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Theam Soon Lim *Editor*

Recombinant Antibodies for Infectious Diseases

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Theam Soon Lim
Editor

Recombinant Antibodies for Infectious Diseases

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Preface

The fate of antibodies and infectious diseases has been entwined since the early days when Emil von Behring and Shibasaburo Kitasato first witnessed the unique toxin neutralization ability of antibodies in sera from immunized rabbits. The hybridoma technology by Köhler and Milstein in the mid-1970s revolutionized the way antibodies can be applied in the future. Their contributions set about the first monospecific antibodies to be generated against a specific target. This brought about major revolutions in the way we carry out basic research, medical diagnostics, and therapeutics. However, the ability to produce murine monospecific antibodies was not the finish article due to major side reactions associated with the use of animal-derived antibodies in humans.

The quest for further improvements in the way antibodies were being generated meant there was a need to generate monoclonal antibodies at a more rapid pace with more human-like characteristics. The biotechnology boom at the turn of the century resulted in rapid advancements in recombinant DNA technology, molecular biology, and DNA sequencing technologies. The technological developments allowed the floodgates of genetic information to open making molecular-based technologies accessible to many laboratories around the world. The knowledge, information, and technological advancements worked symbiotically to fuel the advancements of complementary technologies. Recombinant antibody technology also benefited from this evolution with the improved understanding of genes and mechanisms associated with *in vivo* antibody production.

In the quest to make antibodies more human, researchers sought after new alternative methods to generate recombinant versions of human antibodies. This possibility was only realized with the introduction of phage display technology by George Smith, which allowed the presentation of peptides on the surface of bacteriophages. This allowed for the evolution of the technology to present antibodies on the surface, which catalyzed the growth of human monoclonal antibody technology. Since then, numerous versions of human antibody formats have been developed with astonishing success. As a result, new antibody libraries have been developed and are now an important tool for monoclonal human antibody development work. The technology has allowed for different antibodies to be developed against many different kinds of targets which was otherwise impossible with conventional approaches. The ability to generate fully human monoclonal antibodies at a rapid pace has shaped the pharmaceutical landscape in recent years. This signaled a turning

point for many medical approaches applied at that time and has helped shaped the way modern immunotherapeutics are designed.

The ongoing challenges associated with infectious diseases like antibiotic resistance and the cost for drug discovery meant that an alternative treatment was required. This book provides an in-depth introduction to bacteriophage biology as well as its application for antibody phage display. The book also includes examples of different forms of antibody libraries that are used to tackle the issue of infectious diseases. It also provides a comprehensive list of antibody phage display technologies and the application of antibodies against different infectious agents. In addition to that, the book also includes concepts of computational-based antibody design, antibody engineering strategies, and considerations in the application of antibody-based therapy for infectious diseases.

On a personal note, the treatment of infectious disease is a topic close at heart due to the constant threat it poses around Southeast Asia. This is a key focus area for me personally as part of the antibody technology initiative at the Institute for Research in Molecular Medicine (INFORMM), which is the brainchild of the Malaysian Ministry of Higher Education under the Higher Institutions' Centre of Excellence (HICoE) program together with Universiti Sains Malaysia (USM). It is our aim that this book can provide technical assistance to new start-up laboratories and researchers looking to apply antibody phage display for infectious diseases. We also hope this book will help spur interest and ideas in the field while at the same time expand research focusing on antibody-based therapy for infectious diseases.

I would like to thank the authors whose contributions to this book have allowed it to be a comprehensive guide for antibody phage display in infectious diseases. I would also like to thank Prof. Michael Hust for his guidance and advice throughout the preparation of this book. My scientific career would not have been possible without the influence of great mentors like Zoltàn Konthur and Jörn Glökler. On a personal note, I would like to thank Poi Hong, Hayley, Hayden, and my parents for their support while preparing this book and throughout my scientific career.

Penang, Malaysia Theam Soon Lim

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Abbreviations

%	Percent
ΔG_{Bind}	Binding free energy
T3SS	Type three secretion system
AAC	Antibody–antibiotic conjugate
Ab	Antibody
ACSs	Antibody-secreting cells
ActIIR	Myostatin/activin type II receptor
ADAs	Antidrug antibodies
ADC	Antibody–drug conjugate
ADCC	Antibody-dependent cell-mediated cytotoxic
ADCP	Antibody-dependent cell phagocytosis
ADCVI	Antibody-dependent cell-mediated virus inhibition
ADE	Antibody-dependent enhancement
ADIN	Antibody-dependent intracellular neutralization
AID	Activation-induced cytidine deaminase
AIDS	Acquired immunodeficiency syndrome
AIGIV	Anthrax immunoglobulin intravenous
AMA	Antibody modelling assessment
AMA1	Apical membrane antigen 1
AMBER	Assisted Model Building with Energy Refinement
AMF	*Aspergillus fumigatus* membrane fraction
ANN	Artificial neural network method
APC	Antigen-presenting cells
ART	Antiretroviral therapy
ARTs	ADP-ribosyltransferases
ASCs	Antigen-specific
ASPD	Artificially selected proteins/peptides database
B. anthracis	*Bacillus anthracis*
BCRs	B cell receptors
BDB	Biopanning data bank
BER	Base excision repair
BLAST	Basic local alignment search tool
bnAbs	Broadly neutralizing antibodies
BoNT	Botulinum neurotoxin
bp	Base pairs
BSA	Bovine serum albumin
BsAbs	Bispecific antibodies

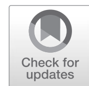
BSL4	Biosafety level 4
C. albicans	*Candida albicans*
CASP	Critical Assessment of Techniques for Protein Structure Prediction
CD4bs	CD4 binding site
CDC	Centers for Disease Control and Prevention
CDC	Complement-dependent cytotoxicity
CDCs	Cholesterol-dependent cytolysins
CDI	*C. difficile* infection
cDNA	Complementary deoxyribonucleic acid
CDR	Complementary determining region
CDT	*Clostridium difficile* transferase
CF	Compactness factor
cfu	Colony-forming unit
C_H	Constant region genes
CH1	First heavy chain constant domain
CHARMM	Chemistry at Harvard Macromolecular Mechanics
CHESS	Centocor: HA-1A Efficacy in Septic Shock
ClfA	Clumping factor A
COGs	Cost of goods
CP	Cysteine proteinases
cryo-EM	Cryo-electron microscopy
CsCl	Cesium chloride
CSF	Cerebrospinal fluid
CSR	Class switch recombination
Dabs	Domain antibodies
DALYs	Disability-adjusted life years
DBP	Duffy binding protein
DENV	Dengue virus
DNA	Deoxyribonucleotide acid
dPNAG	Deacetylated form of poly-β-1,6-N-acetylglucosamine
DSB	Double-strand break
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
E	Envelope
E. coli	*Escherichia coli*
e.g.	For example
EBC	Epstein–Barr virus
EBOV	Ebola virus
EbpC	Major pilus component of *Enterococcus faecalis*
EBV	Epstein–Barr virus
ECLIA	Electrochemiluminescent immunoassay
EF	Edema factor
EGFR	Epidermal growth factor receptor
EHEC	Enterohemorrhagic *E. coli*
ELISA	Enzyme-linked immunosorbent assay
EMV	Extracellular enveloped virions
EPPND	Enhanced pre- and postnatal development

ET	Edema toxin
Fab	Fragment antigen binding regions
FACS	Fluorescence-activated cell sorting
FBI	Federal Bureau of Investigation
Fc	Fragment crystallizable
FcRn	Fc receptor neonate
FDA	US Food and Drug Administration
fHbp	Factor H binding protein of *N. meningitidis*
FML	Fucose–mannose ligand
FR	Framework
FRs	Framework regions
Gb3	Globotriaosylceramide
GC	Germinal center
GD2	Disialoganglioside
GENESIS	Generalized-ensemble simulation system
GIPL	Glycosylinositolphospholipid
GP	Glycoprotein
GP_{CL}	Cleaved glycoprotein
GPI-APs	Glycosylphosphatidylinositol-anchored proteins
GP_{UNCL}	Uncleaved glycoprotein
GRA	Granule antigens
GROMOS	Groningen molecular simulation
GS	Glycine–serine
GVHD	Graft versus host disease
HA	Hemagglutinin
HAMA	Human anti-mouse antibody
HAP	Hospital-acquired pneumonia
HBcAg	Hepatitis B core antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HC	Heavy chain
HCAbs	Heavy-chain-only antibodies
HeV	*Hendra* virus
HIV	Human immunodeficiency virus
Hla	Alpha-hemolysin
hLD_{50}	Mean lethal dose
HSC	Human string content
HSLs	Homoserine lactones
HTS	High-throughput sequencing
HUS	Hemolytic uremic syndrome
IBS	Inflammatory bowel syndrome
IEDB	Immune epitope database
IFA	Immunofluorescence assay
Ig	Immunoglobulin
IgG	Immunoglobulin G
IHC	Immunohistochemical
IMV	Intracellular mature virions
IND	Investigational new drug

IP	Intellectual property
IPP	Ileal Peyer's patches
IRIS	Immune reconstitution inflammatory syndrome
ISAAC	Immunospot array assay on a chip
JCV	Polyomavirus JC
JEV	Japanese encephalitis virus
K_D	Dissociation constant
kDa	Kilodalton
KGB	Komitet Gosudarstvennoy Bezopasnosti
L. acidophilus	*Lactobacillus acidophilus*
LC	Light chain
LcrV	Low-calcium response V antigen
LD	Linear dichroism
LF	Lethal factor
LF	Lymphatic filariasis
LFIA	Lateral flow immunoassay
LPS	Lipopolysaccharide
LT	Lethal toxin
LVS	Live vaccine strain
mAb	Monoclonal antibody
MAC	Membrane attack complex
MD	Molecular dynamic
MDR	Multidrug-resistant
MFP	Membrane protein fraction
MHC	Major histocompatibility complex
MIC	Microneme proteins
MM-GBSA	Molecular mechanics/generalized born solvent area
MM-PBSA	Molecular mechanics Poisson–Boltzmann surface area
MMR	Mismatch repair
MoMp	Mitochondrial outer membrane permeabilization
MPER	Membrane-proximal external region
MPFIA	Magnetic particle fluorogenic immunoassay
MrkA	Type 3 fimbrial shaft subunit of *Klebsiella*
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Multiple sclerosis
MSP	Merozoite surface protein
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NA	Neuraminidase
NAbs	Neutralizing antibodies
NC	Neurocysticercosis
NHEJ	Nonhomologous end joining
nM	Nanomolar
NMR	Nuclear magnetic resonance
NPC1	Niemann–Pick C1
NS	Nonstructural
NSG	Next-generation sequencing
nt	Nucleotide
OPK	Opsonophagocytic uptake and killing

PA	Protective antigen
PACE	Phage-assisted continual evolution of protein
PBL	Peripheral blood lymphocyte
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Phage display
PDA	Protein design automation
PDB	Protein data bank
PEG	Polyethylene glycol
PfHRP2	*P. falciparum* histidine-rich protein 2
PFTs	Beta-barrel pore-forming toxins
pI	Isoelectric point
PIGS	Prediction of immunoglobulin structure
Ply	Pneumolysin
pM	Picomolar
PML	Progressive multifocal leukoencephalopathy
PNAG	Poly-β-1,6-N-acetylglucosamine
PNH	Paroxysmal nocturnal hemoglobinuria
PS	Packaging signal
Psa/Ph6	Pathogenesis
Psp	Phage shock protein
PTM	Posttranslational modification
PTx	Pertussis toxin
PVL	Panton–Valentine leukocidin
QS	Quorum sensing
RA	Rheumatoid arthritis
RAG	Recombination-activating genes
RF	Random forest
RF	Replicative form
RMSD	Root-mean-square deviation
RNA	Ribonucleic acid
ROA	Route of administration
ROP	Rhoptry proteins
RSS	Recombination signal sequences
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription polymerase chain reaction
S. aureus	*Staphylococcus aureus*
SAbDab	Structural antibody database
SAR	Structure–activity relationship
SARS-CoV	Severe acute respiratory syndrome coronavirus
scFab	Single-chain Fab
scFv	Single-chain variable fragment
sdAb	Single-domain antibody
SDRs	Specificity-determining residues
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEB	Staphylococcal enterotoxin B
SHM	Somatic hypermutation

sIBM	Inclusion body myositis
SLE	Systemic lupus erythematosus
SlpA	Surface layer A
SoC	Standard-of-care
SPA	Sequence prediction algorithm
SpA	Staphylococcal surface protein a
SPR	Surface plasmon resonance
spvB	*Salmonella* virulence plasmid factor B
ssDNA	Single-stranded DNA
STEC	Shiga toxin-producing *E. coli*
Stxs	Shiga toxins
SUDV	Sudan virus
SurA	Survival protein A
TB	Tuberculosis
Tc	Transchromosomic
TcdA	Enterotoxin A
TcdB	Cytotoxin B
TCR	Tissue cross-reactivity
TCR	T-cell receptor
TeNT	Tetanus neurotoxin
TG	Thyroglobulin
TLS	Tumor lysis syndrome
TNF	Tumor necrosis factor
TRIM	Trinucleotide-directed mutagenesis
TRIM21	Tripartite motif-containing 21
TSST	Toxic shock syndrome toxin
USAMRIID	US Army Medical Research Institute of Infectious Disease
USFDA	US Food and Drug Administration
VAP	Ventilator-associated pneumonia
VEEV	Venezuelan equine encephalitis virus
VEGF	Vascular endothelial growth factor
V_H	Variable heavy chains
VH	Heavy chain variable domain
VHF	Viral hemorrhagic fevers
VIG	Vaccinia immune globulin
V_L	Variable light chains
VL	Visceral leishmaniasis
VLA1	Human integrin domain I
VP1	Viral protein 1
VSG	Variant-specific surface glycoprotein
Vκ	Kappa variable domain
WEEV	Western equine encephalitis virus
WHO	World Health Organization
WNV	West Nile virus
WNV	West Nile
YFV	Yellow fever virus
ZIKV	Zika virus



1 Filamentous Phage: Structure and Biology

Jasna Rakonjac, Marjorie Russel, Sofia Khanum, Sam J. Brooke, and Marina Rajič

Abstract

Ff filamentous phage (fd, M13 and f1) of *Escherichia coli* have been the workhorse of phage display technology for the past 30 years. Dominance of Ff over other bacteriophage in display technology stems from the titres that are about 100-fold higher than any other known phage, efficacious transformation ensuring large library size and superior stability of the virion at high temperatures, detergents and pH extremes, allowing broad range of biopanning conditions in screening phage display libraries. Due to the excellent understanding of infection and assembly requirements, Ff phage have also been at the core of phage-assisted continual protein evolution strategies (PACE). This chapter will give an overview of the Ff filamentous phage structure and biology, emphasizing those properties of the Ff phage life cycle and virion that are pertinent to phage display applications.

Keywords

Bacteriophage · Filamentous phage · Phage display · Ff structure · Infection mechanism · Ff life cycle

1.1 Introduction

The appearance and mode of assembly of filamentous phage are oddities in the world of bacteriophage, as they do not fit the archetypal head and tail image, and they do not lyse (kill) the host in order to reproduce. Most of what is known about filamentous phage assembly comes from work on *E. coli* bacteriophage Ff (f1, M13 and fd) which are 98% identical at the DNA sequence level and have been studied interchangeably [97, 98, 106, 121, 134]. Like fila-

This article is dedicated to the memory of Peter Model, a pioneer of filamentous bacteriophage research and a greatly admired mentor to students and junior faculty at the Rockefeller University.

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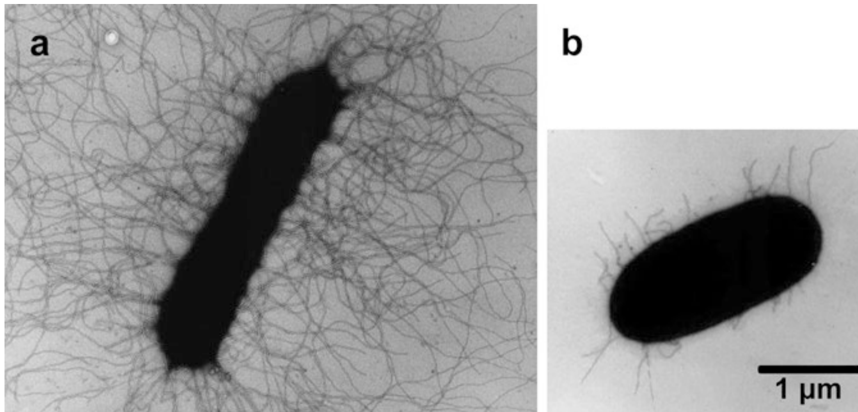

Fig. 1.1 *E. coli* cells assembling the Ff phage. (**a**) Cell infected with a ΔgIII Ff phage. (**b**) Cell infected with a wild-type Ff phage (Reproduced from Ref. [120])

mentous bacterial surface appendages, pili, Ff are assembled by a secretion-like process, aided by ATP and proton motive force [48, 130]. Simultaneous assembly and secretion are executed by a dedicated membrane-embedded assembly machinery, composed of an outer membrane channel and an inner-membrane-embedded, phage-encoded ATPase [48]. Deletion of the phage gene encoding the Ff phage release protein (pIII) results in permanent assembly of multi-length filaments, morphologically converting the Ff into DNA-containing pili (Fig. 1.1; [123]). The factory-like production of Ff phage (1000 phage per cell per hour at the peak of assembly) necessitates production and membrane targeting of millions of major coat protein copies and 300–400 assembly complexes traversing the envelope. Both of these seemingly unsustainable tasks are achieved and endured by *E. coli*, which remains viable. Ff assembly and secretion do have a consequence on cell physiology, causing a doubling of generation time and inducing the phage shock protein (Psp) stress response [14, 82]. A consequence of the extended generation time is a thinner bacterial lawn in comparison to uninfected cells, allowing formation of turbid "plaques". Infected cells form colonies that are small and transparent in comparison to the uninfected cells. Protein and gene nomenclature used for the Ff phage is numerical, expressed in Arabic or Roman numerals. This review will use the Roman numerals.

The virion of filamentous phage is composed of a circular single-stranded DNA (ssDNA) genome in the form of a two-stranded helix, surrounded by a tube formed by thousands of major coat protein (pVIII) subunits (Fig. 1.2; [98]). The tube is composed of the helically arrayed 50-residue α-helical major coat protein pVIII, and is capped at both ends by two different pairs of proteins (pVII/pIX and pIII/pVI).

Ff bacteriophage infect *Escherichia coli* by binding to the tip of the F-pilus, which then retracts [10, 83]. Like Ff, other filamentous bacteriophage appear to bind the retractable pili [8, 69, 103]. The secondary receptor in Ff and several distantly related phage is the TolQRA complex [28, 63]; hence this complex is likely a universal filamentous phage secondary receptor. Gram-negative bacteria are predominant hosts of filamentous phage. Among nearly 100 different filamentous phage that have been identified to date, only two were found to infect Gram-positive hosts [35].

Ff phage replicate as plasmid-like extra-chromosomal replicons (episomes), by a rolling circle mechanism [106]. Whereas many other filamentous phage replicate exclusively as episomes, there is also a large number that integrate into the host chromosome [89]. Regulation of replication, integration and excision in these "temperate" filamentous phage follows a few different strategies, but the common characteristic is that the host survives both the integrated

(inactive) and induced states [78, 103, 125]. Many temperate filamentous phage replicate at extremely low rates, even in the induced state, producing less than one phage per cell per generation. These phage cannot make plaques and are being discovered through bacterial genome and microbial communities' metagenome sequencing. This review will focus on the Ff phage; for a detailed recent review on other filamentous phage please refer to [89].

Ff filamentous phage of *E. coli* have been used extensively in phage display technology and, recently, nanotechnology, due to their resistance to pH extremes, detergents and high temperature [12, 13]. Phage display is a powerful combinatorial technology; it allows identification, amongst billions of peptides/antibodies/proteins, of rare variants that bind to a ligand (or bait) of interest [11, 102, 141]. Ff is the phage of choice for antibody display due to the fact that displayed proteins are folded in the oxidative environment of the *E. coli* periplasm, allowing formation of S-S bridges in the immunoglobulin fold of antibodies [49]. Ff are also suitable for display of bacterial and archaeal membrane, cell-surface and secreted proteins, at genomic and metagenomics scale [24, 52, 108, 109]. Tailed phage, T4, T7 and λ, have also been used in phage display [4, 53, 160]. These phage are more suitable for display of cytosolic and nuclear proteins, given that their virion proteins fold in the reducing environment of the *E. coli* cytoplasm.

Ff phage display technology branched into nanotechnology at the start of the twenty-first century, through selection and display of nanocrystal-nucleating peptides for a number of inorganic materials [16, 66]. The liquid crystalline character of filamentous bacteriophage further expands nanotechnology applications of these viruses [23, 91, 115].

In basic research, Ff-phage-encoded coat and assembly/secretion proteins have served as models for studies of membrane targeting and secretion of proteins in Gram-negative bacteria [92, 136], whereas the phage of pathogenic bacteria have been investigated from the standpoint of bacterial virulence, physiology and effect on biofilm properties [89]. Ff have been used most recently in the phage-assisted continual evolution of proteins (PACE), resulting in e.g. novel polymerases, proteases or transcription factors with novel specificities [15, 47, 116].

1.2 Ff Structure

The genome of filamentous bacteriophage is circular single stranded DNA (ssDNA) which forms an anti-parallel two-stranded helix (similar to A-DNA or B-DNA). Due to the circular nature of the DNA, the helix ends with two loops [36]. In Ff phage one of the two loops is a 32-nucleotide hairpin called the packaging signal (PS), which targets the genome for packaging and initiates filamentous phage assembly [133]. Phosphates along the DNA helix interact with positively charged residues of the major coat protein, which forms a helically symmetrical tube that gives the filamentous appearance to the virion [57, 98, 99]. Due to the absence of complementarity, in the sequences beyond the packaging signal, the Watson-Crick type of pairing is only maintained for about 25% nucleotide pairs. Some filamentous bacteriophage (e.g. Pf1 of *Pseudomonas aeruginosa*) form a helix in which phosphates are in the centre, with the bases on the outside, interacting with the capsid [84].

The virions of filamentous phage are long, flexible filaments 6–7 nm in diameter and of varied length, which depends on the length of the packaged DNA (Fig. 1.2b). If initiation or termination of filament assembly is impaired, multiple genomes can be assembled into long filaments that can extend to up to 20 μm [122]. An additional factor determining length is the rise per nucleotide, or h, that ranges between 0.28 (in Ff phage) and 0.61 nm (in Pf1, in which the DNA helix is in the extended P-DNA-like form) [35]. The virion tube is composed of a helical array of the α-helical major coat protein pVIII (Fig. 1.2). There are two types of virions based on the symmetry of pVIII arrays: class I (5-start helix, C_2S_5) or class II (1-start helix, $C_1S_{5.4}$) [36]. Structures of the major coat protein have been determined for both classes of virions (i.e. in Ff and Pf1 phage) [57, 99]. However, very little is known about the structure of the virion "caps" at the two ends of the filament.

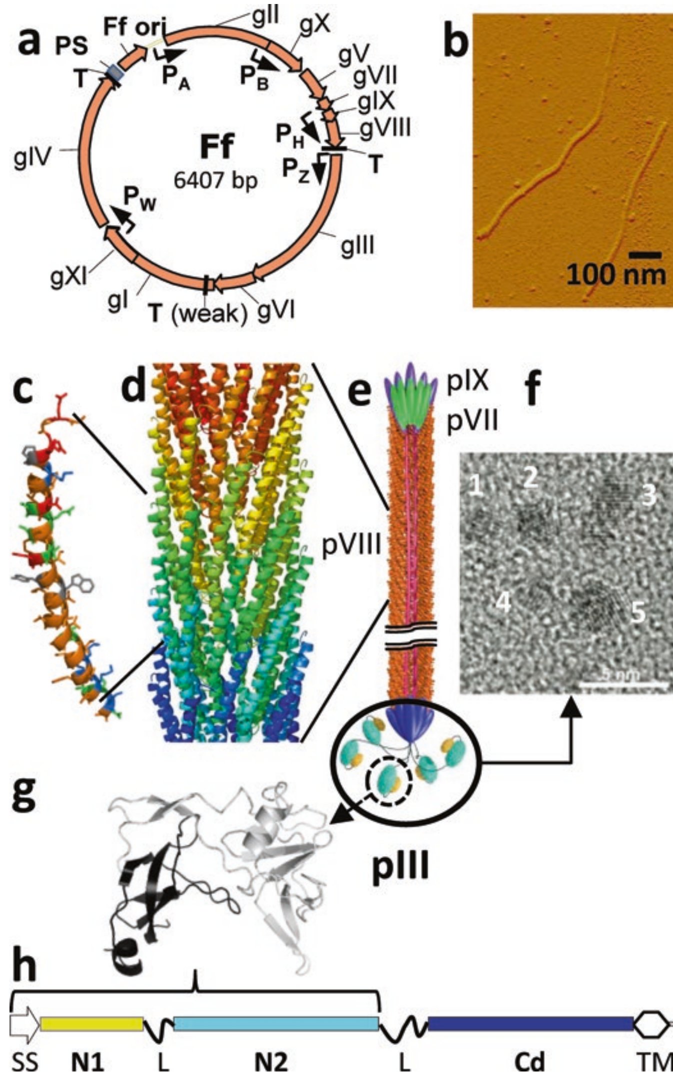

Fig. 1.2 The Ff genome and virion. (**a**) The Ff genome; *gI* to *gXI*, genes (orange block arrows); *PS*, packaging signal; *Ff ori*; origin of replication; $P_{A,}$ $P_{B,}$ $P_{H,}$ $P_{w,}$ P_z; promoters; *T*, terminators. (**b**) Atomic force microscope image of two Ff virions, one (longer) that has encapsidated the helper phage R408 genome, and the smaller that contains a phagemid vector whose genome is smaller (Rakonjac, Russel and Model, unpublished). (**c**) Structure of a pVIII (major coat protein) monomer. (**d**) pVIII arrangement within the filamentous phage capsid. (**e**) Schematic representation of the virion. (**f**) High-resolution TEM lattice fringe images of five ZnS nanocrystals (labelled *1–5*) at the pIII-end of a single Ff virion (From Ref. [81], reprinted with permission from AAAS). (**g**) Ribbon representation of the N1 domain (*dark gray*) and the N2 domain (*light gray*) of pIII. (**h**) Domain organization of pIII preprotein. *SS*, signal sequence, *N1*, *N2*, *Cd*, domains of pIII; G, glycine-rich linkers; TM, transmembrane helix. The images of the pVIII subunit (**c**), the capsid (**d**), and the N1-N2 domains of pIII (**g**) were derived from coordinates of the RCSB PDB database accession numbers 2cOw [99] and 1g3p [87], respectively, using PyMOL [37]

The Ff virions (including the receptor-binding protein pIII) remain intact and infectious after exposure to a broad range of pH and to high temperature [12, 13]. This is of great importance to affinity-screening (biopanning) of phage display libraries which regularly include e.g. pH extremes used for elution of the binders from the cognate 'bait'. Ff are also resistant to ionic detergents below the critical micellar concentration [149]. Ff phage are sensitive to chloroform, to which the tailed phage are generally resistant. However, a peptide displayed on all copies of the major coat protein was identified that rendered the virion resistant to this organic solvent [118]. Filamentous bacteriophage virions contain no lipids [35], a surprising fact, given that all virion proteins are integral inner membrane proteins prior to assembly [44]. At high concentration, filamentous bacteriophage behave as a liquid crystal and can be aligned in a strong magnetic field. These properties of Pf1 phage have been utilized in structural studies of other proteins by nuclear magnetic resonance (NMR) by promoting alignment of the investigated proteins in the magnetic field [163].

