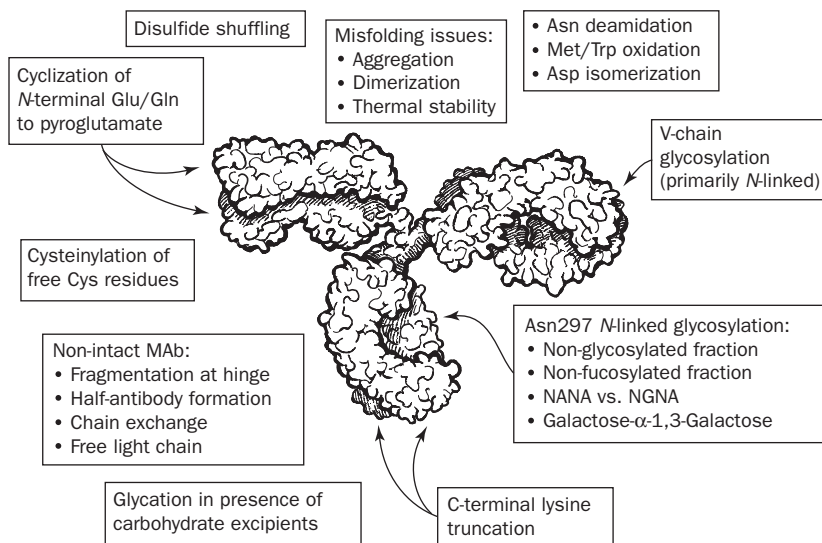


Figure 16.1 Antibody post-translational modification and degradation

Drawing of an IgG with notation of the various post-translational modifications and degradation issues that can be experienced in the discovery and development of therapeutic antibodies. Several of these are described further in the text and Table 16.1.

- aggregation, caused by self-interaction, unfolding, or other protein-association processes;
- lack of solubility at concentrations required for formulation and delivery;
- loss of structural integrity, i.e. fragmentation of the antibody;
- biochemical instability, resulting in post-translational chemical modifications to amino acid residues (e.g. deamidation, oxidation, cyclization, isomerization, glycation) (see Figure 16.2);
- shuffling of disulfide bonds, resulting in multiple isoforms of the molecule;
- V-region glycosylation or undesired glycosylation.

The analysis of proteins for these unwanted modifications is sometimes called “manufacturability” or “developability” analysis (Jenkins et al., 2008; Voynov et al., 2009; Shire, 2009; Conley et al., 2011). This type of process is used by most larger biopharmaceutical companies to ensure that molecules entering pre-clinical and clinical development possess the appropriate characteristics required for development and manufacturing. Once a biologic molecule has been developed for clinical trials, it is critical for that molecule to retain the biochemical and biophysical properties of the well-characterized lead, even when changes are made to the processes used to produce the molecule. The structural and functional comparison of a biologic produced from different batches or made by different processes is called “comparability” or “biocomparability” (Chirino and Mire-Sluis, 2004; Davis et al., 2009; Putnam et al., 2010; Lubiniecki et al., 2011). This chapter describes many of the potential instability or post-translational modification risks encountered with the discovery and development of recombinant therapeutic MAbs and FcFPs.

16.2 Aggregation

Therapeutic MAbs and FcFPs may go through many processes that can potentially lead to aggregation, including low pH elution and low pH holds for viral clearance during purification, ultrafiltration, freeze-thaw cycles, interactions with vials, syringes, and other solid surfaces, and storage over long periods of time at very high concentrations (Hawe et al., 2009; Shukla and Thömmes, 2010; Liu et al., 2010b; Franey et al., 2010; Vázquez-Rey and Lang, 2011). These conditions can exacerbate aggregation of aggregation-prone proteins and can induce aggregation in even reasonably well-behaved proteins. Aggregation of therapeutic proteins can lead to loss

$V_H \rightarrow$	CDR-H1	CDR-H2	
Q ITLKESGPT LVKPTQTITL TCTFSGFSL	<u>T</u> SG MG VSWIR QPPGKALE WL	AH YW DDDKR YNP SL KSR LT	ITKDT SK NQV
		$C_H \rightarrow$	
		ASTKGPSVFP	LAPSSK ST SG GTAALGCLVK DYFPEP VT VS
VL TM NDPV DTATYYCARL D F T YGFAY W	GGTLLVT VS		
	CDR-H3		
WNSGALTSV HTFP AV LQSS GLYSLSSV VT	VPSSSLGTQ T	YICNVN HK PS NTKVDK K YEP KSCDK TH TCP PC PA PELLGG	
PSVFLFPKP KDTL MI SRTP EVTCVVVD VS	HEDPEVK FN W	YVDGVE VH NA KTKPRE EQ YN ST YRVV SV LT VL HQ DWLNGK	
EYRCKV SN KA LPAPIET IS KAKGQ PRE PQ	VYITLPP SR DE	LTKNQVSL TC	LVKG FY PSDI AVEWES NG QP ENNYKT TP PV
DDSDGS FT LY SKLTVDK SR W	QQGNV FS CV	M HEALHN HY T	QKSL SL SPCK

$V_{\kappa} \rightarrow$	CDRL3	CDRL1	CDRL2
DI ~~VMT~~ QSPDS LAVSLGERAT INCRASQSD YNGISVHWY QQKPGQPPKL LIYAASNPES_GVPDFRFGSG SGTDFITIS			
	CK→		
SLQAEDVAVY YCQIIIDPW TFGQGTKVEI KRTVAAPSVF IFPPDEQLK SGTASVVCLL NNFPREAKV QWKVDNALQS			
GNQSOESVTEQ DSKDSTYSLS STLTLKADY EKHKVYACEV THOGLSSPVT KSFNRGEC			

Sequence modified from CNTO3930 for demonstration purposes. Potential problem residues (see also Table 16.1) are noted in bold: N-terminal Q (Gln) residue may be cyclized to pyroglutamate; M (Met) and W (Trp) residues may be oxidized; NXS/T sites (where x is not Pro) may be sites of N-glycosylation; NG (asn-gly) represents a potential deamidation site; DG (Asp-Gly) represents a site of potential isomerization; and C-terminal K (Lys) residue may be clipped off by an exopeptidase. CDRs, which are underlined (Kabat system), represent key sequences that must be tested for post-translational modification because they are involved in antigen binding.

of product activity, induction of immunogenicity, and an increase in the variability of efficacy from patient to patient. Both intrinsic factors such as sequence, hydrophobicity, and isoelectric point of the therapeutic MAb or FcFP, as well as extrinsic factors such as buffers, salts, and formulation excipients, can affect the aggregation potential of the therapeutic protein (Shire, 2009).

Aggregation is one of the most difficult problems to deal with for a candidate antibody or FcFP, so significant efforts have gone into identifying and predicting the causes for aggregation (Pepinsky et al., 2010; Wang et al., 2010b; Engelsman et al., 2011; Lowe et al., 2011a), for removing aggregation-prone sequences (Pepinsky et al., 2010), and formulating proteins to reduce aggregation (Wang et al., 2007; Maity et al., 2009). According to Manning et al. (2010), there are five major mechanisms leading to protein aggregation, as noted below:

- self-association of monomers leading to oligomerization;
- conformational changes in monomers, leading to aggregates;
- chemical modification resulting in aggregation;
- aggregation caused by nucleation or seeding;
- surface-induced aggregation.

Self-association of MAbs often occurs as a result of the intermolecular interactions of exposed highly hydrophobic sequences (Chennamsetty et al., 2009a, 2009b; Voynov et al., 2009; Chennamsetty et al., 2010; Wang et al., 2010b). Thus one method of controlling aggregation of MAbs is to limit the level of hydrophobicity in the exposed CDR sequences (Trevino, 2008). This, of course, is not always easy since hydrophobic amino acid residues such as Trp, Tyr, Phe, and Leu are important for protein–protein interactions (Moreira et al., 2007; Wu et al., 2010), and removal of those sequences often reduces binding to the desired target. Pepinsky et al. (2010) recently demonstrated, using crystallography of aggregating FAbs, that hydrophobic surfaces of a CDR were directly responsible for CDR–CDR and CDR–FR interactions of FAb fragments. They went on to mutate some of the less hydrophobic residues in the CDR and made other compensatory changes such as switching the isotype which resulted in significantly decreased propensity of the MAbs to aggregate while retaining antigen-binding activity (Pepinsky et al., 2010). Not only are amino acid sequences responsible for IgG stability, but glycosylation also plays a role. As noted previously in Chapter 11, glycosylation helps to stabilize the C_H2 domain of the IgG Fc. Kayser et al. (2011a) recently demonstrated that non-glycosylated antibodies were more prone to aggregation than their glycosylated counterparts,

suggesting that the use of non-glycosylated antibodies, as has been proposed by some, may lead to potential aggregation risks.

CNTO607 was demonstrated to be highly soluble as a FAb fragment but poorly soluble and highly aggregating as an IgG (Wu et al., 2010). It was determined by crystal structure, however, that the FAb formed a stable, soluble tetramer in which the CDR-H3 of one FAb interacted with the elbow region of another FAb (Teplyakov et al., 2009). The hypothesis was put forward that this same interaction occurred with the IgGs, but with the resulting oligomers generating an insoluble aggregate (Wu et al., 2010). Modification of CDR-H2 by addition of a glycan that could sterically block the interaction resulted in a highly soluble IgG (Wu et al., 2010).

Over the past few years, several investigators have tried to construct algorithms to determine what sequences or structures would lead to aggregation. Wang et al. (2010b) used the databases from 29 available high-resolution co-crystal structures of FAb-antigen complexes to define short “aggregation prone regions” (APRs). They pointed out that 45% of the CDR-H2 sequences within their database possessed APRs, whereas only 7% of CDR-H3 sequences contained them (Wang et al., 2010b). They also noted the high propensity for APR-causing sequences to be involved in antigen-binding (Wang et al., 2010b). Another structure-based computational approach called spatial-aggregation-propensity (SAP) has been used to measure protein and surface hydrophobicity and its potential contribution to aggregation (Chennamsetty et al., 2009a, 2009b; Voynov et al., 2009; Chennamsetty et al., 2010). This computational approach helped to identify surface patches with excessive hydrophobicity in therapeutic antibodies, some of which were in the constant chains. Some of these were then modified by mutation to improve the solubility and lack of aggregation characteristics of a MAbs (Chennamsetty et al., 2009a, 2009b).

Conformational changes in MAbs occur with partial unfolding of the three-dimensional structure, resulting in exposure of normally buried hydrophobic faces that then may interact causing aggregates to form (Kayser et al., 2011b). Thermal stability, as measured by differential scanning calorimetry (DSC), is one measure that is widely used to determine the overall stability of an antibody (Garber and Demarest, 2007). Garber and Demarest (2007) studied the thermal stability of 17 MAbs by DSC and showed that the midpoint for C_H2 domain unfolding was about 65–70°C, the C_H3 domain was ca. 80–85°C, and the T_m values for the FAbs ranged from 57.2 to 81.6°C. Among those 17 MAbs, there was an overall correlation between lower T_m, poorer expression, poorer stability, and unique amino acid residues in the CDRs (Garber and Demarest, 2007). Unfolding and aggregation can be measured in a variety of ways, including size exclusion

chromatography (SEC), hydrophobic interaction chromatography (HIC) (Wu et al., 2010; Boyd et al., 2011), dynamic light scattering (Luo et al., 2010a), extrinsic fluorescent dye-based unfolding assays (Li et al., 2011), and analytical centrifugation (Chirino and Mire-Sluis, 2004; Li et al., 2011; Kayser et al., 2011b). New approaches for analyzing protein conformational stability include differential scanning fluorescence, using hydrophobicity-probing dyes such as SYPRO® Orange to detect protein unfolding, and differential static light scattering, which probes conformational and colloidal stability (Goldberg et al., 2011; Li et al., 2011).

How can an aggregating MAb or FcFP be saved? There is no single answer to this that fits every situation. In some cases, in fact, the only answer is to move on to another base molecule. There have been studies on fixing aggregating antibodies, however, that highlight some interesting approaches. The first and perhaps most common approach is to remove or change hydrophobic amino acid residues (e.g. Trp, Tyr, Phe, Leu) in the CDRs that often serve as the root cause of aggregation (Wu et al., 2010). This is not always possible, however, as those same hydrophobic amino acid residues may contribute significantly to binding to the antigen (Trevino, 2008; Wu et al., 2010). Other ways to fix aggregating antibodies may be to alter the pI (Shire, 2009; Wu et al., 2010), introduce a V-region glycosylation site (which may be problematic in its own right; Wu et al., 2010), mutate potential problematic residues or sequences back to germline (Luo et al., 2010a), or determine changes computationally that should aid in reducing aggregation followed by experimental testing of the computational designs (Chennamsetty et al., 2009a, 2009b). In summary, it is absolutely critical that a MAb or FcFP development candidate have as few aggregation issues as possible, as this alone can potentially kill a program later in development (Lowe et al., 2011a).

16.3 Lack of desired solubility

Many antibodies are now being developed for indications for which subcutaneous dosing is the most desirable route of administration. With the exception of anti-cancer antibodies, which typically require dosing in the 5–10 mg per kg range (see Chapter 8, Table 8.2), MAbs and FcFPs are being developed for high concentration subcutaneous dosing. Typically, subcutaneous dosing is limited to a volume of 0.5–1.0 mL. Examples of MAbs and FcFPs that are currently being dosed subcutaneously include:

- adalimumab (Humira®) (40 mg/0.8 mL (50 mg/mL))
- etanercept (Enbrel®) (49 mg/0.98 mL (50 mg/mL) or 25.5 mg/0.51 mL (50 mg/mL))
- golimumab (Simponi®) (50 mg/0.5 mL (100 mg/mL))
- denosumab (Prolia®) (60 mg/mL) and
- ustekinumab (Stelara®) (45 mg/0.5 mL (90 mg/mL))

(data obtained from prescribing information for each drug). As can be seen from these examples, for a typical 0.5 mL subcutaneous dose, these antibodies and FcFPs need to be formulated at concentrations of 50–100 mg/mL or higher. Thus solubility of an antibody at high concentration is a critical feature to build into a discovery program, and antibody or FcFP leads that lack that particular characteristic are often discarded in favor of other candidates.

An important approach for measuring protein solubility using a small amount of protein is to assess the 2nd virial coefficient (B_{22}), a term that describes a protein's interactions with itself in a given solvent system, by static light scattering or self-interaction chromatography (SIC) (Le Brun et al., 2010). A related method used successfully for screening solubility is cross-interaction chromatography (CIC) (Jacobs et al., 2010). While Bajaj et al. (2006) cautioned not to over-interpret B_{22} values as a surrogate for irreversible aggregation rates, they do help to understand the solubility characteristics of MAb development candidates. Gibson et al. (2011) have also developed a high-throughput poly-ethylene-glycol (PEG)-based method for assessing the solubility of therapeutic MAb candidates, whereby the addition of PEG reduces protein solubility and the rate of the loss of solubility in the presence of PEG is correlated with the protein's inherent solubility (Middaugh et al., 1979).

16.4 Fragmentation

Upon storage, antibodies have been known to fragment chemically, losing peptides or domains from an IgG, resulting in degradation products (Cordoba et al., 2005). The FAb moiety is the most labile portion of an IgG₁, followed by other fragmentation patterns: FAb > (FAb + Fc) = HHL = HL > HH (where H and L represent heavy and light chains, respectively) (Kamerzell et al., 2011). One of the key cleavage sites is in the upper hinge (SC₂₂₀DKTHTC₂₂₆ (EU numbering); Cordoba et al., 2005) which can occur via either β -elimination or peptide bond hydrolysis, depending on the pH (Kamerzell et al., 2011). The most labile residue in the IgG₁ hinge was found to be the Asp residue (D₂₂₁), at which cleavage occurred on either side at

rates of ~100-fold greater than any other residue in the hinge (Cordoba et al., 2005; Kamerzell et al., 2011). The mechanism for this appears to be acid-enhanced Asp isomerization, which leads to cleavage of the hinge and fragmentation (Hambly et al., 2009). Exposure of IgGs to hydrogen peroxide or other strong oxidants can promote radical formation, leading to peptide cleavage and fragmentation (Yan et al., 2009). Radical-induced cleavage of the IgG₁ hinge often occurs between the two Cys residues (Cys₂₂₆-Pro-Pro-Cys-Pro) with the Cys₂₂₆ (corrected to EU numbering) residue being the site of primary reaction (Yan et al., 2009). It has also recently been shown that His₂₂₄ (corrected to EU numbering) in the upper hinge can mediate a radical-induced cleavage of human IgG₁ and that substitution of Ser or Gln at that position blocked the fragmentation (Yates et al., 2010).

As described in more detail in Chapter 9, normal IgG₄ antibodies form half-antibodies naturally in blood (van der Neut Kolshoten et al., 2007; Labrijn et al., 2009) as well as in cell culture (Reid et al., 2010). Reid et al. (2010) demonstrated that as high as 20–30% of the antibody in a cell culture of a model IgG₄ was present as half-antibody, much of it non-glycosylated. This half-antibody formation can be avoided by incorporating an S228P mutation into the hinge of IgG₄ (Angal et al., 1993), which has been reported to result in nearly 100% elimination of half-antibody formation (van der Neut Kolshoten et al., 2007).

Fragmentation by proteolysis is common for MABs and FcFPs during cell culture production. One of the most common proteolytic degradation reactions is the cleavage of the C-terminal lysine of one or both heavy chains (Dick et al., 2008). Virtually all antibodies possess heterogeneity at the C-terminus of their heavy chains after production in cell culture (Dick et al., 2008; Reid et al., 2010). An analysis of cell culture-produced MABs indicated that ca. 64% of the MAB product had both C-terminal lysines cleaved off, ~23% had one lysine removed, and ~14% still retained both lysines (Reid et al., 2010). Dick et al. (2008) also demonstrated that C-terminal lysine degradation was highly sensitive to the culture conditions used.

16.5 Post-translational amino acid residue modifications

Table 16.1 shows the possible sequences in a candidate therapeutic MAB or FcFP that can lead to biochemical instability that can result in potential activity and developability issues. The most common of these include isomerization, deamidation, and oxidation (Yan et al., 2007; Huhn et al., 2009; Reid et al., 2010). Thus one of the key functions during the discovery and optimization of an antibody as a potential therapeutic candidate is to

Table 16.1 Potential undesired post-translational modifications and other major issues for MAb and FcFPs

Potential issue	Sequence or residues	Reference
<i>Post-translational modifications</i>		
N-glycosylation site in V-regions	Asn-x-Ser/Thr (where x is not Pro)	Wright and Morrison (1993); Leibiger et al. (1999); Wu et al. (2010)
Lack of N-glycosylation at N297	Asn297	Rustandi et al. (2008); Ha et al. (2011)
Aspartate isomerization to isoaspartate	Asp-Gly	Chelius et al. (2005)
Deamidation of asparagine and glutamine to isoaspartate and isoglutamate, respectively	Asn-Gly; Asn-Ser; Gln-Gly Gln-Ser are most prominent	Chelius et al. (2005); Yan et al. (2007)
Methionine oxidation to methionine sulfoxide	Surface-exposed Met residues; particular issue if Met in CDRs	Chumsae et al. (2007); Liu et al. (2008b); Ji et al. (2009)
Tryptophan oxidation to hydroxytryptophans (HTRP), N-formylkynurenine (NFK), and kynurenine (KYN).	Surface-exposed Trp residues; particular issue if Trp in CDRs	Ji et al. (2009); Perdivara et al. (2010)
Pyroglutamate formation from N-terminal glutamate or glutamine	N-terminal Glu or Gln residue	Chelius et al. (2006); Dick et al. (2007); Liu et al. (2011c)
Glycation	Glycation of exposed Lys residues	Zhang et al. (2008); Miller et al. (2011)
<i>Other developability issues</i>		
C-terminal Lys cleavage	C-terminal Lys, when produced in CHO cultures	Dick et al. (2008); Reid et al. (2010)
Disulfide scrambling	Rearrangement of sulfhydryl bonds of Cys residues	Wypych et al. (2008)
Hydrophobicity-driven aggregation	Multiple Trp residues in CDRs	Wu et al. (2010); Wang et al. (2010b)
Half-antibody formation	Interchain disulfide bonds	Angal et al. (1993); Aalberse and Schuurman (2002); van der Neut Kolshoten et al. (2007); Reid et al. (2010)

minimize the number of residues that are labile and prone to chemical instability. Post-translational modification of the antibody can lead to loss of activity, aggregation, degradation, and/or immunogenicity (Huhn et al., 2009). Two fundamental approaches can be taken. The first is to scan the sequence of the V-regions for any undesired residue combinations as shown in Table 16.1 and, if they are present, mutate them to other residues. In some cases, this may result in significant changes to the activity or behavior of the antibody. A second approach is to test the antibody under light, heat, acid, and other potential stress conditions, followed by engineering to remove the significantly labile residues while retaining functional activity (Chumsae et al., 2007; Hambly et al., 2009).

The N-termini of all 51 germline V_H gene products are initiated with either a Gln (31/51) or Glu (20/51) residue. Similarly, of 40 V_K light chain germline genes, 10 possess an N-terminal Glu residue, and of 31 V_λ light chain genes, 20 are predicted to have an N-terminal Gln residue. It is well known that both Glu (Chelius et al., 2006) and Gln (Liu et al., 2011c) with free primary amine group are subject to N-terminal cyclization. Dick et al. (2007) found that 90–100% of N-terminal Gln residues on a model MAb were converted to pyroglutamate over the course of a cell culture run. Chelius et al. (2006) noted that a high percentage of N-terminal Glu would be expected to be modified chemically to pyroglutamate, but that the conversion did not affect the activity of the antibodies they tested. Liu et al. (2011c) noted that the conversion of Glu to pyroglutamate was slower than that of Gln. They and others suggested that N-terminal pyroglutamate formation actually stabilized the proteins and made them resistant to aminopeptidase activity (Chelius et al., 2006; Liu et al., 2011c).

Asparagine and glutamine deamidation occurs by elimination of the amide from the side-chain, resulting in generation of isoaspartate and isoglutamate, respectively (Robinson, 2002). Such a change is associated with a net increase in negative charge for the protein. The rate of deamidation is influenced by the position of the residue in the protein, the neighboring amino acid residue on the carboxyl side, and buffer or medium conditions (Chelius et al., 2005). The most reactive deamidation site is Asn-Gly, and to a lesser extent Asn-Ser, Asn-Ala, and other combinations (Chelius et al., 2005; Manning et al., 2010). Gln-Gly is also an amidation hotspot, although typically less so than Asn-Gly. Asparagine deamidation is an issue with many protein therapeutics and is so prevalent that there are multiple books written on it (Manning et al., 2010) and even a web page built specifically to address it (<http://www.deamidation.org/>). Aspartate isomerization, which is most prevalent with an Asp-Gly pairing, is relatively similar to asparagine deamidation but with no change in net charge.

Oxidation of amino acid residues is most prevalent with residues possessing reactive side-chains such as methionine, cysteine, tryptophan, tyrosine, and histidine (Ji et al., 2009; Boyd et al., 2011). In particular, exposed Met residues can be oxidized to methionine sulfoxide under mild oxidizing conditions or even in buffer (Ji et al., 2009), and further oxidation to the sulfone can occur under strongly oxidizing conditions. Thus exposed Met residues in the CDRs can oxidize and drastically affect antibody activity. Moreover, oxidation of the Met residues in the constant chain, which may be more prone to oxidation than Met residues in the FAB domains (Chumsae et al., 2007), can also have a negative impact. Liu et al. (2008b) demonstrated that oxidation of Met residues in the C_H2 domain lowered the T_m, thus decreasing the overall stability of the antibody. Additionally, Pan et al. (2009) demonstrated that oxidation of Met₂₅₂ in the C_H2 domain and Met₄₂₈ in the C_H3 domain could reduce Protein A binding, thus affecting purification, FcRn binding, and half-life.

Oxidation of tryptophan residues by reactive oxygen species or photo-oxidation can lead to the formation of hydroxytryptophans, kynurenine, and N-formylkynurenine (Ji et al., 2009; Perdivara et al., 2010; Boyd et al., 2011). The most significant factors for Trp oxidation include pH, solvent accessibility, and sequence around the Trp residue (Perdivara et al., 2010). Trp residues in CDRs pose both this potential risk, as well as the risk of making the antibody greasy due to their large surface area and hydrophobicity. Thus, while Trp residues are often found in CDRs and contribute to antibody activity, they need to be carefully analyzed under stress conditions to determine their lability (Ji et al., 2009; Boyd et al., 2011). Perdivara et al. (2010) cautioned, however, to differentiate between possible artifacts such as Trp oxidation during preparation of the antibodies and post-translation modifications.

Recently, it has been demonstrated that the sequence of Asp-Pro, and to a lesser degree, Asp-Gly, can result in peptide bond cleavage and thus fragmentation (Stackhouse et al., 2011). The Asp-Pro sequence has long been known to be a classical chemical cleavage site for peptides, but has not been studied as widely in therapeutic MAbs and FcFPs.

16.6 Instability and isomerization of disulfide bonds

Standard human IgG₁, IgG₂, and IgG₄ have 16, 18, and 16 disulfide bonds and 4, 6, and 4 inter-chain disulfide bonds, respectively (Wypych et al., 2008) (see Figure 16.3). Under optimal conditions, none of these Cys residues is free or unpaired, although unpaired Cys residues are found at

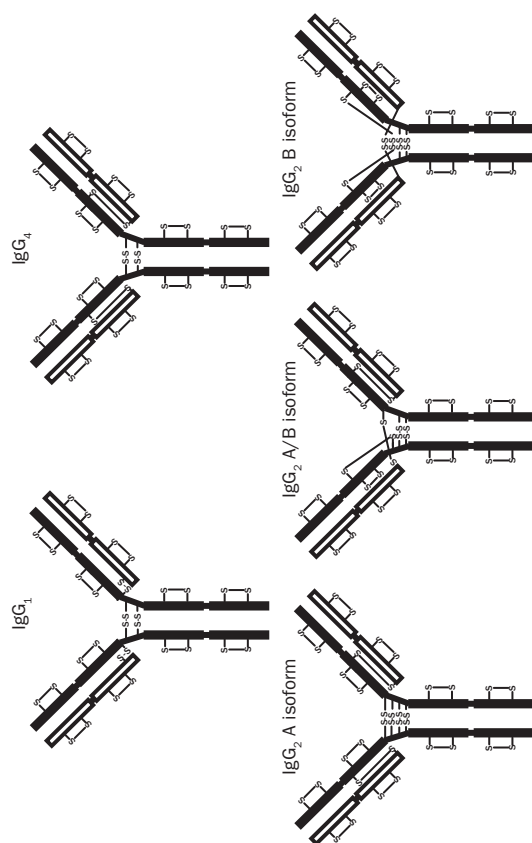


Figure 16.3 Diagram of disulfide bond patterns in IgGs

Intra-chain and inter-chain disulfide bonds of IgG₁ and IgG₂ (top), and three isoforms of IgG₂ (bottom). All numbering is according to the EU numbering scheme. Intra-chain disulfide bonds for all IgGs shown are: V_H, Cys22-Cys92; C_H1, Cys144-Cys200; C_H2, Cys261-Cys321; C_H3, Cys 367-Cys425; V_L, Cys23-Cys88; C_L, Cys134-Cys194 (Steiner, 1985). Inter-chain bonds for IgG₁ are C_κ Cys214-C_H1 Cys220, hinge Cys 226-Cys226, and hinge Cys229-Cys229 (EU numbering; Steiner, 1985). IgG₄ inter-chain disulfide bonds are: C_κ Cys214-C_H1 Cys131, hinge Cys226-Cys226, and hinge Cys229-Cys229 (although intra-chain Cys226-Cys229 disulfide bonding within the IgG₄ hinge is in equilibrium with inter-chain disulfide bonding, as described in detail by Schuurman et al., 2001). IgG₂ undergoes isomerization to generate multiple isoforms as described in the text and recent literature. The IgG₂-A isoform ("classical" isoform) possesses inter-chain disulfide bonds of (all hinge) Cys219-Cys219, and Cys220-Cys220, Cys226-Cys226, Cys229-Cys229 (EU numbering used here; labeled by Wypych et al., 2008, as Cys232-Cys232, Cys233-Cys233, Cys239-Cys239, and Cys242-Cys242 (Kabat numbering)). In the IgG₂ A/B isoform, which is an intermediate formed from the A isoform, the inter-chain bonds are: hinge Cys219-V_κ Cys214, hinge Cys220-V_H Cys131, hinge Cys226-Cys226, and hinge Cys229-Cys229 (EU numbering). The IgG₂ B isoform has inter-chain disulfide bonds of hinge: Cys219-V_κ Cys214, hinge Cys220-V_H Cys131, hinge Cys226-Cys226, and hinge Cys229-Cys229 (EU numbering; based on data provided by Wypych et al., 2008).

low levels in most MAb products (Zhang and Czupryn, 2002; Xiang et al., 2009; Chumsae et al., 2009). Occasionally an unpaired Cys residue may occur in variable chains, although most engineering efforts are designed to eliminate such incidence due to the reactivity and ability to promote aggregation even if buried (Brych et al., 2010). Certain human germlines such as IG VH-7 (Tomlinson et al., 1992) and genes 1-8 and 1-D8 of the IG VK-1 family (Tomlinson et al., 1995) possess unpaired Cys residues in their framework-3 sequences. These, as well as unpaired Cys residues in any CDRs, should be avoided if at all possible.

Liu et al. (2010a) determined the relative susceptibility of all of the disulfide bonds of a human IgG₁ to reduction, and found that inter-chain disulfide bridges between the heavy chain (HC) and the light chain (LC) were the most labile, followed by the two HC–HC disulfide bridges of the hinge (the most N-terminal of which was more susceptible than the other). Of the intra-chain disulfide bonds, those in the C_H2 domain were most susceptible to reduction, followed by those in the V_L, C_L, V_H, and C_H1 domains, and finally the C_H3 domain, which was most stable to reduction (Liu et al., 2010a). They also determined that the LC–HC inter-chain bonds of IgG₁λ were more labile than those of IgG₁κ (Liu et al., 2010a), a result similar to that found by Montañó and Morrison (2002) with IgG₂λ vs IgG₂κ. The C-terminus of C_κ ends in the Cys residue that bridges to the HC, but with C_λ, the disulfide bridging Cys residue is followed by a Ser residue, which is the C-terminal residue of that chain (Liu et al., 2011a). The presence of that Ser residue (or even if changed to an Ala residue) was found to be the cause for the increase in lability of the IgG₁λ HC–LC disulfide bridge. Deletion of that C-terminal residue increased the stability of the IgG₁λ HC–LC disulfide bridge (Liu et al., 2011a).

As noted in Chapter 9, human IgG₂ antibodies have the ability to form multiple structural isoforms due to alternative disulfide paring (Wypych et al., 2008; Martinez et al., 2008; Dillon et al., 2008; Liu et al., 2008c; Allen et al., 2009; Zhang et al., 2010; Lightle et al., 2010) (see Figure 16.3). These isoforms are found not only in recombinant IgG₂ antibodies, but also in IgG₂s isolated from myeloma patients and normal human serum. Thus they are “natural” and an intrinsic property of the human IgG₂ isotype, rather than being the product of recombinant DNA manipulations and/or cell culture production (Wypych et al., 2008). The root cause of this alternative disulfide bridging appears to be due mostly to the presence of two extra disulfide bonds in the hinge of IgG₂s (Martinez et al., 2008). The three major isomers have been labeled IgG₂-A (i.e. “normal” IgG₂ disulfide bridging), IgG₂-A/B, and IgG₂-B (Wypych et al., 2008; Lightle et al., 2010; see Figure 16.3). Additional minor isoforms have also been described,

including IgG₂-B₁ and IgG₂B₂ (Martinez et al., 2008), and, more recently IgG₂-B₃ (Zhang et al., 2010). These isoforms of IgG₂ are generated because the Cys residue in the LC can form a disulfide bond with any of the three different Cys residues in the HC, Cys127 (Cys131; EU numbering) of the C_H1 domain or Cys232 (Cys219; EU numbering) or Cys233 (Cys220; EU numbering) in the hinge region (Allen et al., 2009).

Multiple investigators have demonstrated that the major species of recombinant IgG₂ is not the “normal” IgG₂-A (Wypych et al., 2008; Martinez et al., 2008; Liu et al., 2008c; Zhang et al., 2010). Liu et al. (2008b) demonstrated that B cells produce IgG₂-A, which is converted relatively rapidly in serum to IgG₂-A/B, followed by slower conversion to IgG₂-B, prompting the authors to state that the form might be an indicator of the “age” of an IgG₂ in serum. Liu et al. (2008c) also demonstrated that the clearance of the different forms of IgG₂ were essentially identical. The significance of this is that the different isoforms do not have the same binding activity or functionality. Dillon et al. (2008) compared an IgG₁ isotype with the wild-type IgG₂ and its various isoforms of an anti-IL-1 β MAb. They found that IgG₁ had about a threefold greater activity than classical IgG₂ (IgG₂-A) and its various isoforms, and that the IgG₂-B isoform had about a threefold lower affinity and a threefold higher IC₅₀ than IgG₂-A, indicating that there are significant differences in the *in vitro* biological activity of the various isoforms (Dillon et al., 2008). Allen et al. (2009) generated Cys $\rightarrow$ Ser mutations of several of the IgG₂ Cys residues involved in multiple isoform generation, and showed that the mutant IgG₂ molecules were both stable and biologically active.

16.7 Stability at low pH

MAbs can have pI values typically ranging from 6.8 to 9.1 (Hari et al., 2010). Therapeutic MAbs and FcFPs are typically purified through a series of steps including Protein A binding and elution at low pH (2.5–4.0), a low pH (pH 3.5) hold for viral inactivation, ultrafiltration, and ion exchange chromatography (Liu et al., 2010b; Vázquez-Rey and Lang, 2011). Thus an antibody is exposed to acid pH for extended periods of time during the purification process (Liu et al., 2010b; Vázquez-Rey and Lang, 2011). It is critical, therefore, that MAbs and FcFPs being developed as therapeutic candidates are stable at low pH and do not undergo irreversible unfolding at low pH (Hari et al., 2010). In an analysis of several IgG₁ and IgG₂ MAbs with pI values ranging from 6.8 to 9.1, Hari et al. (2010) showed that IgG₂ is more susceptible to acid-induced aggregation than IgG₁, driven by the instability of the IgG₂ C_H2 domain at low pH. In another example of the

importance of IgG stability after low pH exposure, one IgG was recently shown to become polyreactive after exposure to low pH (Djoumerska-Alexieva et al., 2010), a condition which could potentially lead to undesired side effects via unknown mechanisms. Feige et al. (2010b) have characterized the alternatively folded states (AFS) of MAb domains driven by exposure to low pH. Despite the fact that all antibody domains are comprised of β -pleated sheets, they formed very different AFS at low pH. For example, the V_H domain, which is usually the most unstable domain at neutral pH, was one of the most stable domains in its low pH-induced AFS (Feige et al., 2010b).

16.8 Glycosylation issues

The recognition sequence for N-glycosylation of proteins is Asn-x-Ser/Thr (where x is not Pro). Most natural antibodies are glycosylated to a high extent at N297. Nevertheless, antibodies produced by cell culture often have a small but detectable fraction (~0.5%) that are non-glycosylated (Rustandi et al., 2008). Lack of glycosylation at N297 of a normally glycosylated antibody is not desirable (Tao and Morrison, 1989). Non-glycosylated MAbs have several differences from their normally glycosylated counterparts, including a significantly lower binding to Fc γ Rs (e.g. Bolt et al., 1993; Nesspor et al., 2012), decreased thermostability (Ghirlando et al., 1999), and increased susceptibility to proteolysis (Raju and Scallan, 2006) (see also Chapter 11). The crystal structure of a non-glycosylated IgG was shown to have a “superclosed” conformation, which would lead to lowered Fc γ R binding and lower stability (Feige et al., 2009; see Chapter 11). Non-glycosylated MAbs do, however, bind to FcRn normally and retain the long half-life of a wild-type IgG (Tao and Morrison, 1989). Recently, Ha et al. (2011) demonstrated that hemi-glycosylated antibodies (glycosylated on one heavy chain at N297 but not the other) also had reduced Fc γ R interactions and complement activation but retained normal FcRn interactions and half-life.

While it is both normal and typically desired for antibodies to possess glycans attached to N297 of the C_H2 domain (Chapter 11), it is not desirable to have glycans in the V-regions of therapeutic candidates because of the inherent variability they bring as well as potential interference with antigen binding (Wallick et al. 1988; Wright et al. 1991; Co et al. 1993; Wright and Morrison 1993; Khurana et al. 1997; Leibiger et al. 1999; Wu et al., 2010). A significant fraction of the human and mouse antibody germline genes shown in the IMGT database (Lefranc et al., 2005) possess N-linked glycosylation recognition sequences. Additionally, these sequences also can arise as a result of somatic mutations. As a result, about 18% of

the V_H sequences in the Kabat antibody sequence database contain the recognition sequence for N-linked glycosylation in their V-regions (Co et al., 1993), and approximately 20–30% of mature natural antibodies contain oligosaccharides in their V-regions (Spiegelberg et al., 1970; Wright and Morrison, 1993; Raju, 2008; Jefferis, 2009a). Some of these may be glycosylated in one FAb arm but not the other, leading to differential binding characteristics of the FAb arms (Labeta et al., 1986). V-region glycans can be involved in antigen binding, so in those cases, removal or loss of the glycan may eliminate or reduce binding (Wallick et al. 1988; Wright et al. 1991; Co et al. 1993; Wright and Morrison 1993; Khurana et al. 1997; Leibiger et al. 1999). As differentiated from Fc glycans, which are sandwiched between the two Fc chains, the V-region glycans are fully accessible by various glycan modifying enzymes. Thus V-region glycans often have a complex nature with variable levels of sialylation (Youings et al., 1996; Leibiger et al., 1999), much of which is influenced by the position of the attachment site (Wright et al., 1991; Coloma et al., 1999). V-region glycans also can play a significant role in the half-life and immunogenicity of antibodies (Wright et al., 1991; Coloma et al., 1999; Rudd et al., 1999), whereby incompletely sialylated V-region glycans can be recognized by asialoglycoprotein receptors (ASGP-Rs) which may lead to uptake, degradation, and increased clearance of the antibody (Day et al., 1980; Thornburg et al., 1980; Huang et al., 2006). Finally, V-region glycosylation may decrease antibody expression in cell culture (Gala and Morrison, 2004). Cetuximab (Erbix[®]), which has a glycan attached to the framework-3 sequence of the V_H domain, is the only currently marketed antibody that possesses V-region glycosylation (Qian et al., 2007).

In the presence of glucose, MABs can undergo glycation on exposed Lys residues (Zhang et al., 2008; Miller et al., 2011). Certain hotspots for glycation may be Lys₉₈ in the heavy chain (Miller et al., 2011). As much as 80% of one MAB was glycated at Lys₉₈ when incubated in the presence of 5% glucose for five days at 37°C, although this was an exceptionally high level as compared with other MABs tested (Miller et al., 2011). In another case, however, a fully human MAB was found to be glycated to the 40–60% level, indicating that this process may be more frequent than broadly appreciated (Zhang et al., 2008).

Finally, spurious O-glycosylation of IgGs of FcFPs may occur, depending on the structure of the protein and the host in which it is produced. As described in Chapter 18, *Saccharomyces cerevisiae*, wild-type *Pichia pastoris* (Alder Pharmaceuticals), and “humanized glycan-producing” *Pichia* (GlycoFi; Hamilton and Gerngross, 2007) are being considered or used for manufacturing MABs for therapeutic use. Since both yeasts are capable of O-glycosylation of Ser or Thr residues, there is a chance some IgGs produced

in those systems may contain at least low levels of O-glycosylation, which has the potential to lead to immunogenicity. Martinez et al. (2007) also reported the discovery of an IgG₂ produced in Chinese hamster ovary (CHO) cell culture that possessed a single O-mannose residue attached to Ser₆₆ of the light chain. This suggests that even mammalian production systems may O-glycosylate MABs and FcFPs more than commonly appreciated.

16.9 Immunogenicity

“What about immunogenicity?” This is often the first question asked when a discussion about therapeutic biologics occurs. Thus this issue has been the subject of a wide variety of papers, reviews, and professional conferences, and has been the subject of a cross-industry group that has written multiple white papers on aspects of immunogenicity (Koren et al., 2008; Shankar et al., 2008; Ponce et al., 2009). Fundamentally, with therapeutic MABs and FcFPs being developed today, the most important factor concerning immunogenicity is that it is a co-variate of pharmacokinetics (Büttel et al., 2011), i.e. when immunogenicity against a therapeutic MAB or FcFP occurs, it increases clearance and decreases exposure to that therapeutic (Büttel et al., 2011). Immunogenicity is usually described in terms of *incidence* of immune response against the protein drug by the patient population.

Many factors are involved in the potential immunogenicity of therapeutic MABs and FcFPs, including the presence of B cell and T cell epitopes in the primary amino acid sequence, aggregation, route of administration, genetic background of the patient population, immunological status of the patient population, contaminants that may act as adjuvants, target (soluble vs cell bound), and dosing concentration and frequency (Rosenberg and Worobec, 2004; Mukovozov et al., 2008; Büttel et al., 2011), to name a few of the major factors.