The liquid-crystalline properties of filamentous phage and ability to align them by shear flow [25] have seen them become a basis for diagnostics devices based on linear dichroism (LD), where a signal from aligned filaments, displaying antibodies against e.g. surface antigen of a pathogenic bacterium, changes upon recognition of the cognate bacterial cell [115]. A similar principle was used in design of "phage litmus", where the spectral changes are used to monitor detection of an analyte [111]. Chemical and enzymatic modifications have been applied to Ff phage to further expand the range of their applications as bionanoparticles [please refer to the recent reviews [7, 22, 65]]. The liquid crystalline Ff virions form membrane-like sheets when exposed to high-osmolarity solutions (dextran, PEG) that assume several macroscopic topologies, including disc, sphere, ribbon or more complex star-like disc-ribbon intermediate structure [40, 142].

1.2.1 Minor Virion Proteins

An atomic-resolution structure of the Ff virion caps has not been determined, in contrast to the detailed knowledge of pVIII structure and packing along the filament. Minor proteins pVII and pIX are small, containing only 32 (pVII) and 33 (pIX) amino acids; both proteins are hydrophobic. Like all other virion proteins, they are integrated into the inner membrane prior to assembly; however they have no signal sequence and are thought to spontaneously insert into the membrane [44]. These two proteins are incorporated into the virion at the initiation step of assembly (Figs. 1.2 and 1.5); [44, 59]. Genetic analysis suggests that the packaging signal interacts with the C termini of these two proteins to initiate assembly [133]. Both pVII and pIX have been used as display platforms for scFv's, however in those cases, SecA-dependent signal sequences must precede the scFv in order to target the fusions to the inner membrane and into the virion [54, 55, 71].

Proteins pIII and pVI terminate filament assembly and release the virion from the cell [122, 123]. The largest protein in the virion, pIII, also mediates receptor binding and entry of the phage into the host cell (Figs. 1.2e–h and 1.3). Both pIII and pVI are integral membrane proteins prior to assembly into the filamentous phage virion [9, 44].

The pVI is a 112-residue that is mostly hydrophobic. It is predicted by the TMHMM2.0 [77] to contain three transmembrane α helices, with the N terminus in the periplasm and the C terminus in the cytoplasm. This protein is not exposed on the virion surface [44] and is assumed to serve as a "base" or "adaptor" for attachment of pIII in forming the virion cap. The C-, but not the N-terminus of pVI can be used, however, as a point of fusion for display of peptides [72]. The C-terminus therefore appears to be near the surface of the virion.

The length of the pIII pre-protein (including signal sequence) and mature protein is 424 and

406 amino acids, respectively. It is composed of three domains (N1, N2 and C) separated by long glycine-rich linkers (Fig. 1.2h). Prior to assembly into the virion, pIII is targeted to the inner membrane by its N-terminal signal sequence and anchored in the phospholipid bilayer by a hydrophobic transmembrane α helix near the C-terminus, in a SecYEG and SecA-dependent manner [9]. Given the position of the membrane anchor, most of pIII is localized in the periplasm prior to assembly into the virion; only five C-terminal residues are located in the cytoplasm [33, 34].

The pIII copy number per virion was indicated as a by-product of a nanotechnology application, in which ZnS nanocrystals were nucleated by N-terminally displayed peptides [81]. The TEM of the nanocrystals associated with the virion tip shows five ZnS nanocrystals, corresponding to five copies of pIII (Fig. 1.2f). Since pVI and pIII are equimolar in the virion cap [59], if there are five copies of pIII, there are also five copies of pVI subunits per virion. Thus the distal cap appears to maintain the fivefold axial symmetry of the major coat protein in the filament shaft.

The N1 and N2 domains of pIII interact with the host receptors; the structure of these two domains has been determined using X-ray crystallography and NMR (Fig. 1.1g; [70, 87]). The three-dimensional structure of the C-domain, which is required for assembly of the virion end-cap, termination of phage assembly, formation of a detergent-resistant virion cap and for late steps in phage infection [6, 122], is yet to be determined.

Display on full-length pIII results in up to five fusion copies per virion and is used preferentially for screening of the naïve antibody libraries, where low-affinity binders are expected [60]. However, display at the N-terminus of the mature full-length pIII often leads to proteolysis of the displayed moiety, reducing the copy number of displayed protein per virion, and increasing infectivity of the particles, providing advantage to these variants in amplification steps during library screening, without binding to the bait [101]. It was recently suggested that this problem might be overcome by interdomain display, where the coding sequence for the displayed peptide (protein) is inserted between the pIII N1 and N2 or N2 and C domains [150]. This strategy eliminates clones containing inserts that are susceptible to proteolysis, by rendering the virion non-infectious due to the loss of the N1, or both N1 and N2 domains, depending on the site of the insert.

Not only full-length, but also the truncated pIII, containing solely the C-terminal domain, are used as a platform for display of antibody variable domains. The fusions in this case are inserted between a signal sequence and the pIII C domain, and have to be co-expressed with wild-type pIII in the same cell and co-assembled into the virion [3] in order to allow amplification of the virions i*n E. coli*. The insert in these vectors does not affect infectivity of the virion; however the C-terminal fusions are displayed only in one or two copies per virion, resulting in low avidity. This mode of display is used for screening the libraries derived from immunized individuals, which results in selection of high-affinity recombinant antibodies [2].

An antibody specific for the C-terminal 10 residues of pIII cannot bind to pIII when it is in the virion (Rakonjac, unpublished). Therefore, the C-terminus must be buried within the virion cap. Nevertheless, it was reported that, in conjunction with a long flexible linker, it was possible to display proteins at the C-terminus of pIII, albeit in combination with the wild-type pIII copies [50].

1.3 Ff Gene Organisation, Transcription and Translation

The genome of Ff phage is 6407 nt in length (Fig. 1.2a). The genes required for infection, replication and virion assembly are clustered along the genome in three groups: (i) replication (gII/gX and gV); (ii) virion structure (gVII, gIX, gVIII, gIII and gVI); (iii) assembly/secretion (gI/gXI and gIV), and are organised in two operons [106]. gVII, gIX and gVIII (encoding two minor proteins that initiate assembly and the major coat

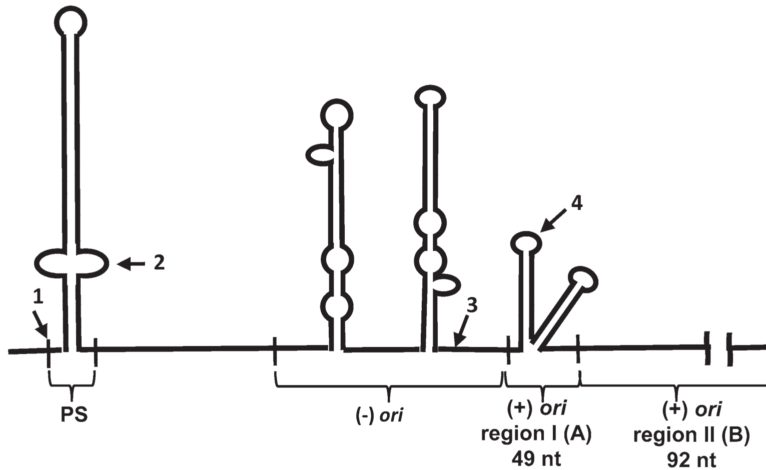

Fig. 1.3 Ff intergenic sequence (IG) containing origin of replication. Packaging signal (*PS*); (−) *ori*, negative strand origin of replication; *(+) ori region I (A)*, the region I of the positive strand origin of replication; *(+) ori region II (B)*, the region II (B) of the positive strand origin of replication; *(1)* stop codon of gIV; *(2)* terminator of gIV mRNA; *(3)* initiation site of (−) strand primer synthesis; *(4)* initiation site of (+) strand synthesis

protein, respectively) are transcribed within an operon that also contains genes gII/gX and gV (encoding replication proteins), whereas gIII and gVI (genes encoding two minor virion proteins that terminate assembly), are in the same operon with downstream gI/gXI and gIV (encoding the secretion/assembly machinery). Two genes, gX and gXI, encode truncated translational products of gII and gI, respectively, and these shorter proteins are required, respectively, for regulation of phage replication and for assembly [62, 106]. Upstream of the replication module, there is an intergenic region (IG; Fig. 1.3) that contains origins for positive (+) and negative (−) strand replication and a packaging signal [162].

The large body of published work on Ff transcription, translation and replication has been used recently for developing a mathematical model of the Ff life cycle and performing simulations of single- and multi-generation infection cycles [143, 144]. A number of early publications on Ff phage were devoted to transcription, resulting in a good qualitative and quantitative picture of transcription and translation (Reviewed by [106]) Both operons, gII-gV-gVII-gIX-gVIII and gIII-gVI-gI-gIV end with strong terminators. Multiple nested promoters, however, result in a number of overlapping transcripts [43, 107]. These multiple transcripts undergo differential turnover and are translated at different rates [58, 79, 104, 106]. In the absence of transcriptional regulators controlling Ff gene expression, these modes of regulation are sufficient to ensure that the requirements for extremely high levels of phage assembly are met. In particular, high level production (10^5–10^6 per cell per generation) is ensured for the ssDNA-binding protein pV and major coat protein pVIII [82, 143, 144].

In contrast to Ff phage that contain no specific regulators of transcription, in lysogenic filamentous phage, dedicated phage- and host-encoded transcriptional regulators determine the level of phage proteins, which is generally much lower than those of the Ff phage even in the induced state (reviewed in [89, 121]).

1.4 Ff Infection

The primary receptor for the Ff filamentous phage is the tip of the conjugative pilus (F); these phage were isolated originally as “male”-specific bacteriophage [68, 85, 96]. The secondary receptor is the TolQRA complex of inner membrane proteins, highly conserved in Gram-negative bacteria [27]. This secondary receptor appears to

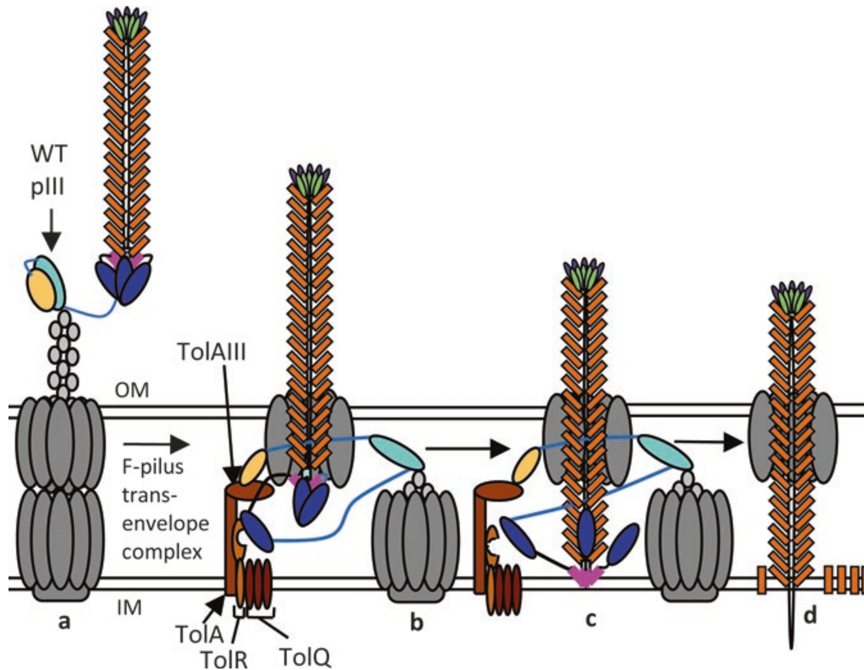

Fig. 1.4 Model of the Ff phage infection. (**a**) Binding of N2 domain (*aquamarine oval*) to the tip of the F-pilus (light-gray circles) and pilus retraction. (**b**) Binding of N1 domain (*yellow oval*) to TolAIII domain (*brown oval*). (**c**) "Opening" of the pIII C-domain and insertion of the C-terminal hydrophobic helix into the inner membrane. (**d**) Entry of phage DNA into the cytoplasm and integration of the major coat protein pVIII into the inner membrane. Steps (*a*) and (*d*) are based on published findings, whereas steps (*b*) and (*c*) are speculative. Symbols: *OM*, outer membrane; *IM*, inner membrane. pIII N1 domain, *yellow oval*; pIII N2 domain, *aquamarine oval*; pIII C-domain, *blue oval*; pIII C-terminal hydrophobic helix (membrane anchor), *pink rectangle*; pIII glycine linkers, *blue lines*; major coat protein pVIII, *orange rectangles*; pVII, *green ovals*, pIX, *purple ovals*; TolA and TolRQ, *brown shapes*; F-pilus and the trans-envelope pilus assembly/retraction system, *gray ovals*. The phage contains five copies of pIII, but for simplicity only one full-length pIII is shown. However, this is consistent with experimental data: N1N2 and C domain operate "*in cis*" and fewer than five functional copies are sufficient for infection [6]

be universal for filamentous phage [63]. TolQRA belongs to a larger trans-envelope Tol-Pal complex involved in cell division and maintenance of cell envelope integrity [17, 56].

The N2 domain of pIII binds to the primary receptor, the tip of the F (or conjugative) pilus, whereas the N1 domain binds the periplasmic domain III of TolA (Fig. 1.4; [38, 126]). Binding to the F pilus induces a conformational change in pIII by cis-trans isomerization of Pro_{213} within the N2 domain [42]. This releases the N1 domain from the N2 domain, exposing the TolA binding site on the N1 domain [42, 88, 126]. If the F pilus itself, or the F-pilus-binding domain of pIII are absent, the infection efficiency decreases by about 1000-fold, but it is not completely abolished. In contrast, TolQRA and the cognate pIII domain are absolutely required for infection [27, 28, 126, 135].

A common characteristic of pili that serve as primary receptors for filamentous phage is the ability to retract towards the cell surface, bringing the filamentous phage close to the secondary receptor located in the periplasm [80, 90]. It had been thought that the retraction of the F pilus was induced by the binding of Ff phage, however recent evidence shows that the F pilus undergoes spontaneous oscillatory extension and retraction cycles [26]. The events that follow pilus retraction and allow pIII to gain access to TolA, which is on the other side of the outer membrane, are unknown, simply because the mechanics of the F pilus conjugative machine is poorly understood. Pilus retraction followed by a set of unknown

events, somehow ushers pIII (and presumably the virion cap) across/through the outer membrane and into the periplasm, where the N1 domain can interact with the periplasmic domain of TolA [88, 126]. The post-receptor binding steps of infection are also unknown. All three proteins of the TolQRA complex, and a functional pIII C-domain covalently linked to the N1N2 domains, are absolutely required for phage infection, which ultimately results in entry of the phage ssDNA into the cytoplasm and integration of the major coat protein into the inner membrane [6, 27, 145, 152]. The C domain of pIII is predicted to be α-helical; three predicted C-terminal helices (two amphipathic and the third, hydrophobic anchor) are required for phage entry [5]. The organization of α helices in the C domain resembles α-helical pore-forming toxins; this is supported by threading using the algorithm I-TASSER [161], which selected the membrane insertion domain (domain T), of diphtheria toxin as the top-ranked template for modeling of the C domain [21].

Interestingly, expression of the pIII N2 domain alone in F+ *E. coli* abolishes conjugation as well as infection with another F-pilus-specific phage (small ssRNA phage f2 or MS2) that binds along the sides of the pilus. These two phenotypes are suggestive of a pIII-mediated pilus assembly inhibition [10]. Given that the F-pilus is up to 4 μm in length, inhibition of its assembly by the N2 domain is strategically very important to securely dock the virion to the host cell envelope and make the infection irreversible.

An approach using chimeric pIII molecules showed that the primary receptor-binding domain (N2) of pIII from phage IF1 and CTXφ allow Ff to infect hosts of the matching specificity with high efficiency: I-pilus containing *E. coli* and TCP pilus-containing *V. cholerae*, respectively [64, 86]. The efficiency of infection, mediated by chimeric pIII proteins, however, decreases by several orders of magnitude if the distance between the three domains (N1, N2 and C) is changed by inserting additional domains [64, 100]. Despite very low N1 domain conservation, these phage use the conserved TolA protein as a secondary receptor, and this appears to be sufficient to trigger $pIII_{Ff}$ C domain-mediated entry.

1.5 Ff Replication

Ff filamentous phage genomes replicate as episomes, by a rolling circle mechanism, one strand at a time. This is also true for other non-integrative filamentous phage and for the "induced" replication of lysogenic filamentous phage (Fig. 1.5). The lysogenisation upon ssDNA entry does not apply to the Ff phage; it is discussed in recent reviews [32, 89, 121].

Ff phage of *E. coli* are the source of detailed knowledge about the rolling-circle replication mechanism [106]. The origin of replication is located in the intergenic region of about 500 nt (Figs. 1.2a and 1.3). Upon entry of the positive (+) strand ssDNA into the host, the (−) strand is synthesized, creating a dsDNA genome (Fig. 1.5). Synthesis of the (−) strand is primed by a short RNA primer synthesised by *E. coli* RNA polymerase, which binds to the (−) origin of replication, a palindromic sequence that forms a hairpin whose stem is a mimic of a typical −35 and −10 promoter sequence [67]. The (−) strand is then extended by DNA Polymerase III, creating a double-stranded circular DNA, called replicative form or RF. Supercoiled RF and replication protein pII (a strand transferase) are required to initiate replication of the (+) strand from the (+) origin of replication. The replication protein pII forms a nick in the (+) strand origin of replication (the position called initiator or I). The resulting 3′ end serves as a primer for (+) strand synthesis by DNA polymerase III. Replication terminates when pII encounters the terminator sequence of the (+) origin of replication, with a strand-transferase reaction, joining the 5′ and 3′ ends of the newly synthesised (+) strand to regenerate the intact double-stranded template (or the RF form), and releasing the "old" (+) strand (Fig. 1.5) [106]. The (+) origin of replication is composed of A and B segments, of which A is absolutely essential for replication. Disruption or deletion of the B domain decreases phage production to 1%; however compensatory mutations that result in an increase of replication protein pII amount in the cell can restore a wild-type-like level of phage production (see below).

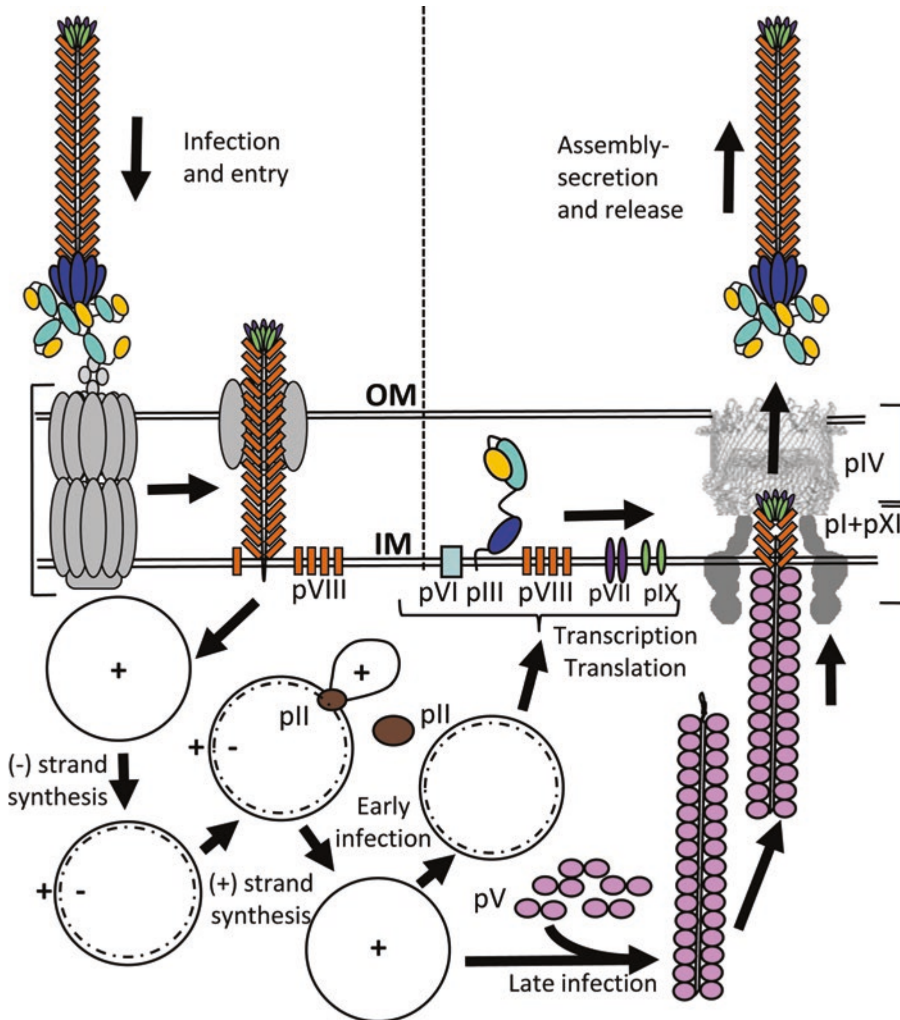

Fig. 1.5 The Ff phage life cycle. Upon infection, the ssDNA (the + strand) enters into the cytoplasm, while the pVIII major coat protein integrates into the inner membrane. Synthesis of the negative (−) strand is initiated at the negative strand origin of replication by RNA polymerase, which generates an RNA primer and is then released from the template [159]. Host DNA polymerase III uses this primer to replicate the complete negative strand. Positive strand synthesis is initiated by pII (*brown oval*), which creates a nick in the + strand of the dsDNA replicative for at the positive origin of replication. Supercoiling and formation of a stem-loop structure of the positive (+) origin of replication is required for this step (not shown in the figure). Rolling circle replication then ensues, one strand at a time. During the initial period of viral infection, new positive strands are used as templates for synthesis of negative strands, resulting in an increase in copy number of the dsDNA replicative form (RF). The RF serves as a template for production of phage proteins. Phage proteins II, V and X remain in cytoplasm and mediate genome replication and formation of the packaging substrate. Proteins pI, pIV and pXI form a transport complex spanning the inner and outer membrane (*gray shapes*). Virion proteins pVII, pIX, pVIII, pVI, pIII are inserted into the membrane prior to their assembly into phage particles. Later in the infection, positive strands are coated by dimers of the phage-encoded single-stranded DNA binding protein pV to form the packaging substrate and brought to the cell membrane assembly/export complex (pI/pXI and pIV) for assembly and export. The pIV channel is modelled based on the structure of a homologue, GspD (RCSB PDB database accession number 5wq8.1 [157] using the SWISSMODEL software [1], and presented using the Vector NTI 3D viewer. The structure of the inner membrane complex (*gray silhouette*) has not been determined; it is drawn based on the cryo-EM structure of the type III secretion system inner membrane component [94]. Symbols: *OM*, outer membrane; *IM*, inner membrane. pIII N1 domain, *yellow oval*; pIII N2 domain, *aquamarine oval*; pIII C-domain, *blue oval*; pIII glycine linkers, *black lines*; major coat protein pVIII, *orange rectangle*; pV, *pink circle*, pII, *brown oval*, pVI, *sky-blue rectangle*, pVII, *green oval*, pIX, *purple oval*

Given that pII recognises an upcoming (+) origin as a terminator, duplication of Ff (+) origins results in production of short ssDNA circles which are packaged into short virions called "defective interfering particles" [45, 124]. These short replicons (that do not encode any proteins) severely reduce replication of the full-length phage, decreasing production of phage-encoded proteins and overall titres of the full-length phage and defective interfering particles. Interference-resistant phage were selected, whose replication was not affected by the presence of defective interfering replicons in the same cell. These phage contained mutations in the 5′ untranslated portion of gII mRNA and in the coding sequence [46]. Interference resistance has permitted development of "helper" phage, used in conjunction with hybrid "phagemid" vectors that carry the complete Ff origin of replication and packaging signal, in addition to a plasmid origin [129, 153]. The interference-resistant helper phage are less affected by the phagemid replicon in the same cell than a wild-type Ff phage. This in turn allows effective production of phage proteins and high titres (10^{11}–10^{12}) of the phagemid-containing phage-like particles (or phagemid particles). The titre of the helper phage itself is between 1/10 and 1/100 of the phagemid particles and as a result it cannot form plaques on the lawn of *E. coli* cells that contain phagemids. Phage display phagemid vectors were instrumental in broadening the size and flexibility of the antibody libraries [2, 3, 93, 139].