Anti-antibody responses have been described as human anti-murine antibodies (HAMA), human anti-chimeric antibodies (HACA), and human anti-human(-ized) antibodies (HAHA) (Mirick et al., 2004; Hwang and Foote, 2005). The increase in human sequence content in therapeutic IgGs from murine (0% human, e.g. Orthoclone OKT3®), to chimeric (~67% human, e.g. rituximab (Rituxan®), infliximab (Remicade®)), to humanized (~95–100% human, e.g. omalizumab (Xolair®), bevacizumab (Avastin®)), to human (~100% human, e.g. adalimumab (Humira®), golimumab (Simponi®)) has resulted in significant declines in incidence of immunogenicity across the board (see Table 16.2). The two largest jumps occurred in moving from murine to chimeric antibodies (reduction of ~80+% to ~20% immunogenicity)

Table 16.2 Immunogenicity of example marketed therapeutic MABs and FcFPs^a

US trade name (generic name)	Target	Major indication	Protein format	Percent immunogenicity	Ref.
Orthoclone OKT3® (Muromonab-CD3)	CD3 on T cells	OTR	Murine IgG _{2a}	44–89% of 90 patients, depending on assay	b
ReoPro® (Abciximab)	gpIIb/IIIa on platelets	CVD	Chimeric FAb	5.8% after first exposure; 27% after second exposure; 44% after fourth exposure	PI
Rituxan® (Rituximab)	CD20 on B cells	NHL, RA	Chimeric IgG ₁ κ	1.1% (4/356) of NHL patients; 11% (273/2578) of RA patients	PI
Zenapax® (Daclizumab)	IL-2Rα	OTR	Humanized IgG ₁	14% overall incidence; 34% incidence in pediatric patients	PI
Synagis® (Palivizumab)	RSV F-protein	RSV (infant)	Chimeric IgG ₁ κ	0.7% after fourth injection; anaphylaxis observed rarely (in <1/100,000 patients)	PI
Remicade® (Infliximab)	TNF-α	RA, Crohn's disease	Chimeric IgG ₁ κ	20–51% of patients, depending on dose and indication	PI
Herceptin® (Trastuzumab)	HER2/Neu	Breast cancer	Humanized IgG ₁ κ	0.1% (1/903); no allergic reaction	PI
Enbrel® (Etanercept)	TNF-α, TNF-β	RA	FcFP; IgG ₁ -Fc	Range of 3.6–8.7%; all non-neutralizing	PI
Simulect® (Basiliximab)	IL-2Rα	OTR	Chimeric IgG ₁ κ	1.1% (4/339 patients) in one study; 1.4% (2/138 patients) in second study	PI
Campath-1H® (Alemtuzumab)	CD52 on B- and T cells	Leukemia	Humanized IgG ₁ κ	8.3% (11/133) low titer non-neutralizing; 2% (2/133) neutralizing; 2% (4/211) low titer after multiple dosing	PI**
Humira® (Adalimumab)	TNF-α	RA, Crohn's disease	Human IgG ₁ κ	Overall 5% (58/1062); 1% when taking other immune-suppressants, 12% when taken alone	PI
Amevive® (Alefacept)	CD2	Psoriasis	FcFP; IgG ₁ Fc	3% (40/1357); low titer	PI
Xolair® (Omalizumab)	IgE	Asthma	Humanized IgG ₁ κ	<0.1% (1/1723 patients)	PI

Table 16.2 Immunogenicity of example marketed therapeutic MABs and FcFPs^a (*cont'd*)

US trade name (generic name)	Target	Major indication	Protein format	Percent immunogenicity	Ref.
Raptiva® (Efalizumab)	CD11a, α -subunit of LFA-1	Psoriasis	Humanized IgG ₁ κ	6.3% (67/1063 patients)	PI
Erbix® (Cetuximab)	EGFR	Colorectal cancer	Chimeric IgG ₁ κ	5% (49/1001), all non-neutralizing	PI
Avastin® (Bevacizumab)	VEGF	Colorectal cancer	Humanized IgG ₁ κ	0/500 patients (negative for high titer)	PI
Tysabri® (Natalizumab)	α 4 subunit of α 4 β 1 or α 4 β 7	Multiple sclerosis	Humanized IgG ₁ κ	~9% of patients; all neutralizing <i>in vitro</i>	PI
Orencia® (Abatacept)	CD80/CD86	RA	CTLA4-FcFP-modified Fc	1.7% (34/1993 patients); 5.8% (9/154) after treatment halted, of which 4% (6/154) were neutralizing	PI
Lucentis® (Ranibizumab)	VEGF-A	Wet AMD	Humanized IgG ₁ κ Fab	1–8% of patients with low titers after 12 to 24 months of treatment	PI
Vectibix® (Panitumumab)	EGFR	Colorectal cancer	Human IgG ₂ κ	<1% (2/612) by ELISA assay; 4.1% (25/610) by Biacore assay; <1% (1/350) were neutralizing	PI
Soliris® (Eculizumab)	Complement C5	PNH	Humanized IgG _{2/4} modified Fc	2% (3/196 patients)	PI
Arcalyst® (Rilonacept)	IL-1 β , IL-1 α , IL-1RA	CAPS, Muckle Wells syndrome	FcFP; IgG ₁ Fc	35% (19/55) of which 5 (9%) were neutralizing	PI
Nplate® (Romiplostim)	TPO-R	Thrombocytopenia	FcFP; IgG ₁ -Fc-peptide	8% (17/225) patients had pre-existing antibodies; 10% (23/225) had antibodies after treatment; 0.4% (1/225) neutralizing	PI
Simponi® (Golimumab)	TNF- α	RA	Human IgG ₁	Overall 4% (57/~1425); 2% in presence of methotrexate; 7% in absence of methotrexate	PI

Stelara™ (Ustekinumab)	P40 subunit of IL-12 and IL-23	Psoriasis	Human IgG ₁	3% (33/1,198) positive in one study; 5% (38/743) positive in second study; 48–90% of data were considered to be “non-conclusive” due to assay	PI
Actemra® (RoActemra in EU) (Tocilizumab)	IL-6R	Castleman’s disease; RA	Humanized IgG ₁	2% (46/2,876); 1% (30/2,876) were neutralizing; 5/2,876 patients had hypersensitivity reaction	PI
Removab® (Catumaxomab)	EpCAM and CD3	Malignant ascites; cancer	Rat IgG _{2b} -mouse IgG _{2a} hybrid	5% (71/132) before fourth infusion; 87% of patients, one month after last infusion	PI
Cimzia® (Certolizumab pegol)	TNF-α	RA	PEGylated humanized FAb	Overall 8%; 3% when taking other immunosuppressants, 11% when taken alone	PI
Ilaris® (Canakinumab)	IL-1β	CAPS	Human IgG _{1κ}	0% (0/60 patients after extended therapy)	PI
Arzerra™ Ofatumumab	CD20 on B cells	B-cell chronic leukemia	Humanized IgG _{1κ}	(0%) 0/79 patients after 8 or 12 weeks of therapy	PI
Prolia™; XGEVA™ (Denosumab)	RANK-ligand	Osteoporosis	Human IgG ₂	<1% (55/8113) after up to 5 years of treatment; none were neutralizing	PI
Benlysta™ (Belimumab)	BLyS	Lupus (SLE)	Human IgG _{1λ}	0.7% (4/563) patients at 10 mg/kg; 4.8% (27/559) at 1 mg/kg	PI
Yervoy™ (Ipilimumab)	CTLA4	Malignant melanoma	Human IgG _{1κ}	1.1% (11/1024 patients); none were neutralizing	PI
Nulojix™ (Belatacept)	CD80/CD86	Organ rejection	CTLA4-FcFP- modified Fc	2% (8/~400) patients; at least 3 of which had neutralizing antibodies	PI

Abbreviations: AMD: age-related macular degeneration; CAPS: cryopyrin-associated periodic syndrome; CVD: cardiovascular disease; EpCAM: epithelial cell adhesion molecule; EU: European Union; FcFP: Fc fusion protein; EGFR: epidermal growth factor receptor; NHL: non-Hodgkin’s lymphoma; TNF: tumor necrosis factor; OTR: organ transplantation rejection; PI: prescribing information from manufacturer; PNH: paroxysmal nocturnal hemoglobinuria; RA: rheumatoid arthritis; RANK: receptor activator of nuclear factor kappa-B; RSV: respiratory syncytial virus; SLE: systemic lupus erythematosus; TPO-R: thrombopoietin receptor; VEGF: vascular endothelial growth factor.

^a Unless otherwise stated, all data from prescribing information provided by manufacturers, given in the Works Cited under the trade name (e.g. Cimzia® prescribing information, 2011; etc.).

^b Kimball et al. (1995).

and from chimeric to humanized MAb (reduction of ~20% to <10% immunogenicity). Most researchers in the field agree that increase in human-ness of an antibody should result in an overall reduction in the rate of immunogenicity (Koren et al., 2002; Pendley et al., 2003; Hwang and Foote, 2005; Mukovosov et al., 2008; Almagro and Strohl, 2009; De Groot and Martin, 2009). On the other hand, Getts et al. (2010) have recently argued that the increase in human-ness of an antibody in its own right is not so highly correlated with lowered rates of immunogenicity, and that the evaluation needs to be made on an antibody-by-antibody basis. Similarly, Harding et al. (2010) point out that humanized, and even fully human, antibodies can still possess sequences in the CDRs that provoke immunogenicity in humans. Most MABs being developed today are either humanized or fully human MABs that would be expected to have in the range of 0.1–10% immune responses (HAHA) among patients (Dasgupta et al., 2008) (see Table 16.2). One note to make from the data shown in Table 16.2 is that these are data included in the prescribing information for each of these therapeutic molecules and are typically data obtained in Phase II clinical trials, so they may not represent longer-term treatment. One case in point is the immunogenicity observed with adalimumab (Humira®), which was reported to be, on average, about 5% (Humira® prescribing information, 2012). A recent study by Bartelds et al. (2011), however, paints a very different picture, with reports of up to 28% incidence of immunogenicity and a concomitant reduction in clinical benefit with longer-term use of Humira®. Krieckaert et al. (2010) also show examples in which anti-MAB antibody responses against therapeutic MABs are significantly higher than reported in the prescribing information packages. These data would support the contention that human-ness alone is not enough to result in low immunogenicity, and that a variety of other factors, e.g. aggregation, formulation, dosing, etc., should be considered carefully in developing new antibodies. One other issue concerning the reporting of immunogenicity rates is that the assay sensitivities are not consistent for different drugs which makes direct comparisons difficult (Aarden et al., 2008). Furthermore, the presence of the drug itself can confound assay results due to significant interference issues (Hart et al., 2011).

In most cases, the antibodies generated against a drug after the treatment of patients with a therapeutic MAB or FcFP are low titer and non-neutralizing. In these situations, the anti-drug antibody (ADA) response may have little or no effect on the efficacy of the protein therapeutic (Purcell and Lockey, 2008; Getts et al., 2010). Sometimes, however, these non-neutralizing ADAs may increase the clearance rate and thus have a negative impact on PK-PD, limiting exposure of the patient to the drug (Purcell and

Lockey, 2008; Putnam et al., 2010). One approach to this result has been to increase dosing to overcome the loss of drug, although this approach was recently suggested to be futile (Bartelds et al., 2011). In other cases, the ADAs may be neutralizing and eliminate efficacy of the drug, which typically results in patients needing to find other drugs to treat their condition. In rare occasions, treatment with a MAb or FcFP may result in formation of IgE ADAs against the product which can result in anaphylactic reactions, which can span the range from mild to rather severe reactions (Descotes and Gouraud, 2008; Purcell and Lockey, 2008; Maggi et al., 2011).

One adverse event that has occurred with several MAb or FcFP candidates is injection site reactions, in which swelling, redness, and pain occur at the injection site (Descotes and Gouraud, 2008; Maggi et al., 2011). As described in Chapter 10, there is some indication that CDC may be linked with injection site reactions (Van der Kolk et al., 2001; Tawara et al., 2008), so removal of CDC activity by Fc modification of development candidates (Chapter 10) may be worth consideration, where it is not required for activity or efficacy of the MAb or FcFP.

How can a discovery team best reduce the risk of immunogenicity in a candidate MAb or FcFP? There are several approaches taken by many groups. The first is to generate a sequence in the candidate MAb that has the highest degree of “human-ness” possible (Lazar et al., 2007; Almagro and Strohl, 2009; Bernett et al., 2010; Fransson et al., 2010; Parker et al., 2010). This can be done through the humanization of non-human V-region sequences, the replacement of non-human sequences with human sequences, and the conversion of framework sequences to human germline sequences (see Chapter 6). The second includes an *in silico* analysis of the primary sequence to search for sequences that might contain potential T cell epitopes (Koren et al., 2007; Jones et al., 2009; Parker et al., 2010; Bryson et al., 2010). The potential T cell epitopes can then be removed and the antibodies are analyzed for binding functionality. While most major antibody development organizations carry out an analysis of this type, it is not sufficient alone to completely eliminate T cell epitopes since not all human leukocytes antigen (HLA) types have been completely characterized yet. Another version of this is to have the epitopes of the candidate MAb or FcFP analyzed by T cell analysis, which gives a more robust characterization of potential T cell epitopes (Van Walle et al., 2007; Wullner et al., 2010). Unfortunately, pre-clinical animal models are very poor predictors of immunogenicity in humans, and can even be highly misleading (Bugelski and Treacy, 2004). Antibodies shown to be highly immunogenic in non-human primates have been demonstrated to have low incidence of immunogenicity in humans, and vice versa. Thus the true predictability of

immunogenicity in humans really only comes in Phase II when enough patients of various types are exposed to the drug candidate.

Beyond primary sequence analysis and remediation, the most important factors in generating a low- or non-immunogenic MAb or FcFP candidate are absence of aggregation and degradation products (deamidation, oxidation, fragmentation, etc.; Manning et al., 2010; Engelsman et al., 2011; see Table 16.1). Thus the concept of carrying out “developability” or “manufacturability” assays of lead candidates, testing for solubility, aggregation, biochemical degradation, and intact protein, has become a standard in the industry (Jenkins et al., 2008; Voynov et al., 2009; Shire, 2009; Conley et al., 2011). In the end, the key in today’s environment is to make potent, well-behaved (highly soluble, little to no aggregation, unlikely to undergo chemical changes) antibodies that are as human-like as possible (Almagro and Strohl, 2009).

For the treatment of autoimmune diseases such as rheumatoid arthritis, immunomodulatory drugs such as methotrexate or azathioprine are often administered either before or coincidentally with a therapeutic MAb or FcFP (Maini et al., 1998; Krieckaert et al., 2010). While it has long been assumed that the co-treatment of patients with methotrexate and therapeutic MAb or FcFP results in lowered rates of immunogenicity (e.g. Humira® has an immunogenicity rate of 12% in the absence of methotrexate but 1% when co-administered with methotrexate (Humira® prescribing information, 2012)), Krieckaert et al. (2010) showed that in eight out of ten examples cited, no effect on immunogenicity rates were observed for either Humira® or Remicade® dosed either in the presence or absence of methotrexate or azathioprine. Thus there are still some questions as to whether co-administration of methotrexate truly reduces immunogenicity rates.

16.10 Biocomparability

One of the key issues in the development of a biologic that, by definition, is made in a living cellular system (rather than by chemical synthesis) is that the biologic is not a pure chemical that can be defined exactly by a chemical structure. Thus the comparability of the molecule from production run to production run is a critical feature of the development process. An alteration in the process may result in physicochemical changes to the biological molecule such as amino acid sequence (e.g. C- or N-terminal changes, proteolytic clipping) and/or post-translation modification (glycosylation patterns, various chemical changes to amino acid residues (see section 16.5)) which could potentially result in changes in efficacy, safety, and/or

immunogenicity (Chirino and Mire-Sluis, 2004; Büttel et al., 2011). The International Committee for Harmonization (ICH) has developed a guideline, ICH 5QE, on comparability called “Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process” (<http://www.fda.gov/OHRMS/DOCKETS/98fr/2004d-0118-gdl0001.pdf>) that defines, explains, and guides investigators on comparability issues in biologics. According to ICH 5QE, “The goal of the comparability exercise is to ensure the quality, safety, and efficacy of DP (drug product) produced by a changed manufacturing process, through collection and evaluation of relevant data to determine whether there might be any adverse impact on the DP due to the manufacturing process changes.” The key to a comparability study, as summarized by Lubiniecki et al. (2011), is to understand the impact of changes in a protein that might affect its functionality in a “molecular background of naturally occurring heterogeneity.” The EMEA also has put out a guideline document for comparability (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003935.pdf). These comparability guidelines will clearly have an impact on the approaches taken for biosimilar antibodies (Davis et al., 2009; see also Chapter 19). Additionally, in February 2012, the US FDA issued draft guidance for the development of biosimilars in the United States (<http://www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/Biosimilars/default.htm>). The EMEA and US guidelines will combine to set the stage for the development of biosimilar monoclonal antibodies.

Typically, the comparability criteria are set based on multiple batches so that run-to-run variation can be assessed and a reasonable window of product variability can be established. Key to this process, however, is that the product variation cannot affect the pharmacology of the molecule. To this end, comparability assays for antibodies typically include analysis of the chemical structure of the antibody as well as analysis of the activities of the two major functional regions of the molecule, the FAb arms and the Fc. Thus analytical assays such as N297 glycan and peptide mapping analysis by mass spectroscopy, binding analyses by ELISA and high-sensitivity binding approaches (e.g. Biacore, Proteon), and Fc analyses such as binding to FcγRs/FcRn are typically run to assess the comparability of MAbs or FcFPs derived from different cell culture and purification batches. Additionally, it is important to establish that the molecules from different batches function similarly, so cell-based functional assays are also often part of the comparability assay process. The issue with cell-based assays is often the variability of the assays due to drift in the cell lines used

for the assays. Lubiniecki et al. (2011) showed comparability studies for two different MABs and described in detail the assays used to determine comparability between batches of the MABs. The assays included:

- SDS-PAGE and capillary SDS (size, subunit size, purity);
- isoelectric focusing (IEF) and capillary IEF (charge distribution);
- ion exchange HPLC (charge profile);
- oligosaccharide mapping (N297 glycan analysis);
- peptide mapping (amino acid residue integrity);
- mass spectrometry (chain and amino acid residue integrity);
- cell-based bioactivity (FAb functionality);
- human FcγRI, human FcRn, and human FcγRIIb (CD32b) binding assays (Fc functionality);
- soluble C5b-9 generation (complement activation assay);
- size exclusion-HPLC (SE-HPLC; to determine chain integrity);
- differential scanning calorimetry (thermal stability of domains);
- sedimentation velocity (multimerization and aggregation);
- measurement of subvisible particles (solubility);
- circular dichroism spectroscopy (proper folding); and
- animal pharmacokinetics (half-life, and thus structure-function of Fc).

(Lubiniecki et al., 2011)

A significant key to the assays used to assess comparability is that they should be orthogonal in nature, i.e. they should measure different aspects of the biologic and its function (Lubiniecki et al., 2011). Chirino and Mire-Sluis (2004) also list a series of assays that can be used in comparability studies, some of them different from the assays described by Lubiniecki et al. (2011).

Significant changes that have been made to a product or the process to make that product, such as the rederivation of a new cell line using the same host, a switch from one cell line to another (e.g. change from NS0 to CHO production lines), a change from one facility to a new facility, or process changes such as starting with a perfusion process and changing to fed-batch, can result in changes significant enough to warrant *in vivo* studies to determine comparability in pharmacokinetic profiles, potency, and *in vivo* efficacy (Chirino and Mire-Sluis, 2004; Lubiniecki et al., 2011), and, if serious enough, sometimes bridging clinical trials (Putnam et al., 2010). The timing of process or product changes also has a significant impact on the types of comparability studies that are required. Typically, if process changes are made prior to pivotal registration clinical trials, the

comparability studies may be kept to analytical, functional, and animal PK or PK/PD studies (Putnam et al., 2010). Acceptance criteria for comparability in PK studies generally require that 90% confidence intervals of the geometric mean ratios are between 0.8 and 1.25 for PK endpoints such as the area under the curve (AUC) and $C_{\max}$ (Putnam et al., 2010). If the changes occur during or after pivotal trials are held, it is likely that additional bridging clinical studies may be required. Thus it has been suggested that the earlier major changes can be made in the development process, the better it might be for the development program (Chirino and Mire-Sluis, 2004; Putnam et al., 2010; Lubiniecki et al., 2011). Putnam et al. (2010) provide useful examples of the timing of process changes and likely studies required for comparability assessment.