Plasmid constructs that contain closely positioned tandem origins of replication, of which the second is a mutant that can only terminate the (+) strand ssDNA replication, have been shown to result, in the presence of a helper phage, in production of short phage-like particles where the genome corresponds to the ssDNA between the two origins [41]. The shortest particles produced in this manner contained a 221-nucleotide genome and were less than 50 nm in length [148]. These short particles were recently functionalised through modification of helper phage for use in lateral flow diagnostic assays and as vaccine carriers [137, 138].

The fate of released (+) strands after one round of rolling-circle replication depends on the stage in the infection cycle (Fig. 1.5). Early in the infection they serve as templates for additional (−) strand synthesis, increasing the copy number of the double-stranded replicative form (RF) to ~50 per cell. The copy number of the RF decreases again as the infected *E. coli* undergoes successive divisions, progressively decreasing the particle production per generation [82]. The RF is template for transcription of viral genes, one of which is the ssDNA-binding protein pV. Once sufficient pV accumulates in the cell, (+) strands are diverted from replication by acquiring a coating of pV. Binding of pV to DNA is initiated at the base of the packaging signal hairpin loop. Each pV dimer binds to two strands of ssDNA; zipping-up the single-stranded circular DNA into a pV-ssDNA filament [61]. The (+) strand-pV filament is a substrate for virion assembly (Fig. 1.5). pV is also a regulatory protein that inhibits translation of replication proteins pII and pX [104] and (−) synthesis [51]. Mutations of pV that prevent binding to the cognate binding site in the 5′ UTR of the gII mRNA and mutations of the pV binding site that result in increased pII production have been found to compensate for a replication defect in the phage vectors (e.g. R218, fdTet, M13KE and M13mp1; [93, 104, 110, 141, 158]), which all contain inserts in the B region of the (+) origin of replication.

1.6 Ff Assembly

Properties that set filamentous phage apart from most other bacteriophage are that the virion proteins are targeted to the inner membrane prior to being assembled into the filament-like virion by a secretion-like process (Fig. 1.5). Targeting of the major coat protein to the membrane is a very efficient process, resulting in pVIII becoming one of the dominant proteins in infected cells (together with pV and the host-encoded phage-shock protein, PspA). This is unusual, since the inner membrane protein translocation complex, SecYEG, typically limits the amount of protein that can be

integrated into the inner membrane. Perhaps the fact that pVIII is Sec-independent and uses an alternative translocon, YidC [136], helps overcome the SecYEG bottleneck to allow overproduction and membrane integration of pVIII.

Assembly of filamentous phage is a secretion-like process akin to pilus assembly or toxin secretion through dedicated trans-envelope protein "secretion systems". Assembly is initiated by interaction of the packaging signal with two minor proteins, pVII and pIX, and with the inner membrane assembly complex (via pI) [133]. Assembly then proceeds by dissociation of pV from ssDNA on the cytoplasmic side of the inner membrane, and association of pVIII at the level of inner membrane until the whole ssDNA helix is covered by pVIII.

In the course of assembly, the virion proteins translocate from the inner membrane into the growing phage filament, which is lipid-free [35]. During the switch from membrane to virion, the assembly machinery must catalyse conversion of protein-phospholipid to protein-protein interactions [117]. Adding to the complexity of filamentous phage assembly is a strict requirement for coordinated ssDNA helix translocation across the inner membrane with association of the major coat protein subunits. During this process DNA serves as an axis around which the helical array of the coat protein is assembled [130].

Once the DNA is completely covered with pVIII, addition of two minor proteins, pIII and pVI, forms the terminating cap of the virion and releases the phage from the cell [122]. If pIII or pVI are absent, the infected cell shows what appears to be hundreds of pili-like structures, containing multiple sequentially packaged Ff genomes, emanating from its surface (Fig. 1.1a).

Virion assembly is energised at the inner membrane. The pI subunit of pI/pXI complex contains an essential ATP-binding Walker motif; furthermore, in a semi-permeable assembly system it was shown that the assembly requires both ATP and proton motive force [48]. A large outer membrane channel protein, pIV, which interacts with the pI/pXI complex, completes the trans-envelope assembly machinery [128]. pIV belongs to a family of outer membrane proteins, called secretins, together with the outer membrane components of type II and type III secretion systems, as well as the type IV pilus biogenesis system [76]. Cryo-EM analyses of many secretins revealed that they are composed of up to 15 copies of radially arranged identical subunits, and that they have large diameters (6.4–8 nm) interrupted by a septum or gate [reviewed by [76]]. Recent near-atomic resolution structures of the secretins from the type II and type III secretion systems revealed a novel type of outer membrane channel structure, a double-barrel; the internal septum or gate is formed by two β-hairpins emanating from the walls of the inner barrel [156, 157]. The septum-forming portions of the hairpins correspond to the "Gate 1" and "Gate 2" regions previously identified by positive selection for "leaky" mutants in pIV (and a type II secretion system homologue PulD) [75, 147]. A detailed structure of an "open" secretin is still not available, however from the lower-resolution structures of the type III secretin containing a lodged substrate [94] it is clear that the secretin gate and inner barrel undergo major structural rearrangements in order to accommodate their large substrates.

Interestingly, although phage assembly is a more complex process than protein secretion, the filamentous phage assembly complex, containing only three proteins, is remarkably simple, in contrast to the type II and III secretion systems and type IV pilus biogenesis system that boast of 15 or more different protein components [29, 73, 95].

Assembly of the f1 filamentous phage requires the presence of thioredoxin, a bacterial protein that normally reduces disulphide bonds in proteins. The reducing activity of thioredoxin is not required, however the reduced, not oxidised, conformation functions in f1 assembly. Details of the thioredoxin role in f1 assembly are not known, however it has been hypothesised that it confers processivity to dissociation of pV from the f1 DNA as it reaches the assembly site. Reduced thioredoxin confers processivity to the g5p DNA polymerase of the T7 phage by binding to it and sequestering a cysteine residue in its thumb domain [151]. Interestingly, a missense mutation

in the cytoplasmic portion of pI (asparagine 142 to tyrosine) renders the f1 assembly independent of thioredoxin [131, 132], suggesting that pI is involved in the thioredoxin function. In contrast to f1, the pI residue 142 in M13 and fd phage is a histidine and their assembly is independent of thioredoxin [131].

1.7 Effect of Ff on *E. coli* Physiology

The most prominent characteristic of filamentous phage is the absence of host lysis. The high production of Ff phage is a result of extremely efficient use of host resources, as recently modelled [143, 144]. Most of the phage progeny are produced in the first six generations after infection; production tapers off in subsequent generations due to a low level of Ff DNA replication in the infected cells [82]. It was predicted through simulation that increasing the amount of the host DNA polymerase III or the phage replication protein pII in the cell would make it possible to increase replication and production of particles over 20 generations by a factor of 3. If experimentally proven correct, such manipulations would have the potential to increase productivity of Ff phage particles for practical applications.

In accordance with the extremely productive replication of phage DNA (1000 ssDNA per cell per generation), increased phosphorylation of several proteins including chaperone DnaK [127] has been observed.

Even more impressive, four million copies of the major coat protein pVIII [82] per infected cell are targeted to and transiently anchored in the inner membrane via YidC translocon prior to incorporation into the virions [136]. Although most of pVIII leaves the cell by being assembled into virions, it accumulates early in the infection before the assembly starts (10–12 min post-infection). This accumulation is accompanied by a stark change in the membrane lipid composition, with cardiolipin increasing and phosphoethnolamine decreasing relative to uninfected cells [155]. Furthermore, in the infection with the Ff phage mutants that do not produce pI and pIV assembly complexes, the cardiolipin increase is drastic, reaching 30% of the membrane lipids, as opposed to 5% in uninfected cells. These cells accumulate cristae-like inner membrane arrangements which increase in parallel with accumulation of pVIII [113, 140]. The membrane accumulation is pVIII-dependent (it does not occur in a gene VIII mutant Ff infection) and it appears to be the result of a major decrease in cardiolipin turnover, presumably through sequestration by millions of pVIII copies, and increased synthesis at the expense of ethanolamine synthesis [18, 19].

Even in the wild-type Ff infection the burden of up to 400 trans-envelope phage-assembly complexes (Fig. 1.1; [123]) causes envelope stress in the host, particularly in the stationary phase of growth [154]. This stress is relieved by induction of the inner membrane stress-response pathway called the phage shock protein (Psp) response [105]. The Psp response is not limited to *E. coli* infected with Ff phage, but is also induced by the type III secretion system, as determined in *Salmonella enterica* Sv. Typhimurim and *Yersinia enterocolitica* [31, 74]. The main trigger of the Psp response was, interestingly, shown to be mistargeting of the outer membrane channel, the secretin, to the inner membrane [30], rather than the massive production of the major coat protein.

Even though infection with wild-type Ff phage is not lethal, infection with mutants in which phage assembly is severely compromised result in the death of the host [119]. The exceptions are non-polar mutations of genes encoding assembly termination proteins pIII and pVI, which continue to assemble and extrude copious amounts of cell-associated long filaments [122], and mutations of the gene encoding replication protein pII that do not support phage replication [119]. In a surprising twist, however, “helper plasmids”, replicons in which the Ff origin of replication was replaced by a theta-replicating plasmid origin of replication, have been successfully constructed [20]. Survival of cells containing these plasmids, despite the presence of about 15 copies of a replicon that encodes all Ff proteins in the absence assembly, is surprising. It suggests that production of phage proteins is tolerated in

the absence of phage assembly, as long as the Ff single-stranded DNA is not being produced. Therefore, single-stranded Ff phage DNA (or a portion thereof) in the host cell in combination with one or more specific phage proteins and in the absence of assembly is required for killing the host.

Expression of pIII and pVIII fusions for phage display is an additional burden to the host cell, due to interference with phage assembly or infection; in large phage display libraries there are often "censored" variants that are lost in the course of amplification [39]. The toxic effect of fusions is enhanced in host strains that have *recA* mutation (e.g. XL1-Blue or DH5αF′), hence the preferred hosts for use in phage display are RecA-positive strains such as TG-1 and K91 [146].

Being non-lytic, yet productive, the Ff filamentous phage have evolved to successfully balance their own reproduction and host survival/dissemination, avoiding a prey-predator type of relationship with bacteria. Infection with Ff phage is permanent, with only one cell per ten generations losing all phage replicons [82]. Nevertheless, all cells in a bacterial population infected with wild-type Ff remain infected over many generations due to re-infection of cells that have lost the Ff replicon by the phage released from infected cells.

Infected cell physiology is also changed by expression of the minor virion protein pIII which triggers retraction of the F-pilus and blocks the TolQRA secondary receptor. Furthermore infected cells have a "leaky" outer membrane, resulting in increased release of periplasmic β-lactamase from the pIII-expressing cells and protection of surrounding ampicillin-sensitive cells [10]. Ff infection may be beneficial in protecting the host from superinfection by the lytic phage that use one or both of these receptors, thereby protecting the host from killing [114]. One such example is f2 (MS2, R17 or Qβ), a ssRNA phage that binds along the side of the F-pilus and has a lytic infection cycle [85, 112]. Increased β-lactamase release, on the other hand, may result in increased protection and survival of surrounding sensitive bacterial cells in the environment, if the Ff-infected cells have acquired a replicon encoding a β-lactamase-encoding sequence.

1.8 Conclusions

Accumulated knowledge about Ff transcription, translation, replication and assembly has permitted mathematical modelling and simulation of the Ff life cycle. In contrast, mechanistic details of infection and assembly remain poorly understood and are awaiting structural information on the complexes of proteins in the *E.coli* envelope that catalyse infection and assembly, as well as the structure of the virion cap, which is involved in both entry and release of the Ff phage. Furthermore, deeper understanding of physiological changes that *E. coli* undergoes upon Ff infection may be helpful in understanding how the cell copes with production of millions of integral membrane protein pVIII and several hundred assembly sites that pierce the bacterial envelope.

Ff phage, stable over a broad range of pH and temperatures, and highly productive, are the particles of choice for construction and screening of combinatorial libraries displaying peptides and proteins. They are particularly suitable for displaying antibodies, given that the displayed proteins fold in the oxidizing environment of *E. coli* periplasm, where the S-S bridges of the antibody variable domains are formed.

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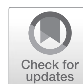

2 Antibody Phage Display

Hyunbo Shim

Abstract

Antibody phage display has become an indispensable tool for the discovery and optimization of target-specific monoclonal antibodies suitable for demanding applications including therapeutic reagents. The *in vitro* nature of the technology enables the rapid and efficient identification of specific binders, as well as greater control over selection parameters that facilitates the isolation of antibodies with unique, desirable functional characteristics. In this chapter, the technological background and the state of the art in the field of antibody phage display is discussed.

Keywords

Phage display · Antibody library · scFv · Fab · Bacteriophage · Synthetic antibody library · Natural antibody library · Immune antibody library

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2.1 Introduction

Antibodies are an integral part of the vertebrate immune system that arise as a response to antigenic challenge and protect the body by binding to and neutralizing the antigen. They exert their protective activity by binding to their target with high affinity and specificity, and these characteristics have been widely exploited by researchers and physicians for more than a century [33, 46, 62]. The advent of hybridoma technology that enabled the generation of target-specific monoclonal antibodies [42] has been pivotal to the explosive growth of the antibody research and commercialization, as this provided a powerful method to produce molecularly defined, renewable sources of antibodies against practically any type of antigen. In particular, the field of therapeutic antibody technologies has been growing at a remarkable rate since the regulatory approval of the first therapeutic antibody in 1986 [78]. Antibodies are large molecules (~150 kDa) capable of blocking protein-protein interaction, have a long serum half-life (up to 3 weeks for IgG1), are highly specific for their targets with low off-target binding, are reasonably stable and can be produced in large quantities (up to several grams per liter of culture) from mammalian cells. All these characteristics make antibodies an attractive modality for pharmaceutical development, especially in disease areas such as cancer and autoimmune diseases of which cell-cell

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interactions or ligand-receptor interactions play a crucial role in the pathogenesis.

However, the hybridoma technology is not without its shortcomings. Most critically, the method produces a murine monoclonal antibody, which elicits human anti-mouse antibody (HAMA) response when administered in human patients. Several antibody engineering techniques such as chimerization [64], humanization [1, 17, 22], and deimmunization [26] have been successfully applied to murine antibodies to produce engineered monoclonal antibodies with low immunogenicity suitable for therapeutic applications. More recently, technologies that enable the generation of fully human antibodies, such as transgenic mice [51] and phage display [14, 36], became available. These antibodies have amino acid sequences indistinguishable from antibodies of human origin, and supposedly have lower immunogenicity than antibodies of murine (or other animal) origin.

Antibody phage display is of special interest because it enables the generation of target-specific antibodies *in vitro* and can overcome some of the limitations of animal immunization-based antibody generation methods. In this chapter, the technological details of antibody phage display and antibody libraries will be discussed. Additionally, discussions on the design and characteristics of several antibody phage-display libraries are provided.

2.2 M13 Filamentous Bacteriophage

Most antibody phage display systems utilize M13 filamentous bacteriophage [89]. Like other bacteriophages, an M13 phage particle consists of phage DNA and a protein capsid. The capsid is composed of approximately 2,700 copies of pVIII major coat protein that form the cylindrical side of the phage particle, and several copies of four other minor coat proteins at either end of the phage filament (pIII and pVI at one end; pVII and pIX at the other). Inside the protein capsid is a single-stranded circular phage DNA that encodes the five capsid proteins as well as other phage proteins required for the amplification and assembly of the bacteriophage.

The infection of *E. coli* by M13 bacteriophage begins with the attachment of the phage to the bacterium *via* the interaction between the pIII minor coat protein and the F pilus of the bacteria. More specifically, the N2 domain of pIII on one end of the bacteriophage interacts with the pilus. This triggers the retraction of the pilus, which brings pIII in contact with TolA on the bacterial inner membrane. Subsequently pIII gets inserted into the inner membrane and phage DNA is injected into the cytoplasm [40]. Phage DNA is replicated inside the bacteria and the phage proteins are expressed, which either form the protein capsid of the M13 phage or play a role in the replication, assembly, or secretion of the bacteriophage. M13 bacteriophage is non-lytic; i.e. replicated phage particles escape the host bacterium without lysing it, although infected bacteria show slower growth rate than uninfected ones. Consequently, M13-based phage display libraries can be stored as frozen *E. coli* stocks before, during, or after biopanning, which provides easier handling and storage options than other systems that are based on lytic phages [23, 24].

Another advantage of M13 bacteriophage as a platform for antibody library display is that it is highly stable and can withstand high temperature, prolonged storage, desiccation, acidic conditions, or disinfectant treatment [15]. Phage-displayed antibody libraries can be stored frozen for more than several months, or kept refrigerated if the phages were extensively purified by CsCl gradient or other methods [58, 93] and free of contaminating proteases which could cleave the displayed antibody from the phage. The extraordinary stability of M13 bacteriophage allows the employment of a wide range of experimental parameters during library selection, including temperature, pH, and solvent composition.

2.3 Display of Antibody Fragments on M13 Bacteriophage

Full-length antibodies in the immunoglobulin format are large, complex, multi-subunit proteins with many disulfide bonds, not suitable for prokaryotic expression or phage display. Consequently, smaller antigen-binding fragments such as scFv (single chain fragment variable) or Fab (fragment antigen-binding) are the preferred formats for phage display. The Fab molecule is a disulfide-linked heterodimer of an Fd fragment, an approximately 25 kDa subunit consisting of a heavy chain variable domain (VH) and the first heavy chain constant domain (CH1), and a light chain (also ~25 kDa) consisting of a light chain variable domain (VL) and the light chain constant domain (CL) (Fig. 2.1a). The two subunits (Fd and LC) are held together by a disulfide bond at their C-termini. Fab molecules in most cases retain their binding activity upon conversion to full-length immunoglobulin (usually IgG) and vice versa, making this a preferred format when constructing immune antibody libraries (see below). However, the scFv molecule is a small (~25 kDa) protein consisting of a single VH and a VL domain linked together by a short, flexible peptide linker of ~15 amino acids (Fig. 2.1b). It can be made either in VH-linker-VL or VL-linker-VH orientation, and while both formats have successfully been employed in antibody library construction, the former appears to be more frequently utilized [5, 34, 43, 48, 86]. The scFv format can influence antigen binding in some cases; the binding activity can occasionally be lost upon conversion of scFv to IgG or *vice versa*, and the changes in VH/VL orientation and/or linker length may also affect the antigen binding activity [27]. This is in contrast to the Fab format which does not have a linker nor the orientation issue and generally does not suffer the loss of binding activity upon reformatting [18]. The scFv format is also prone to dimerization or oligomerization [90] which may complicate the binding kinetics. On the other hand, scFv generally is expressed in higher levels than Fab in *E. coli*, and is small enough to be sequenced in full by a single sequencing reaction (~750 base pairs), which make the early screening process more efficient.

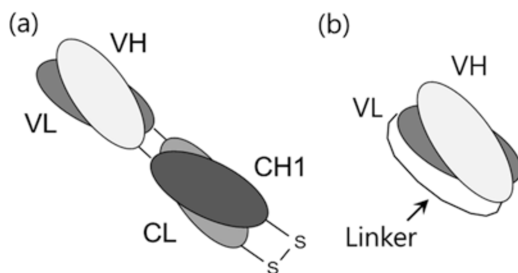

Fig. 2.1 Structures of antibody fragments employed in antibody phage display. (**a**) Fab (Fragment antigen-binding) is a heterodimeric protein composed of Fd (VH-CH1) and the light chain (VL-CL). The two chains of a Fab molecule are disulfide-bonded at their C-termini. (**b**) scFv (single chain fragment variable) consists of a VH and a VL domain connected by a peptide linker. The domains can be arranged in either orientation (VH-VL or VL-VH), and the length and the sequence of the linker may also be varied

Antibody fragments are generally displayed on M13 bacteriophage as a fusion to the N-terminus of pIII minor coat protein. The pIII minor coat protein consists of N-terminal domains (N1 and N2) and a C-terminal domain. The N-terminal domains are required for the infectivity of M13 phage, whereas the C-terminal domain participates in the capsid assembly. The N-terminal domains are also required for the inhibition of superinfection by another phage [12]. As *E. coli* cells need to be superinfected by helper phage during biopanning, many antibody libraries are designed to be displayed on truncated pIII lacking the N-terminal domains [5, 35, 91]. Phage antibody libraries displayed on intact, full-length pIII [37] require the suppression of the expression of antibody-pIII fusion protein (e.g. suppression of *lac* promoter by glucose) to allow helper phage superinfection.

Most antibody libraries are cloned in a phagemid vector that has a *gIII* (the gene encoding pIII; either full-length or the C-terminal domain only) at the 3′ end of the antibody cloning site, an f1 origin of replication for single stranded replication, a double-strand origin of replication, and a gene for antibiotics resistance. *E. coli* cells infected by antibody-displaying phages do not have the phage genes required for phage assembly except *gIII* in the phagemid. The

other genes need to be supplied by the helper phage which superinfects the phagemid-infected bacteria. Helper phages are engineered variants of the wild-type M13 bacteriophage that generally have an antibiotics resistance gene (usually for kanamycin) and a defective phage packaging signal that allows preferential packaging of the phagemid vector to the helper phage DNA. Because helper phage DNA has a copy of *gIII*, the phage particles produced after helper phage superinfection contain copies of both wild-type pIII expressed from helper phage DNA and antibody fragment-fused pIII originating from the plasmid. Also the antibody fragment may be proteolytically cleaved from pIII in the periplasm. As a result, the display valency of antibody fragments on phage surface is low, and a majority of phage particles do not display any antibody fragments [16, 91]. While this may sound problematic, the working density of antibody phage libraries is typically 10^{12}~10^{13} cfu/mL, which is 10–100 times greater than most large antibody libraries, therefore the low display level does not significantly compromise the performance of the library. Also the low display level means prevalently monovalent display which favors the selection of high affinity binders [66]. Nonetheless, it is sometimes advantageous to have a higher level of display, especially when performing the first round of biopanning during which there is a greater risk of losing unamplified target-binding clones. There are a number of engineered helper phages with defective *gIII* which, when used to superinfect *E. coli*, help produce phage particles with pIII originating preferentially from the phagemid [4, 69, 80], hence increasing the level of display and the display valency of antibody fragments on phage.

While the pIII display is the most prevalent form of antibody phage display, other capsid proteins have also been utilized as a display platform. While the major coat protein pVIII is frequently used for the display of peptide libraries, antibody fragments are too large for pVIII display [16]. On the other hand, the minor coat proteins pVII and pIX on the opposite side of pIII have successfully been employed in antibody phage display [32, 52, 74]. However, these display platforms are not as widely used as the pIII display. Part of the reason may be that pIII was the display platform that was successfully demonstrated to work by the first antibody libraries [8, 38, 55], and most of the subsequently constructed libraries utilized the proven technology. Additionally, pVII and pIX display systems differ from pIII in display levels and phagemid packaging efficiency [52], which may further discourage their use in antibody phage display.

2.4 Selection of Target-Binding Clones from Antibody Libraries by Phage Display

Phage-displayed antibody libraries with as many as ~10^{11} individual clones can be rapidly panned and screened against an antigen to yield target-specific binders in just 2 weeks. The speed, along with the *in vitro* nature of the selection process and the ability to produce fully human antibodies, make phage display a very potent technology for the generation of monoclonal antibodies suitable for therapeutic development. The panning (or “biopanning” to distinguish the process from the mining method) involves the addition of the antibody library to immobilized antigen, washing out of unbound phage antibody clones and elution of the bound phages, and amplification of the selected clones by infecting *E. coli* with the eluted phage. The amplified phage is then subjected to the next round of panning, and after several such rounds individual clones are screened for target binding (typically by ELISA) (Fig. 2.2). While the general principle of antibody library panning is quite straightforward, there are a variety of different methods for each step of panning.