Interactions of human IgGs with non-human systems

Abstract: During the discovery and early development of therapeutic antibodies, animal models are used to determine the pharmacology, efficacy, mechanisms of action, and potential toxicity of the candidate antibody. Thus the interaction of the candidate antibody with the immune system of these animals, including mice, rats, non-human primates (NHPs), and other species, is critical to understanding the efficacy and toxicity in humans. Human IgGs interact with rodent Fc γ Rs much more differently than with rodent IgGs, so understanding those interactions is key to the development of antibodies. NHPs are usually used as the species of choice for GLP-toxicology studies of human therapeutic antibody candidates. While NHPs have an immune system relatively similar to that of humans, significant differences such as antibody-induced cytokine responses are found, which are still relatively poorly understood. To improve antibody development processes, better understanding of the interaction with human antibodies with these non-human systems needs to be attained.

Keywords: mice, rats, non-human primates (NHPs), surrogate antibodies, Fc γ receptors, mouse strains, antibody-dependent cellular cytotoxicity (ADCC)

17.1 Introduction

Antibodies developed as therapeutic MAb candidates typically bind to their targets with very high specificity and affinity. It is not unusual to find antibodies that can bind a target protein but not related proteins that differ by only a few amino acids. While this is highly desirable to eliminate off-target activity, a problem often associated with small molecules that typically lack the exquisite selectivity of MAbs, this level of selectivity also can translate into a development candidate that may lack the ability to bind to non-human primate, mouse and/or rat homologs of the target. This

scenario, which has occurred many times over the years, can create issues when dealing with non-clinical pharmacology, toxicology, and reproductive studies (Foulkes, 2002; Wu et al., 2006; Chapman et al., 2007; Tabrizi and Roskos, 2007; Bussiere, 2008; Longstaff et al., 2009; Wakefield et al., 2011). Under these conditions, a surrogate antibody must be obtained that will bind in a pharmacologically relevant manner to non-human homologs of the target such as those from the cynomolgus monkey, mouse, and/or rat (Arnet and Viney, 2007; Bussiere, 2008; Wakefield et al., 2011). In most cases, both binding (K_D) and physiological functionality (e.g. IC_{50}) for the non-human target need to be within about tenfold of the human system to be relevant.

In today's discovery scenario, considerable efforts are typically made to obtain physiologically relevant cross-binding and pharmacological functionality of the development MAb candidate on both the human and the cynomolgus monkey homolog of any particular target. For example, when using phage display discovery technologies, panning now is typically carried out by alternating between the human and cynomolgus monkey antigens, so that hits are more likely to bind both. Likewise, immunization of mice often entails priming with the human antigen followed by boosting with the cynomolgus monkey antigen, again with the idea of generating antibodies that will cross-react to antigens of both species. Minimally, this allows for the GLP-pre-clinical toxicology studies to be run in cynomolgus monkeys, and if there are relevant disease models in cynomolgus monkeys for the biology, some pharmacological readouts may also be achieved using that species (Chapman et al., 2007; Bussiere, 2008). Nevertheless, running pharmacology experiments in cynomolgus monkeys is generally quite expensive, so also having mouse or rat cross-reactivity and functionality is highly desirable, but generally more difficult to attain (Chapman et al., 2007). Moreover, reproductive toxicology studies require both elongated in-life studies and analysis as well as analysis of the therapeutic mechanism of action on embryonic development. For these studies, it is often preferable to have MAbs that can be studied in rodent models of reproductive toxicology, requiring both binding and pertinent pharmacological activity against the rodent target. For these studies, surrogate antibodies are very often required if the therapeutic does not bind and function well in rodents, which happens frequently (Wu et al., 2006; Wakefield et al., 2011).

Very rarely does a potential therapeutic MAb bind the homologous human, cynomolgus monkey, mouse, and rat targets with similar potency and functionality. In fact, antibodies originally isolated from mouse hybridomas will typically not cross-react with the mouse homolog because of tolerance to "self." In these cases, it is necessary to generate anti-mouse

and/or anti-rat surrogate antibodies that bind the human target homolog and function similarly to the therapeutic antibody candidate (Wu et al., 2006; Wakefield et al., 2011). The key to generating surrogate MABs, whether they be for rodent species or for non-human primates, is that the pharmacological activity and potency of the surrogate MAB must mirror that of the therapeutic MAB. Thus the epitope, affinity, *in vitro* functionality, and interaction of the isotype with the host immune system of the surrogate MABs must all be carefully taken into consideration, making the generation of a good surrogate MAB nearly as rigorous as that of a therapeutic MAB program. In this chapter, the interaction of human antibodies with the NHP and rodent immune systems will be discussed, and the implications of human IgGs binding or not binding non-human Fc γ Rs will be assessed.

17.2 Non-human primate IgGs and Fc γ receptors

17.2.1 NHP IgG isotypes

Whether surrogate antibodies are obtained, or whether the human(-ized) therapeutic candidate cross-reacts and is therefore used in monkey studies, a significant factor that is often underappreciated is the potential for different responses in the monkey to the human isotype. NHPs have the same isotypes as humans, IgG₁, IgG₂, IgG₃, and IgG₄ (Scinicariello et al., 2004), and based on the knowledge we have today, it seems reasonable to use the same human isotypes in studies with NHPs as with humans (Shearer et al., 1999; Scinicariello et al., 2004). Nevertheless, there are some differences between NHP IgGs and human IgGs and their cognate Fc γ Rs that are worth mentioning, as noted below.

The overall sequence identity between human and NHP IgG isotypes of the same isotype is reasonably high (identities for IgG₁, IgG₂, IgG₃, and IgG₄ are 90%, 88%, 88%, and 90%, respectively) (Scinicariello et al., 2004), but the hinge sequences of human and NHP IgGs are more divergent, as shown in Figure 17.1. The human and NHP IgG₂ hinge sequences exhibit significant sequence divergence, suggesting that the immunological activities of a human IgG₂ in rhesus macaques might be different than in humans. While the key residues for Fc γ R binding in the lower hinge of human and rhesus IgG₁ are more highly conserved than the residues in either the upper hinge or the other isotypes, these differences do highlight that the IgG–Fc γ R interactions are likely to be subtly different in NHPs than in humans.

	Upper Hinge	Core Hinge	Lower hinge
HulG ₁	EPKSC--DKTHT	CPPCP	APELLGGP
RhlgG ₁	EIKTCGGSKPPPT	CPPCP	APELLGGP
HulG ₂	ERK	CCVECPPCP	AP-PVAGP
RhlgG ₂	GLP	CRSTCPPCP	A-ELLGGP
HulG ₄	ESKYGPP	CPSCP	APEFLGGP
RhlgG ₄	EFT--PP	CPPCP	APELLGGP
EU:	216	226	231 238

Figure 17.1 Comparison of hinge regions of human vs NHP IgGs

Hinge sequences from rhesus IgG₁, IgG₂, and IgG₄ compared with the human counterparts, indicating that the NHP sequences are not identical (data from Scinicariello et al., 2004). Of particular interest is the lower hinge of rhesus IgG₂, which appears to be more IgG₁-like (EU numbering is shown at the bottom; the gaps have been rearranged to align better with IgG₁).

17.2.2 NHP FcγRs

The sequences of human and cynomolgus monkey FcγRs are highly conserved, with only about 8–10% amino acid sequence variation (FcγRI, 90% identical; FcγRIIa, 89% identical; FcγRIIb, 92% identical; FcγRIIIa, 91% identical; γ chain, 99% identical) (Presta and Namenuk, 2005). Within these sequences, there were both some key similarities and interesting differences. While the cynomolgus monkey γ-chain used in FcγRI and FcγRIIIa localization and activity was 99% identical to its human counterpart (85/86 residues identical; the one change is conservative), the ITAM motif (YxxLx₁₂YxxL) of cynomolgus monkey FcγRIIa was subtly different from that of the human FcγRIIa ITAM motif (Presta and Namenuk, 2005). Whether this translates into activity differences is as yet unknown. Similarly, using low-resolution ELISA assays, all of the human IgG isotypes bound similarly to human and cynomolgus monkey FcγRs (Presta and Namenuk, 2005). On the other hand, Rogers et al. (2006) demonstrated by FACS analysis that human IgG₁ and IgG₂ antibodies bound to NHP FcγRIIIa, which was demonstrably different than to human FcγRIIIa. Additionally, key differences in the sequence of NHP FcγRIIIa from the human sequence are found in residues A135L, H153E, V176I (first letter indicates human sequence; second letter is NHP mutation), all three of which have been shown to be involved in IgG binding (Sondermann et al., 2000). Finally, NHPs have a homolog of human FcγRIIIa (CD16a), found on monocytes and NK cells as in humans, but not an apparent homolog to human FcγRIIb (Rogers et al., 2006), suggesting that the interaction of a human IgG₁ with NHP neutrophils might be very different to what is observed with human neutrophils.

Differences have been observed in the reactivity of human IgGs in human versus NHPs (Walker et al., 2011b). In the most glaring case, the anti-CD28 agonist MAb, Tegenaro TGN1412, created a massive and injurious cytokine storm in human volunteers but showed no similar effect in GLP-toxicology studies using NHPs (Stebbins et al., 2007; Longstaff et al., 2009). Many studies have concluded that TGN1412 acted as a super-agonist against human activated T cells, particularly of the CD4-type, but not against a macaque CD4⁺ T cell population that apparently does not express CD28 (Eastwood et al., 2010). Nevertheless, there have been some questions about whether the Fc of TGN1412, which was a human IgG₄ isotype, might have been involved in the induction of the cytokine storm (Jefferis, 2007, 2009b). One recent study suggests that interaction of the anti-CD28 IgG₄ antibody with FcγRI on vascular endothelial cells and monocytes and CD28 on monocytes and NK cells may have led to a clustering effect that stimulated TNF-α production, leading to the cytokine storm (Sandilands et al., 2010). If this hypothesis plays out, even only partially, then the interaction of human IgG₄ with NHP receptors may have been different enough not to have observed the downstream effects. Thus data from studies conducted on the effects of normal or modified human IgGs in either rhesus or cynomolgus macaques need to be viewed with these potential differences in mind (Scinicariello et al., 2004). It is clear that the understanding of how human antibodies interact with the NHP systems is not as well understood as it should be, considering that most antibodies are studied in NHP models for both non-GLP toxicology and GLP toxicology studies.

17.3 Mouse IgGs and Fcγ receptors

17.3.1 Rodent IgG isotypes

Both mice and rats have IgG, IgA, IgD, IgE, and IgM classes of antibodies, generally similar to those found in humans. The mouse and rat IgG isotypes, however, differ in both naming convention and activity from their human counterparts (Clark, 1997). Mice have IgG₁, IgG_{2a}, IgG_{2a/c}, IgG_{2b}, and IgG₃ isotypes, whereas rats have IgG₁, IgG_{2a}, IgG_{2b} and IgG_{2c} isotypes. The numbering of IgG subtypes in rodents is completely unrelated to that of human IgGs (Strohl, 2011).

Mouse and rat IgG₁ isotypes are most closely related to those of human IgG₄, which is generated as part of a Th2 response. BALB/c mice, which are predominantly Th2 shifted, produce mostly an IgG₁ response to antigen

stimulation (Slack, 1987). Mouse and rat IgG₁ isotypes do not bind C1q in a physiologically significant manner similar to human IgG₄ (Strohl, 2011). Mouse and rat IgG₁ antibodies, however, are not known to carry out FAb arm exchange as shown for human IgG₄ antibodies.

Mouse IgG₁, however, does not bind to the mouse high-affinity receptor FcγRI (Baudino et al., 2008b) (see Table 17.1), differentiating it from human IgG₄, which functions through human FcγRI. Mouse IgG₁ binds the low-affinity activating receptor mouse FcγRIII (see Table 17.1) and can mediate immune responses including phagocytosis *in vivo* (Hazenbos et al., 1998; Hamaguchi et al., 2006). As compared with other mouse isotypes, mouse IgG₁ has a three amino acid deletion (N233, L2334, E235) that eliminates binding to mouse FcγRIV or C1q (Baudino et al., 2008b). Rat IgG₁ and IgG_{2a} share a similar deletion (Clark, 1997) and possess activities similar to mouse IgG₁ (Brüggemann et al., 1989b). Rat IgG_{2a} is also most closely aligned with mouse IgG₁ (Vernersson et al., 2004), and is a poor inducer of ADCC (Clark, 1997).

Table 17.1 Binding of mouse and human antibodies to mouse Fcγ receptors

Antibody isotype	K _D values for IgG isotype binding to mouse FcγR (in nM)			
	MuFcγRI	MuFcγRII	MuFcγRIII	MuFcγRIV
Mouse IgG ₁	nb ^a	300 ^b , 830 ^a	3200 ^b , 4800 ^a	Nb ^{ab}
Mouse IgG _{2a}	26 ^a	1800 ^a , 2390 ^b	1460 ^b , 1800 ^a	35 ^b , 71 ^a
Mouse IgG _{2b}	nb ^b	450 ^b , 900 ^a	1550 ^b , 1600 ^a	59 ^b , 63 ^a
Mouse IgG ₃	nb ^{ab}	nb ^{ab}	nb ^{ab}	nb ^{ab}
Human IgG ₁	110 ^c , 600	nb ^b , 1100 ^c , 84 ^g	nb ^b , 157 ^g	30 ^c , 300 ^b , 536 ^d , 79 ^g
Human Fc modified IgG ₁ (XMAb5574) ^e	2 ^c	100 ^c	nd ^c	0.2 ^c
Mutant 18 ^f	nd	106 ^g	113 ^g	19 ^g

Abbreviations: nb: no detectable binding; nd: no data.

Notes:

^a Baudino et al. (2008b).

^b Nimmerjahn et al. (2005).

^c Horton et al. (2008).

^d Bruckheimer et al. (2009).

^e S239D,I332E mutant of IgG₁ that increases binding to activating FcγRs and confers stronger ADCC and ADCP than wild-type IgG₁.

^f Mutant 18 – F243L, R292P, Y300L, V305L, P396L; Stavenhagen et al. (2007).

^g Based on dimeric avidity measurements instead of straight affinity (most other researchers have not observed human IgG₁ to bind mouse FcγRIII; Baudino et al., 2008b).

Mouse IgG_{2a}, which is generally considered the mouse isotype closest in activity to human IgG₁, is generated as the primary response to antigen stimulation in Th1-shifted C57BL/6 mice (Slack, 1987). IgG_{2a} binds mouse FcγRI and FcγRIV in the 25–50 nM range, binds mouse FcγRIII and FcγRII in the 2 μM range (see Table 17.1) and binds C1q to activate the complement cascade via the classical pathway. Residues LLGGP at positions 234–238 of mouse IgG_{2a} have been shown to be required for binding to high affinity mouse FcγRI (Baudino et al., 2008b). An anti-red blood cell mouse IgG_{2a} isotype has been shown to induce anemia at low doses in mice via the low-affinity receptor, FcγRIII. At higher doses, that antibody induced a more severe disease through additional FcγR activities with the high-affinity receptors mouse FcγRI and FcγRIV (Baudino et al., 2008a). The rat IgG_{2b} isotype is most closely related to the mouse IgG_{2a}, both in activity (Brüggemann et al., 1989b; Clark, 1997) and sequence similarity (Vernersson et al., 2004; Strohl, 2011). Similar to mouse IgG_{2a}, rat IgG_{2b} binds mouse or rat FcγRI (Clark, 1997), mediates ADCC via rat FcγRIII, and activates complement via the classical pathway (Bindon et al., 1988).

Mouse IgG_{2b} binds mouse FcγRIV in the 60 nM range, binds FcγRIII in the 2 μM range (see Table 17.1), and binds C1q to activate the complement cascade via the classical pathway (Strohl, 2011). Mouse IgG_{2b} may have stronger complement activation activity than IgG_{2a}, possibly through its interaction with C3 and C4 rather than C1q which binds both isotypes well (Giorgini et al., 2008). Different from mouse IgG_{2a}, mouse IgG_{2b} does not bind the high affinity receptor mouse FcγRI, and binds FcγRII in the 500 nM range (Baudino et al., 2008a). It is thought that mouse IgG_{2b} plays an intermediate role in response to antigen stimulation, i.e. a milder Th1-type response than occurs with mouse IgG_{2a}. An anti-red blood cell IgG_{2b} MAb induced anemia at both low and high doses via both the low-affinity mouse FcγRIII and the high-affinity FcγRIV (Baudino et al., 2008a), whereas in other models IgG_{2b} activity appeared to be dependent on FcγRIV exclusively (Nimmerjahn and Ravetch, 2008).

Similar to the human IgG₂, mouse IgG₃ is the predominant isotype found to respond to T-independent antigens such as polysaccharides (Díaz de Ståhl et al., 2003). As such, IgG₃-deficient mice are more susceptible to pneumococcal infections than wild-type mice (McLay et al., 2002). The mouse IgG₃ isotype appears to be a more potent activator of both the classical and alternative complement pathways than human IgG₂. Since mouse IgG₃ does not bind any mouse FcγR in a physiologically relevant manner (see Table 17.1), complement activation appears to be its primary mechanism for antigen neutralization and activation of the immune response system (Ding et al., 2008). The rat IgG_{2c} isotype is similar in

sequence, as well as in its function as the response to T-independent antigens, to mouse IgG₃ and human IgG₂ (der Balian et al., 1980; Yoo et al., 2003). Rat IgG_{2c} also binds C1q and activates complement (Bindon et al., 1988). Both mouse IgG₃ and rat IgG_{2c} tend to form dimers, similar to human IgG₂, albeit by what appear to be different mechanisms (Greenspan and Cooper, 1992; Strohl, 2011). Since all three of these forms are used to defend against pathogens with recognized repeating structures such as bacterial polysaccharides and cell wall components, it is possible that this dimerization capability is evolutionarily selected (Strohl, 2011).

17.3.2 Rodent Fcγ receptors

Rodent FcγRs are generally different from human FcγRs, although some broad similarities can be found (see Figure 17.1). Rodent immune cells express four different types of Fcγ receptors, including the activating receptors, FcγRI (CD64), FcγRIII (CD16), and FcγRIV (CD16-2), and the inhibitory receptor FcγRII (CD32) (Nimmerjahn et al., 2005; Nimmerjahn and Ravetch, 2008) (see Tables 17.1 and 17.2). Mouse activating receptors, FcγRI, FcγRIII and FcγRIV, interact with an ITAM-bearing common gamma chain (FcRγ) to make receptor complexes, similar to those of human FcγRI and FcγRIIIa (Figure 17.2) (Nimmerjahn and Ravetch, 2008). Just as with the human receptors, the mouse ITAM-containing FcRγ chain is essential for effector cell responses such as ADCC and ADCP (Nimmerjahn and Ravetch, 2008). FcRγ knockout mice, which do not express the activating receptors FcγRI, FcγRIII, or FcγRIV, are resistant to autoimmune disorders such as glomerulonephritis and induced inflammatory conditions such as collagen-induced arthritis (Nimmerjahn and Ravetch, 2008). The mouse inhibitory receptor, FcγRII, bears its own ITIM motif, similar to that of human FcγRIIb (Figure 17.2).

Mice and rats both have a high-affinity, activating FcγRI, similar in sequence and function to human FcγRI (Figure 17.2). Mouse FcγRI is found on macrophages, monocytes, neutrophils, eosinophils, and dendritic cells (Table 17.2), similar to human FcγRI distribution in humans (Lazar and Desjarlais, 2009). The only mouse isotype to bind mouse FcγRI is IgG_{2a}, which binds with an affinity of about 25 nM (Baudino et al., 2008b), similar to the binding of human IgG₁ to human FcγRI (Bruhns et al., 2009). Thus IgG binding to FcγRI in both rodents and humans is likely to be in the monomeric state (Giorgini et al., 2008). A key natural function of FcγRI is to facilitate antigen presentation to T cells by professional antigen-presenting cells. It has been demonstrated that in the presence of FcγRI

Table 17.2 Distribution of Fcγ receptors on mouse immune cells and identity of IgGs that bind those mouse immune cells

Human cell type	Human Fcγ receptor distribution	Mouse cell type	Mouse Fcγ receptor distribution	IgGs that bind mouse receptors	
				With high affinity	With low affinity and/or inhibitory
MO*	FcγRI, FcγRIIIa, FcγRIIb	MO	FcγRI, FcγRIV	MulG _{2a} , MulG _{2b} , HulG ₁	—
MA	FcγRI, FcγRIIIa, FcγRIIb, FcγRIIIa	MA	FcγRI, FcγRII, FcγRIII, FcγRIV	MulG _{2a} , MulG _{2b} , HulG ₁	MulG ₁
NK	FcγRIIc, FcγRIIIa	NK	FcγRIII	HulG ₁	MulG ₁ , MulG _{2a} , MulG _{2b}
NP**	(FcγRI), FcγRIIIa, FcγRIIb, FcγRIIb	NP	FcγRI, FcγRII, FcγRIII, FcγRIV	MulG _{2a} , MulG _{2b} , HulG ₁	MulG ₁
DC	FcγRI, FcγRIIIa, FcγRIIb, FcγRIIIa	DC	FcγRI, FcγRII, FcγRIII, FcγRIV	MulG _{2a} , MulG _{2b} , HulG ₁	MulG ₁
BC	FcγRIIb	BC	FcγRII	MulG ₁ , MulG _{2b}	MulG _{2a} , HulG ₁
EO	FcγRI, FcγRIIIa, FcγRIIb, FcγRIIb	EO	FcγRI, FcγRII, FcγRIII	MulG _{2a}	MulG ₁ , MulG _{2b} , HulG ₁

* About 10% of blood human monocytes express CD16, and this subset resembles tissue macrophages. CD16 negative monocytes are typically considered "classic" monocytes (Gordon and Taylor, 2005).