For antigen immobilization, passive adsorption on a plastic surface is easy, simple, and one of the most commonly used methods. Protein molecules can be adsorbed on activated surfaces (e.g. Maxisorp® surface) through multiple non-covalent interactions which, when combined, are stable enough to retain the protein on the surface during the panning experiment. These surfaces are usually produced by irradiation/oxidation of

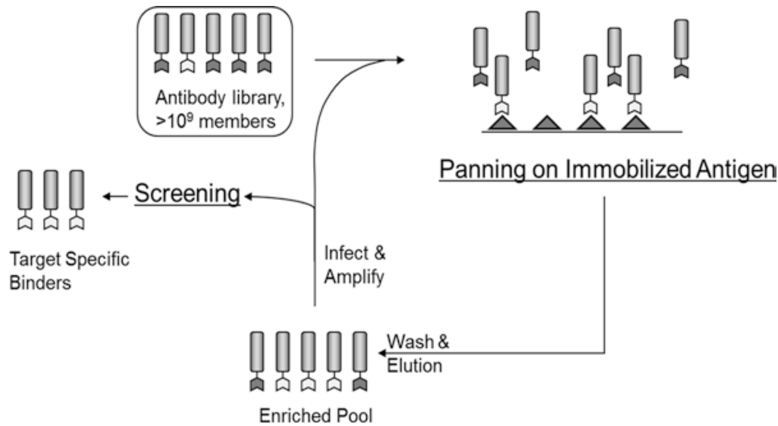

Fig. 2.2 Identification of target-specific binder by phage display biopanning of an antibody library. A large phage-displayed antibody library is added to surface-immobilized antigen. Unbound phage clones are washed off, and the bound clones are eluted and amplified by infecting *E. coli*. Target-specific binders are sufficiently enriched after several rounds of panning, and can be identified from the panning output by ELISA screening

polystyrene which creates hydrophilic patches on the initially hydrophobic plastic surface. Protein molecules are adsorbed on the surface through both hydrophilic (ionic or hydrogen bond) or hydrophobic interaction. Protein antigen (generally 0.1 ~ 10 μg/mL but may be higher or lower depending on the nature of the antigen and experimental design) diluted in a buffer is added to a plastic test tube or a microtiter plate with the activated surface. The dilution buffer should be free of detergents (such as Tween-20) that may interfere with adsorption, and needs to have a proper pH. Bicarbonate/carbonate buffer with pH > 9 is commonly used [81] to increase the net charge of the protein molecule and facilitate hydrophilic interactions with the surface, however the optimal pH may be different depending on the isoelectric point (pI) of the protein. Also, other common buffers such as phosphate-buffered saline (PBS) work reasonably well in most cases. While the passive adsorption is a simple and easy way to immobilize the antigen, it has several drawbacks. For example, antigens with a low molecular weight, such as haptens or peptides, do not get adsorbed efficiently on the plastic surface and need to be conjugated to a carrier protein such as bovine serum albumin (BSA). Also, the adsorption is usually accompanied by some degree of structural change of the antigen, and there is a possibility that the antibodies isolated against a passively adsorbed antigen may not recognize the free antigen in solution.

Another common immobilization method utilizes biotin-streptavidin (or avidin) interaction. The target antigen is conjugated with biotin, and surface-immobilized streptavidin is used to capture the antigen on the surface. The main advantage of this approach is that the binding interaction occurs in solution phase and not on the surface, hence more faithfully emulating the antibody-antigen interactions in physiological contexts. Also, the adsorption-induced conformational change of the antigen is not an issue, although biotinylation itself may mask functionally relevant epitopes or otherwise affect the physicochemical properties of the antigen. After solution phase incubation of the library with the biotinylated antigen, microbeads (e.g. agarose or paramagnetic beads) coated with streptavidin (or avidin) are added to the mixture. A molar excess of streptavidin to biotin should be added in order to maximize the retrieval of binders. The library also needs to be depleted of streptavidin binders prior to the addition of the biotinylated antigen, by pre-incubating the library with fresh streptavidin-coated microbeads. Proteins can be biotinylated by chemical reaction through lysine (a primary amine) or cysteine (a thiol) side chain, or post-translationally biotinylated *in vivo* by an *E. coli* strain expressing the protein biotin ligase

BirA [19]. Haptens and short peptides can be synthetically biotinylated.

Whole cells expressing a surface antigen can be used as an antigen for biopanning [29, 76, 88]. The isolation of target-specific antibodies using whole cells as the antigen is not straightforward since the cells express a great variety of surface molecules, and only a tiny fraction of the isolated cell binders will actually bind the target molecule. Overexpression of the target antigen and/or depletion of non-target binders is generally required in order for cell panning to be successful. The main advantage of the whole cell panning approach is, if successful, it can generate antibodies that bind to the target antigen in a more physiologically relevant environment.

After the incubation of the library with the antigen, unbound phages are washed out using a wash buffer. Typically the buffer is at physiological pH (such as PBS) and contains a non-ionic detergent such as Tween-20, however buffers with different pH or ionic strength can be used to facilitate the preferential isolation and enrichment of clones with desired binding properties. Wash stringency can also be controlled by changing the temperature and/or incubation time. Clones with a slow off-rate can be selectively enriched by incubating the bound phages in a wash buffer with an excess amount of a competitor (such as soluble antigen or an antibody that binds to the desired epitope) [54, 92]. In general, a more stringent wash condition favors the enrichment of high-affinity binders, however the diversity of the panning outputs may decrease as a result because the extensive wash cycles may remove binders with weaker affinity. In many cases the main objective of antibody library panning is not merely to produce antibodies with very high affinity but to isolate antibodies that bind to a specific epitope or have a desired function. Because these characteristics cannot be identified during panning, it is usually preferable to generate as many binders as possible regardless of their affinity, and subject them to a functional screening assay to identify clones with the desired characteristics. For this reason, high-stringency washing conditions are not frequently employed, especially during the initial rounds of panning, unless the library has already been enriched with binders (e.g. immune libraries or focused libraries for affinity maturation) or high-affinity binders are required regardless of the epitope.

For the elution of bound phages, pH shock by either an acidic or basic solution is most frequently used. Alternatively, phages can be eluted by proteolytic cleavage of the antibody fragment from pIII [67]. The CysDisplay™ technology links the antibody fragment to pIII through a disulfide bond, which can be reduced by the addition of dithiothreitol (DTT) to liberate the phage from the immobilized target [71]. While these alternative elution methods require extra phagemid components such as a protease cleavage site or non-native cysteine residues, they may be advantageous in minimizing the elution of nonspecifically bound phages. Target-specific binding phages can also be preferentially and competitively eluted using an excess amount of the antigen or a competing antibody [57], however there is a risk of elution bias against high-affinity binding clones if the concentration or affinity of the competitor is not high enough.

The eluted phages are subsequently amplified in *E. coli* hosts. The infection by M13 bacteriophage requires the F pili encoded by the F plasmid [68], and *E. coli* strains such as TG1, ER2537, ER2738, and XL1-Blue that carry the F plasmid are widely used as phage display hosts. Many of these strains also carry the *SupE44* mutation that suppresses the amber stop codon (UAG). Certain phagemid vectors (e.g. pComb3, pHEN, and pCANTAB [20, 37, 73]) have an amber codon before the beginning of *gIII* sequence, and antibody fragments can be displayed on phage or solubly expressed from the same vector by using an amber suppressor or non-supressor strain, respectively. After the infection by the eluted phages, *E. coli* cells are superinfected by helper phage and cultured overnight, and the amplified phages selected from the previous panning round are recovered from the growth medium.

After several rounds of panning, the phage pool is enriched with target-binding clones. Individual bacterial colonies from the panning

output are grown, and specific binders are screened by ELISA or other screening assays. ELISA screening can rapidly be performed using unpurified antibody fragments, either solubly expressed or displayed on phage. Soluble scFv or Fab can be directly produced from the panning output colonies if the phagemid has an amber codon between the antibody gene and *gIII*, because the amber codon suppression by *SupE44* mutant strains used in panning is incomplete. For the phagemid systems without the amber codon, phage ELISA can be performed for initial screening of the output clones [45], or additional steps such as the excision of *gIII* and re-ligation of the phagemid are required for the soluble expression of antibody fragments [82]. Target-specific clones as identified by ELISA can further be validated by DNA sequencing, cell sorting, immunoblotting, or other binding or functional assays.

2.5 Antibody Libraries for Phage Display

Immunoglobulins are large (~150 kDa), multi-subunit protein with many intra- and interchain disulfide bonds, which make them challenging to express in a bacterial host. For this reason antibody phage display libraries are in most cases constructed in either scFv or Fab formats which are much smaller (~25 kDa for scFv and ~50 kDa for Fab). Both formats have been successfully employed in the construction of large, high-quality antibody libraries [7, 41, 55, 71, 86].

The diversity of antibody libraries can be derived from either natural or synthetic sources, or both. B-cells from animals are the natural source of antibody diversity; antibody libraries can be prepared from either immunized or unimmunized animals. From B-cell cDNA, V(D)J-rearranged variable domain genes can be amplified by PCR using a set of primers designed to cover the whole antibody sequence diversity [43, 55, 72, 86]. A subsequent series of overlap extension PCRs yields a large scFv or Fab repertoire, which is then cloned to a phagemid vector and transformed to *E. coli*. Natural antibody libraries from immunized animals are a highly effective tool for the generation of high-affinity monoclonal antibodies, especially from the species for which hybridoma technology cannot be easily applied. Following immunization, serum is drawn from the animal and tested for the presence of target-specific antibodies by ELISA or immunoblotting. Tissues containing B-cells (most frequently spleens, but other sources such as blood can also be used) are harvested, RNA is isolated and cDNA is synthesized by reverse transcriptase using oligo-dT primer or a gene-specific primer annealing to the immunoglobulin constant region. Primer sets for the PCR amplification of variable regions typically consist of 5–10 forward primers and a smaller number of reverse primers for each of variable heavy and light domains [2], although the exact numbers vary depending on the primer design and the animal species. The individual combinations of these forward and reverse primers result in a large number of reactions, some of which may not yield detectable amount of amplified DNA product presumably because the variable gene usage varies among individual animals. An immune library does not need to be as large as naïve libraries which typically contain 10^9 or more independent clones, because the immunized repertoire is already highly enriched with target binders. However, the library construction process removes the VH-VL pairings that existed within intact B-cells, and the resulting library is a mixture of randomly combined VH and VL, of which only a small percentage of clones have the same VH-VL pairings of the original immune repertoire. While dominant clones can easily be recovered by phage display even with this random chain shuffling, rarer clones may be lost if the size of the immune library is too small. A library size of 10^7–10^8 can routinely be obtained by electroporation, which is large enough for most immune library constructions.

Naïve (or non-immunized) antibody libraries can also be constructed by essentially identical methods from B-cells of unimmunized animals. Human antibody libraries have been constructed using B-cell-containing tissues such as spleen, bone marrow, blood, and tonsil [48, 72, 86]. These naïve human antibody libraries are excel-

lent sources of fully human antibodies suitable for therapeutic applications, and many such antibodies are in clinical use or in various stages of therapeutic development [28, 70]. Because these libraries are supposedly unbiased, they need to have a considerably larger size than immune antibody libraries in order to generate target-specific binders. A naïve antibody library typically has >10^9 independent clones, and some contain 10^{11} clones or more [72], from which specific, high-affinity binders against virtually any types of antigens can be isolated. Probably the practical limit of phage antibody library size is around 10^{11} because of the logistical limitation of bacterial transformation, culture, and storage. Ideally performed electroporetic transformation may produce 10^8–10^9 transformants, and hundreds of such transformation are required for a library of 10^{11} clones. Also, the number of *E. coli* cells in library culture needs to be much larger than the number of independent clones in the library in order to retain the full library diversity with minimal omission of clones. This means that at least several liters of bacterial culture is needed for the amplification and phage rescue of a library with 10^{11} members (10^{11} *E. coli* cells roughly corresponds to a liter of mid-log phase culture). Also, ~10^{13} phage particles would be needed to represent the full diversity of such libraries (see above). Phage display selection is typically performed in a small volume (~ 1 mL), and phage concentrations significantly higher than 10^{13} cfu/mL would be difficult to achieve or handle.

Antibody diversity can also be generated by synthetic methods. Early examples of synthetic antibody libraries used degenerate oligonucleotides to create random CDR sequence [8, 63]. While the libraries thus constructed work well in producing specific target binders, the strategy lacks fine control over amino acid composition of CDRs and many unnatural or undesirable sequences get incorporated in the resulting library. Codon-based synthetic methods such as trinucleotide phosphoramidite synthesis [87] or Slonomics® [85] enable the incorporation of any combinations of amino acids at desired frequencies at any position. It is therefore possible to emulate the amino acid composition of natural CDRs, however the random combinatorial concatenation of the trinucleotide units may still result in unnatural CDR sequences. A number of recent synthetic antibody libraries have been constructed using these methods [83, 94], including HuCAL® series of antibody libraries by MorphoSys AG [41, 65, 71] from which many late-stage therapeutic antibody candidates have been derived [9–11].

Virtually all previously reported synthetic antibody libraries, including ones described above, relied on the random combination of mono- or trinucleotide units to generate the CDR sequence diversity, which inevitably introduced a certain percentage of undesirable or unnatural sequences. More recently, a synthetic antibody library with predesigned, non-combinatorial synthetic CDR diversity has been reported [5], maximizing the control over library sequence diversity. Thousands of individual CDR sequences that closely mimic natural human CDRs but without potentially detrimental post-translational modification (PTM) motifs were designed and synthesized on oligonucleotide arrays. The array-synthesized oligonucleotides were chemically stripped from the surface, amplified by PCR, and assembled with framework regions to yield the scFv library with highly natural synthetic CDR diversities without undesirable PTM motifs. Also, synthetic and natural antibody sequences can be combined in one antibody library to create semi-synthetic antibody libraries. Different semi-synthetic formats have been reported, such as natural CDRs grafted in a synthetic framework sequence [79], or synthetic VL and CDR-H3 diversities combined with a natural VH repertoire [35].

2.6 Therapeutic Antibodies by Phage Display

Earlier therapeutic antibodies had mostly been produced by hybridoma technology and engineered to chimeric or humanized antibodies, however more recently phage display technology is extensively utilized to generate and develop

fully human therapeutic antibodies and candidates [31, 75]. The earliest and best known example is adalimumab (Humira®), a fully human anti-TNF-α antibody for autoimmune and inflammatory disorders [61]. Unlike other phage display-produced antibodies isolated directly from large antibody libraries, adalimumab was generated by guided selection [39], using a hybridoma-derived murine anti-TNF-α antibody MAK-195 as a template. Variable heavy and light chains of the parental murine antibody were paired with a repertoire of human light and heavy chains, respectively, and TNF-α binders were selected from these libraries by phage display. The selected human light and heavy chains were paired to produce a fully human anti-TNF-α antibody that recognizes the same epitope as MAK-195, which eventually became adalimumab after affinity maturation (see below). Adalimumab has been the best-selling drug worldwide since 2012, with 2015 revenue of over 14 billion U.S. dollars.

There are several other approved therapeutic antibodies generated by phage display. Ramucirumab (Cyramza®) is an anti-angiogenic agent targeting VEGFR2 derived from a large naïve Fab library, and is approved for the treatment of cancers in combination with chemotherapy [53, 77]. Another antibody derived from a naïve Fab library, necitumumab (Portrazza®) binds to and neutralizes EGFR, and is approved for the treatment of advanced non-small cell lung cancer [47, 84]. Belimumab (Benlysta®) against BLyS (B-lymphocyte stimulator) inhibits B-cell activation and is used for the treatment of systemic lupus erythematosus (SLE), a chronic autoimmune disease characterized by the production of self-reactive antibodies [25]. Raxibacumab (ABthrax®) against *B. anthracis* protective antigen was approved for the prophylaxis and treatment of inhaled anthrax [56]. Both belimumab and raxibacumab were isolated from large naïve human scFv libraries. There are also a number of other antibodies in late stages of clinical development for a variety of indications and targets, and many more in phase I or II clinical studies and preclinical development stages [31]. These late-stage development candidates include tralokinumab, a fully human antibody from a naïve scFv library targeting IL-13, being developed for the treatment of inflammatory and allergic diseases including ulcerative colitis and asthma [59]. Another such candidate, gantenerumab is a high-affinity anti-amyloid-beta antibody derived from a synthetic human Fab library, and is being developed as a therapy for Alzheimer's disease [13]. Bimagrumab is also from a synthetic Fab library and targets myostatin/activing type II receptor (ActIIR), and is in phase III clinical trial for sporadic inclusion body myositis (sIBM), a degenerative skeletal muscle disease [44].

Antibody phage display technology is also often employed in affinity maturation. Therapeutic antibody development process may require *in vitro* affinity maturation, because the affinities of the antibodies obtained from immunized animals or from antibody libraries are often not high enough for therapeutic development. While a dissociation constant (K_D) of approximately 10^{-10} M is considered to be the affinity ceiling for naturally occurring antibodies [30], many hybridoma-derived antibodies bind to their target antigen with weaker affinity. Also, murine antibodies need to undergo humanization in order to be developed as therapeutic agents, a process that in many cases results in an unintended decrease in affinity. Antibodies derived from antibody libraries by phage display or other *in vitro* display technologies may have suboptimal affinities, although high-stringency biopanning of a large, high-quality antibody library can produce antibodies with subnanomolar dissociation constants [65, 72]. Ranibizumab (Lucentis®) is a humanized anti-VEGF antibody and its development is a good example of the application of phage display in the engineering and optimization of a murine-derived lead antibody [3, 60]. Ranibizumab originates from a murine anti-VEGF antibody A4.6.1, the same monoclonal antibody from which another anti-VEGF therapeutic antibody, bevacizumab (Avastin®), was humanized. The CDRs of A4.6.1 were first grafted onto human V_H3-Vκ1 frameworks. The affinity of the resulting Fab clone hu2.0 was estimated to be >7 μM, or more than 4000 times

weaker than the parental clone A4.6.1. In order to enhance the binding affinity of hu2.0, a number of key residues in the framework regions were randomized, and the resulting library was displayed on phage and panned against VEGF to yield the clone h2.10 with the K_d of 55 nM. Further rational mutations and phage display selection in the FRs and CDRs produced the clone Y0317 (ranibizumab) with ~100-fold improvement in K_D over bevacizumab or A4.6.1 [21]. Phage display-derived therapeutic antibodies such as ramucirumab [54] and belimumab [6] have also been affinity-matured *in vitro* by phage display selection.

2.7 Closing Remarks

Phage display has several unique advantages as the technological platform for the generation of therapeutic antibodies. It is one of the most widely used technologies for the production of fully human antibodies with supposedly low immunogenicity. Also, target-specific antibodies can be readily isolated by phage display against antigens that are not suitable for animal immunization because of e.g. high toxicity or low antigenicity due to high homology between human and murine proteins.

It needs to be mentioned, however, that although antibody phage display has many advantages, to date more fully human therapeutic antibodies have been derived from transgenic mice than by phage display [50]. Because both antibody phage display and the transgenic mice that produce human antibodies were available at about the same time (early- to mid-1990s), this bias is not likely due to the chronological order of the appearance of the technologies. One of the possible reasons might be because transgenic mouse-derived fully human antibodies already have undergone *in vivo* selection for properties such as stability and aggregation propensity [49], they have better developability as biotherapeutics. Also, transgenic mice-derived antibodies generally do not require additional *in vitro* affinity maturation, decreasing their development timelines considerably [50]. Some of the more recent phage antibody libraries were designed so that the antibodies isolated from them have desirable bio/physicochemical properties [5, 83], and it is expected that continued technological development in the area will take more phage display-derived therapeutic antibodies into the clinic.

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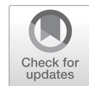

3 Naïve Human Antibody Libraries for Infectious Diseases

Soo Khim Chan, Anizah Rahumatullah, Jing Yi Lai, and Theam Soon Lim

Abstract

Many countries are facing an uphill battle in combating the spread of infectious diseases. The constant evolution of microorganisms magnifies the problem as it facilitates the re-emergence of old infectious diseases as well as promote the introduction of new and more deadly variants. Evidently, infectious diseases have contributed to an alarming rate of mortality worldwide making it a growing concern. Historically, antibodies have been used successfully to prevent and treat infectious diseases since the nineteenth century using antisera collected from immunized animals. The inherent ability of antibodies to trigger effector mechanisms aids the immune system to fight off pathogens that invades the host. Immune libraries have always been an important source of antibodies for infectious diseases due to the skewed repertoire generated post infection. Even so, the role and ability of naïve antibody libraries should not be underestimated. The naïve repertoire has its own unique advantages in generating antibodies against target antigens. This chapter will highlight the concept, advantages and application of human naïve libraries as a source to isolate antibodies against infectious disease target antigens.

Keywords

Naïve antibody library · Infectious diseases · Monoclonal antibodies · Phage display

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3.1 Introduction

The two halves of the human immune system is divided as the innate and adaptive immune system, with the former being less specific as suppose to the latter. The innate immunity is the first line of defence against infections casting a wide protective net against foreign proteins. The work horse of the innate immune response is mostly present before the onset of infections and are not disease specific. The cellular and molecular components associated with the innate immune response like lysozyme, interferons, complement and toll-like receptors function by means of recognizing different classes of molecules unique to frequently encountered pathogens [1]. On the other hand, the adaptive immune system is highly

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specific and is capable of recognizing specific foreign microorganisms and antigens to selectively eliminate them from the body. It differs from the innate immune response, as it is mainly a reaction towards a specific challenge. The adaptive immune response showcases four critical attributes that allows it to be effective in response to an infection. It shows high antigenic specificity, requires the generation of an elevated diversity of recognition entities, exhibits immunologic memory to allow a heighten response towards subsequent encounters of the same pathogen and permits recognition of self from non-self to elevate the risk of inappropriate response to self-components [2]. More importantly, one should note that the innate and adaptive immune responses actually functions in sync in a cooperative manner instigating a more efficient combined response than the individual response [1, 3–5].

The adaptive immune response has two major groups of cells, mainly antigen-presenting cells (APC) and lymphocytes. APC like macrophages and dendritic cells do not exhibit antigen-specific receptors but they function by processing and presenting the antigens to the antigen-specific receptors on T-cells. Lymphoytes are categorised further into two distinct cell types, namely the B-lymphocytes and T-lymphocytes [6]. B-lymphocytes are essential components that protect us against invasive antigens from the environment. The B-lymphocytes upon interaction with target antigens will proliferate and produce soluble forms of the B-cell receptors commonly known as antibodies [7]. The human immune system has the ability to produce a diverse collection of unique antibodies targeting a wide range of targets [8, 9]. These antibodies are circulating in the blood and lymphatic system to encounter foreign antigens [10]. The manner by which B-cell receptors are capable of demonstrating high target specificity is hypothesised using the clonal selection theory.

At the molecular level, B-cells undergo several complex stages of development to become fully activated antibody producing cells. However, the complex diverse nature of antibody development involves genetic rearrangement and somatic hypermutation, which is crucial for the immune system to fight off any possible foreign antigens encountered [11]. In this chapter, we will give a short overview of B-lymphocyte development including the repertoire generation processes. In addition to that, we will also highlight the concept and utilisation of the naïve B-lymphocyte repertoire in phage display library generation focusing on infectious diseases.

3.1.1 Diversification of B-Cell Repertoire

B lymphocytes, named after their discovery from bursa of Fabricius or bone marrow are differentiated from pluripotent hematopoietic stem cells [12]. Pre-B cells are generated from progenitor cells (pro-B cells) and migrate into the fetal liver during early embryonic development [13]. In the fetal liver, they develop and mature into B lymphocytes which mainly settles in epithelia, lung and gut-associated lymphoid tissues [14]. B-cell development at the early bone marrow-dependent stages is concentrated along the functional rearrangement process of the heavy chain (HC) and light chain (LC) gene segments independently. This rearrangement process is capable of fabricating an extended B-cell repertoire, which is responsible for expressing a diverse pool of antibodies with an estimated diversity above 10^{12}.

In general, the generation of diverse antibodies by the B-cell can be classified to two stages comprising of pre- and post-antigen stimulation. The repertoire that exist at pre-antigen stimulation is categorised as the preimmune repertoire with an estimated 10^{12} unique antibody molecules found in the human body. This initial repertoire is sufficient to isolate antibodies against a wide range of antigens [2]. The diversity of the preimmune antibody repertoire is generated by the allelic diversity of the variable gene segments, V(D)J recombination, junctional diversification, pairing of both heavy and light chains, and, receptor editing [15].

The variable gene (V-gene) of the HC is a result of the rearrangement of three different gene segments, being the variable (V), diversity (D)

and joining (J) segments. However, the LC gene is formed by the combination of only two gene segments, the V and J gene segments. Each gene segment exist in multiple copies and the selection of different combination of the gene segments for construction of the V-gene is random. This gene rearrangement allows the formation of the initial diversity of the antibody repertoire [16]. Human heavy chain V regions have at least 56 functional V gene segments, 23 D gene segments, and 6 J gene segments [17].

V(D)J recombination is the primary somatic gene rearrangement of V, D, and J gene segments prior to antigen exposure [15, 18]. This mechanism happens in variable regions of HC and LC which encodes the antigen recognition sites. The sequence of formation allows the HC to be assembled prior to the LC [19]. V(D)J recombination is initiated by the introduction of DNA double-strand breaks using recombination activating gene (RAG) proteins (RAG 1 and RAG 2) at specific recombination signal sequences (RSS). The absence of these RAG proteins can lead to the failure of lymphocyte development at progenitor stages where V(D)J recombination occurs [20, 21]. The recombination process is guided by a set of conserved flanking DNA sequences located next to the gene segments to ensure correct recombination. These flanking DNA sequences are known as RSS which are conserved heptamer and nanomer sequences separated by a spacer which is either 12 or 23 bp in length [4, 19]. The joining of two gene segments during V(D)J recombination will adapt the 12/23 rule. The V and J gene segments are flanked by a pair of 23 bp spacers while D gene segments have 12 bp spacers flanking at both ends. Therefore, this allows the joining of D gene segments to both V and J gene segments but ensures that V gene segments will not combine with J gene segments directly [4]. Binding of RAG proteins to the RSS allows the formation of complexes to initiate a DNA double-strand break (DSB) in between the gene segments and RSS. The breaks are later joined by nonhomologous end-joining (NHEJ) proteins by the addition or deletion of nucleotides. This addition and deletion to the genes will further enhance the diversity of the variable regions by junctional diversification. However, not all genes produced after junctional diversification are functional, which will eventually lead to the death of these B cells [2]. The gene diversification process can also lead to the formation of B cell receptors that target self-antigens. This population of B cells that targets self-antigens will undergo a further editing process termed as receptor editing. Receptor editing functions to eliminate self-reactive B cells from the system. These B cells will be stimulated to undergo further V(D)J recombination in order to edit the receptors which further enhance the diversity of antibodies [18].

There is further antibody gene diversification that occurs post-antigen exposure with the ultimate aim to further diverse the repertoire. Upon exposure to antigens, antibodies perform a second wave of diversification to enhance the binding affinities against antigens via affinity maturation. Cumulative point mutations in both V regions of heavy and light chains induce affinity maturation of antibodies [2]. Somatic hypermutation (SHM) and class switch recombination (CSR) are the examples which lead to affinity maturation of the antibodies upon stimulation by antigens [22]. SHM diversifies the V regions and alter the affinity of antigen binding sites while CSR changes the heavy chain constant (C_H) region to generate different antibody isotypes (IgA, IgD, IgE, IgG, and IgM) [23, 24]. Both SHM and CSR are initiated by somatic mutations in V regions and constant regions respectively [25, 26]. It was also reported that activation-induced cytidine deaminase (AID) is required by both SHM and CSR to trigger the mechanisms [27, 28]. AID is a 24 kDa protein that deaminates cytidine residues on single-stranded DNA (ssDNA), not RNA or double stranded DNA (dsDNA) [23]. AID causes the formation of DNA lesions which is later repaired by multiple DNA repair pathways such as base excision repair (BER), mismatch repair (MMR), and NHEJ proteins [29]. The role of AID in the diversification process is evident as AID deficient mice and humans are unable to carry out SHM and CSR [30]. Despite happening at the same stage of B

cell differentiation, SHM and CSR are different biochemical processes which are mediated by different sets of enzymes [24]. SHM occurs in the germinal centers (GCs) while CSR can occur either inside or outside GCs [31–33].

SHM is a region specific mechanism which begins 150–200 base pairs (bp) downstream from the transcription start site (TSS) and is detected as far as 2 kb within the intronic region between J and C exons [34, 35]. AID deaminates cytosine residues in the V regions where the products of deamination lead to uracil-guanine (U-G) mismatch. This mismatch is later repaired by DNA repair mechanisms such as base excision repair and mismatch repair [36]. Incorporation of point mutations by SHM in V(D)J exons of both HC an LC helps to improve antibody affinities [37]. SHM occurs at a frequency of 10^{-5} to 10^{-3} mutations per base pair per generation, which is about a million times greater than the mutation rates in other genes [38]. Generally, single base substitution is preferable than insertions or deletions (indel) in SHM. Also, SHM has been reported to favour certain motifs such as WRCY (W = A or T, R = A or G, C, Y = T or C), and its reverse complement RGYW motifs [24, 39]. Therefore, SHM is active in CDR regions where these motifs are largely presented [40, 41]. About two third of SHM induced substitution mutations are transition mutations as they are more preferred in SHM [42]. Antibodies that have undergone SHM will produce higher affinity antigen receptors to compete with lower affinity or inactive antibodies. This selection process enables higher affinity antibodies against target specific antibodies to be concentrated [3].

CSR is a mechanism that allows the antibody isotypes to be switched to initiate different mechanisms at different sites in the body. CSR switches antibody isotypes via DNA deletion and recombination on the IgH constant region upon exposure to an antigen. Therefore, CSR is vital in antibody maturation against infections and vaccines. Defective CSR has been associated with a range of diseases [43–45]. CSR occurs between switch (S) sequences that are located upstream of each constant region genes (C_H) exon except for Cδ exons [46, 47]. Different immunoglobulin isotypes are encoded by different C_H exons which are arranged in the order of Cμ, Cδ, Cγ, Cε and Cα in heavy chains [48]. Thereby, replacement of C_H exons with Cγ, Cε and Cα could give rises to IgG, IgA or IgE respectively. However, the V regions of antibodies will remain intact and unchanged [32, 49]. IgD is the only immunoglobulin isotype of the five that is generated via alternative splicing of the primary transcripts that encode IgM but not via CSR [48].

The diversity generated via these molecular mechanisms is predominantly responsible for the assorted repertoire of the antibody genes. This variation provides the flexibility of the antibody repertoire to generate antibodies against a wide array of antigens with varying specificities and affinities. As the antibody repertoire is a reflection of the threats encountered by the immune system, different repertoires are formed as a result of that. Therefore variations in antibody repertoire in individuals of similar infections and different infections are expected. This will have a direct influence on the repertoire of antibodies that can be isolated from different antibody libraries used for phage display.

3.1.2 Antibody Phage Display Technology

Historically, the application of antibodies for biomedical applications was first shown with the application of antisera from hyperimmunized animals to treat botulism and diphtheria [50]. The use of antisera highlighted the potential of antibodies although in a polyclonal level as a magic bullet to target infections for treatment. It was not until the introduction of hybridoma technology that production of monoclonal antibodies (mAbs) was realised. Hybridoma technology requires the fusion of immortal myeloma cells with antibody producing spleen cells to generate a hybridoma exhibiting characteristics of both myeloma and spleen cells. This characteristic allows for the infinite generation of mAbs *in vitro* [51]. However, the approach requires the use of murine derived spleen cells post-immunization which have been reported to trigger human anti-mouse

antibody (HAMA) response upon administration. This greatly limits the therapeutic applications of murine mAbs in humans which may result in reduced efficacy over time and in some cases anaphylaxis [52]. The complications associated with the use of animal derived mAbs resulted in a string of technological advancements like CDR grafting and antibody humanization to reduce the "murine-nature" of the antibodies generated. Ultimately, the ability to generate fully human antibodies was perceived as the solution for this issue.

The introduction of phage display technology revolutionized the way antibodies was to be produced *in vitro* in the twentieth century. The application of *in vitro* display technologies like phage display has allowed the generation of fully human mAbs in greater amounts and in a more controlled manner [53, 59–61]. However, phage display is no longer the only display method available for the production of human mAbs. Examples of other *in vitro* display technologies that can be used for mAb generation are ribosomal display [54], yeast surface display [55, 56], bacterial surface display [57, 58] and mRNA display [62, 63]. The introduction of the xenograph mice technology for human antibody generation also assisted in the generation of monoclonal antibodies with the prospect of affinity maturation [64]. Phage display is a promising *in vitro* display method that has been utilised successfully to isolate target specific antibodies by exploiting the Ff phages or filamentous phages [65]. The important advantages of phage display that makes it a preferred alternative is the robustness, simplicity, and stability of phage particles which allows the selection of desired antibodies against different targets under predefined conditions [66].

Phage display technology was first introduced by George Smith in 1985 to display peptides on the surface of phage particles as a fusion to the coat proteins [67] which was ultimately exchanged with antibody fragments for the isolation of mAbs [68]. Due to the non-lytic characteristic of filamentous phages (f1, fd, M13), they are commonly used in phage display to infect gram negative bacteria carrying the F pilus [69]. The fundamental concept of phage display lies in the direct physical linkage between genotype and phenotype [70]. The proteins or peptides encoded by the genotype are usually displayed on the phage surface by fusion with the phage coat proteins pVIII (p8) and pIII (p3) [71, 72] even though fusion to coat protein pVII [73, 74] and pIX [74, 75] have also been reported. The N-terminal of p3 was found to be vital for phage infectivity [76]. Hence, the protein of interest fused to p3 affects the efficiency of phage infectivity and propagation [77]. Phage display takes advantage of the natural process of phage infection and propagation by replicating the process with the aid of a helper phage system when using a phagemid vector. The helper phage is used in combination with the phagemid system to provide the necessary coat proteins as well as wild-type p3 for phage packaging as shown in Fig. 3.1. The phagemid vector is unique in that it is designed to contain the features from both a bacterial expression plasmid as well as a phage replication plasmid. The phagemid is normally designed to harbour the antibody gene as a fusion to gIII. Therefore the helper phage will function as the source of the remaining wild-type proteins required for phage replication, morphogenesis and assembly [78].

The process for mAb generation by antibody phage display requires the presence of antibody libraries harbouring approximately 10^9 to 10^{11} phage particles, each presenting an unique antibody clone [79]. The physical isolation and enrichment of target specific mAbs from the diverse library is performed via biopanning. Biopanning is a term coined to describe the sieving process of positive clones from the diverse library and subsequent augmentation of the positive population. The biopanning process involves several repetitive cycles of binding, washing, and amplification of the positive phage clones until a predominant population is present [70]. The process involves the immobilization of the target antigen on various solid surfaces such as polystyrene plates, microbeads [80], nitrocellulose blots [81], column matrix [81] or immunoassay tips [59]. The antibody phage library is then introduced to the bound target antigen and left to incu-

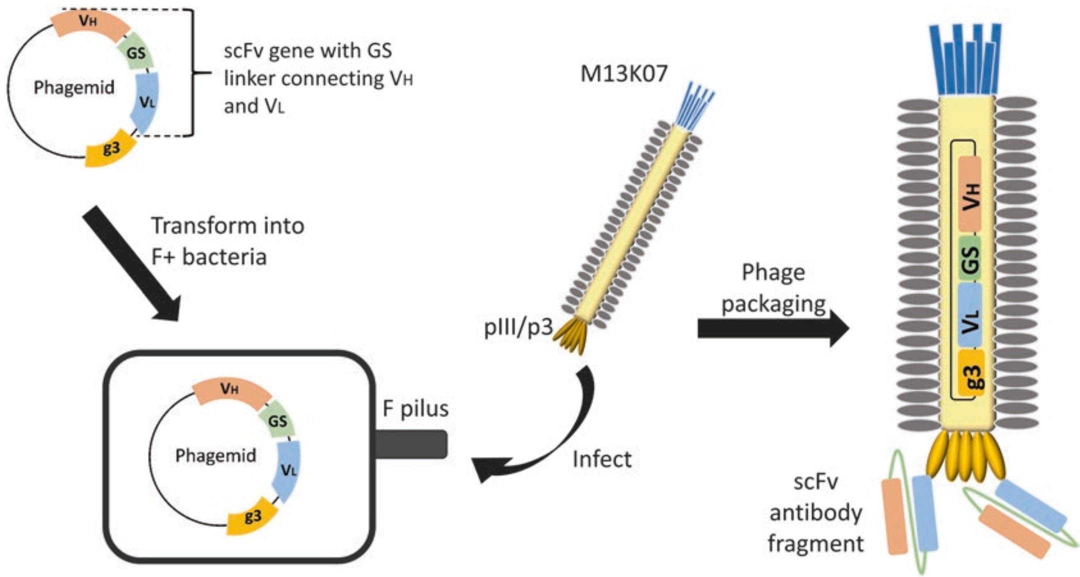

Fig. 3.1 Schematic representation of phage particles bearing antibody fragments for display. The gene encoding the scFv fragment is fused to gIII in a phagemid before introduction into a bacteria carrying the F pilus. Infection of scFv fused gIII phagemid with a helper phage (M13K07) allows the helper phage to provide all necessary proteins required for phage packaging

bate to allow affinity based capture of the antibody bearing phage particles to the antigens. The unbound antibody-phage particles are then removed by washing. The remaining bound phage particles are then eluted using acid-based elution or enzyme based digestion. The eluted phage particles are then amplified by re-infection with *E. coli* for a fresh cycle of phage packaging. The new population of phage particles are then ready to be used for the ensuing round of biopanning. This cycle is repeated a few rounds to ensure a continuous enrichment of the targeted population of phage particles. Varying the wash and elution conditions can customize the characteristics of the isolated mAbs. This is to simulate different environments and stringencies to isolate mAbs featuring a particular characteristic.

The major limitation associated with the *E. coli* folding machinery is its ability to provide better display efficiencies of smaller versions of the antibody fragments instead of the full antibody format on phage surfaces [82]. However, the presentation of full length IgG using bacterial periplasmic display was previously demonstrated to yield antibodies with nanomolar range of binding affinities [83]. Even so, the use of smaller antibody fragments has a distinct advantage in terms of tissue penetration. Smaller antibody fragments are able to penetrate tissues with a higher efficacy without compromising their affinities and specificities. The diminutive size of antibody fragments that works in favour for bacterial expression host allows for an easier and faster expression of recombinant antibodies [84, 85]. Several common antibody formats displayed on phage includes the single-chain fragment variable (scFv) [86, 87] fragment antigen binding (Fab) [88, 89] and domain antibodies [85]. The scFv format is the preferred antibody format used for phage display as it is not prone to degradation and is easier to be expressed in its functional form by bacteria [90, 91]. The scFv format is made up of the variable heavy chain (V_H) and variable light chain (V_L) domains interconnected with a glycine-serine (GS) linker [70]. The application of linkers has also provided new Fab molecules in the form of single chain Fab (scFab) fragments for phage display [88]. However, the choice of fragment to be used for library generation will be dependent on the downstream appli-

cation of the antibodies. Possibilities to engineer new formats allow grafting of the variable domains from any format to be engineered to other subsequent formats. This freedom for format exchange allows for improved applications of antibodies for biomedical applications. Even so, the choice of format used will also have to take into account the ability to clone a large diverse collection of the clones to form a library for phage display selections. In addition to that, format conversions from scFv to full IgG have been reported to cause a loss of target affinity largely due to conformational changes in the structure post-conversion. Therefore, proper consideration and design is required before format exchange of antibody fragments is carried out [92].

3.2 Construction of Naïve Antibody Library for Phage Display

As highlighted earlier, the basic requirement for antibody phage display is the accessibility of an antibody library for screening. Antibody libraries are useful assets for antibody development programs as they can be applied for various targets for multiple applications. In general, antibody libraries are classified into four categories, namely the naïve, immune, semi-synthetic and synthetic libraries. The classification of the antibody libraries are mainly influenced by the source of antibody V genes for library generation [93]. Naïve antibody libraries are constructed using V genes from non-immunized donors [93, 94]. Immune antibody libraries however are constructed utilizing antibody V genes from immunized donors or infected individuals [93]. Synthetic and semi-synthetic libraries are constructed using synthesized DNA oligonucleotides where the repertoire diversity is devoid of any natural immune maturation processes [95]. This chapter will focus on the principles and characteristics of naïve libraries including the application of antibodies derived from naïve libraries in combating infectious diseases.

Naïve antibody libraries are constructed using B cells of unimmunized or healthy donors, normally focusing on the IgM isotype. Naïve libraries are supposedly able to be used for the isolation of mAbs against any antigen [96]. The process involved in the generation of naïve antibody libraries is well established and has been described in numerous publications [97–100]. The human natural antibody repertoire is approximately 10^{11} in size, which is a thousand times larger than the murine naïve antibody repertoire [101]. However, antibodies isolated from naïve antibody libraries usually exhibit lower affinities compared to antibodies isolated from immunized libraries. This is because the naïve repertoire would not have undergone *in vivo* affinity maturation to produce higher affinity antibodies [96]. During antibody library generation, several critical aspects that are monitored includes library size and repertoire diversity in determining the quality of the library [102]. In the case of naïve libraries, larger library sizes are preferred to ensure higher affinity mAbs are isolated. The correlation between larger library sizes with higher affinity mAbs has been reported in several instances as high affinity clones were successfully retrieved from larger size libraries compared to smaller size antibody libraries [93, 103, 104].

The actual source where antibody genes are retrieved from is a vital consideration during the construction process of antibody libraries. Antibody genes could be sourced from peripheral blood mononuclear cells (PBMC), bone marrow, tonsil, and, spleen [105]. However, B cells at various stages have different degrees of mutation. B cells obtained from the bone marrow are the most naïve while B cells from tonsils are the most mutated. The degree of mutation will have an adverse effect on the repertoire of the library. Therefore, the highly mutated V genes from tonsils is not an ideal choice for naïve antibody library construction but suitable for immune libraries [106]. Naïve B cells are better represented by the raw V(D)J recombination in the bone marrow which have yet to encounter any antigens. The diversity of a naïve antibody library

is largely dependent on the number of unique antibody sequences that is successfully cloned into the library [61]. B cells are usually taken from peripheral blood for the construction of human antibody libraries as the method for extraction is less invasive compared to bone marrow extraction [107]. Peripheral blood contains a high proportion of naïve B cells, making peripheral blood a very good source for B cells [61]. The first naïve antibody library reported was constructed using peripheral blood lymphocytes (PBLs) of unimmunized donors with an estimated library size of greater than 10^7. The library was successfully used to isolate soluble antibody fragments against haptens with good affinities [108].

The general concern associated with naïve antibody libraries is the true nature of the sample naivety. This is due to the subjective nature of the term healthy, as it is unlikely that donors have never been infected throughout their lifetime making it realistically impossible to obtain "truly naïve" samples [94]. This is because healthy individuals would have recovered from a prior infection or have been immunized at some stage in their life. Vaccinations have been reported to increase antigen-specific repertoires which could result in a skewed repertoire being generated [109, 110]. In addition, memory cells in the immune system are capable of recognizing past infections which could contribute to changes in the antibody repertoire [94, 111]. The common considerations for naïve library sample collection includes healthy donors without immunosuppressive treatments as well as antigen exposure history [112], age [113, 114], genotype and chromatin structure [115, 116].

The general protocol for the construction of naïve antibody libraries using PBMC is demonstrated in Fig. 3.2. Naive antibody library construction starts with blood sample collection from a healthy population. B cells from whole blood can be isolated using the Ficoll-Hypaque density gradient centrifugation by utilizing the density differences between mononuclear cells

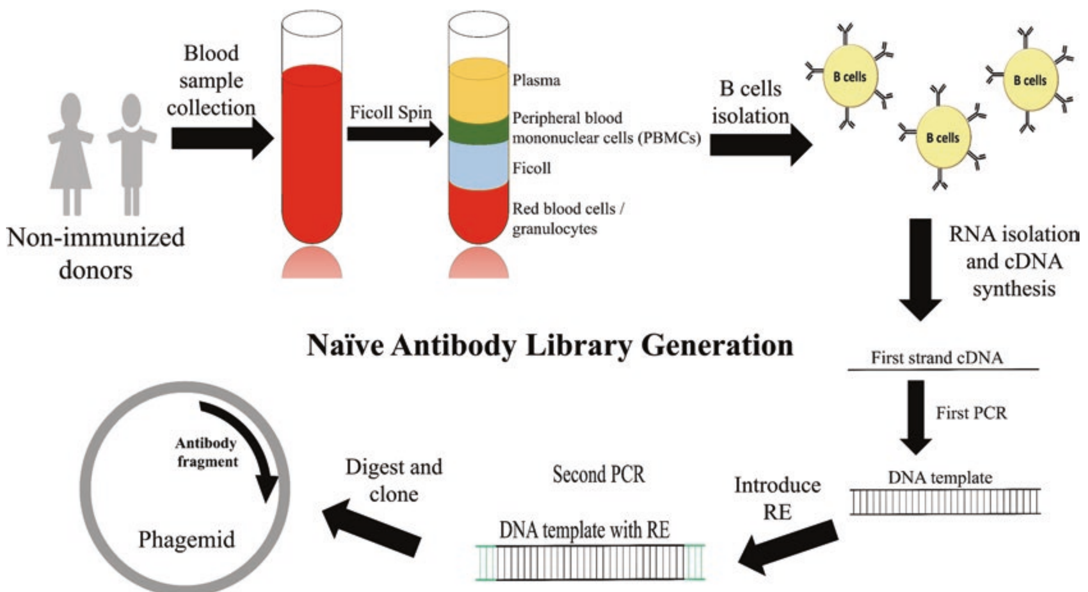

Fig. 3.2 General steps involved in naïve antibody library generation. Generation of naïve antibody library starts with blood sample collection from healthy donors. Total RNA is then prepared and used as template for reverse transcription into first strand cDNA by using gene specific primers. The cDNA is then amplified by polymerase chain reaction (PCR) to generate DNA amplicons. Second round of PCR is performed on these DNA samples to introduce cut sites for restriction enzymes. The amplicons are then cloned into a phagemid and transformed into suitable bacteria cells

with other elements in the blood for separation [117]. Mononuclear cells such as lymphocytes, monocytes, and platelets stay on top of the Ficoll layer because they are not dense enough to penetrate the Ficoll layer. Granulocytes on the other hand sediment to the bottom of the Ficoll layer due to increased density upon contact with the Ficoll medium. Red blood cells also sediment to the bottom of Ficoll solution due to aggregation by Ficoll. Mononuclear cell layer is washed and centrifuged to remove platelets that remain in the supernatant [117].

Upon obtaining B cells, mRNA is isolated immediately and reversed transcribed into cDNA. Reverse transcription of mRNA can be carried out using either random hexamer primers or antibody specific primers. Antibody specific primers such as IgM constant region specific primers allow the cDNA synthesis of antibody sequences from the IgM isotype. However, priming using random hexamers allows all the five antibody classes (IgA, IgD, IgE, IgG, IgM) to be amplified for a highly diverse repertoire. Therefore, random hexamers are being used more often to synthesize first strand cDNA from B cell mRNA for the generation of naïve antibody libraries [105]. In most cases, the preferred isotype for naïve antibody library generation is IgM. However, up to 40% of the circulating memory cells expresses IgM that may not be naïve due to somatic hypermutation upon exposure to antigens [61].

Upon obtaining B cell cDNA, variable (V) regions of the heavy chain (V_H) and light chain (V_L) are amplified using a defined set of primers that encompasses the human antibody gene repertoires. Different primer sets have been published over the year for the amplification of all V genes [118, 119]. However, V gene primer designs have evolved over the years due to additional information available with improvements in sequencing technology and bioinformatics analysis. An improved database of antibody V gene sequences from next generation sequencing has shed much light into antibody gene usage and V gene family coverage. The addition of new antibody gene sequences has allowed for new primer sets to be designed with improved gene coverage [67]. Improved gene coverage of the primer set has a great influence on the repertoire of the final antibody library generated. This helps to ensure all possible antibody genes are represented in the library as well as increasing the possible combinations of the HC and LC genes.

The first V gene amplification is usually done with gene specific primers without the introduction of any flanking regions. Amplification bias during PCR is a common concern that could influence the final repertoire of the library. Antibody gene usage is not evenly distributed with variations resulting from previous infections with additional variations from individual immune responses. Therefore having a highly representative primer set is important to ensure that unique gene families or poorly presented templates are also amplified [61]. A second round of PCR amplification is performed to introduce the desired restriction sites for cloning into the phagemid vector. There are several different strategies to construct a naïve antibody library. The most common approaches used for the construction of naïve antibody libraries is either by two-step/three-step cloning [94, 120] or PCR assembly [108] as shown in Fig. 3.3 In two-step cloning, light chain genes are first amplified to introduce the glycine-serine linker before cloning into the phagemid vector to establish an intermediate library. Cloning of the amplified heavy chain genes to the intermediate library is done to form a full scFv format library. The orientation of which V gene to clone first is influenced by the higher diversity of the HC CDR3 regions [121]. In some cases, three-step cloning is used to construct naïve antibody libraries. In this approach, independent V_H and V_L libraries are first cloned to function as intermediate libraries. Then the libraries are digested and ligated together to form the full antibody library containing both V_H and V_L repertoires [102]. In PCR assembly, the V_H and V_L genes are amplified to introduce the GS linker before cloning. Then, PCR assembly is used to assemble the three fragments to form the full scFv format. The assembled fragment is then digested with restriction enzymes before cloning it upstream of the gene III coat protein in the phagemid [97].

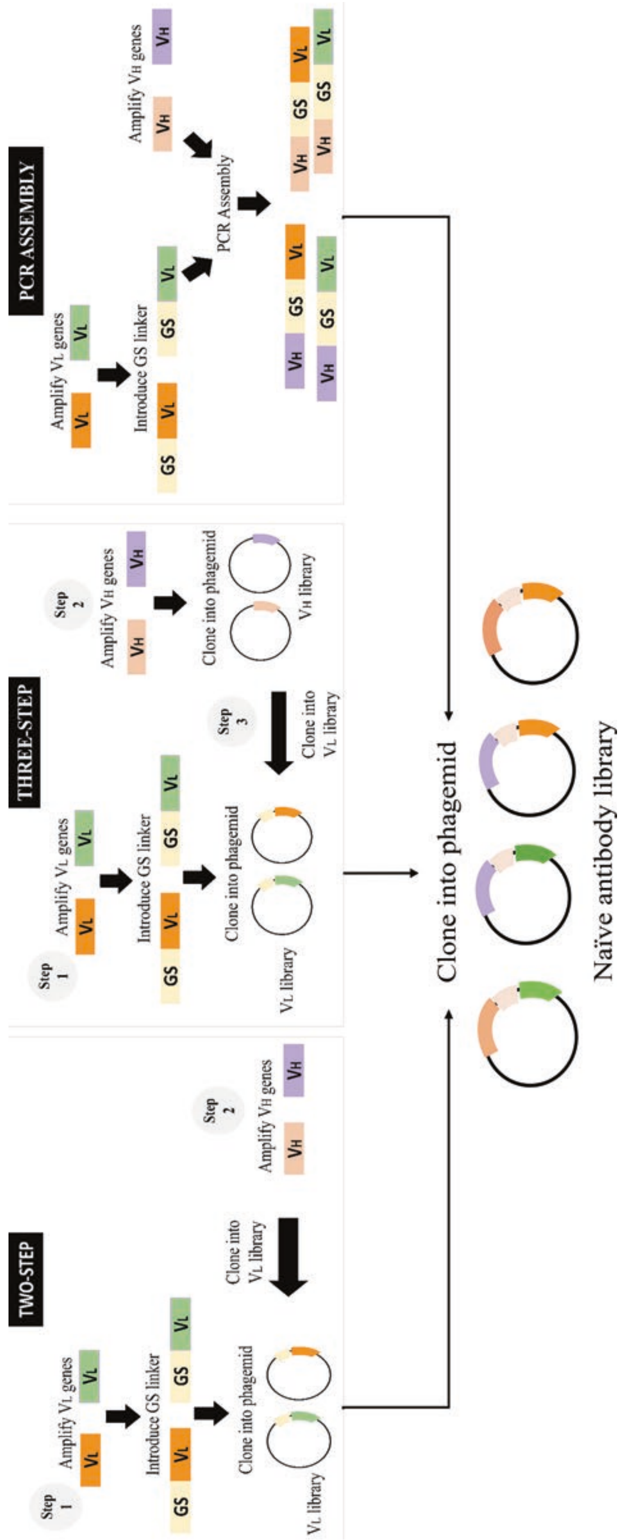

Fig. 3.3 Possible approaches for naïve library generation

Ligated products of antibody sequences and phagemids are transformed into bacterial cells carrying the F pilus such as XL1-Blue MRF'[97], ER2738 [122], TOP10F'[123], *E. coli* SS 320-M13cp [124, 125] and TG1 [120, 126]. The choice of cells that are used must have high transformation efficiencies. Even so, a high number of transformation is required to overcome the limitation of *E. coli* transformation in order to generate large naïve antibody libraries [127]. The library diversity is normally calculated based on the number of colonies formed from serial dilutions of transformants that are plated out on 2xYT agar and incubated overnight at 37 °C [97, 128]. Library developers often target for the highest library size as possible. However, this is an uphill task due to the limitations mentioned earlier. Even so, the average size for naïve antibody libraries is in the range of 10^9 or higher. The direct correlation between library size with antibody affinity has spurred the need to generate large library sizes in order to isolate high affinity antibodies without the need to further improve the affinities [61].