**"Normal" circulating neutrophils do not express CD64, rather it must be induced *in vivo* or *in vitro* with G-CSF.

Notes: Mouse IgG₃ does not bind any of the mouse FcγRs with any discernable affinity. The human cell type and FcγR distribution patterns are provided as comparators. Abbreviations: BC: B cells; DC: dendritic cells; EO: eosinophils; Hu: human; MA: macrophages; MO: monocytes; Mu: mouse; NK: natural killer cells; NP: neutrophils.

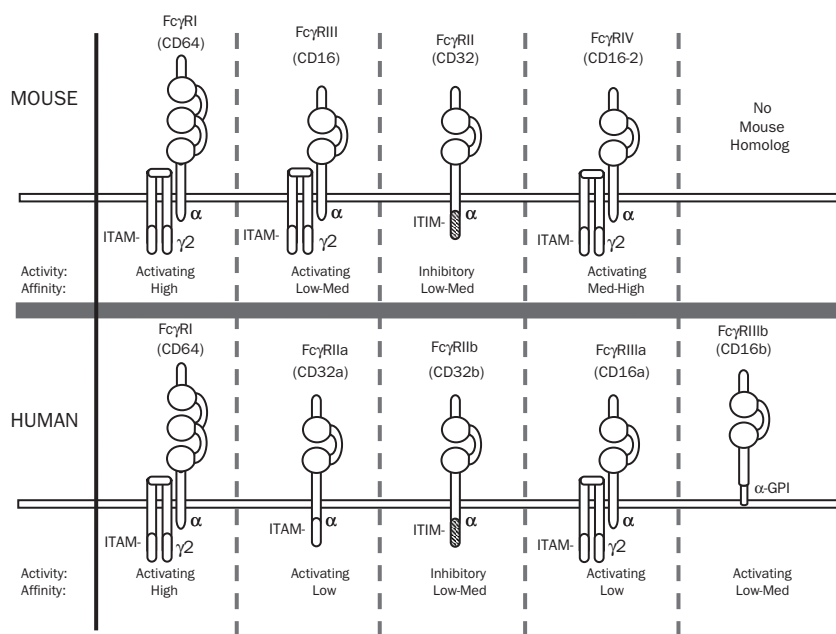


Figure 17.2 Rodent vs human FcγRs

Model of the structures of the mouse Fcγ receptors (top) compared with their human Fcγ receptor homologs (bottom).

Abbreviations: ITAM: immunoreceptor tyrosine-based activation motif; ITIM: immunoreceptor tyrosine-based inhibition motif; α: alpha chain of receptor; α-GPI: α-glycosyl-phosphatidylinositol (linked receptor); γ2: Fcγ receptor.

signaling, 100-fold less antigen is required than in the absence of FcγRI, suggesting a “vaccine effect” against FcγRI-cross-linked antigens (van der Pol and van de Winkle, 1998). FcγRI knockout mice are impaired in phagocytosis of immune complexes, immune complex-dependent antigen presentation, protection from bacteria, and endocytosis of IgG (Ioan-Facsinay et al., 2002). These knockout data strongly support a role for FcγRI in phagocytosis.

Both rats and mice possess an inhibitory FcγRII receptor that is structurally and functionally similar to human FcγRIIb (see Figure 17.2 and Tables 17.1 and 17.2) (Nimmerjahn and Ravetch, 2008; Baerenwaldt and Nimmerjahn, 2008). Rodent FcγRII is a monomeric receptor in which the ITIM is harbored directly in the cytoplasmic domain of the receptor (Nimmerjahn and Ravetch, 2008). In mice, FcγRII is found on B cells, mast cells, basophils, macrophages, eosinophils, neutrophils, dendritic cells, and

Langerhan's cells (see Table 17.2). Fc γ RII-knockout mice are highly susceptible to a variety of autoimmune conditions, e.g. lupus erythematosus (Lin et al., 2006), indicating the balancing role the inhibitory receptor plays in immune signaling homeostasis (Baerenwaldt and Nimmerjahn, 2008).

Mice and rats do not possess a direct equivalent of the low-affinity, activating Fc γ RIIa (CD32a) receptor found in humans and other primates (Figure 17.2). Rodent Fc γ RIII receptor is a low-affinity, multi-subunit activation receptor that is related phylogenetically to human Fc γ RIIa, although with a different cell distribution pattern (Strohl, 2011; see Table 17.2). While murine Fc γ RIII has been named in the literature as "CD16," it is apparent that mouse and rat Fc γ RIII are not necessarily the functional equivalents of human CD16 (Fc γ RIIIa) (Nimmerjahn and Ravetch, 2008). Mouse Fc γ RIII is present on macrophages, neutrophils, eosinophils, platelets, dendritic cells, Langerhan's cells, and, importantly, NK cells (Nimmerjahn and Ravetch, 2008). This makes Fc γ RIII the sole activating receptor found on mouse NK cells (see Table 17.2), and thus the key receptor in mice responsible for driving NK-mediated ADCC in mice. In this way, murine Fc γ RIII mirrors the activity of human Fc γ RIIIa (Lazar and Desjarlais, 2009; Syed et al., 2009). Mouse isotypes IgG₁, IgG_{2a}, and IgG_{2b} bind Fc γ RIII in the 1.5–4 μ M range, suggesting that activation requires binding by cross-linked antigen-antibody complexes, similar to the biology of the human low-affinity receptors, Fc γ RIIa and Fc γ RIIIa (Jakus et al., 2008). Fc γ RIII knockout mice are unable to mount a complete immune response, even though they increase Fc γ RIV expression to compensate for loss of Fc γ RIII (Syed et al., 2009). This demonstrates the key synergistic nature of mouse Fc γ RIII and Fc γ RIV for the interactions with mouse IgG_{2a} and IgG_{2b} to mount a complete immune response (Syed et al., 2009).

Mice and rats have been found to possess a novel Fc receptor, Fc γ RIV (CD16-2) (Figure 17.2), which is 63% identical in sequence to human Fc γ RIIIa (CD16a) (Nimmerjahn et al., 2005; Strohl, 2011). Like murine Fc γ RI and Fc γ RIII, Fc γ RIV interacts with the FcR- γ chain to form an activating IgG-binding receptor. While Fc γ RIV has been called an "intermediate affinity" Fc receptor, both mouse IgG_{2a} and IgG_{2b} isotypes bind Fc γ RIV with affinities of ca. 50–60 nM, as compared with their binding to the mouse high-affinity receptor Fc γ RI (25 nM range) (Strohl, 2011). Bruhns et al. (2009) have defined a high-affinity Fc receptor as having a K_D of about 110 nM, which is close to 100 nM, a value which had been used traditionally as the cut-off. Moreover, both mouse IgG_{2a} and IgG_{2b} were recently shown to bind to Fc γ RIV as monomers, thus functioning as a high-affinity receptor for those antibodies (Mancardi et al., 2008).

Syed et al. (2009) showed that Fc γ RIII and Fc γ RIV work closely together to mediate immune responses in response to binding mouse IgG_{2a} and/or IgG_{2b}. Additionally, Nimmerjahn et al. (2010) demonstrated using Fc γ RIV knockout mice that Fc γ RIV is a critical receptor for the immune responses mediated by mouse IgG_{2a} and IgG_{2b}, much more so than mouse Fc γ RIII. Lazar and Desjarlais (2009) suggest that murine Fc γ RIV plays a role similar to human Fc γ RIIa based on its distribution and antibody binding characteristics. While there are some parallels, murine Fc γ RIV has unique properties that make it substantially different in its role from human Fc γ RIIa. Mouse Fc γ RIV was also recently shown to bind mouse IgE in the ~2–7 μ M range (Mancardi et al., 2008). Additionally, antigen-bound IgE immune complexes were found to be capable of displacing receptor-bound IgG on macrophages, resulting in induction of cytokine secretion (Hirano et al., 2007; Mancardi et al., 2008). Human Fc γ RIIIa, the closest human relative to mouse Fc γ RIV (Figure 17.2) (Hirano et al., 2007), was shown to lack the ability to bind human IgE, further demonstrating differences between human Fc γ RIIIa and mouse Fc γ RIV (Mancardi et al., 2008). Several additional similarities exist between mouse Fc γ RIV and human Fc ϵ RI, including quaternary structural similarities and tissue distribution, which led Mancardi et al. (2008) to suggest that mouse Fc γ RIV is a key low-affinity receptor for IgE in mice. Nevertheless, given the critical nature of Fc γ RIV in the activity of mouse IgG_{2a} and IgG_{2b} antibodies (Nimmerjahn et al., 2010), it is clear that Fc γ RIV plays a central role in IgG-mediated immune responses in rodents. Thus rodent Fc γ RIV appears to be quite different from any other Fc γ R previously described (Hirano et al., 2007; Mancardi et al., 2008; Nimmerjahn et al., 2010).

17.3.3 Mouse in vivo studies for anti-tumor activity of human antibodies

One of the key issues in studying either the pharmacology or safety of human antibodies in rodents is the fact that human IgG isotypes do not function the same in rodents as they do in humans. Most of the information on the use of human IgG isotypes in rodents is focused on human IgG₁ (see Table 17.1) (Strohl, 2011), but even in the case of human IgG₁, additional information is needed to fully understand the function in mice. There are multiple factors that make it difficult to fully appreciate the function of a human IgG₁ in rodents, including the different strains of mice used which have very different phenotypes (see Table 17.3), the still evolving knowledge on Fc γ R functionality in rodents, the differential distribution of Fc γ Rs in

Table 17.3 Mouse strains often used to study human antibody responses*

Mouse type	Characteristics	Cell types available	References
BALB/c	Th-2 cytokine shifted; predominant antibody response is IgG ₁	All immune cells, including NK, MO, MA, DC, NP, BC, TC	Slack (1987)
C57BL/6	Th-1 cytokine shifted; predominant antibody responses are IgG _{2a} , IgG _{2b}	All immune cells, including NK, MO, MA, DC, NP, BC, TC	Slack (1987)
Nude mice	FOXP1 KO; lack thymus, no T cells, so no CD8 T cell responses or T-dependent Ab responses due to lack of CD4 T cells; can only mount T-independent Ab responses, so poorly matured Abs	MO, MA, NP, NK	Fu and Chaplin (1999)
SCID mice	Recessive prkdc** mutation; cannot mount adaptive immune response due to dysfunctional V(D)J recombination	MO, MA, NP, NK	Bosma et al. (1983); Fulop et al. (1990)
Beige mice	Defect in NK cells; no NK cell activity; no NP-mediated cytotoxicity; no CD8 cytotoxic T cell response	MO, MA	Roder (1979); Roder and Duwe (1979); Roder et al. (1979); Saxena et al. (1982)
Beige/SCID mice	SCID and beige mutations; very low B and T cells, defective in NK, NP, and CD8 cell functions	MO, MA	—
NOD mice	(Non-obese diabetic); defective Idd3 locus resulting in defective IL-2 production; NOD mice have a T cell and NK cell defect due to IL-2 deficiency, but still have residual innate immunity with neutrophils and monocyte/macrophages	All immune cells, including NK, MO, MA, DC, NP, BC, TC	Makino et al. (1980); Anderson and Bluestone (2005)
NOD/SCID mice	NOD-SCID lack T, B, or NK cells; retain functionally intact neutrophils and macrophages	MA, NP	Kinoshita et al. (2011)
IL-2R γ null mice	Lack γ -common chain (required for IL-2, 4,7,9,15,21 signaling); lack NK cell activity, lowered B cells, lowered $\gamma\delta$ T cells	MO, MA	Cao et al. (1995)
NOG or NSG mice	Combination of NOD, SCID, and IL-R γ null mice; most immune-compromised mice available; lack B and T cells; lack NK, MO, MA, NP cells	Lacks all immune cells	Shultz et al. (2005)

*Sincere appreciation to Randall Brezski for his help with this table.

Abbreviations: BC: B cells; DC: dendritic cells; EO: eosinophils; MA: macrophages; MO: monocytes; NK: natural killer cells; NP: neutrophils; prkdc: protein kinase, DNA activated, catalytic polypeptide; TC: T cells.

mice versus humans (see Table 17.2), and the interaction of the FAb binding activity to the target and the Fc binding activity in those environments.

As demonstrated in Tables 17.1 and 17.2 and Figure 17.2, the distribution of Fc γ receptors on rodent immune cell types differs significantly from the human system (Nimmerjahn and Ravetch, 2008). Mouse Fc γ RIII is the only known Fc γ receptor present on mouse NK cells (Nimmerjahn and Ravetch, 2006; Inoue et al., 2007; Nimmerjahn and Ravetch, 2008). Depending on the models used, depletion of NK cells may (McEarchern et al., 2007) or may not (Ofiazoglu et al., 2007, 2009) significantly alter cell-mediated cytotoxicity in mice. Human IgG₁, which is used for most of the mouse xenograft studies, binds all four mouse Fc γ receptors (activating receptors Fc γ RI, Fc γ RIII, and Fc γ RIV, and inhibitory receptor Fc γ RII) (see Table 17.1) and therefore interacts with every major immune cell that can engage and kill cancer cells using one mechanism or another (see Table 17.2).

It is clear from a variety of studies that different immune cell populations in mice are capable of carrying out human IgG₁-mediated tumor cell killing. As early as the late 1980s, it was demonstrated that chimeric mouse-human antibodies containing either the human IgG₁ or human IgG₄ constant regions were capable of interacting with mouse effector cells such as granulocytes and macrophages to direct cell lysis *in vitro* and inhibit tumor growth *in vivo* using human SW948 colon carcinoma cells in a xenograft nude mouse model (Stemplewski et al., 1988). In comparison, antibodies with the same variable regions and either human IgG₂ or IgG₃ constant regions in those experiments failed to mediate cell lysis or inhibit tumor growth in those models (Stemplewski et al., 1988).

The severe combined immunodeficient (SCID) mouse (see Table 17.3) has been used for a wide variety of xenograft tumor models. That mouse strain cannot mount an adaptive immune response against foreign antigens, but still retains functional monocytes, macrophages, neutrophils, and NK cells, making it a good choice for studying the effects of human IgG₁ MABs in tumor models. In one study, macrophages were found to be critical in the treatment of xenografted tumors in SCID mice. Treatment of CD30⁺-tumor xenografts in C.B-17 SCID mice with SGN-30, a human anti-CD30 IgG₁, resulted in lengthened mouse survival whether or not NK cells had been pre-depleted, whereas pre-depletion of macrophages prior to treatment led to significantly greater mouse death (Ofiazoglu et al., 2007). Similarly, the MAB-related cell-killing activity of SGN-40, a humanized anti-CD40 MAB, in CB-17/lcr SCID mice was also demonstrated to be driven by macrophages and not NK cells or neutrophils (Ofiazoglu et al., 2009). Thus, in these studies, human IgG₁ antibodies killed tumors in mice via Fc γ R-mediated ADCP. McEarchern et al. (2007) demonstrated that an anti-CD70 c1F6 MAB,

containing human IgG₁ constant domains, required the activity of neutrophils, macrophages, and NK cells to kill disseminated CD70⁺ non-Hodgkin's lymphoma cells in SCID mice. Depletion of any of the three cell pools, or modification of the IgG₁ Fc to silence effector functions, resulted in rapid mouse death from the tumor cells (McEarchern et al., 2007).

In a study of the anti-CD20 IgG₁ MAb rituximab using disseminated Raji lymphoma cells in SCID mice, interaction of the MAb with FcγRIII on neutrophils and CD20 on the target cells was determined to be the primary mechanism of target cell killing and associated mouse survival. NK cells contributed a small fraction of the activity (Hernandez-Ilizaliturri et al., 2003). Similarly, the anti-CD52 MAb, alemtuzumab, was demonstrated to protect SCID mice in both disseminated and subcutaneous xenograft models mostly via the activity of neutrophils, with modest NK cell activity (Siders et al., 2010). For the most part, macrophages, dendritic cells, and neutrophils appear to be the major cell types in SCID mice carrying out human IgG₁-mediated cell killing activities (Inoue et al., 2007; Minard-Colin et al., 2008). These cells in mice contain the full complement of mouse Fcγ receptors (see Table 17.2). Additionally, antibody-antigen complexes involving both mouse FcγRIII and FcγRIV appear to stimulate mouse neutrophils much in the same way that human neutrophils are activated by antibody-antigen complexes involving both human FcγRIIa and FcγRIIb (Jakus et al., 2008). Nimmerjahn and Ravetch (2008) recently concluded that, given current information, it appears that the IgG-dependent, cell-mediated activities in mice are mediated by granulocytes, monocytes, or macrophages, not NK cells, primarily via the activity of FcγRIV. On the other hand, Bruckheimer et al. (2009) claimed that both a wild-type human IgG₁ version and an Fc-engineered form of the anti-EphA2 MAb, 3F2, killed targeted cancer cells in mouse models primarily by NK-mediated ADCC. They demonstrated that the antibody worked much better in SCID mice which have NK cells than in NOD/SCID mice lacking NK cell functions (Bruckheimer et al., 2009). Since mouse NK cells only contain FcγRIII and do not appear to be functionally equivalent to human NK cells for analysis of ADCC by human IgG₁ antibodies, Shiokawa et al. (2010) developed a new model for assessment of ADCC in mice. They seeded NOD/SCID/γc^{null} (NOG) mice with purified human NK cells and demonstrated *in vivo* ADCC activity of the anti-CD20 MAb rituximab using CD20⁺ Daudi cells as the xenografted tumor cells (Shiokawa et al., 2010). Similarly, Ito et al. (2009) utilized a similar model to establish a "humanized mouse" in which PBMCs from a patient were used to repopulate NOG mice to determine the functionality of the non-fucosylated anti-CCR4 MAb KM2760 (Ito et al., 2009). This same group utilized the

humanized NOG mouse model to demonstrate the CDC activity of a high-CDC version (IgG₁/IgG₃ chimeric form called “113F”) of the anti-CD20 human antibody, rituximab (Sato et al., 2010).

It is expected that additional models will be developed and tested to improve the predictability of pre-clinical mouse models for clinical efficacy.

In summary, this chapter has described the current state of information concerning interactions of human IgG isotypes with non-human Fcγ receptors, cell types, and models. It is clear that one significant area in which knowledge is incomplete is the interaction of human IgGs with the NHP immune system. This seems incredible, given that NHPs are used for virtually all GLP-toxicology studies with therapeutic antibodies. Additionally, the development of fully “humanized” mouse immune systems in NSG mice, which today are still in an early phase of development, will help to study human IgG interactions with the human immune system in mice. This will be particularly important for the development of next-generation tumor models. Today’s tumor models, which are largely subcutaneous xenografts in immune-deficient mice, lack the immune system interaction and the human tumor microenvironment, making those systems both highly artificial and poorly informative.

Cell line development

Abstract: Most biologics are manufactured using a living cell system, typically either of mammalian, yeast or bacterial origin. The most widely used mammalian cell system is Chinese hamster ovary (CHO) cell culture, which results in antibodies that are glycosylated at asparagine-297 (N297). Murine myeloma cell lines such as NS0 and SP2/0 also have been used but are largely being phased out by high cell density, fed-batch CHO cell culture. The most widely used expression system for CHO cells has been the glutamine synthetase (GS) system, which was pioneered by Lonza. Alternative expression systems for IgGs include the yeasts *Pichia pastoris*, which has been engineered by GlycoFi (Merck) to produce human glycans, and *Saccharomyces cerevisiae*. IgGs not glycosylated at N297 can be produced in *Pichia pastoris* and *Escherichia coli*. The keys to the upstream manufacturing system are high titers in a reasonable timeframe and quality of the product obtained after cell culture or fermentation.