3.3 Isolation of Naïve Antibodies from Human Antibody Library Against Infectious Diseases

There is a growing concern in terms of global health with the emergence of new infectious diseases and the re-emergence of old diseases. This situation is intensifying concerns that these diseases might eventually become pandemics [129, 130]. The advent of antibody based technologies has a direct impact on global health as antibodies are useful biomolecules for diagnostics and therapeutic applications. The application of antibodies in diagnostics allows for a major role in disease management and early detection systems. This is critical in the fight against infections and disease prevention. In the last decade, the identification of antibiotic resistance has highlighted the vulnerability of the human population towards new infections. This highlighted the need for new alternative treatments which includes antibody based therapies [131]. Antibody based therapies appear to be a promising solution for infectious diseases due to its high specificity and the flexibility it provides in terms of modifications allows for direct programming of specific mechanisms for the treatment of specific diseases [132].

Generally, immune libraries are preferred for the generation of antibodies against infectious disease related targets due to the skewed antibody repertoires as a result of exposure to the infection [133]. In addition to that, high affinity antibodies could be isolated from immune libraries due to *in vivo* affinity maturation from antigen presentation [96]. Therefore, the preferential repertoire against a particular disease means that libraries of smaller sized repertoires are sufficient. One major drawback for immune libraries is the inability for it to be applied across a wide range of antigens. This means that new libraries have to be constructed each time antibodies against antigens from different infections have to be generated. The major advantage of naïve libraries is the huge diversity that does not show bias towards any antigen making it a universal source of antibodies against any target antigen [96, 133].

3.3.1 Naïve Library Derived Antibodies Against Target Antigens of Infectious Agents

The use of antibody based therapies started as early as the 1890 when sera from immunised animals was applied to cure bacterial infections with *Clostridium tetani* and *Corynebacterium diphtheriae* [134]. Since then, it has also been applied for other diseases such as tetanus, hepatitis A and B, measles, rabies, varicella, and vaccinia [135]. Over the years, the developments in antibody technology allowed for antibodies to be produced at a faster rate *in vitro*. This contributed to the increase of antibody discovery projects against many different diseases. Table 3.1 highlights the application of naïve human antibody libraries against infectious diseases caused by virus, bacteria, fungal and parasites.

Table 3.1 Monoclonal antibodies against a list of infectious agents

Infectious agent	Target	Antibody format	References
Bacterial			
Bacillus subtilis IFO 3336	Native spores	scFv. Fab	Zhou et al. [139]
Lactobacillus acidophilus ATCC 4356	Intact bacteria	scFv	Close et al. [143]
Salmonella typhi	Hemolysin E antigen	scFv	Lim et al. [94]
Bacillus anthracis	Protective antigen (PA83)	scFv	Cirino et al. [140]
Mycobacterium tuberculosis	85 B	scFv	Fuchs et al. [141]
Virus			
H5N1 influenza virus	Hemagglutinin (HA)	Fab	Lim et al. [145]
Venezuelan equine encephalitis virus (VEEV)	Intact virus particles	scFv	Kirsch et al. [162]
Dengue	Nonstructural (NS) protein 5	Fab	Zhao et al. [152]
Dengue	Nonstructural (NS) protein 3	Fab	Moreland et al. [151]
Rabies virus		scFv	Pansri et al. [156]
Hendra virus	Envelope G glycoprotein	Fab	Zhu et al. [157]
Fungal			
Candida albicans 3153A	Heavy and light chain variable regions	scFv	Haidaris et al. [160]
Fungal	Aflatoxin-BSA	scFv	Moghaddam et al. [163]
Aspergillus fumigatus	Glycosylhydrolase Crf2	scFv	Schütte et al. [164]
Parasite			
Plasmodium falciparum	Histidine rich protein 2 (rPfHRP2)	scFv	Leow et al. [165]

Antibody generation using traditional methods that require a living host is difficult when dealing with targets that are toxic to the host. This can be overcome by using naïve antibody libraries coupled with antibody phage display technology to allow the isolation of antibodies against antigens which are toxic or detrimental to the host [79]. For instance, hemolysin E (HlyE) toxin produced by *Salmonella enterica serovar* Typhi is a pore forming toxin which is antigenic to humans, may pose a challenge for conventional antibody generation techniques [136]. However, soluble scFv mAb against HlyE toxin was successfully isolated from a 2×10^9 naïve human scFv library constructed from 90 healthy donors. The donors consisted of equal distribution of donors from three ethnic groups (Malay, Chinese, and Indian) and equal gender distribution. To enhance the diversity of the library, combinatorial mixing of V_H and V_L chains were performed where heavy and light chains were randomly mixed. The naïve library was generated by two-step cloning by inserting the light chains followed by heavy chains [94]. The ability to harness the contrasting immune responses from different individuals resulting in varying gene rearrangements, gene pairings and usage allows for the improved diversity of the naïve library. This improved diversity ensures higher repertoire for antibody generation with improved affinities. The large library size also has a significant role in determining the antibody quality isolated from the library.

Another primary example of the application of naïve libraries is the ability to generate antibodies against the spores of *Bacillus anthracis* which can result in death post inhalation. The spores of *B. anthracis* is a potent biological threat which was abused as a bioweapon in 2001 when anthrax spores were intentionally mailed to Washington, DC [137, 138]. The toxic nature of the spores makes immunization for antibody generation

impossible as it would most likely kill the host. Therefore, isolation of human antibodies capable of binding to live native spores is possible using a naïve scFv library. Biopanning of a naïve scFv library isolated two clones with differences in specificity and affinity to the spores of the Bacillus strains as well as recognizing different epitopes. Analysis of both clones revealed a bias in V_H-V_L pairing in the repertoire after exposure of the spores to the human immune system. A naïve Fab library was later constructed by combining V_H and V_L genes from the isolated positive scFv clones with the aim to reduce the cross reactivity with other Bascillus strains. Chain shuffling technique was introduced to the Fab library to increase the diversity of V_H and V_L genes. Clones isolated from Fab library showed much lower cross reactivity with spores from other strains [139]. The process was envisioned from the panning of the human naïve scFv library against the spores of *Bacillus subtilis* IFO 3336 [139].

Another published work on *Bacillus anthracis* was focus at the protective antigen (PA83) which is essential for the anthrax toxicity mechanism. Several scFvs binding to PA83 were isolated from a naïve library where the highest binder exhibited K_d of 50 nM. The library was generated via three-step cloning where both the V_H and V_L genes were first cloned in separate plasmid vectors to generate separate V_H and V_L libraries. The advantage of individual libraries is to supply endless material for scFv assembly [108]. The size of naïve scFv library constructed was 6.7×10^9 [140].

A naïve human antibody library has also been used to isolate antibody fragments against tuberculosis (TB) antigens [141]. TB appears to be one of the leading fatal microbial infectious diseases worldwide caused by *Mycobacterium tuberculosis* [142]. A naïve library constructed using B cells from 44 donors of Caucasian, African, Indian and Chinese origin was used. Two-step cloning was applied to clone both the heavy and light chains of antibodies to create two libraries with the same heavy chain repertoire but differ in the light chain (lambda and kappa) repertoire. The libraries were validated with 110 antigens to ensure the quality of the libraries [121]. The libraries were used to pan against the antigenic 85 B complex of Mtb. Five scFv clones were successfully isolated and confirmed with DNA sequencing. Four clones have lambda light chain and one with kappa light chain [141].

The ability of phage display methodology to be applied to an array of different target types allows for the isolation of antibodies against various targets. A naïve antibody library was also used to pan against intact bacteria for antibody generation. The naïve scFv library isolated species-specific antibodies against *Lactobacillus acidophilus* ATCC 4356A prior to FACS quantification and genome sequencing. Generation of this naïve scFv library incorporated the use of cre-lox system which is able to generate a highly diverse library with the size of 3×10^{11}. The phagemid with the lox site was used to infect Cre recombinase expressing bacteria, where a high number of phagemids were able to enter a single bacterium where shuffling between V_H and V_L occurred. The lox site was introduced between V_H and V_L genes to act as the scFv linker. Selection of lox site was restricted to a single basic amino acid to reduce proteolysis, removal of stop codons and is least hydrophobic in nature. Analysis showed the isolated scFv recognised the surface layer A (SlpA) protein of *L. acidophilus* which is abundant and the antibody demonstrated high degree of specificity [143].

Hemagglutinin (HA) regions of influenza viruses are responsible for antigenicity of influenza viruses. Extensive mutation of these regions enabled the emergence of multiple antigenically distinct H5N1 strains [144]. To overcome this issue, targeting conserved regions of protein outside the antigenic sites becomes one of the option to avoid virus escape. A combination of panning strategies was performed to isolate Fab-phage antibodies away from HA antigenic sites, towards the conserved regions of HA. Thirty three Fab antibody fragments against multiple HA were successfully isolated from naïve human Fab phage display library HX01 (Humanyx Pte Ltd., Singapore). Five out of these 33 antibodies exhibited specificity towards the conserved region HA2 which is vital for fusion of viral and cell membrane [145].

Aside from isolating antibodies against the intact bacteria, naïve antibody phage library was also applied successfully to isolate antibodies against intact virus particles. Human naïve scFv library HAL4/7 was applied to pan against intact virus particles of Venezuelan equine encephalitis virus (VEEV). This is very interesting as most publications have shown isolation of naïve antibodies against virus proteins and not the intact virus particles [146–148]. The size of the naïve scFv library applied is 5×10^9 and was subjected to three rounds of panning. Preselection step was performed by incubating the phage library with concentrated supernatant of non-infected Vero cells. To minimize false positive results, soluble scFvs were used in ELISA instead of scFv phage. The 11 isolated scFvs were analysed by integrative database of germ-line variable genes from immunoglobulin loci of human (VBASE2). Results demonstrated the variable domains of light chains consisting of 1, 2, 3, and 6 while 1, 3, and 4 for heavy chains.

Dengue virus of the *Flaviviridae* family has a single stranded positive strand RNA as the genome. Post translational modification of the polypeptide give rise to three structural proteins (capsid, membrane, envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [149, 150]. Non-structural 3 (NS3) protein and non-structural 5 (NS5) proteins are essential in viral replication which are subdivided into few distinct functional domains. Targeting these domains provides important information about dengue replication and for the development of inhibitors against dengue virus [151, 152]. Naïve human Fab library HX02 (Humanyx Pte Ltd., Singapore) was used to screen for NS3 binders. NS3 proteins were biotinylated to bind to streptavidin magnetic beads prior to panning. Concentration of immobilised NS3 proteins was reduced for each round with an increase in the washing steps during panning. Ten unique clones were obtained and observed to bind to the protease domain, helicase domain, or both the domains for different dengue serotypes. One clone 3F8 demonstrate the ability to bind with NS3 proteins from all dengue serotypes (DENV1-4) and has a high binding affinity (15 nM) in ELISA as well as surface plasmon resonance (SPR) with K_D 0.5 nM [151]. Similar panning strategy was applied to isolate antibodies against the NS5 proteins. However, the six unique clones isolated were specific only to dengue virus serotype 3. Hence, panning was performed with alternate dengue serotypes to enrich clones which were able to recognise NS5 proteins for all serotypes. Two cross-reactive Fabs (5M1 and 5R3) and one DENV3 specific Fab (5M3) were obtained. The clones were incubated with an array of overlapping 15-mer peptides spanning the domain regions to map the epitope binding sites [152]. The ability to isolate cross-reactive clones against several serotypes as well as serotype specific antigens shows the flexibility afforded when using naïve antibody libraries.

Various endeavours have been taken to select good binders from antibody phage libraries against various antigens. Approaches such as increased library sizes [96, 100], enhanced cloning strategies [153], and improve panning protocols [154] have been demonstrated to retrieve good binders. Another approach reported the use of protease sensitive helper phage KM13. This method allows high throughput screening of naive library for a wide range of antigens [155, 156]. The protease-cleavage sequence was inserted between domain 2 and domain 3 of pIII to eliminate background contributed by helper phage. With this KM13 helper phage, one or two rounds of selection would be needed since the number of selection did not increase the chance to obtain new binders. Naïve scFv against rabies virus was successfully isolated from a human naïve scFv library with the size of 1.5×10^8 individual clones. Rabies virus is considered a difficult target due to the presence of limited amount of target in the mixture which might contribute to the enrichment of background or non-specific binders [156].

Hendra virus (HeV) belongs to the *Henipavirus* genus of the *Paramyxovirinae* which depends on two major membrane-anchored envelope glycoproteins (G and F) for infection. This virus can cause mortality in both animal and human hosts by infecting cells with the envelope

glycoproteins fused to cell membranes. Naïve Fab antibody library with an approximate size of 10^{10} was used to pan against the soluble form of glycoprotein Hendra G (sG). The library was constructed by collecting peripheral blood B cells of 10 healthy donors. Seven unique Fabs isolated from the library demonstrated longer V_H CDR3 among stronger binders compared to weakly bound Fabs. Isolated Fabs produced measurable inhibitory activity in reporter gene assay and inhibited syncytium formation. Later, conversion of a particular potent Fab to full antibody format exhibited 100% neutralizing potency with 12.5 μg/mL and 98% with 1.6 μg/mL [157].

Candida albicans is a very common pathogen to humans which usually attacks immunocompromised individuals with weak immune systems [158]. It has been reported that protein and carbohydrate moieties of *C. albicans* cell wall elicit strong immunological responses in humans [159]. Hence, isolation of antibodies which are able to bind to these moieties could lead to a better understanding of antibody reactivity towards these surface antigens for therapeutic purposes. A combinatorial phage display scFv library was used to pan against the surface antigen of *C. albicans*. The combinatorial library consists of two scFv libraries where one derived from lambda (λ) 2 family and another one derived from kappa (κ) 3 family. Panning of antigens used roughly 10^{12} from each phage library where panning with V_L λ2 and V_L κ3 library managed to obtain enrichments of 20-fold and 300-fold respectively. Three scFv clones were isolated and confirmed to bind with blastoconidia surface antigen via immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA), and western blot [160].

Malaria is a life-threatening disease caused by *Plasmodium* parasites that is transmitted by mosquitoes. Out of the five species, *Plasmodium falciparum* is the most common and lethal type which leads to morbidity and mortality [161]. *P. falciparum* histidine-rich protein 2 (PfHRP2) is a common candidate for the detection of *P. falciparum* infection which remains in the bloodstream up to 28 days upon infection. A naïve scFv library which was applied for isolation of antibodies against protective antigen PA83of *Bacillus anthracis* [140] mentioned earlier was also used to pan against PfHRP2. This highlights the flexibility afforded by naïve libraries to be used against various disease targets simultaneously.

3.4 Potential Applications of Antibodies Derived from Naïve Antibody Libraries

Monoclonal antibodies are recognised as an important class of drug especially against autoimmune diseases, cancers and infectious diseases [156, 166]. To accommodate the increased interest in biomedical applications of antibodies, antibody phage display technology has provided an alternative for rapid discovery and broad utilisation of novel, highly specific and fully human antibodies [167].

Since the introduction of monoclonal antibody technology by Kohler and Milstein in 1975, antibodies have become important biomolecules due to their capability to be applied in various fields, and most importantly in diagnostic, therapeutics, and vaccine development [168]. The potential of applications of naïve antibodies isolated from naïve libraries in diagnostics had been demonstrated in few publications. Isolated scFv and Fab antibody fragments against native spores of *Bacillus subtilis* IFO 3336 were labelled with two different fluorescent dyes (FITC or rhodamine-*N*-hydroxysuccinimide) for direct and indirect detection by fluorescence microscope. The detection signal was greatly amplified when fluorescent dye was labelled to the high-copy number of phage coat protein pVIII of the antibody phage particles. Direct detection of individual spores was made possible by fluorescent-labelled antibody-phage clones [139]. These spore binding antibodies could be further incorporated into other detection systems such as high-flow-rate fluidized bed as solid phase capture [169], electrochemiluminescent immunoassay (ECLIA) [170], and magnetic particle fluorogenic immunoassay (MPFIA) [170].

Another example was demonstrated by *L. acidophilus* species specific scFv isolated with phage display technology. The resulting scFvs isolated was applied in FACS for enrichment of *L. acidophilus* which resulted in more than 99.8% genome coverage. It was envisioned that this species specific antibodies could be applied to enrich low abundance organism in a community which could lead to better taxonomic identification and genome recovery prior to genome sequencing [143].

Conversion of antibody fragments into full IgG format or Fc format is always preferable for therapeutics [171]. Naïve Fab antibody fragments isolated against HA region of H5N1 virus were converted into full human IgG format. These antibodies showed binding to conserved region HA2 and exhibited promising neutralization efficacy [145]. Another publication reported potential therapeutic applications against Hendra virus and Nipah virus via conversion of the most potent naïve Fab into IgG1. Full-length IgG1 format has been shown to have much better cell fusion inhibitory activity with 50% lesser inhibitory concentration required [157]. The naïve scFv clones against 85B antigen of TB converted into scFv-Fc (yumab) format was able to serve as a potential biomarker for the diagnostic of TB. Validation of binding between this scFv-Fc antibody and 85B antigen was performed with titration ELISA and sandwich ELISA. The lowest detection limit achieved was 5 ng/mL and 10 ng/mL respectively. Later, these antibodies were put onto the platform of lateral flow immunoassay (LFIA) for detection of 85 B by conjugating the antibodies to 40 nm colloidal gold. The assay was able to detect the antigen at ≤ 5 ng/mL. Mtb culture filtrate was also successfully detected using immunoblot, analysed by reducing gel analysis via Tape station [141].

Despite the low instances of naïve antibody libraries being applied for infectious diseases, the antibodies derived from these libraries could be applied in a similar fashion for infectious disease therapy like those in oncology or autoimmunity. Antibodies can play a crucial role in neutralizing the infectivity of a virus by blocking binding sites or receptors on the virus or host cells. These class of antibodies are known as neutralizing antibodies (NAbs). Neutralization is defined as the reduction in viral infectivity via the binding of antibodies to viral surface particles which leads to the blocking of viral replication processes [172, 173]. Viral infection requires the attachment of the virus to the host cell membrane but different viruses may utilize different approaches for infection. Naked viruses (adenoviruses and papillomaviruses) are able to penetrate the host cell membrane to enter the cytoplasm or inject their genome through the membrane [174]. While enveloped viruses (dengue viruses, zika viruses, ebola viruses) have to fuse their envelopes with hosts' membranes for attachment [175–177]. Therefore, antibodies binding to influenza virus membrane protein is able to impede the fusion of influenza viruses with host's cell membrane [178].

Targeting viral envelope proteins of envelope viruses is becoming an attractive option for both diagnostic and therapy since the envelope proteins are expressed on viral surface and are therefore accessible for antibody attachment [179]. For example, dengue envelope (E) protein is a common target for neutralizing antibodies and also vaccine development since it contains few neutralising epitopes and binding motifs for virus entry into host cells [180]. A human Fab mAb 5J7 was shown to bind to all four dengue serotypes in enzyme-linked immunosorbent assay (ELISA) but could only neutralize dengue serotype 3. This mAb is able to neutralise 50% of dengue virus at nanogram level. Cryo-electron microscopy (cryo-EM) showed one Fab is able to bind across three E proteins and engage only domains that are crucial for infection. Moreover, the 5J7 antibody could prevent the virus from entering the host cells after attachment. The pre-attachment neutralization test showed 100% efficiency with 10 ng μL $^{-1}$ of antibodies [181].

In some cases, polyclonal antibodies work better in detection of viral proteins such as dengue NS2B protein. NS2B protein works as a cofactor for NS3 protease activity [182]. NS2B protein is small in size making it difficult to target when the whole virus particle is used. Polyclonal antibodies against NS2B proteins

were generated via gene or protein immunisation in mice. Western blotting and immunofluorescence assays confirmed the polyclonal antibodies generated are able to recognise both the native and denatured form of NS2B protein [183]. In certain cases such as ebola virus (EBOV) infections, an antibody cocktail is used as NAbs. Administration of antibody cocktails instead of single antibodies has shown promising results on non-human primates. Several antibody cocktails against EBOV were developed in recent years, which includes ZMAb [184], MB-003 [185], ZMapp [186] and the combination of mAB114 with mAb100 [187] that demonstrates successful therapeutic activity. ZMapp, the improved version of two antibody cocktails (MB-003 and ZMAb) reported 100% survival rate when administered to rhesus macaques despite the treatment being initiated only after 5 days of infection [186]. The antibodies in the cocktail are found to target several vulnerable sites on the glycoprotein (GP). Neutralizing antibodies target the base of GP while the non-neutralizing antibodies will bind to the glycan cap and mucin-like domains which functions as an external domain for the viral attachment and fusion [188–192]. This allows for a cumulative effect on the neutralization of virus replication and prevents virus survival. The latest antibody cocktail of mAb114 and mAb100 was successful in protecting nonhuman primates against the Ebola virus disease which includes viremia. The combination of both antibodies function to target different regions of the Ebola virus to improve its efficacy. The antibody mAb100 was found to recognize the base of the Ebola virus GP trimer which prevents access to the cathepsin-cleavage loop and prevents the proteolytic cleavage of the GP that is required for virus entry. However, mAb114 was able to interact with the glycan cap and inner chalice of the GP. The antibody remains associated to the glycan cap even after proteolytic removal. This aids to inhibit the binding of the cleaved GP to its receptor. The combination effect of the two antibodies provides the basis of virus neutralization and protection against the Ebola virus [187].

The cocktail approach by using several different antibodies with varying specificities and functionalities offer a broader coverage to prevent the escape of the virus [193]. Aside from EBOV infections, antibody cocktails are also being applied in other viral infectious diseases such as rabies virus [194], SARS coronavirus [195], HIV [196], hepatitis B virus [197] and other viruses [198]. The application of naïve antibody-cocktails is no longer something new, with the formulation and commercialization of different naïve antibody cocktails for separation of B cells and T cells from samples (Merck, BDbiosciences, Miltenyi Biotec). However, applications of naïve antibody cocktails in therapies especially infectious diseases has yet to be realised in clinical settings.

One of the major attractions in using antibodies for therapy is the ability of antibodies to induce cytotoxicity. Antibodies are able to lyse the target molecules by triggering the antibody dependent cell mediated cytotoxic (ADCC), or complement dependent cytotoxic (CDC) activity. ADCC uses immune-effector cells such as macrophages and natural killer cells while CDC function by activating a cascade of complement proteins [199]. As an example, Rituximab (Rituxan®) targeted against the pan-B-cell marker CD20 induces cell death [200]. In this regard, several other pathogens such as varicella, tetanus, Respiratory Syncytial Virus (RSV), rabies and Hepatitis B has been reported to show successful prophylactic use of antibodies in exposed individuals [43, 135]. In cases of immunodeficient or immunosuppressed individuals especially in HIV infected patients, antibody therapy can be a viable option for therapy because it can provide immunity to other infectious pathogens without giving rise to any further T-cell stimulation which promotes HIV growth. In addition, antibodies can be given to infections where no vaccines are available, for example, Ebola or Marburg viruses.

Antibody-drug conjugates (ADCs) are complexes of antibodies covalently joined with potent or cytotoxic drugs using various conjugation techniques. This conjugates are widely applied in cancer therapies due to their capability to differentiate healthy and disease state tissues [201]. In 2015, a novel antibody-antibiotic conjugate was developed to eliminate intracellular *S. aureus*,

one of the major causes of bacterial infection in the world. The anti-*S. aureus* antibodies were screened and carefully selected from peripheral blood of patients recovering from various *S. aureus* infections and linked to highly efficacious antibiotics via capthesin-cleavable linker. The conjugate eliminates *S. aureus* via two mechanisms: (1) engulf by host cells and let intracellular proteases cleave and release the antibiotic in active form; (2) binding of conjugate to bacterium and deliver antibiotic within phagosome to kill the bacterial. This conjugate was able to kill *S. aureus* inside human macrophages, endothelial and epithelial cell lines [202]. Naïve antibody-drug conjugate would be a new avenue for therapy, not only in infectious diseases but also other diseases like cancer and autoimmune diseases. Conjugating drug or other potent molecules with naïve antibodies might be a promising way to overcome the limitations possess by intact naïve antibodies and conventional antibiotic treatments. Naïve libraries will likely prove to be a promising diagnostics and therapeutics tool for infectious diseases in the coming future due to their great diversity and immune-system-independent nature which gives endless possibilities to isolate antibodies targeting a wide array of disease antigens [156].

3.5 Conclusion

Antibody based therapies have indeed been on a comeback trail after the disappointment of murine mAbs which provoke undesired immune response in humans upon administration. Despite a lot of mAbs have been approved for clinical usage, only one mAb (Palivizumab) has been approved for treatment of infectious disease. Antibody based therapies for infectious diseases are still underdeveloped and neglected due to the lack of research interest compared to cancer and other pandemic diseases. Immunised antibody libraries have always been preferred for the development of antibodies against infectious disease antigens. Skewed repertoires of immunised libraries allow isolation of high-affinity binding antibodies. However, with the rate of emerging and re-emerging infectious diseases, generation of disease specific immunised libraries is not a feasible option. Hence, a good quality naïve antibody library with a satisfactory size and diversity becomes an impeccable tool to generate antibodies against infectious diseases. Despite the antibodies isolated from naïve antibody libraries might not have satisfactory binding affinities, the advancement of molecular technology could be utilised to overcome these limitations. We believe, with the aid of newer technologies, better quality human naïve libraries could be constructed for the isolation of better quality antibodies to combat the infectious diseases. Naïve antibody phage libraries will have a foreseeable role to play in the journey to overcome the challenges posed by infectious agents.

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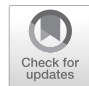

4 Immune Human Antibody Libraries for Infectious Diseases

Soo Khim Chan and Theam Soon Lim

Abstract

The incident of two children in Europe who died of diphtheria due to a shortage of anti-toxin drugs has highlighted the need for alternative anti-toxins. Historically, antiserum produced from immunised horses have been used to treat diphtheria. Despite the potential of antiserum, the economical and medial concerns associated with the use of animal antiserum has led to its slow market demise. Over the years, new and emerging infectious diseases have grown to be a major global health threat. The emergence of drug-resistant superbugs has also pushed the boundaries of available therapeutics to deal with new infectious diseases. Antibodies have emerged as a possible alternative to combat the continuous onslaught of various infectious agents. The isolation of antibodies against pathogens of infectious diseases isolated from immune libraries utilising phage display has yielded promising results in terms of affinities and neutralizing activities. This chapter focuses on the concept of immune antibody libraries and highlights the application of immune antibody libraries to generate antibodies for various infectious diseases.

Keywords

Immune libraries · Antibody libraries · Monoclonal antibodies · Phage display

4.1 Introduction

Antibody based therapies has in theory been in existence since the nineteenth century when sera from immunised animals were used for treatment of infectious diseases. Sera of immunised animals or humans was successfully used to treat a variety of bacterial infections such as *Corynebacterium diphtheriae*, *Streptococcus pneumoniae*, *Neisseria meningitides*, *Haemophilus influenzae*, *group A streptococcus*, and *Clostridium tetani* [21, 22]. However, the administration of these heterologous sera has led to several complications namely toxicity in hosts caused by the formation of antigen-antibody complex [20, 23]. In addition to that, the amount of antibodies to be administered needed to be

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strictly regulated as high doses was found to produce a prozone-like effect, giving lesser protection than less antibody [53]. Sera therapy was later abandoned after the introduction of antimicrobial chemotherapy which claimed to be more advantageous in terms of efficacy and toxicity [17, 22].

In 1975, the generation of monoclonal antibodies was first initiated with the introduction of the hybridoma technology [76]. Hybridoma technology requires the B cells of immunised animals to be isolated from spleen cells before fusion with myeloma cells. The product of the fusion is called hybridoma cells, representing cells with hybrid characteristics. The hybridomas are then able to exhibit both the immortal nature of myeloma cells as well as the antibody producing function of B cells in a continuous culture [114]. This allowed for the first time a truly monospecific antibody to be produced *in vitro*. Despite the advantage of producing large quantities of monospecific antibodies, hybridoma technology fell short of expectations when the immunogenicity of murine antibodies triggered human anti-mouse (HAMA) responses in humans. Moreover, hybridoma technology suffered from the restricted antigen panel as animal immunisation meant toxic antigens, non-immunogenic targets as well as self-antigens were not possible targets [121].

The introduction of phage display as a means for physical display of peptides on the surface of bacteriophages opened new avenues for antibody generation. *In vitro* display methods has aided the evolution of antibody generation methodologies where high quality antibodies are capable of being isolated without the need of animal immunisation [13]. Many alternative *in vitro* display methods have been developed and successfully applied to isolate antibodies against a wide range of antigens. Other *in vitro* display methods such as ribosome/mRNA display [59, 104, 120], bacteria display [136, 147], yeast display [10, 115] and lytic phage (T7 or Lambda) [67] have emerged as a result of the success paved by phage display. Although different in terms of the presentation mechanisms, the basic concept of the methods is similar where the genotypic information remains physically attached to the phenotypic product on display. Even with the array of alternative display approaches, phage display remains one of the most commonly used *in vitro* selection for antibody generation [7].

The entire antibody phage display process requires the construction of antibody libraries. Generally, classification of antibody libraries can be divided to two main possibilities (immune and single-pot libraries) depending on the source of antibody repertoires [68, 166]. The naïve, synthetic and semi-synthetic libraries are classified as single-pot libraries where the antibody repertoires are either obtained from healthy donors or synthesised artificially. Single-pot libraries are capable of producing antibodies against virtually any target antigen due to their naïve repertoire. Contrary to single-pot libraries, immune libraries have a higher tendency to deliver antibodies against antigens of a particular disease. This is mainly due to the repertoire of the library that is obtained from disease infected or immune donors as a result of the immune response [121]. As many individuals have received vaccinations in their lifetime and have undergone a continuous cycle of falling and recovering from sickness, this condition has an adverse effect on the natural representation of antibody repertoires in individuals. The B cells collected from these "healthy" individuals will most likely not be a true presentation of the naïve state of the antibody repertoires. Therefore, it is theoretically impossible to obtain a truly naïve healthy repertoire from donors for library generation. This will have a profound influence on the definition of what an immune library actually is. An immune library should be defined as the collection of the antibody repertoire from clinically or recently infected donors [90]. This allows immune libraries to have a skewed antibody repertoire which is useful in isolating antibodies against diseases specific targets [79, 89]. Therefore, immune libraries are typically smaller in size and have lower diversities (10^6–10^8) compared to naïve libraries (10^8–10^{10}) making them physically easier to be constructed [125]. The projected rate of

antibody isolation in different libraries is approximately one binder in 10^3–10^6 antibodies in immune libraries as compared to 10^7–10^9 in naïve libraries [50]. In addition, high affinity antibodies are reported to be likely isolated from immune libraries due to the affinity maturation process underwent by antibodies upon exposure to antigens.

4.1.1 Interplay of Antibody Phage Display and Infectious Diseases

Phage display technology has been widely applied in various fields such as vaccine development [1], isolation of recombinant antibodies [50, 91, 127], peptide identification [25], identification of immunogenic proteins and biomarkers [107, 178] and epitope mapping [136, 159]. The broad applicability of phage display is due to the robustness and stability of phage display technology in investigating protein-protein interactions under harsh conditions. Majority of antibody phage display studies are focused on oncology, autoimmunity and inflammatory disease targets [134]. However the application of antibody phage display in infectious diseases is still under reported probably due to a lack of research attention. Up till today, only one mAb (Palivizumab) has been approved for infectious disease which is licensed to prevent respiratory syncytial virus while numerous mAbs are still under development and in clinical trials [62]. This is in vast contrast to the number of mAb in the market and under development for oncology and autoimmune diseases.

Microorganisms, such as bacteria, viruses, parasites or fungi can cause infectious diseases [144]. The diseases can be spread, directly or indirectly, from one person to another whereas zoonotic diseases are infectious diseases of animals that can cause disease when transmitted to humans. These microorganisms harm the human body by releasing toxins or enzymes that disrupts or damages the normal host functions [109]. Today, infectious diseases remain as one of the leading causes of human mortality worldwide despite the availability of vaccines [66]. This is due to the rapid evolution of infectious pathogens especially viruses that pose a serious threat to humans. Infectious diseases are observed to spread rapidly and more frequently nowadays [60]. The Asian Pacific region is particularly a hotspot for infectious diseases due to the richness of biodiversity such as birds and mammal species [102]. With the rising concern of superbugs such as methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant (MDR) *Pseudomonas aeruginosa*, the need for alternative anti-infective agents has increased [66]. Pathogenic targets identified by antibodies could generally be classified as either whole bacterial cells or accessible molecular targets. The advantage of using intact bacterial cells is that isolation of antibodies could target any potential target on the cell surface which had not been identified before. Molecular targets on the other hand provide potential antibody recognition sites. Binding interaction established between cell surface antigens and antibodies are favourable because they disrupt bacterial division [92], colonization and virulence [129]. Antibodies are seen as an ideal candidate against filoviruses due to safer, effective and most importantly, it could be administered before and after exposure [118, 139].

In recent years, the use of immune antibody libraries to isolate antibody fragments against target antigens for infectious diseases has intensified. The skewed antibody repertoire of an immune library is a source of interest for the use of this library in infectious diseases. The biopanning process involves several rounds of binding, washing, rescue and re-amplification which is a standard practice in phage display processes [89]. Antibody phage display is useful in investigating the interactions between host and pathogens to underpin the underlying mechanism of infections. Recently, antibody phage display is coupled with next generation sequencing (NGS) platforms to allow a high-throughput and deeper characterization of the immune repertoire from individuals [8, 96]. Studies of immune responses towards an infection can help to unveil the interactions between antibodies and specific epitopes of various pathogens which is crucial in

the development of vaccines and antibody-based therapeutics [105]. Screening of potent antibodies based on sequence frequency could be done with NGS to improve the chances of isolation of good binders and reduce the loss of any potential binders. Also, the quality control process of antibody library generation could be improved by employing NGS to identify biasness during DNA amplification and presentation of antibody on phage [130].

4.2 Immune Antibody Libraries

The library construction stage is a crucial step in the antibody generation process that will have a direct influence on the quality of antibodies that could be isolated. Therefore, several considerations have to be taken into account before constructing immune libraries. The main considerations are the source of B-cells, antibody format to be used and type of display system. B-cells can originate from several sources such as peripheral blood, spleen, mucosal surfaces, bone marrow and lymph nodes. Most of the studies conducted on B cells were taken from peripheral blood due to the less invasive nature or sampling and easier accessibility of blood samples from donors. However, only 2% of all B cells are present in the peripheral blood which does not yield good coverage of the prospective antibody repertoire for immune library construction [172]. Spleen and lymph nodes are secondary lymphoid tissues rich in B cell sources with a fair portion of the B cell population having undergone affinity maturation making it a good source of material for immune library construction [156]. However, this may not be an option for the development of immune human antibody libraries.

Antibody formats used in phage display include single chain fragment variable (scFv) [91], fragment antigen-binding (Fab) [176], domain antibodies (VH) [26], and variable domain of heavy chain (VHH). And more recently, a bivalent display format was successfully achieved to resemble the natural behaviour of natural IgGs [85]. Generally, small antibody fragments are chosen to be displayed on phage surface due to the limitation of *E. coli* folding machinery [146]. Fragment antigen-binding (Fab) and scFv are most commonly used due to their favourable expression properties in *E. coli* periplasm [121, 177]. Fab is functionally more stable as compared to scFv due to the additional interface of the constant domain [45, 124]. Hence, it was found that Fab resulted in lower aggregation with higher monomeric proportions. Moreover, Fab format allows conversion into full length IgG without impairment of function [121]. However when looking at the practicality of library generation, scFv libraries are easier to be constructed as it requires only one overlap PCR to assemble V_H and V_L as compared to Fab which requires two. Hence, less time and money is needed in scFv library constructions [9].

The selection of intact full-length antibody molecules from libraries has not yet been well demonstrated apart from the work on the E-clonal antibodies using bacterial periplasmic display. In this situation, the conversion of the smaller recombinant fragment is required from isolated antibody fragments if full-length antibody molecules are desired. Nevertheless, the advantages of smaller-sized antibody fragments should not be ignored as it allows for better tissue penetration which is impossible with full-length antibodies [70, 173]. Another advantage is the lower production cost required due to the lack of glycosylation and smaller size [108]. Therefore utilization of smaller recombinant antibody formats in essence provides added advantages and efficiency for phage display.

4.3 Application of Immune Antibody Libraries for Infectious Diseases

The immune library repertoire is made up of a pool of affinity matured antibodies shaped by V-D-J recombination, somatic hypermutation (SHM) and class switch recombination (CSR) upon exposure to an antigen. Polarization of V genes in antibodies towards certain antigens results in a skewed antibody repertoire against a

particular group of antigens [54]. This generates a specific pool of antibodies targeting antigens of a particular disease. This allows immune libraries the ability to generate affinity rich antibodies against targets of a particular disease the library was developed for. Many antibodies have been successfully isolated from immune antibody libraries against infectious diseases. The size of immune antibody libraries is generally small in the range of approximately 10^6–10^8 [100]. In the case of a 10^8 immune scFv library that was constructed from five lymphatic filariasis (LF) individuals, the library was sufficient to generate monoclonal antibodies targeting a LF specific antigen. Profiling of six unique monoclonal antibodies obtained from this library revealed a bias in V, D, and J gene segment usage as well as combinations. In addition to that, an increase in neutral and small amino acids such as glycine, proline, serine and threonine was demonstrated in those enriched clones [127]. This preferential use of particular gene segments and amino acid distribution is likely a direct result of affinity enrichment of antibodies by the immune system post exposure to the disease.

More importantly, immune antibody libraries are advantageous for antibody isolation against toxic substances such as tetanus neurotoxin (TeNT). TeNT is produced by *Clostridium tetani* that results in tetanus infections. Passive administration of polyclonal antibodies derived from animals immunized with the tetanus toxoid adjuvant has been the mainstay therapy of choice [151]. Unfortunately, this treatment is costly, poses a high risk of infectious disease transmission and is associated with adverse immunological effects due to the origin of anti-toxin from non-homologous species [131, 151]. Therefore, fully human antibodies provide an ideal alternative for many TeNT like therapy targeting toxins. This concept was applied with the construction of an immune antibody library from volunteers who have high antibody titers against tetanus toxoid. Human antibodies against the heavy chain of TeNT were successfully isolated from the library. The isolated scFv antibodies exhibited high binding affinities which is a crucial feature for therapeutics [160]. The binding affinities improved by almost ten times after the antibodies were converted to full length IgG due to stability enhancement [110]. The use of immune antibody libraries for TeNT is just an example of the advantages it provides for antibody generation against targets that are harmful to the host. Similar work was also done to isolate antibodies against heavy chains (HCs) and light chains (LCs) of botulinum neurotoxin (BoNT) A and B. Immune antibody phage library was first constructed by immunising macaques with the recombinant HCs and LCs of BoNT A and B. The best scFvs or scFv-Fcs against the HCs and LCs respectively were isolated based on assays. Isolated best scFvs were later produced as full size IgG by using human germline sequences as template. Researchers observed combinations of both the best antibodies against HCs and LCs provided full protection in mice [142]. The application of immune antibody libraries for infectious diseases has gained momentum with applications for more deadly diseases like human immunodeficiency virus (HIV), ebola virus (EBOV), zika virus and influenza virus [52].

4.3.1 Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) was first discovered 30 years ago. Despite the introduction of antiretroviral therapy (ART) to fight against HIV infection, it still remains as a pandemic worldwide [28, 138]. This is due to the low accessibility of therapy which requires lifetime administration to suppress HIV infections [149]. According to UNAIDS report [155], a total of 36.7 million people are living with HIV and about 17 million people are on ART (UNAIDS [155]). There are two types of HIV, namely HIV-1 and HIV-2 with HIV-1 being the predominant HIV that is responsible for worldwide acquired immune deficiency syndrome (AIDS) epidemic. HIV-1 could be further classified into four groups (M, O, N, and, P) based on the geographic distributions but all produce similar clinical symptoms. Group M of HIV-1 has 11 subtypes and is associated to global HIV epidemic [117]. On the

other hand, HIV-2 is rare and less virulent compared to HIV-1 [2, 3]. The presence of many HIV subtypes brings about different challenges in HIV eradication as most of the isolated antibodies are unable to neutralize distinct HIV strains [152]. Some HIV-infected patients develop neutralising antibodies against a broad spectrum of HIV viruses years after infection [40, 55, 158].

Antibodies can act against HIV replications via three mechanisms: neutralization, antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated virus inhibition (ADCVI). Most of the antibodies act using the first mechanism via binding of antibodies to cell-free virus which subsequently blocks the attachment of viruses to host cells [111]. The human scFv isolated from immune antibody phage libraries have been shown to harbour the potential to produce neutralizing antibodies against HIV by binding to the virus. These immune libraries for the generation of neutralizing antibodies (NAbs) were constructed using valuable genetic material from HIV-infected patients [12, 80, 153]. Studies done with these NAbs revealed the vulnerable spots of HIV envelope glycoproteins providing key areas for antibody targeting [18, 148]. The antibody candidates VRC01, NIH45-46, and 3BNC117 are examples of naturally occurring antibodies isolated from HIV-1 infected patients that binds to the CD4-binding site (CD4bs) of gp120 and induce conformational changes similar to those induced by CD4 receptor [46].

HIV envelope glycoprotein is synthesised as a 160 kDa precursor (gp160) which are digested by cellular proteases into gp120 and gp41 [35, 49]. Gp120 and transmembrane molecules gp41 are crucial in mediating the binding with CD4 receptors and chemokine receptors (CCR5 or CXCR4). Gp 120 is held on viral surface via non-covalent interaction with gp41 [101]. Binding between these envelope glycoproteins and gp41-gp120-CD4 complex impedes viral attachment to host cells leading to a reduction in HIV replication [81, 95]. Therefore it was interesting for antibodies to be isolated from immune libraries against different parts of HIV envelope glycoproteins such as V3 region [80, 153], gp41 [119, 153], gp120 [12], and, gp140 [64, 103]. Mutational studies showed the three amino acids located at the C-terminal of the CDR3 loop are related to the binding affinities and neutralizing ability of antibodies to gp120. Hence, any change in this motif would be sufficient to reduce the neutralizing ability of these antibodies as well as resulting in lower binding affinities [75].

The second mechanism is thru the initiation of antibody-dependent cell-mediated cytotoxicity (ADCC) against HIV by utilizing the complement recruitment ability of the fragment crystallisable (Fc) region to trigger cell lysis [111]. The use of non-neutralizing antibodies to trigger ADCC were observed to successfully slow down disease progression of HIV [77, 87]. The killing efficiency is likely to correlate to antibody binding and accessibility of Fc region [16]. Monoclonal antibody A32 was an example of potential ADCC mediator that recognises the conformational epitope of gp120 in C1 and C4 regions. It was observed that A32 antibodies were able to trigger ADCC activity four to six fold higher than two other anti-gp120 antibodies (17b and 2G12) [47].

The third mechanism is antibody-dependent cell-mediated virus inhibition (ADCVI) which reduces viral replication via cytokines and secondary interaction between FcR and virus [111]. ADCVI has been associated with spread rate reduction of both cell-free and cell-associated viruses [111]. Conventional methods linking two scFvs by using a G4S peptide failed to mediate ADCVI activity despite retaining the immunoreactivity with gp41 and neutrophils. In contrary, bi-Fab antibodies have been found to show significant ADCVI activity against few tested isolates. Fab fragment is structurally longer with the presence of the first constant domain of Fc allowing higher flexibility in bridging neutrophils and infected cells [175].

Despite the promising neutralizing activities and binding affinities of isolated antibodies against HIV envelope glycoproteins, HIV remains a challenging target. This is due to the diverse subtypes (clades) of HIV and circulating recombinant forms [19]. Besides, glycan shields at the vicinity of the antibody recognition sites

has restricted the binding capabilities of antibodies [5, 128]. Viral envelope glycoprotein lacking glycan moieties and mutation at its domains was demonstrated to produce higher titres of neutralizing antibodies compared to wild type viruses [11, 132]. Studies showed many of the broad neutralizing antibodies isolated could recognise the epitopes formed by these glycan moieties [71, 72, 113]. Hence, these glycan shields are defined as alternative targets for broad neutralizing antibodies and vaccine development to elicit antibody responses. Information of the mimetic epitopes recognised by the neutralizing antibodies is useful in designing the vaccine to perform similar neutralizing functions [65].

4.3.2 Zika Virus

Zika virus (ZIKV) was first identified in 1947 from monkeys in the Zika forest of Uganda [37, 38]. ZIKV is categorised in the flavivirus genus which includes dengue virus (DENV), West Nile (WNV), Japanese encephalitis (JEV) viruses and yellow fever virus (YFV) [83]. ZIKV outbreaks have been associated with Guillain Barré syndrome to microcephaly and even death [6]. Envelope (E) protein of zika virus (ZIKV) serves as the primary target for NAbs [143, 150]. ZIKV E protein is divided into three main domains which are the DI, DII, and DIII [33]. DI domain links DII domain and DIII domain together. The DII domain utilizes its fusion loop to interact with the endosomal membrane [14, 99]. Antibodies that recognise the DII domain have been observed to be more cross-reactive with a lower neutralizing ability despite still being able to provide *in vivo* protection [27, 33, 157]. DIII domain contains vital epitopes which are recognised by NAbs and is important for viral attachment [14, 99]. Different mAbs with distinct neutralizing activities were observed to target the DIII domain of E protein of ZIKV [181].

Recently, ZIKV-C10 antibody isolated from an immunised donor was touted as an ideal candidate for ZIKV therapy. Investigations on the interaction between ZIKV-C10 antibody with the E protein at extracellular (pH 8.0), early (pH 6.5), and late endosomal conditions (pH 5.0) allowed a clearer understanding of the neutralization mechanism of this antibody. ZIKV-C10 antibody was observed to recognise an intra-dimer epitope which covers all the three domains (DI, DII, and DIII). As the pH decreases, ZIKV-C10 antibody was observed to impede the attachment of virus to the host cells which prevents structural rearrangement of E proteins that is crucial for viral infection [180]. Recently, ZIKV-117 isolated from ZIKV infected donors was also reported to exhibit neutralizing ability by binding to surface E glycoprotein of ZIKV. The Fab antibody was observed to bind to vicinity dimers as well as the surface E glycoprotein which prevents the formation of fusogenic trimers in acidic environment of endosomes [58].

However, titers of NAbs isolated from several animal species against DIII were lower than expected due to inaccessibility of DIII on viral particles. In contrast, murine antibodies with shorter CDR3 regions and distinct amino acid composition were found binding to DIII epitopes in a specific manner [30]. ZIKV specific antibodies would be useful to distinguish ZIKV from other flaviviruses in diagnostic assays. Since DIII has been applied in the development of various flavivirus vaccines, NAbs against DIII domain could serve as an ideal candidate for the design of domain and epitope specific vaccines against of ZIKV infection [93, 141]. Analysis of gene segments of antibodies binding to DIII revealed segment VH3-23 paired with VK1-5 are preferred. This B cell also effectively target against dengue virus serotype 1.

As dengue virus (DENV) and ZIKV are closely related, they also share substantial antigenic overlaps with an overall amino acid sequence similarity of 53.9% which allows them to have superimposable structures [122]. Recent publications showed immune sera isolated from DENV infected patients were able to exhibit varying degrees of neutralization effect against ZIKV and enhancement of ZIKV infection at the same time. This antibody-dependent enhancement (ADE) portrayed by antibodies from DENV immune sera bound to ZIKV epitope were unable to neutralise the virus but promote ADE instead

[36, 116]. ZIKV has been considered as the fifth serotype of DENV where the cross reaction between these two needs to be considered in vaccine development to avoid ADE in both infections [36]. Recently, a human monoclonal antibody ZIKV-117 isolated from ZIKV infected donors was reported to exhibit therapeutic potential and more importantly does not show cross-reactivity with other flaviviruses. ZIKV-117 was shown to neutralize all the tested ZIKV strains from Africa, Asia, and the Americans, making it an ideal candidate for therapy [140].

4.3.3 Ebola Virus

The first Ebola outbreak was documented in 1970s along the Ebola River in Africa where the Ebola virus (EBOV) was named after [29]. EBOV infections results in severe and lethal hemorrhagic fever in both humans and nonhuman primates [179]. The EBOV outbreak in 2014 at West Africa was declared as the most severe in history where the number of EBOV affected people surpassed the previously combined recorded cases [4]. EBOV could be genetically categorised into five genera: Zaire (EBOV), Sudan (SUDV), Bundibugyo (BDBV), Reston (RESTV) and Tai Forest (TAFV) viruses [78]. EBOV, SUDV, and BDBV have been largely associated to the past EBOV outbreaks in human history [15].

The surface glycoprotein (GP) of EBOV is the only protein exposed on the viral surface. It mediates host cell attachment and fusion which makes it an important target for NAbs [179]. Few publications have demonstrated the isolation of monoclonal antibodies having potent neutralizing activities against the GP of EBOV [32, 133, 179]. Epitope mapping revealed there are three major vulnerable sites on GP surface including the glycan cap, mucin-like domain and GP base where GP1 and GP2 subunits interact [106]. Previous publication showed EBOV infected patients maintained circulating antibodies against GP of EBOV after 11 years of infection due to the remaining serologic memory of B cells. Isolated mAbs from these patients demonstrated higher binding affinities than previously reported ZMapp cocktail [165] and prototypic EBOV GP-specific mAb KZ52 [94, 123]. The four isolated mAbs (mAb 100, mAb 114, mAb 165, and mAb 166) showed 100% inhibition of EBOV. Also, mAb 100 and mAb 114 were able to mediate *in vitro* ADCC at 0.03 μg/mL through the Fc receptor since Fc LALA mutations abrogated ADCC activity. LALA mutation is alteration of both leucine residues at position 234 and 235 of antibody Fc into alanine residue to confirm the Fc role in the mediation of cell killing activity [31, 63].