Keywords: cell line development, Chinese hamster ovary (CHO) cells, SP2/0 cells, NS0 cells, human embryonic kidney (HEK) 293 cells, glutamine synthetase (GS) expression system, dihydrofolate reductase (DHFR), cost of goods, transfection, *Escherichia coli*, yeasts, *Pichia pastoris*, *Saccharomyces cerevisiae*

18.1 Introduction

One of the key features that distinguishes biologics from small molecules is that biologics are manufactured by cell-based systems, whereas small molecules are manufactured by chemical synthesis processes. This results in a second fundamental difference. Biologics are a heterogeneous product purified from either the cells or cell culture medium. Small molecules can be chemically defined in the strictest sense (i.e. by a chemical formula such as $C_xH_xO_xN_xS_x$). Jefferis (2009a) estimated as many as 500 different potential species could be present in a typical cell culture system based on glycosylation and post-translational heterogeneity. Therefore a therapeutic

MAB or Fc fusion protein is a truly heterogeneous population of molecules, dominated by a few major species, if one includes post-translational modifications (e.g. C-terminal lysine clipping, methionine oxidation, deamidation, etc.) which could contribute additional minor species (see Chapter 16). With that in mind, the current status of MAB and Fc fusion production by cell line development will be discussed in this chapter.

In the early days, expression vectors, for instance pSV2, were used with murine myeloma cell lines, such as SP2/0, to generate recombinant stable cell lines (Shin and Morrison, 1989). The next cell expression system to gain popularity was Chinese hamster ovary (CHO)-strain DG44, using a dihydrofolate reductase gene as the selection system (Cacciatore et al., 2010). In more recent years, Lonza CHO cell lines, in particular CHO-K1SV, utilizing the glutamine synthetase (GS) selection system (Birch and Racher, 2006; de la Cruz Edmonds et al., 2006), has seen more widespread use. Current levels of MAB expression using the CHO-based systems routinely hit 1–2 g/L, with ranges of 3–5 g/L antibody production typically achieved after cell cloning and process optimization in a fed-batch culture (Kalwy et al., 2006; Kelley, 2009; personal communications with several bioprocess colleagues in the industry). Productivities up to ~10 g/L have been reported in extended fed-batch culture with extensive process optimization (Kelley, 2009). Two major advances have helped to fuel the increase in titers over the past decade, the selection of highly productive cell lines that grow well in suspension culture and generate product at high specific productivity (Q_p), and the refinement of defined media completely devoid of animal cell components and serum (Shukla and Thömmes, 2010).

18.2 Process summary

While, for brevity, this chapter will not delve into details of the processes involved in the manufacturing of MABs and Fc fusion proteins, it is worth noting and referencing the processes themselves. Shukla and Thömmes (2010) have outlined three major phases of MAB production: (1) generation of the inocula; (2) cell culture; and (3) product recovery and downstream processing. Similarly, Kelley (2009) has laid out ten consensus steps that cover approximately the same range of processes.

All biologic manufacturing begins with establishing a large inventory of cryopreserved cells as a Master Cell Bank (MCB). The MCB is the source of all cells used in clinical and commercial production for the entire life of the product. A single MCB vial is expanded and cryopreserved as a Working Cell Bank (several hundred vials). The WCB vials are used to inoculate

production runs and ensure consistency over numerous years. Cryopreservation, recovery and growth of the frozen cells, and expansion of the seed culture train are highly controlled and documented processes to ensure consistency (Kelley, 2009; Shukla and Thömmes, 2010).

Cell culture production processes are typically carried out in bioreactors from 500 L to 25,000 L in size. The industry has transitioned to fed-batch cultures but there are numerous products using perfusion (continuous) cultures. Production runs are generally reproducible, but deviations remain a constant problem. Recently there has been an emphasis on in-line process controls and automation to increase reproducibility (Kelley, 2009). The adoption of chemically defined media and feeds seems to be the most important contributor to process consistency and increased productivity for mammalian cell culture. Most new processes are developed using chemically defined media and feeds, which also increase product safety, i.e. reduce adventitious viruses or lot-to-lot variability risks from animal-derived components (Li et al., 2010; Rita Costa et al., 2010; Clarke et al., 2011).

The industry is moving towards adopting a two-step chromatography process with single capture and polishing steps following clarification of the spent cell culture medium. The capture and concentration step of MAbs or Fc fusion proteins is accomplished by affinity resins such as Protein A or Protein G. MAbs or Fc fusion proteins are subjected to low pH elution from Protein A or G columns followed by a hold period at low pH that acts as a viral clearance step. The polishing step is typically an anion and cation exchange chromatography, followed by ultrafiltration as a final viral removal procedure (Kelley, 2009; Shukla and Thömmes, 2010). MAbs or Fc fusion proteins need to withstand these processes, i.e. pH changes and sheer stress, without substantial aggregation or degradation, as described in Chapter 16. For example, one IgG was recently shown to become polyreactive after exposure to low pH (Djoumerska-Alexieva et al., 2010) potentially leading to undesired side effects via unknown mechanisms.

Productivity, antibody quality, purification efficiency, and cost of processes are often hotly debated topics as they relate to cost of goods (COGs). Current estimates for COGs for bulk active product ingredient (API) are generally in the \$100/g to \$300/g range for upstream manufacturing processes producing >2 g/L. Future downstream processing improvements could potentially drive COGs below \$100/g (Kelley, 2009). Typical manufacturing titers in the biopharmaceutical industry these days range ~3–5 g/L with ~14-day fed-batch culture processes (Kelley, 2009). At or above 3–5 g/L, the major contributor to COGs tips toward downstream purification. These productivities are reasonable for most manufacturing processes until more cost-effective purification procedures are developed (Farid, 2007; Kelley, 2009).

18.3 Key issues in cell line development

The major issues facing cell line development today have not changed dramatically over the last 15 years and still include selection of the (1) host cell line, (2) expression vector(s), and (3) process parameters. Our understanding of these general concepts has been expanded to include:

- host cell selection and engineering for enhanced product characteristics or process robustness;
- cell line stability over time;
- product quality, e.g. glycosylation patterns or lack thereof;
- gene and vector optimization;
- transfection and selection processes including targeting integration into chromosomal hot-spots;
- chemically defined media in place of extracts and animal-based ingredients;
- process optimization or perfusion processes improves cell viability or production time to increase productivity and product quality.

A few of these will be discussed in more detail below, but recent reviews by Anderson and Reilly (2004), Birch and Racher (2006), Kelley (2009), Rodrigues et al. (2010), Durocher and Butler (2009), Rita Costa et al. (2010), Shukla and Thömmes (2010), and Li et al. (2010) delve into these topics in greater detail.

18.4 Choice of cell line

The choice of host cell line must be aligned with the type of biologic to be expressed, regulatory experience, and the company's internal knowledge base, manufacturing capacity, and risk tolerance. Other factors that enter into the choice of the cell line to be used for manufacturing include expression characteristics, manufacturing platform (e.g. fed-batch vs perfusion), scalability of cell culture, titer needs, glycosylation requirements, requirements for secondary processing, proteolytic sensitivity of the product, cost-of-goods, and operational flexibility.

The bulk of the 151 biologics approved for manufacturing as of 2009 are produced in mammalian cells (~39% of all biologics), *E. coli* (~30%), *Saccharomyces cerevisiae* (18.5%), or hybridomas (~11%) (Ferrer-Miralles et al., 2009). Insect cells and transgenic animals each comprise less than

1% of the methods used for manufacturing of biologics today (Ferrer-Miralles et al., 2009). Fifteen of 37 currently or formerly marketed MAbs and Fc fusion proteins are produced in CHO cells, 15 are produced in murine myelomas (NS0 or SP2/0), four are produced by hybridoma or quadroma cell culture, and three are produced in *E. coli* fermentation culture (see Table 18.1).

Perhaps the two biggest issues in choosing a cell line for MAb or Fc fusion protein production are glycosylation and regulatory experience. CHO cells offer perhaps the best of both worlds. CHO cells provide a simple and close-to-human glycosylation pattern with the strongest industrial history and regulatory acceptance. Moreover, most of the major biopharmaceutical companies are either committed to CHO as their platform or are considering it as a platform. It is likely that more and more commercial manufacturing platforms will be based on CHO cells.

While there have been several attempts to produce MAbs and/or Fc fusion proteins in transgenic animals (e.g. goats, cows – Baruah et al., 2006; Durocher and Butler, 2009), land plants (e.g. tobacco, corn – Law et al., 2006; Rademacher et al., 2008; Durocher and Butler, 2009), or aquatic plants (e.g. *Lemna* – Cox et al., 2006; Woodard et al., 2009), none of these has resulted in late-stage clinical candidate MAbs or Fc fusion

Table 18.1 Cell lines and vectors for antibody and Fc fusion protein manufacturing

Production cell line	Antibodies manufactured using cell line	
	Number	Antibodies
CHO (hamster)	15	Denosumab, Rilonacept, Panitumumab, Tocilizumab, Abatacept, Bevacizumab, Adalimumab, Omalizumab, Efalizumab, Alefacept, Ibritumomab tiuxetan, Alemtuzumab, Rituximab, Trastuzumab, Etanercept
NS0 (mouse)	6	Ofatumumab, Natalizumab, Gemtuzumab ozogamicin, Daclizumab, Palivizumab
SP2/0 (mouse)	9	Golimumab, Canakinumab, Ustekinumab, Eculizumab, Nimotuzumab, Cetuximab, Infliximab, Basiliximab, Abciximab
<i>E. coli</i>	3	Certolizumab pegol, Romiplostim, Ranibizumab
Murine hybridoma	3	Tositumumab, Edrecolomab, Muromonab-CD3
Mouse-rat quadroma	1	Catumaxomab

proteins that seem marketable. Exceptions occur and it should be noted that ATrynâ (human antithrombin; GTC Biotherapeutics), manufactured from transgenic goats, was approved by the FDA in February 2009.

18.5 Mammalian cell lines

18.5.1 CHO

As mentioned above, CHO cell lines have become the industry standard for the production of therapeutic MAbs and Fc fusion proteins. CHO suspension cultures are likely to become even more widely used in the future due to their long safety record, excellent growth and productivity in serum-free medium, and good selection systems (Rita Costa et al., 2010). CHO now has a quarter-century of regulatory history since it was first approved as a manufacturing cell line for tissue plasminogen activator in 1987. A typical CHO culture produces antibodies containing glycans comprising ~35% G0F, ~35% G1F (two isomers), ~16% G2F, and ~15% minor glycoforms (Arnold et al., 2006). About 5–10% of the glycans are sialylated and another ~5% are typically non-fucosylated, as described in more detail in Chapter 11. Recently, it was demonstrated that abatacept (Orencia®), which is produced in CHO cell culture, contains oligoglycans with the immunogenic galactose- α -1,3-galactose (Bosques et al., 2010). This demonstrates that CHO cells contain the gene necessary for galactose- α -1,3-galactosylation and that cultures can be selected “accidentally” to produce proteins with this undesired glycan.

There are two significant industry-relevant CHO processes used for the expression and production of MAbs and Fc fusion proteins. The first utilizes CHO strains DG44 or DUXB1, which are deficient in dihydrofolate reductase (DHFR) activity required for nucleotide metabolism (Li et al., 2010). The expression vector carries the gene(s) of interest and the DHFR selection gene. The advantage of DHFR is that the MAb-encoding genes can be amplified with increasing DHFR inhibitor, methotrexate, concentrations which result in high productivities (Derouazi et al., 2006; Cacciatore et al., 2010). The downside to this process is that the DHFR amplification process adds significant time to the selection of stable clones (Chusainow et al., 2009).

The CHO-K1 strain and its many variants, which are DHFR sufficient, make up the second major group of CHO cell lines. The industry standard is quickly becoming Lonza’s unique oligoclonal suspension cell line CHO-K1SV used with the glutamine synthetase (GS) selection system (Li et al., 2010).

Culturing GS-positive clones in glutamine-free medium encourages the scavenging of ammonia byproduct to produce glutamine. Lonza's CHO GS system allows for methionine sulfoximine (MSX, an inhibitor of GS) selection of high-producing, stable clones without amplification (de la Cruz Edmonds et al., 2006; Li et al., 2010).

18.5.2 Mouse myeloma cell lines

Mouse myeloma cells have been used to produce MABs since the early 1990s (Knight et al., 1992). Fifteen different MABs and Fc fusion proteins are currently manufactured by the mouse myeloma cell lines, NS0 and SP2/0, a similar number as currently manufactured in CHO cells. One of the primary selection systems used with NS0 cells is the GS system mentioned above. NS0 cells are naturally deficient of GS, which makes selection in the absence of MSX straightforward. The other primary method used for selection with these cell lines is antibiotic resistance (see Table 18.2). Murine myeloma cell lines are generally not as robust as CHO cells and do not reach productivity levels near what is possible in CHO fed-batch processes. Murine cell lines also produce MABs containing galactose- α -1,3-galactose, which is known to be immunogenic in humans (Chung et al., 2008; Durocher and Butler, 2009). It was recently demonstrated that ~22% of patients treated with SP2/0 cell line-derived cetuximab (Erbix[®]) experienced hypersensitivity reactions with the first dose (Chung et al., 2008). Murine cell lines can produce MABs with terminal N-glycolneuraminic acid (Neu5Gc) oligosaccharides, which are also potentially immunogenic in humans (Padler-Karavani et al., 2008; Ghaderi et al., 2010).

18.5.3 Rat myeloma cell line

The rat myeloma cell line YB2/0 has been used as a fusion partner for the generation of rat hybridomas for several years. Recently, this cell line has been developed as a host for the manufacturing of recombinant MABs. One of the key features of YB2/0 is its ability to produce antibodies with low fucose oligosaccharides, resulting in MABs with high ADCC functionality (see Chapter 11; Sib  ril et al., 2006; Kanda et al., 2006a; Teylaert et al., 2011). Currently, there are at least two clinical candidates being produced by the French biotechnology company LFB using YB2/0 cell lines for the treatment of cancer where increased ADCC activity is desired.

Table 18.2 Examples of selectable markers for antibody and Fc fusion protein gene expression

Selectable marker	Selection type	Selection reagent	Cell type and notes
Dihydrofolate reductase (DHFR)	Metabolic	Methotrexate (MTX)	DHFR-negative CHO strains such as DG44 and DUXB11; requires additional gene amplification step
Glutamine synthetase (GS)	Metabolic	Methionine sulfoximine (MSX)	GS-negative cell lines such as NS0, or GS-sufficient cell lines in presence of strong MSX selective pressure
Hypoxanthine phosphoribosyltransferase (HPRT) OSCAR™	Metabolic	HAT medium which blocks <i>de novo</i> purine biosynthesis	Combined selection and amplification in single step (Melton et al., 2001)
Puromycin acetyltransferase	Antibiotic resistance	Puromycin	Puromycin-sensitive cell line
Bleomycin N-acetyltransferase	Antibiotic resistance	Bleomycin	Bleomycin-sensitive cell line
Hygromycin phosphotransferase	Antibiotic resistance	Hygromycin	Hygromycin-sensitive cell line
Aminoglycoside phosphotransferase	Antibiotic resistance	Neomycin	Neomycin-sensitive cell line

18.5.4 HEK293

Human embryonic kidney (HEK293) cells are probably the most widely used cells in the biopharmaceutical industry, but their use is typically limited to experimental studies and transient transfection. Large-scale HEK 293 transient transfections have been developed to obtain multi-gram MAb quantities for important non-GLP pre-clinical studies (Liu et al., 2008a). The only protein approved for manufacturing using HEK293 cells was Xigris® (drotrecogin alfa (activated)), an activated Protein C for the treatment of septic shock (Durocher and Butler, 2009). HEK293 cells are capable of properly processing Protein C by cleavage of the pro-protein and

gamma-carboxylation to achieve the activated final product (Durocher and Butler, 2009). One potential advantage for manufacturing MAb and Fc fusion proteins in HEK293 cells would be to reduce immunogenic reactions against non-human epitopes. CHO, BHK, and murine cell lines can produce proteins containing non-human glycans which are potentially immunogenic in some people (Padler-Karavani et al., 2008). A hybrid cell line, HKB11, derived from the fusion of HEK293 with a human B cell line, has been used to express experimental therapeutic proteins with human glycans and higher productivity (Cho et al., 2003; Mei et al., 2006). There have been no efforts to produce MABs or Fc fusion proteins in HKB11 to date.

18.5.5 PER.C6®

PER.C6® is a human embryonic retina cell line transformed by the adenovirus E1 gene (Fallaux et al., 1998). PER.C6® has been used to produce several adenoviral-based vaccine product candidates (Havenga et al., 2006), experimental MABs (Jones et al., 2003; Tchoudakova et al., 2009; de Kruif et al., 2010), and monoclonal IgMs (see Chapter 9; Tchoudakova et al., 2009). Recently, de Kruif et al. (2010) demonstrated the ability of a single PER.C6 cell to produce three different MABs simultaneously, which could potentially lead to a novel production system for multiple MABs in the same product, as described in Chapter 13. At least 14 different PER.C6®-based products, many of which are vaccine-based, are in early-stage clinical trials (Durocher and Butler, 2009). Precautions must be in place to control for accidental viral infections of human cells, e.g. HEK293 and PER.C6®, from operators. This is a potential risk since infections could occur anywhere from cell line development through to manufacturing.

18.5.6 BHK cells

Baby hamster kidney (BHK) cells are currently used for vaccine production as well as production of a few recombinant proteins such as Factor VIIa (NovoSeven®) and FVIII (Kogenate®). Factor VIII, a clotting factor used to treat hemophilia, is perhaps the most challenging therapeutic protein to manufacture as it is a large (~300 kDa) complex protein containing 25 potential N-linked glycosylation sites, several potential O-linked glycosylation sites, 6 tyrosine O-sulfation sites, and 7 disulfide bonds (Soukharev et al., 2002).

BHK is appropriate for FVIII production because of its ability to add sulfate to the tyrosine residues which is required for its biological function (Moore, 2003). There has been little to no interest in producing MAbs or Fc fusion proteins in BHK cells despite the success of vaccine and complex therapeutic protein production.

18.5.7 Engineering cell lines for greater fitness

The recent sequencing of the CHO and hamster genomes has sparked renewed interest in engineering mammalian cell lines to improve fitness for production of MAbs. In the past, the addition of butyrate to defined cell culture medium to increase the specific productivity of MAbs was considered the easiest method to manipulate production cell lines. But butyrate decreased cell growth and induced apoptosis (Jiang and Sharfstein, 2007). Since then, several culture-based and genetic approaches have attempted to counteract the negative effects of butyrate with little success (Rossi et al., 2011). Approaches have largely focused on improving growth rate and/or viability through resistance to apoptosis (Dorai et al., 2010; Rossi et al., 2011), growth under hypothermic conditions (i.e. at cooler temperatures) (Sunley et al., 2008; Han et al., 2009a), or a decrease in lactate production at high cell density (Dorai et al., 2009). Many, if not all, of these critical features can be achieved through primary selection and screening during cell line development. That is, appropriately designed cell line development screens can identify desired cell phenotypes, e.g. high viability and productivity. Another major hurdle for cell line engineering is successful media/feed and process optimization that can result in robust and highly productive cell cultures without chemical additives, genetic manipulation, or access to the CHO genome.

18.6 Microbial cells

18.6.1 Introduction

Microbial cells have been widely used for the production of recombinant protein therapeutics with a long track record. The primary advantage of microbial expression systems is their typically fast growth rates and robust fermentation characteristics, which allow for higher productivity, faster turnaround, and fewer contamination issues (Chartrain and Chu, 2008;

Makino et al., 2011). The disadvantage of microbial systems is their limited ability to modify proteins, e.g. glycosylation, and fold proteins with chaperones similar to human cells.

18.6.2 *Escherichia coli*

E. coli was used to produce the first recombinant protein, human insulin, and approved for marketing in 1982. *E. coli* remains the standard cell line for many simple non-glycosylated recombinant proteins. Advances in antibody expression and purification from *E. coli* have led to three marketed antibodies, certolizumab pegol, romiplostim, and ranibizumab, produced in *E. coli* fermentations (see Table 18.1). Thus the regulatory, safety, genetic, physiological, and fermentation pathways for *E. coli* expression of MABs are well worked out and are essentially non-issues.

The first description of the expression of full-length IgGs in *E. coli* was in 2002 by Simmons et al. (2002). Production levels of up to 1 g/L of intact IgGs has been reported for *E. coli* since Simmons demonstrated expression, proper folding, and periplasmic targeting of several non-glycosylated IgGs (Simmons et al., 2002; Reilly and Yansura, 2010). *E. coli* production levels could eventually compete with the best of the mammalian systems in the near future with additional expression and process enhancements.

The major hurdle for MAB expression in *E. coli* has been the lack of appropriate glycosylation and the effector functionality attributed to Fc glycans (see Chapters 10 and 11; Simmons et al., 2002; Tao and Morrison, 1989; Walker et al., 1989; Bolt et al., 1993). Fortunately, not all MABs require glycosylation for efficacy. The anti-CD3 MAB, orelizumab, currently in Phase III clinical trials for the treatment of type I diabetes, was generated as a non-glycosylated MAB to reduce effector function (Bolt et al., 1993). Sazinsky et al. (2008) recently demonstrated that certain mutants of IgG₁ (e.g. IgG₁-K290E, S298G, T299A, K326E) result in non-glycosylated IgGs with effector functions comparable, and perhaps better than, standard glycosylated IgG₁. So it is possible now to produce IgGs in *E. coli* with a wide range of potential effector functions.

The peptibody romiplostim (NPlate®) is manufactured in *E. coli* as a non-glycosylated Fc fusion protein to which a TPO mimetic peptide is fused on the C-terminus (see Chapter 14; Kuter, 2007; Kumagai et al., 2007). Amgen has at least three peptibodies in clinical trials that are produced in *E. coli* (see Table 14.1). Amgen's decision to use *E. coli* as the primary production host for their peptibody platform (Sutherland, 2004) demonstrates the alignment of product type and the appropriate expression system.

Recently, George Georgiou and his colleagues have built libraries of intact IgGs in *E. coli* for the purpose of screening potential therapeutic targets (Mazor et al., 2007, 2009; Makino et al., 2011). This technology will combine library screening, lead identification, and cell line development into a single step. They have optimized a variety of promoters, chaperones, and other conditions for enhanced dicistronic expression of IgGs in *E. coli* (Makino et al., 2011). With these tools in hand, it is not unreasonable to expect that companies would begin to adapt *E. coli* more broadly as a host for MAbs and Fc fusion proteins.

18.6.3 Yeasts

The yeasts *Saccharomyces cerevisiae* and *Pichia pastoris* constitute the other industry-relevant microbial systems that have been explored significantly for the production of MAbs and Fc fusion proteins (Ferrer-Miralles et al., 2009). There is a long history of commercial production of therapeutic proteins in *S. cerevisiae*, including hormones, e.g. insulins and human growth hormones, and virus-like particles, e.g. the major capsid protein L1 of human papillomavirus types 6, 11, 16, and 18 for the vaccine Gardasil®. As of 2009, all 28 approved yeast-produced human therapeutic and prophylactic proteins are manufactured in *S. cerevisiae* (Ferrer-Miralles et al., 2009). As mentioned in Chapter 4, three biotechnology companies, GlycoFi, Adimab, and Alder, have focused on developing yeasts to either engineer or produce MAbs. While Adimab has focused on using yeasts as MAb discovery tools (Weaver-Feldhaus et al., 2004; Rakestraw et al., 2009), GlycoFi (a subsidiary of Merck & Co.) and Alder Pharmaceuticals have focused on development of the yeast platform for the manufacturing of antibodies.

Yeast produces proteins with potentially immunogenic oligoglycans comprised primarily of mannose residues. Biotech companies Alder and GlycoFi have taken dramatically different approaches to yeast manufacturing of MAbs. Alder Pharmaceuticals produce non-glycosylated MAbs in *P. pastoris* and, thereby, avoid the issue. Alder's ALD518 (BMS-945429) is furthest along in Phase II clinical trials in collaboration with Bristol-Myers Squibb. GlycoFi has completely re-engineered the glycoprotein pathways in *P. pastoris* (Hamilton et al., 2006) to generate MAbs with fully human glycosylation patterns (Li et al., 2006a). The glycans produced in GlycoFi's engineered *P. pastoris* produce single glycans or a major glycan with a few minor species. GlycoFi's glycosylation pattern is significantly

less complicated in structure than the oligoglycans attached to MAbs produced in mammalian cells. The glycans are inherently non-fucosylated and ideal for therapies requiring high ADCC because GlycoFi engineered the human glycosylation in *P. pastoris* from the bottom-up (Zhang et al., 2011).

18.7 Multiple cell lines in single batches

The Danish biotechnology company Symphogen has pioneered methods for the production of multiple monoclonal antibodies in a single bioreactor with the concept to manufacture poly-monoclonal antibody products (Nielsen et al., 2010). The idea is to use their multi-antibody therapeutic approach to target diseases, such as microbial infections and cancer, in which a broad array of antibodies against multiple epitopes may be used to clear the invasive cells.

The Symphogen process requires the isolation and characterization of multiple monoclonal antibodies against a single target or possibly related targets. Key to this technology is the site-specific integration (called Sympress™) of the MAb producing genes into identical sites in the CHO chromosome for each different clone. Creating identical clones for each MAb, in theory, should balance the productivity of each clone in the polyclonal cell cultures (Wiberg et al., 2006). A separate master and working cell bank for each MAb expressing cell line is generated. An optimized seed train from each working cell bank is important for the consistent production of multiple monoclonal antibodies from different cell lines in a single batch cell culture (Nielsen et al., 2010). Using this approach, Symphogen has taken Sym001 (Rozrolimupab), a poly-monoclonal product candidate containing 25 individual MAbs targeting human Rhesus D antigen, into Phase II clinical trials (Nielsen et al., 2010). The most significant issue for this type of product is the ability to analyze to a high degree of accuracy and precision all 25 MAb components that make up Rozrolimupab and set release specifications (Frandsen et al., 2011). Batch-to-batch variability is a significant challenge with this technology. Nielsen et al. (2010) showed results for nine batches of a poly-monoclonal product candidate containing six MAbs targeting vaccinia virus and demonstrated similar, albeit not identical, component mixtures. This emphasizes the difficulty in producing a consistent product of multiple MAbs from a single batch.

18.8 Gene and vector optimization and selectable markers

18.8.1 Expression systems and selectable markers

High-level expression of genes encoding MABs or Fc fusion proteins is relatively standard with strong promoters/enhancers to generate high-quality expression vectors in combination with robust hosts and optimized media. The most widely used promoters/enhancers are derived from viruses, such as the cytomegalovirus (CMV) promoter, and simian virus 40 (SV40) promoter (Rita Costa et al., 2010), or the non-viral promoter for elongation factor 1 α (EF1 α) from CHO cells (Li et al., 2010). Transcript stability and efficient translation is dependent on a proper polyadenylation signal and most are derived from the bovine growth hormone gene or SV40 (Rita Costa et al., 2010). Mammalian codon optimization of the MAB or Fc fusion coding regions is an industry standard (Carton et al., 2007).

One of the more significant issues with respect to vector optimization for MAB production is the choice of a single vector containing both heavy and light chains and their respective promoters/enhancers (Li et al., 2007a), or cotransfection with two separate vectors, each containing one of the genes (Schlatter et al., 2005). The obvious key is to obtain balanced expression of the heavy and light chains. Since the site of integration can dramatically affect expression, a single vector may be preferred (Li et al., 2007a). Currently, the field tends to lean towards use of cotransfection of two vectors but that may change as Lonza double-technology is embraced.

A wide variety of selectable markers has been used in mammalian expression vectors, including both metabolism- and antibiotic-based selections (see Table 18.2). The first utilizes CHO strain DG44 or DUXB1, which are deficient in the dihydrofolate reductase (DHFR) activity required for nucleotide metabolism (Li et al., 2010), and a vector-borne DHFR gene. Cell selections are made using culture medium lacking hypoxanthine and thymidine. The advantage of this system is that the MAB-encoding genes can be amplified by the addition of increasing concentrations of the DHFR inhibitor, methotrexate (MTX) (Chusainow et al., 2009). This process requires additional time for the amplification stage of stable clones, and clones are not stable in the absence of MTX selection resulting in the eventual loss of productivity (Chusainow et al., 2009).

Glutamine is an essential amino acid and cell cultures require the glutamine synthetase (GS) gene for glutamine production in glutamine-free defined medium. A vector-borne GS gene is used in cells that either lack endogenous GS (e.g. NS0 cells) or are made GS-deficient by the addition

of the GS inhibitor methionine sulfoximine (MSX) (Rita Costa et al., 2010). The result is that only transfectants with additional copies of GS can survive the selection process. The GS-based system has the advantage over the DHFR-based system based on a shorter timeline to stable cell selection and high productivity without substantial gene amplification. GS-positive cells also scavenge ammonia from the medium to generate glutamine and thereby reduce a harmful byproduct of metabolism.

A third metabolic system that has been developed, but not yet commercialized, is the OSCAR™ system from the University of Edinburgh. For this system, the hypoxanthine phosphoribosyltransferase (HPRT) gene, which is required for purine salvage pathways, is incorporated into disabled miniplasmids and the cells are grown in HAT medium, which blocks *de novo* purine biosynthesis. Because HPRT expression is limiting and selection requires its expression, both selection and amplification must occur simultaneously (Melton et al., 2001).

Several antibiotic-based selectable markers have also been used, as shown in Table 18.2. One of the key issues with antibiotic selection is the relative “strength” of the resistance. Antibiotics act as a binary selection for resistance with little ability to titrate, and therefore amplify, selection. One approach to improving antibiotic selections is to partially impair, or weaken, the antibiotic resistance mechanisms so that amplification is required to obtain complete resistance. This approach was used successfully to improve recombinant protein production by mutating the neomycin phosphotransferase gene used for selection to reduce enzymatic activity (Sautter and Enenkel, 2005).

18.8.2 Transfection and selection processes

There is a variety of methods used to transfect cells with DNA encoding MABs or Fc fusion proteins, including electroporation (Canatella et al., 2001), lipofection with cationic liposomes (Bombelli et al., 2005), polyfection with the cationic reagent polyethyleneimine (Derouazi et al., 2004), or the use of calcium phosphate (Chenuet et al., 2008). The field is moving toward use of the lipofection and polyfection approaches for the preparation of cells for research reagents. Cell line development for commercial cell lines larger remains with the older transfection technology (calcium phosphate and electroporation) because of simplicity, freedom to operate, and regulatory confidence.

18.8.3 *Transient vs stable transfection*

Transient transfection is typically used at the laboratory scale to obtain reasonably quick production of antibodies and Fc fusion proteins (Derouazi et al., 2004). Typical yields for transient transfections range from 10 to 30 mg/L, although both lower and higher values are sometimes obtained based on methodology or expressability of specific proteins (Liu et al., 2008a). Yields as high as 140 to 300 mg/L have been reported (Codamo et al., 2011; Rajendra et al., 2011). The most significant issues with transient transfection are the production of bulk plasmid (e.g. Cheng et al., 2011) and the method for transfection itself. The industry is adopting the use of cationic reagents such as polyethyleneimine (PEI) and similar approaches for high efficiency transient transfection (Sun and Zhang, 2010). Mammalian cell lines used for transient transfection include HEK293, COS, CHO, and BHK cells (Rita Costa et al., 2010). Transient transfection methods are usually limited to less than 10 L but can be used up to 40–50 L in wave bags.

Transient transfection has not been used for manufacturing, nor has it been used widely for the production of GMP materials. One of the key issues for the use of transient transfection is the difference in MAb glycosylation from transient transfection of HEK293 cells versus stable cell lines such as CHO cells. A intermediate process between transient and stable cell line development called “bulk stable” is becoming more widely used. Bulk stable cultures start with the same process for stable cell line generation but the non-clonal pool is used instead of screening individual clones (Ye et al., 2010). Bulk stables can produce MAbs at 100–1,000 mg/L.

18.9 Other industry trends

Cell line development is not the hotbed of innovation that it used to be. The standardization of pharmaceutical development with platform processes has penetrated into cell line development and largely stopped innovation. It is absolutely necessary to increase cell culture and product quality consistency in order to build efficiencies in development. The large biopharmaceutical companies have established hosts, expression systems, media, and processes with internal expertise and regulatory histories. Deviations from the established development platforms may introduce significant costs, delays, and risk.

There are a few industry trends that are worth mentioning. The adoption of single-use components throughout the production process, exemplified

by disposable bioreactors, will have the greatest impact on biological manufacturing (Mahajan et al., 2010; Rodrigues et al., 2010). Single-use technology will significantly increase manufacturing flexibility and reduce costs. It is likely only a matter of time before the industry embraces end-to-end single-use technology for GMP manufacturing.

The other significant trend is the increased interest in “-omics.” Genomics, transcriptomics, proteomics, and metabolomics have been touted as the keys to understanding production cell lines and manufacturing processes. It is too early to tell whether these analyses will lead to cell lines with greater productivity, stability, or consistency, but it is an effort that is well worth watching.

Issues facing therapeutic monoclonal antibodies for the future

Abstract: While the market for recombinant monoclonal antibodies and Fc fusion proteins is currently excellent, and the industry pipeline is rich with new candidates in all phases of discovery and development, there are several challenges facing the industry. The first is that the methods used for constructing high-quality humanized and fully human MAbs are near the end of their patent life and will be commoditized over the next few years. Additionally, the number of novel validated targets is of concern, as is the level of competition on hot targets, the potential limitations of reimbursement by third-party payors and governments, and the impending glut of follow-on MAbs and Fc fusion proteins that will hit the market in starting in the next few years. Some significant trends that appear to be developing include greater development of bispecific antibodies, antibody-drug conjugates, and companion diagnostics, which should help to identify the most appropriate patient populations for treatment. Additionally, new markets are expected to open up in growing economies, such as Brazil, Russia, India and China. How companies respond to these various scientific and business challenges will define their future as producers of innovative MAbs and Fc fusion proteins of the future.

Keywords: technology commoditization, follow-on biologics (biosimilars), competition, third-party payors, reimbursement, validated targets, BRIC (Brazil, Russia, India, China) countries, “pipeline in a product,” SWOT (strengths, weaknesses, opportunities, threats) analysis

19.1 Introduction to the future state

The field of recombinant therapeutic MAbs, which took hold in the industry in the late 1990s with the introduction to the market of Rituxan®, Remicade®, Herceptin®, and Enbrel®, is now finding itself to be heading toward maturity. This can be evidenced in several ways, including the fact

that all of the large traditional pharmaceutical companies have now embraced therapeutic MABs and have spent billions of dollars acquiring the technologies, pre-clinical and clinical candidates to fill their pipelines, and clinical trials to move those molecules towards marketing approval. Additionally, many of the large pharmaceutical companies have publicly stated that their portfolios going forward will be substantially balanced between biologics and small molecules, i.e. becoming true “biopharma companies,” something that would have been unheard of for many of those same companies just a decade ago. Finally, several of the initial wave of blockbuster MABs and FcFPs will be going off patent in the next few years, which will start a new wave of biologics – the follow-on MABs and FcFPs.

Some clear trends for the future of MABs and FcFPs have been noted in this book. Others that are not discussed here may be just as important. The list below and the following sections describe a few of these topics:

- commoditization of MAB and FcFP technologies;
- effect of follow-on biologics (FOBs) for MABs and FcFPs;
- competition/differentiation (first/best in class effect);
- effects of small molecules in “traditional biologics” areas (e.g. Pfizer oral molecule in advanced clinical trials for the treatment of RA);
- continued need for novel, pre-clinically validated targets for MABs and FcFPs;
- third-party payor pressure;
- pipeline in a product concept;
- companion diagnostics to specify patient population – even in clinical trials;
- multi-specific (multi-targeting) and multi-antibody products;
- antibody and Fc fusion protein toxin and other small molecule conjugates;
- tissue targeting such as getting antibodies across the blood–brain barrier;
- need for sophisticated antibody engineering just to stay in the game;
- marketing to the BRIC countries and the rest of the world to increase.

The following sections briefly highlight the environmental influences that should have a significant impact on the MABs and FcFPs business, from discovery, through development, to marketing. Sections 19.2–19.6 highlight those influences that will challenge the business, followed by several opportunities that may positively impact the business in sections 19.7–19.11. Finally, the chapter and book is summarized in section 19.12 with a short SWOT (strengths, weaknesses, opportunities, threats) analysis and epilogue (section 19.13).

19.2 Commoditization of the core underlying technologies

Many of the underlying technologies that have fueled the MAB revolution, *per se*, were invented in the mid-to-late 1980s and 1990s, and thus many of these are now beginning to come to the end of their normal patent life, making many of these technologies available to a broader group of companies. Some of these include phage display technologies, humanization technologies, technologies for transgenic animals producing human antibodies, and so forth. Additionally, as technology has advanced, many companies have figured out pathways to get around some of the earlier patents, expanding the technology base considerably. For example, a decade ago, there were only two options for making human antibodies with transgenic animals, Medarex, with their HuMAB® mouse technology, and Abgenix, with their XenoMouse® transgenic mouse technology. Today, Abgenix is part of Amgen and Medarex is part of Bristol-Myers Squibb, which in theory might appear to limit access to these types of technologies. But this is not the case. In recent years, Regeneron has developed a transgenic mouse producing human antibodies, and several new companies are building such capabilities now (see Chapter 5) which will be broadly available within the next year or so.

19.3 Impact of follow-on MABs and FcFPs

Biosimilars are biologics that are essentially produced to be the same as the innovator biologics. The reason they are called biosimilars, or follow-on biologics (FOB), however, is that because they are complex biological molecules produced in cell cultures, they are by definition not identical.

The US established new guidelines in 2009 for the approval of FOBs under the “Biologics Price Competition and Innovation (BPCI) Act,” which contained provisions for an abbreviated regulatory approval pathway for “generic” versions of biological medicines (i.e. biosimilars) in the US. According to the legislation, a “biosimilar,” or follow-on biologic (FOB), product was defined as a biological product that “is highly similar to the reference product notwithstanding minor differences in clinically inactive components” and for which “there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity and potency of the product.” Perhaps the most important issue in the biosimilar field is the concept of interchangeability (Davis et al., 2009). According to the legislation, a biological product can be “interchangeable” with the innovator’s reference biological product if:

- “it meets the criteria for being biosimilar to the reference product;”
- “it can be expected to produce the *same clinical result* as the reference product *in any given patient*;”
- “the risk in terms of safety or diminished efficacy in *alternating or switching* between use of the biological and reference product is not greater than the risk of using the reference product without such alternation or switch.”

The risk with interchangeability is that treatment with an innovator's biologics, followed by treatment with an FOB to the innovator, and then retreatment with the innovator's biologic may lead to immune responses and other adverse events due to subtle differences between the two products. Thus the FDA is considering holding biologics to a higher standard of similarity to obtain a claim of interchangeability (Kozlowski et al., 2011).

The biotechnology industry is rapidly becoming a mature industry with several therapeutic proteins such as epoetins, insulins, and human growth hormones already off patent protection, and several more reaching the end of their patent life within the next 4–5 years. DataMonitor (2011) project that approximately 30 branded biologics worth \$51 billion will lose patent exclusivity by 2015, and that the global biosimilars market will grow from \$243 million in 2010 to \$3.7 billion by 2015.

FOBs have already been on the market since 2006, with the approval in the European Union (EU) of Omnitrope® (somatropin; Sandoz) and Valtropin® (somatropin; Biopartners). As of February, 2011, there are now 14 biosimilars approved in the EU for four innovator reference products, Neupogen® (seven EU follow-on biologics (FOBs)), Eprex® (two EU FOBs), Genotropin® (one EU FOB), and Humatrope® (one EU FOB). Japan and Australia each have approved two FOBs, in addition. In the BRIC (Brazil, India, Russia, China) countries combined, over 100 FOBs have been approved over the past several years, with FOBs for innovative filgrastim, epoetin alpha, and interferon alfa being the most prevalent (DataMonitor, 2011). In the US, only four follow-on versions of protein therapeutics originally approved under the FDC Act have gained approval under section 505(b)(2), including Omnitrope™, Fortical™, Hylenex™, and Glucagen™. However, the approvals in the US are limited to those original products approved as NDAs under the FDC Act, not the majority of protein therapeutics approved under the PHS Act.

With the 2009 legislation, the US is slowly moving towards the definition of pathways for the approval of FOBs. The FDA has recently published an opinion paper on FOBs, stating that it is the “totality of evidence,” including the product complexity, formulation, stability, and biochemical and

functional characterizations, that will be considered using a “risk-based approach” when reviewing applications for FOBs (Kozlowski et al., 2011). Additionally, the US FDA recently issued draft guidelines for the development of biosimilar therapeutic proteins in the United States (<http://www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/TherapeuticBiologicApplications/Biosimilars/default.htm>). These guidelines will set the stage for the first round of biosimilar monoclonal antibodies, which are expected to hit the market within the next several years.

Table 2.4 in Chapter 2 points out that 13 MABs and FcFPs for five targets, TNF- α , VEGF, CD20, HER2, and EGFR, hold nearly \$57 billion worth of value based on 2011 sales. At least one product for each one of those targets is expected to go off patent in the US and/or EU within the next 5–7 years, so there will be incredible value that will be made available globally for FOBs against these top targets. Of these, one of the first major MAB/FcFP targets for FOB manufacturers appears to be Rituxan® (known as MABThera® in the EU) which is reported to be coming off patent in the EU in 2014 and in the US in 2015 (Carey, 2011). Rituxan® was approved in 1997 and achieved more than \$6.6 billion in revenues worldwide in 2011 (see Chapter 2, Table 2.6). Multiple companies have already started making and developing rituximab FOBs, including Teva, Sandoz, Dr. Reddy, and Viropro, to name a few (Carey, 2011).

The next five years will be a critical period for decisions regarding “complex” glycosylated FOBs which include MABs and FcFPs. These decisions fall into three broad categories:

- decisions by the innovators on how to deal with their biologic going off patent and the new competition they will face;
- decisions by the FOB producers, many of whom are now also large biopharma companies (e.g. Merck, Novartis, Pfizer); and
- decisions by regulatory agencies on how to deal with the follow-on complex biologics.

19.4 Competition

The first trend, i.e. steep competition, is already evident from the large number of players making and developing therapeutic MABs and FcFPs against the hot targets (see Chapter 8, Table 8.4). As documented in Chapters 2 and 8, as of June, 2012, there are already 40 MABs and FcFPs on the market, at least another 35 in Phase III clinical trials, and over 320 more

candidates in earlier phase trials, targeting only 185 unique molecular targets. In recent years, about 40 new Phase I MAb and FcFP candidates were being added to the clinical programs each year. In the meantime, most companies are increasing their efforts on biologics discovery, often at the expense of their small molecule portfolios, so it is clear that the MAb and FcFP field will become even more competitive than it already is. This will result in a scenario much like that currently seen in the small molecule arena, i.e. the first-in-class and the best-in-class MAbs or FcFPs will likely take the bulk of the market share, with several other molecules fighting for the leftovers. Thus being the first or finding a way to differentiate from all of the competitors are the most likely routes to success as the competition becomes steeper. Nevertheless, the pathways to potential differentiation during discovery and early development, which typically include potency, specificity, dosing frequency and route of administration, properly adjusted Fc activity, and safety, are well known and followed by most of the major players today. Thus the bar for competition gets higher, and the mechanisms to make a “differentiated” molecule pre-clinically become more difficult to define.

Being first, on the other hand, often means discovering the target in the first place, or being the first to find the academic or small biotech company which has discovered and studied the target and its biology. This leads to the next trend – the paucity of novel, pre-clinically well validated targets.

Competition can also come from non-biologics sources. One of the key competition issues that will be watched in the next few years is the effect of Pfizer’s tofacitinib (CP-690550), an orally bioavailable, small molecule inhibitor of Janus kinase (JAK), which is in late-stage clinical trials for rheumatoid arthritis and other indications (Tak and Kalden, 2011). The question that is being considered is whether a once-daily oral pill will take significant market value from the anti-TNF- α inhibitors, or from newer biologics (e.g. Orenia®; Actemra®, etc.) that offer different mechanisms of action for treatment of RA (Tak and Kalden, 2011).

19.5 The continued need for, and limitation of, novel pre-clinically validated targets

As mentioned in detail in Chapter 8, the field is in need of new, pre-clinically well validated targets. The lack of novel, pre-clinically validated targets for MAbs and FcFPs is now reaching a critical stage. While it appears from a surface analysis that there should be plenty of additional pharmacologically relevant targets to be found (see Chapter 8), those

targets nevertheless need to be identified, studied, and either validated or discarded as potential MAB and FcFP targets.

The need for new, validated targets is apparent and it appears from the analysis in Chapter 8 that potential targets should be available for discovery and validation. The question is who should do that work? Despite the almost universal call for increased innovation in the industry, most large pharma have decided to abandon the area of target discovery, with the intention of leaving that part of the business to academic investigators and small biotechnology companies. The general feeling is that this early target discovery and validation work should be done external to the pharma sector and would be available for in-licensing once it reached a level of maturity. Thus this places the onus for target discovery and validation on academia and small, start-up biotechnology companies, both of whom rely on external funding sources to carry out their efforts. For academics in the United States, those funding sources are the NIH, NSF, and other government funding agencies, whose budgets are being squeezed leading to fewer grants being awarded per year. Other governments across the world are seeing similar budgetary constraints on funding academic research that can lead to new target validation. Small biotechnology companies rely mostly on venture capital investments to drive their discovery efforts, a funding pool that also has receded in the past several years, leading to an overall shrinkage of investment opportunities and thus fewer small biotech companies being started. Thus in the next 5–10 year time frame, where will all of the new targets come from? One approach would be for the large biopharmaceutical companies to re-invest in target discovery, perhaps in more of a collaborative manner with small biotechs and academics. Lessl et al. (2011) state just such a case for a program started at Bayer called “Grants4Targets.” More of these types of programs will be required to fund the gap between current funding levels and those needed to help fill the validated targets pipeline. Additionally, academic centers have become involved in drug discovery themselves. A recent analysis of small molecule drug discovery in US academia revealed that 78 different organizations had small molecule discovery units, focused mostly on cancer and infectious diseases (Frye et al., 2011). These and similar groups working on large molecules such as MABs and FcFPs offer hope for the discovery of novel targets in the next decade. Nevertheless, as Kessel (2011) points out, in-licensing of targets or early development candidates will not solve the entire problem. The key remains for the large biopharmaceutical industry to help fill the gap left by reduced government funding for academia and venture capital funding of small biotech companies. As noted above, there are several potential mechanisms for this. Most large biopharmaceutical

companies have very active “external innovation” groups that tie ever more closely with academic and small biotech groups in an effort to maximize the interactions between academic target and drug discovery and the industry.

In an overview of pharma R&D productivity, Paul et al. (2010) contrasted two different models of drug discovery and development – the current pathway which is relatively linear and development oriented versus the “quick win, fast fail” approach that would rely on a significantly increased drug discovery effort to push several molecules, mechanisms of action (MOA), and pharmacologies into pre-clinical toxicology and first in human studies to more quickly understand a variety of MOAs. In theory, this new approach, which faces at least some level of pharma cultural resistance, would push more of the costs towards the front end of the discovery and development process, but should result in better understood drugs making it to the more costly late stages (Paul et al., 2010). Finally, Paul et al. (2010) suggest that increasing the efforts on early target validation and early POC studies could raise the PTRS of Phase II compounds to as high as 50% (for small molecules; equivalently greater for large molecules such as MABs and FcFPs). This approach, which is very appealing, would require a complete reversal of the industry trend away from in-house discovery efforts.

An orthogonal approach from that mentioned above to increase targets for MABs and FcFPs would be to broaden the scope of targets being addressed by those molecules. We are already seeing more MABs being applied to the pharmacology of GPCRs (see Table 8.6), and it is likely that this trend will continue. Additionally, the combination or conjugation of MABs or FcFPs with small molecules, as pioneered at CovX (now Pfizer; see Chapter 15) is an area worth further exploration. These are areas ripe for increased efforts using MABs and FcFPs.

19.6 Payor pressure

One of the less discussed but potentially more significant pressures on the biologics business in general is the increase in pressure from third-party payors and governments. Most MABs and FcFPs cost more than \$10,000 per course or per annum, with a few costing as much as \$100,000 per course or per annum, making them some of the most expensive pharmaceutical treatments available. The analysis in Chapter 2 (see Figure 2.2) suggests that there will be over 65 MABs and Fc fusion proteins within the next 4–5 years, which will have two significant effects on the marketplace. First, the overall market for MABs and FcFPs is expected to reach somewhere between \$75 and \$90 billion

over the next five years or so. This is a positive measure for the industry and for patients who need the medicines, but it also represents a more than 50% increase in the overall price tag for these medicines. Some governments have already begun to reign in costs by imposing strict guidelines on reimbursements and by deciding not to reimburse some medicines. A significant recent example of this is the decision by Britain's National Institute for Health and Clinical Excellence (NICE) to reject payment for Lucentis® as a treatment for diabetic macular edema, stating that they did not consider the treatment as cost-effective for treating the condition. NICE has, however, recommended Lucentis® for macular degeneration. NICE has also developed an algorithm for cancer drugs that takes into consideration a calculation of "quality adjusted life year" (QALY) which essentially puts a price on one's life. Without arguing for or against such policies, the fact is that they will continue to evolve and most likely in the direction of placing greater emphasis on cost savings (Strohl, 2009a), putting more pressure on the biopharmaceutical business, which is facing ever-increasing development costs for all drugs, including MABs and FcFPs. As noted earlier in this chapter, these pressures will force difficult decisions about the continuation of development if a MAB or FcFP is neither first-in-class nor appearing to be best-in-class.

19.7 Pipeline in a product concept

As development costs continue to rise, and as new, well validated targets become more and more difficult to find, biopharmaceutical companies are expanding their efforts to increase the breadth of indications for the products that they already have. Perhaps the best example of this is Remicade®, which is now approved for 16 different disease indications, including Crohn's disease, pediatric Crohn's disease, ulcerative colitis, rheumatoid arthritis (in combination with methotrexate), ankylosing spondylitis, psoriatic arthritis, and plaque psoriasis (Remicade® prescribing information, 2011). It is expected that more biopharmaceutical companies will attempt to extend the indications for their MABs and FcFP medicines to all possible disease states for which the drug makes medical sense.

Significantly, this approach also takes advantage of the safety database that needs to be accumulated in Phase III trials (<http://www.accordclinical.com/clinical-study/types-of-clinical-trials/phase-iii-clinical-trials/>). For a new medicine, large or several Phase III trials need to be run to accumulate enough data to understand the safety risks of the new molecule being tested. Once that database has been established for the first indication,

additional indications can be investigated clinically and potentially approved if the data warrant without the need to go back and collect a new safety data set. This makes add-on indications even more attractive in an environment in which it is already difficult to find safe, efficacious new drugs with novel mechanisms of action.

19.8 Companion diagnostics and patient segmentation

Treatment with Herceptin® is only prescribed for those patients who score at least a 3+ on the HER2 diagnostic test such as HercepTest® (Herceptin® prescribing information, 2010). Thus Herceptin® is being given to patients who are most likely to respond to the drug, thereby segmenting the population. This was one of the first examples of a significant trend in the pharmaceutical industry, and particularly in the future of treatment with MABs and FcFPs. Another example is the finding that patients with the KRAS mutation are not likely to respond to anti-EGFR MAB therapy (Jimeno et al., 2009; De Roock et al., 2010). Retrospective analyses of colorectal cancer patients treated with Erbitux® revealed that those patients with a mutation in the kinase KRAS did not benefit from Erbitux treatment (Jimeno et al., 2009). Up to 40–50% of all patients with colorectal cancer carry KRAS mutations (De Roock et al., 2010). Thus the National Comprehensive Cancer Network (NCCN) now recommends testing for KRAS mutations before initiating treatment with either of the anti-EGFR antibodies, cetuximab or panitumumab, for metastatic colorectal cancer or non-small cell lung cancer, and has recommended that alternative therapies be considered for patients with KRAS mutations (NCCN, 2008a, 2008b). Yet, this does not appear to be enough. While at least seven different mutations of KRAS are screened before assigning treatment with anti-EGFR MABs, many patients who carry the wild-type KRAS allele also do not respond to anti-EGFR treatment (De Roock et al., 2010). It is now recognized that other mutations in related pathways, including BRAF, NRAS, and PIK3CA genes, also significantly correlate with reduced response rates, suggesting that genotyping those genes also may help to improve selection of patients for treatment (De Roock et al., 2010). Both the known KRAS risk factor and the relatively recently understood mutations like BRAF spell out the increased need to segment patients who will benefit the most from antibody treatments, both from a cost-effectiveness standpoint (Blank et al., 2011) as well as identifying best treatment approaches.

19.9 Treatment with multiple antibodies and bispecific antibodies

As documented in Chapter 13, there are already nine examples of clinical trials in which multiple antibodies are packaged together in a single dosage. So far, most of these are for infectious disease agents, but a few of them are cancer targets. This approach, combined with the renewed interest in bispecific antibody approaches, is a significant departure from the first wave of monoclonal antibodies of the late 1990s and early 2000s, but is a next step evolution after serum therapy and specific immunoglobulin IVIG therapy. For difficult-to-treat disorders such as cancer and infectious microorganisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), multi-antibody approaches may be the path to future success.

To that end, it should be noted that acquired immune deficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV) was discovered about 30 years ago. For years, an AIDS diagnosis was considered a death sentence. Today, 30 years later, thanks to HAART (highly active antiretroviral therapy) combination therapy introduced in the late 1990s, most HIV patients can live a relatively normal life with a reasonable life expectancy. HAART therapy consists of a cocktail of at least three antiviral agents with at least two different mechanisms of action, e.g. protease inhibitor mixed with integrase inhibitor and non-nucleoside reverse transcriptase inhibitor (NNRTI). Similarly, it has become standard practice in cancer treatment to treat with two or more orthogonal treatment regimens (e.g. Erbitux® plus cisplatin and 5-fluorouracil therapy for patients with metastatic squamous cell head and neck carcinoma). How long will it be before cocktails of antibodies, targeting different orthogonal pathways, will become standard treatment for at least some forms of cancer? The answer to that lies both in research of the correct combinations as well as improvement in cost containment so that the combination therapeutic will not be cost-prohibitive. Hopkins (2008) described the future of drug discovery and development as network pharmacology with just this concept in mind. In his concept, pathway interactions, synthetic lethality solutions to cancer, multi-target treatment of microorganisms, and other poly-pharmacological approaches will help to overcome multiple mutations, the formation of resistance, and bypasses by redundant biochemical pathways (Hopkins, 2008). It is highly likely that MABs, mixtures of MABs, and combinations of MABs and small molecule approaches will be the keys to success in the treatment of cancer and severe infectious diseases.

Bispecificity and dual targeting antibodies (Kontermann, 2012; Gu and Ghayur, 2012), as well as trifunctional and multi-functional antibodies (Hess et al., 2012) are some of the leading trends for the next generation of therapeutic antibody products. Just in the last year, at least six new multi-specific antibodies have entered clinical trials, with many more candidates shortly behind. Over the next decade, multi-functional and multi-specific antibodies and the poly-pharmacology they bring with them could become a standard in the field rather than an exception.

19.10 MAb and FcFP conjugates

As documented in Chapter 15, the interest, technology, and development of antibody-drug conjugates have increased significantly over the past decade to the point now where most major players are either involved in making and developing antibody drug conjugates or are thinking about how to enter that arena. This is a quickly evolving area that should, within the next few years, expand from antibody-drug toxin conjugates for cancer treatment and imaging reagents to antibody or Fc conjugates with small molecules and peptides for a wide variety of disease indications other than oncology. The acquisition of CovX by Pfizer in 2008 is the first significant step in that direction, with the expectation that interest in this area will continue to expand. The most significant limitations to the expansion of MAb or Fc drug conjugates beyond the confines of cancer are cost and delivery, which would be limited to parenteral administration.

Therapeutic cancer antibodies to date have not shown quite the potency that was desired. One of the leading forms of the next-generation antibodies will be antibody conjugates, which offer the potential for increasing the killing index for antibodies in the field of oncology. Over the past decade, advances have brought the field to the point of understanding many of the “rules” to apply to antibody conjugates, and those rules are now being incorporated into a wide variety of new clinical candidates. It has been reported that in 2012, Genentech alone will place up to nine novel antibody toxin conjugate candidates into clinical trials, in an effort to further pin down what works and what does not in this advancing field.

19.11 Biopharma in 2020 – the focus on BRIC

The major current markets for therapeutic MAbs and FcFPs are in the United States, the United Kingdom, a few of the wealthier European

countries, and Japan. Thus there are millions of patients in need of biologic therapeutics who are not getting access to them, mostly because of the high cost of biologics and the underdeveloped healthcare systems in some countries. Brazil, Russia, India and China, known together as the “BRIC” countries with rapidly developing economies, are increasing their healthcare efforts across the board. Over the next decade, it is expected that there will be increasing pressure to provide expensive biologics drugs to patients in those countries for a fraction of the prices obtained in the developed economies. Additionally, those governments may find novel approaches to obtain innovator biologics drugs at costs that can be borne by their economies. While it is not easy to estimate the impact the BRIC markets will have on MABs and FcFPs in the short term, it is almost certain that by 2020, the BRIC countries will have a significant impact on global sales of both innovator and FOB MABs and FcFPs. Most large pharmaceutical companies are responding to this global change by collaborations or expansion of their organizations into these major growth areas, particularly China.

19.12 SWOT analysis of therapeutic MABs and FcFPs

Table 19.1 shows a SWOT (strengths, weaknesses, opportunities, threats) analysis for MABs and Fc fusions. The most significant strengths include the ability to engage targets unavailable (at least, to date) to small molecules, remarkable efficacy, long half-life, overall lack of toxicities and off-target issues, and interaction with the immune system. The greatest future opportunities include expansion of the various modes of delivery to include intra-nasal, inhaled, and potentially even oral delivery, the ability to tune Fc-immune system interactions to the desired biology, the potential for multi-specificity (i.e. ability to engage multiple therapeutic targets with a single MAB or FcFP), and potentially tunable pharmacokinetics, resulting in MABs that could have dialed-in half-life values from days to possibly months, allowing for better tuning of dosing frequency with the therapeutic need. These features combined together result in the routine construction of “fit-for-purpose” MABs and FcFPs as we head into the future.

The key weaknesses of MABs and FcFPs include the fact that they can only engage a subset of total potential targets (i.e. extracellular and cell-surface targets), that they cannot be administered orally in pills, that there is the potential for injection site reactions and immunogenicity, and the relatively higher cost of goods, which especially for high-dose MABs

Table 19.1 SWOT analysis of therapeutic MAb and FcFPs

SWOT category	Characteristics
Strengths	• Wide range of indications • Extracellular or cell-bound targets that cannot be easily addressed with small molecules (e.g. protein–protein interactions) • Less frequent dosing due to long half-life • Absence of off-target activity and associated toxicities • Efficacy • Interaction with the immune system which allows for amplification of the functional activity and elimination of the target
Weaknesses	• Overall lack of knowledge about some MAb target biology which can lead to the low probability of success (POS) for Phase IIa proof-of-concept (POC) trials • Lack of oral delivery • Limitation to extracellular and cell-surface targets • Costs to patients and/or third-party payors • Potential for immunogenicity and injection site reactions • Requirement for cold chain
Opportunities	• Potential for improvements in delivery (e.g. transdermal, oral, intra-nasal) • Improved subcutaneous dosing self-administration devices • Modified Fc; fine-tuning interaction with immune system • Tunable pharmacokinetics, leading to predictable longer or shorter half-life molecules • Multi-specificity and multiple antibodies in a vial (e.g. ability to engage multiple targets while retaining long half-life) • MAb conjugates, including non-toxin-based conjugates • Tissue targeting, e.g. ability to move MAbs across blood–brain barrier • New markets (e.g. BRIC countries) and marketing strategies
Threats	• Small molecules functioning in same pathways or disease states as biologics • Continued safety concerns in a post-Tegenero TGN1412 era • Third-party payor restrictions on reimbursement • Follow-on-biologics as threats to development of innovator biologics • Limited number of high-quality targets leading to intense competition on a subset of popular targets (e.g. TNF-α, CD20, IGF-1R)

can drive the need for high prices. The most significant threats for the discovery and development of innovative, novel MABs and FcFPs include the limited number of new, novel targets for MABs and FcFPs, the impending increased market pressures from third-party payors, the threat of highly efficacious small molecules that could enter markets currently dominated by biologics (Ziegelbauer and Light, 2008), and the threat of follow-on (i.e. biosimilar) and bio-better MABs and FcFPs that can have a significant impact on several MABs coming off patent over the next decade, although the regulatory environment supporting this still remains unclear today (Williams and Strohl, 2009).

19.13 Epilogue

The overall field of biologics is being led by the great expansion in MABs and FcFPs. The market for MABs and FcFPs is expanding at \$7 billion per year, resulting in an expected market value by 2015 of about \$75 to \$90 billion. As of June, 2012, 40 MABs and FcFPs have been approved for marketing in either the US or EU, with an approval rate between 2000 and 2010 of approximately 2.2 new biological entities (NBEs) per year. Additionally, there are at least another 35 MABs and FcFPs currently in Phase III clinical trials, of which 75% (based on historical POS) are expected to be approved for marketing. This would yield about 65 total marketed MABs and FcFPs by the 2015 timeframe, which should fuel even further growth in market value heading toward 2020. By 2016, seven of the top ten pharmaceuticals of any kind by market value will be MABs or FcFPs. There are several challenges ahead, however, including patent expirations, the growth of the FOB market, third-party payor pressures on pricing and reimbursements, and increasing complexity and less information on potential new targets. Despite these challenges, there are many reasons to be excited about the continued expansion and growth of MABs and FcFPs, as documented throughout this book. Some of these include the development of Fc-modified “fit-for-purpose” MABs and FcFPs, clinical development with new molecular formats including bispecifics, antibody fragments, and novel constructs, and the expansion of antibody conjugates. We look forward to an exciting new decade of innovation and incorporation of technologies into NBEs.

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