More recently, the 'trojan horse' antibody concept was introduced against EBOV infections that allows antibodies to target GPs of all five EBOV genera [162]. Previously, most of the NAbs developed are only targeting a single EBOV. ZMapp™ is an example of an antibody cocktail which is specific to EBOV Zaire but not to the other two deadly viruses (Sudan and Bundibugyo) which caused major outbreaks. With this 'trojan horse' antibody strategy, researchers are able to hit the weak spot of EBOV replication by using bispecific antibodies to hitch a ride on EBOV to enter lysosomes. Binding of virus to the host cell membrane causes the outer membrane to develop into a lysosome to surround the virus. In order to escape from the lysosome, the virus uses host proteases to digest EBOV surface glycoprotein to reveal epitopes which binds to the Niemann-Pick C1 (NPC1) protein of the host cell. NPC1 is embedded in the lysozyme membrane and vital for transportation of LDL-derived cholesterol into cells. Hence, NPC1 is being benefited by virus to escape into cells for multiplication [88]. To solve this issue, researchers developed two pairs of bispecific antibodies where each pair consists of two antibodies that are linked into a single molecule. One set of bispecific antibody (mAb-548) binds to host's NPC1 and another set of bispecific antibody (MR72) binds to EBOV surface glycoprotein. To deliver MR72 and mAb-548 into endosomes, monoclonal antibody FVM09 was fused to both the MR72 and mAb-548 to form FVM09-MR72 and FVM09-548 bispecific antibodies. FVM09 recognises the linear epitope of glycoprotein cap of all known EBOV strains

while the virus is outside the cell which enables the bispecific antibodies to hitch a ride into the lysosome [74]. By targeting the NPC1 (lock) and NPC1-binding protein (key), this strategy avoid the EBOV from interacting with NPC1 and escaping into the cytoplasm [162]. Another interesting work done on EBOV was focused at defining the vulnerability sites of EBOV to prevent the next outbreak. This is due to previously isolated mAbs that are species-specific which limits their usage. By studying the blood of EBOV infected survivors across the years, researchers successfully discovered the first natural human antibodies (ADI- 15878 and ADI-15742) which was able to work against the three major disease-causing EBOVs (Zaire, Sudan, and Bundibugyo). These two antibodies are able to bind to virus surface glycoproteins which are vital for entering lysosome. Binding of antibodies to these glycoproteins avoid the virus from entering the cytoplasm which stops the virus from replicating in cells [163].

4.3.4 Influenza Virus

Infections of influenza virus have resulted in substantial number of hospitalizations and a high mortality rate worldwide especially in children due to their vulnerability to the infections [82, 126]. Influenza A and B are envelope RNA viruses which are responsible for current influenza epidemics but influenza A is more severe which leads to pandemics. However, influenza A viruses are restricted to birds, human, horse, pigs and sea mammals while influenza B is only reported to circulate in humans and seals [82]. Despite the availability of antiviral drugs (M2 inhibitors and neuraminidase) against influenza, emergence of antiviral drugs resistance strains has spurred the urge for alternatives [34, 84].

Influenza virus has two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) which are embedded in the membrane envelope. HA consists of two glycosylated polypeptides (HA1 and HA2) linked by disulphide bonds. HA mediates binding to sialic acid receptors on host cell membrane and triggers membrane fusion between virus and host cells [56]. The globular head of HA consists of protruding peptide loops. Binding of anti-HA against these loops can prevent host cell attachment and cell entry. However, due to the variance in peptide loops among virus strains, these anti-HA are only able to neutralize closely related immunogens [61, 174]. NA on the other hand destroys sialic acid containing receptors on host cell membrane to release virus progeny [97]. HA subtypes (H1, H2, and H3) and NA subtypes (N1 and N2) have been associated to extensive influenza outbreaks [135]. The three main human influenza outbreaks (1918 H1N1 Spanish, 1957 H2N2 Asian, and 1968 H3N2 Hong Kong) have resulted in almost 100 million deaths worldwide [43]. Neuraminidase (NA) is the second major glycoprotein located on the surface of influenza virus A and B but absent in influenza C virus [48]. Despite anti-NA drugs have been used for more than a decade, studies about NA characterisations in the context of humoral immunity are scarce [168]. Anti-NA antibodies are reported to inhibit viral replication efficiency, reduce severity of disease and induce a long-term and cross-protective immunity [69, 168].

The rapid mutation of amino acids in HA globular protein has resulted in the availability of novel strains that are no longer responsive to the vaccines. Hence, annual re-design and re-administration of vaccines which induces NAbs are required [73]. However, antibodies against a highly conserved HA stem prevents fusion of host and virus membrane in low pH environment of late endosomes [57]. Broadly NAbs against influenza viruses have been summarised in a recent publication [82]. The conserved residues within the receptor binding site (RBS) of HA (130 loop, 150 loop, the 190 helix, and the 220 loop) has always been targeted by CDR of antibodies [82]. It is a broad and shallow pocket which is located in the head domain of the HA1 subunit [145]. Interaction of antibodies with HA mimicking its original receptor could neutralize the influenza viruses [164]. S139/1 antibody is an example of antibody isolated from Aichi68/H3 immunised mice which uses its HCDR2 to bind to the RBS to compete with the endogenous sialic

acid receptor. Hydrophobic residues (Leu52, Ile54, and Met56) of HCDR2 interact with highly conserved Trp153 and Leu194 of HA which functions for receptor binding. Aromatic residues within LCDR3 and HCDR3 stabilise the interacted complex via interaction with 150 loop and 190 helix respectively. Interestingly, antiviral activity of S139/1 antibody was greatly improved with avidity. Bivalent S139/1 IgG was three orders of magnitude higher as compared to monovalent Fab in neutralization activities [86].

On the other hand, CH65 and C05 Fab antibody use their HCDR3 to insert into the RBS on HA1. For CH65 antibody, all the three HCDR with LCDR1 and LCDR3 are involved in the interaction between CH65 and HA globular head while HCDR3 alone takes up 47% of the complete interface. It was observed that the tip of HCDR3 of CH65 highly mimics the sialic-acid surface that interacts with HA [164]. C05 antibody uses a particularly long HCDR3 (24 residues) to penetrate the RBS. This length of HCDR3 is very rare and is presumed to be vital for antigen recognition due to its accessibility to constraint epitopes. Long HCDR3 could adopt very unique conformation such as a "hammerhead" structure as portrait by PG9 and PG16. C05 inserts HCDR3 β-hairpin into the RBS to overlap the glycan receptor in order to avoid virus attachment [44].

Neutralizing antibodies against highly conserved HA stem was discovered in 1993. It was discovered that these broadly NAbs which binds to the HA stem regions were developed from IGHV1-69 germline gene [154]. By preventing pH-induced conformational changes that triggers membrane fusion between viruses and host cell, these antibodies performed neutralization against influenza viruses [57, 82]. Since then, substantial effort has been invested to isolate NAbs against the highly conserved HA stem region [42, 161, 171]. These antibodies are reported to be less potent in neutralizing but yet potent in inducing ADCC as compared to antibodies targeting the globular head of HA [39]. Moreover, HA stem region is well-conserved across distinct influenza subtypes with a lower mutation rate compared to the HA head [51].

Many antibodies against the HA stem region have been isolated from phage display libraries using samples obtained from infected or influenza-vaccinated donors [41, 51, 169, 170]. Neutralizing antibodies against the HA stem are categorised into two types (Type 1 and Type 2) depending on the epitopes they recognised on the HA stem. Type 1 antibodies bind to the F subdomain of HA while Type 2 antibodies bind to the fusion peptide and the outermost β-strand preceding helix A of HA. CR9114, CR2621, Mab 3.1 and F16 are examples of type 1 antibodies while CR8020 and CR8043 are Type 2 antibodies [51]. The differences between the antibodies binding to the same epitopes are the regions of antibodies that come in contact with highly conserved HA stem region. For example, CR9114 does not use the light chain but all HCDR loops [112, 137] and framework 3 (FR3) to bind, however clone F16 uses VH3-30 and light chain [41]. The F16 antibody depends heavily on HCDR3 for binding but Mab 3.1 depends on HCDR1, 3 and also FR3 [170]. Recently, a fully human monoclonal antibody 3E1 was isolated from peripheral blood mononuclear cells of a A(H1N1)09pdm vaccinated volunteer. This antibody was shown to bind to both the F subdomain and outermost β-strand preceding helix A of HA stem of H1N1 and H5N6 [161].

Isolation of antibodies using phage display remains critical especially under non-physiological conditions *in vivo*. Phage has high resistance towards extreme temperature and pH conditions which could not be achieved by other *in vitro* display methods [167]. Moreover, phage display could isolate binders within a shorter period of time, which makes it an ideal strategy to tackle sudden outbreaks. This is only feasible if disease specific immune antibody libraries are readily available for use. Fully human antibodies generated from phage display could be administered immediately without much adverse effects as compared to antibodies from animal origins or small molecule development [24]. Hence, phage display could complement other techniques for combating infectious diseases such as existing antibiotics and antivirals. Previous publications

have demonstrated the efficacy of antibody cocktails to yield optimal protection for infectious diseases due to the reduction of escape mutants. However, generation of multiple antibodies to make a cocktail is laborious and costly. The high cost of antibody-based therapy compared to chemically synthesised antibiotics is a great challenge for health care worldwide. Antibody-based treatment with existing antibiotics or antivirals could be the niche area to combat infectious diseases by providing a multi-dimensional approach to treatment [62, 98].

4.4 Conclusion

Immune antibody libraries are powerful platforms for the isolation of high affinity binders against certain antigens due to the preferential nature of the antibody repertoires after diversification. However, low accessibility and availability of human immune repertoire has slowed the development of antibodies against infectious diseases. Introduction of antibody phage display technology has largely underpinned the study of infectious diseases due to the ability of antibody development to be performed *in vitro*, devoid of animal use. Also, the versatility and robustness of phage display has yielded many vital findings for the development of potential vaccine and antibody therapeutics. Today, with the aid of phage display technology coupled with the advancement of high throughput sequencing, characterization of antibody repertoire after exposure to infectious diseases could be performed. In depth analysis at the genomics level of antibody repertoires could further pinpoint the interaction of antibody genes with antigens during an immune response. Despite the vast potential of recombinant antibody technology in tackling infectious diseases, rapid mutation of infectious pathogens has always allowed infectious agents to have the upper hand in this tussle. Therefore, we envision that immune antibody libraries can provide researchers a slight advantage in the near future when dealing with emerging and re-emerging infectious diseases.

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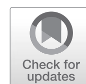

5 Synthetic Antibodies in Infectious Disease

Gang Chen, Sachdev S. Sidhu, and Johan Nilvebrant

Abstract

Rapid spread of microbial resistance and recent outbreaks of viral disease have led to renewed interest in antibody-based therapies for infectious diseases. Synthetic antibody libraries displayed on phage offer unique advantages over traditional immunization-based antibody generation, including full control over library design and selection conditions. The technology has matured beyond natural antibodies and is capable of providing novel insights into infectious disease and can generate novel antibodies that cannot be produced by the natural immune system. This chapter gives an overview of recombinant antibody library technology with an emphasis on our work using a highly successful synthetic single framework Fab library. We demonstrate its utility in targeting viruses and bacterial toxins in five case studies.

Keywords

Synthetic antibody · Fab · Phage display · *in vitro* selection

5.1 Introduction

Already in the late nineteenth century before the chemical nature of antibodies was known, antibody-based immunotherapy for the treatment of bacterial infections was adopted in the form of serum therapy [64]. Serum from immunized animals provided the first effective treatment option against infections with *Clostridium tetani* and *Corynebacterium diphtheriae* [64]. Behring was awarded the Nobel Prize in Physiology and Medicine in 1901 for his work on providing serum-therapy treatment of diphtheria. Although administration of heterologous sera was associated with several side effects, toxicity and variable efficacy, it was widely used because of the lack of alternatives. However, following the introduction of antibiotics in the 1930s, this practice was largely abandoned [17]. Antibiotics have ever since provided a cheap and safe antimicrobial treatment option, which has led to broad and general use in human and veterinary medicine. Due to the low discovery and development rate of

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novel antibiotics and vaccines, modern-day anti-infective therapy faces significant challenges including widespread microbial resistance, development of multi-resistant strains, emergence of new pathogens and infections in immunocompromised patients. Infectious diseases continue to be a leading cause of human mortality and disability worldwide despite the availability of many effective vaccines [27].

Recent advances in antibody engineering hold promise to reintroduce antibody therapy for infectious disease. The high affinity, specificity and flexibility of antibody-based treatments have led to renewed interest in their use in both pre- and post exposure treatment of infections. A key strength of antibody therapeutics is that technologies exist to increase their clinical potential by improving existing properties or endowing them with new activities [16]. Compared to alternative strategies, including probiotics, phage therapy and immune stimulation, antibody therapy has a history of safe use and a high degree of technical feasibility [27]. However, antibody drugs are expensive to develop and manufacture and high doses are typically required. Moreover, the market for a pathogen-specific drug is smaller than for a broad-spectrum antimicrobial agent. Nevertheless, antibodies may still be attractive for commercial development for multidrug resistant microorganisms or new pathogens for which no effective treatments exist. Therapeutic antibodies may also be used in combination with existing antibiotics and have been explored in the form of antibody-antibiotic conjugates [96].

Monoclonal antibody (mAb) therapy has been highly successful in the treatment of cancer and autoimmune diseases. Widespread antibiotic resistance of bacteria as well as frequent emergence of immune-escape mutant viruses have necessitated novel avenues for treatment, thus increasing the interest in pathogen-specific immunotherapies. However, as of November 2016 only four out of over fifty approved therapeutic monoclonal antibodies were for infectious disease indications. mAb development targeting infectious diseases has mainly focused on bacterial toxin-mediated diseases and viral diseases with no available vaccines or effective drugs [110]. Palivizumab (Synagis®), which targets respiratory syncytial virus (RSV) infections in high-risk children, was one of the first therapeutic monoclonal antibodies approved by the United States Food and Drug Administration (USFDA) (in 1998). Raxibacumab (Abthrax®; 2012) and obiltoxaximab (Anthim®; 2016) for the treatment of inhalational anthrax and bezlotoxumab (Zinplava™; 2016), which targets *Clostridium difficile* toxin B, have been approved more recently. Several reviews have reported history and progress in anti-infective mAb development, mostly using natural repertoires [8, 133, 70, 55, 86, 99, 135, 147, 163].

The immune system has evolved to enable humans to produce antibodies to practically all pathogens. Antibodies are produced by B cells of the adaptive immune system and can neutralize and eliminate infectious agents and associated toxins. Antibodies are separated into five classes – IgA, IgD, IgE, IgG and IgM – based on the structure of their constant regions. Because of their high stability, ability to trigger effector functions, favorable pharmacokinetics and ability to be transported to the placenta, antibodies of the IgG class and IgG1 isotype are generally preferred as the basis for new antibody-based therapies. An IgG is a Y-shaped molecule comprised of two heavy chains and two light chains, which associate to form a heterotetramer with two identical antigen-binding sites (Fig. 5.1). Interactions with antigen are mediated by six hypervariable loops, three each on the variable heavy (V_H) and variable light (V_L) domains. The constant region is responsible for initiating effector functions that lead to the removal or destruction of the pathogen or cells harboring the pathogen.

Antibody fragments (Fig. 5.1) have an increasing clinical importance [113] and are commonly used in the engineering of antibody properties. The fragment antigen binding (Fab) is a heterodimer consisting of the light chain and the variable and first constant domains of the heavy chain. A single chain fragment variable (scFv) consists of the light and heavy variable domains connected by a linker. Compared to scFvs, Fabs are generally more stable and binding activity is better retained upon conversion to full-length

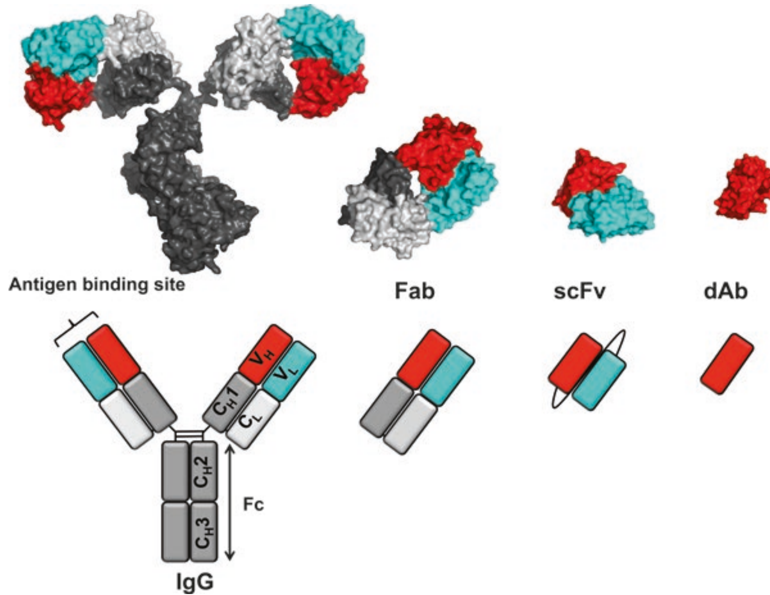

Fig. 5.1 Structure of human IgG and fragments. Constant (C) and variable (V) domains of the heavy and light chains in the heterotetrameric IgG molecule are indicated in the schematic representation. Heavy and light chains are shown in dark or light gray, respectively. V_H is shown in red and V_L in cyan. The following antibody fragments are indicated: Fc (fragment crystallizable), Fab (fragment antigen binding), scFv (single-chain fragment variable) and dAb (domain antibody). Surface representations were generated in PyMOL from PDB 1IGT [62], 2FJF [39], 1P4I [162] and 3B9V [7]

IgG. Single domain formats derived from variable [74, 118]) or constant domains [160, 161] represent the smallest human antibody fragments. The modular architecture of antibodies has been exploited to create a large number of alternative formats with various valences and antigen-binding specificities and has also enabled fusion to other proteins or toxins to further expand functionality.

Compared to small molecule drugs, antibodies have several advantages including high versatility, low toxicity, pathogen specificity, enhancement of immune function and favorable pharmacokinetics. Antibodies can mediate antimicrobial function through several mechanisms including viral neutralization, toxin neutralization, uptake and destruction by phagocytic cells via opsonization, prevention of microbial attachment, lysis through complement-dependent cellular cytotoxicity (CDC) and activation of immune cells to kill infected cells through antibody-dependent cellular cytotoxicity (ADCC) [57]. Pathogen-specific antimicrobial agents have a potential advantage because they are less likely to induce broad resistance among non-targeted microbes and are unlikely to disturb the healthy microbiome of the host. Thus, antibodies may act directly against the pathogen, exert indirect function by neutralizing toxic products of infection or enhance the efficacy of the host immune system [17]. However, drawbacks include high cost, limited usefulness against mixed infections and need for early and precise diagnosis [17].

Immortalizing B cells derived from immunized animals, which is the basis for the hybridoma technology, enables production of large amounts of homogeneous antibodies with defined class, isotype and specificity. The first step towards fully human therapeutic monoclonal antibodies was the production of monoclonal murine antibodies by hybridoma technology (e.g. the anti-CD3 monoclonal murine IgG2 antibody OKT3®, muronomab, approved in 1986 for the prevention of organ transplant rejection). However, low success rate in drug development

due to high immunogenicity and weak interaction with human complement and Fcγ receptors [16] prompted the development of chimeric antibodies, which consist of human constant domains with murine variable regions (e.g. rituximab, Rituxan®; 2006) and humanized antibodies, where mainly the complementarity determining regions (CDRs) are non-human (e.g. omalizumab, Xolair®; 2003). The first fully human antibody (adalimumab, Humira®) was approved in 2002. A human mAb is defined as having variable domains that are entirely derived from human antibody repertoires. Today fully human antibodies can be generated by phage display [107], transgenic mice carrying human antibody genes followed by hybridoma [14, 102], or through direct cloning of immunoglobulin encoding transcripts by single cell PCR [143]. Most antibodies that now enter clinical development are completely human and are derived from phage-display technology or transgenic mice, although several candidates currently under development are chimeric or humanized [114, 127].

Several drawbacks of classical immunization-based methods can be overcome by using *in vitro* selection systems with phage or other display platforms [49]. *In vitro* display physically links an antibody fragment to its encoding DNA and enables screening of libraries containing billions of unique variants. Antigen-binding clones are enriched through rounds of selection and amplification. Following screening of individual clones, the sequences of promising variants are immediately available. Antibody phage display for the generation and selection of antibody libraries displayed in scFv [106] or Fab format [66, 71] revolutionized the field of antibody engineering in the early 1990s. Importantly, *in vitro* display enables full control over the environment where selection takes place and the epitopes that are targeted. Unstable or toxic proteins that are not suitable for immunization can be targeted using appropriate conditions and selections can be designed to deplete antibodies that recognize fusion tags or cross-react with control proteins. Moreover, clones that display cross-species binding can be preferentially isolated by selecting on orthologs of relevance for future testing in animal models of disease or selection can be directed to epitopes of interest by introducing competitors. In contrast, when mining natural immune repertoires *in vivo* it is difficult to raise antibodies against epitopes that are highly conserved across species since antibodies that are reactive to self are eliminated. The transgenic methods suffer from the same restrictions on the target space due to tolerance and the need for immunogenic sequences and epitopes.

Human recombinant antibody repertoires provide a rich source for mAbs, and they are constructed from collections of human immunoglobulin genes that encode human heavy and light chains and essentially represent an immune system in a test tube. Following immunization, B cells that express a suitable antibody undergo clonal expansion in lymphatic organs. Mutation and selection mechanisms preferentially expand cells that express antibodies with high affinity and specificity for the immunogen. Libraries that sample natural diversity from lymphoid organs or peripheral blood of immunized animals or human subjects that have recovered from infection are referred to as ‘immune’ repertoires. For example, libraries constructed from human donors have generated neutralizing antibodies to human immunodeficiency virus (HIV) [71] and hepatitis C virus [50]. Since humans cannot be exposed to antigens at will, the available immune repertoires are limited to those induced by infections, vaccinations, autoimmunity and alloimmunity [9]. Naïve libraries are constructed using rearranged V-genes from B cell sources from non-immunized donors and mimic natural repertoire diversity. When natural diversity is incorporated in recombinant libraries, the combinatorial assembly of antibody heavy and light chains creates V_H-V_L combinations that were not part of the natural donor repertoire. In contrast to natural repertoires, synthetic repertoires generally contain artificial sequences that are not encoded in the human genome and cannot be generated by natural gene rearrangements and somatic hypermutation. However, antibodies derived from synthetic repertoires may be indistinguishable from endogenous human mAbs depending on the design. In the first antibody

libraries with synthetic components, a single V_L was combined with a set of rearranged V_H domains in a scFv format [67] and a library based on a human anti-tetanus toxoid mAb with a randomized CDR-H3 was used to select binders to different antigens [5]. Libraries that blend naturally rearranged CDR sequences with synthetic diversity are sometimes referred to as semi-synthetic, for example by grafting of CDRs amplified from donors onto a single framework in combination with synthetic diversity [65]. By contrast, fully synthetic libraries are entirely designed and engineered *in vitro* and contain precisely defined diversity incorporated from synthetic oligonucleotides [136].

Natural antibody paratopes have been optimized for specialized biological functions, and thus, they do not necessarily have optimal affinity, specificity or biophysical properties. Accumulated knowledge about antibody sequence, structure, function, and biophysical and biological properties now allows researchers to design highly sophisticated synthetic libraries to produce antibodies that can rival or surpass natural antibodies in terms of functionality. Synthetic libraries follow many different design strategies that vary in the number of framework regions used and the regions targeted for diversification. Synthetic libraries generally diversify multiple positions known to contribute to antigen recognition by using all or a defined subset of amino acids. The theoretical library diversity typically exceeds the practical limitations of phage display in terms of library transformation and screening. Libraries can mirror natural diversity by randomizing CDR positions at the center of the binding site and using a set of V-gene segments to provide a low level of diversity in peripheral positions [56]. Alternatively, CDR positions known to be involved in binding based on structural information can be diversified in a single framework [137]. To accommodate diverse paratope conformations required for the recognition of various epitopes with different shapes, some library designs utilize several heavy and light chain framework regions. For example, the HuCAL Platinum library [124] uses 49 framework combinations of consensus designed V_H and V_L domains [76] and the Ylanthia library [144] uses 36 fixed V_H-V_L pairs based on natural prevalence, canonical CDR structures, expression yields, stability and aggregation propensity.

The single framework approach offers the advantage that frameworks with high stability and low immunogenicity can be chosen and, by using modular design features, initial clones can rapidly be affinity-matured and reformatted. Frameworks can be optimized for high protein stability and production, and undesired motifs for post-translational modification can be removed. In contrast to inherently complex natural antibodies, this strategy enables the establishment of high-throughput antibody generation procedures. In fact, libraries with single frameworks using both natural and synthetic CDR diversity have been reported to be capable of generating antibodies to diverse antigens and epitopes [139, 90]. Similar studies have demonstrated that heavy chain diversity alone can be sufficient to generate high affinity synthetic antibodies [104, 137]. Using the framework of the humanized antibody Herceptin, Lee et al. found that restricted diversity in CDR-H1 and -H2 combined with high chemical and length diversity in CDR-H3 yielded highly functional Fab libraries [90]. Interestingly, libraries in which diversity was limited to as few as four [36] or two [10, 35] residue types in solvent accessible CDR positions have shown that a very restricted set of amino acids is sufficient to produce diverse and functional antibody paratopes.

These libraries not only demonstrate the versatility of synthetic repertoires, they also provide important insights into molecular recognition. For example, Tyr has been shown to be optimal for mediating favorable antigen contacts whereas Ser and Gly are effective in providing conformational flexibility to allow bulky Tyr residues to achieve optimal contacts [10, 82]. Such cooperation between large and small residues is critical for optimal molecular recognition and also illustrates that chemical diversity can be restricted without compromising function. Notably, similar minimalist strategies can generate functional binding surfaces also on non-antibody scaffolds [81].

These insights have been used in an iterative design process aimed to optimize synthetic anti-

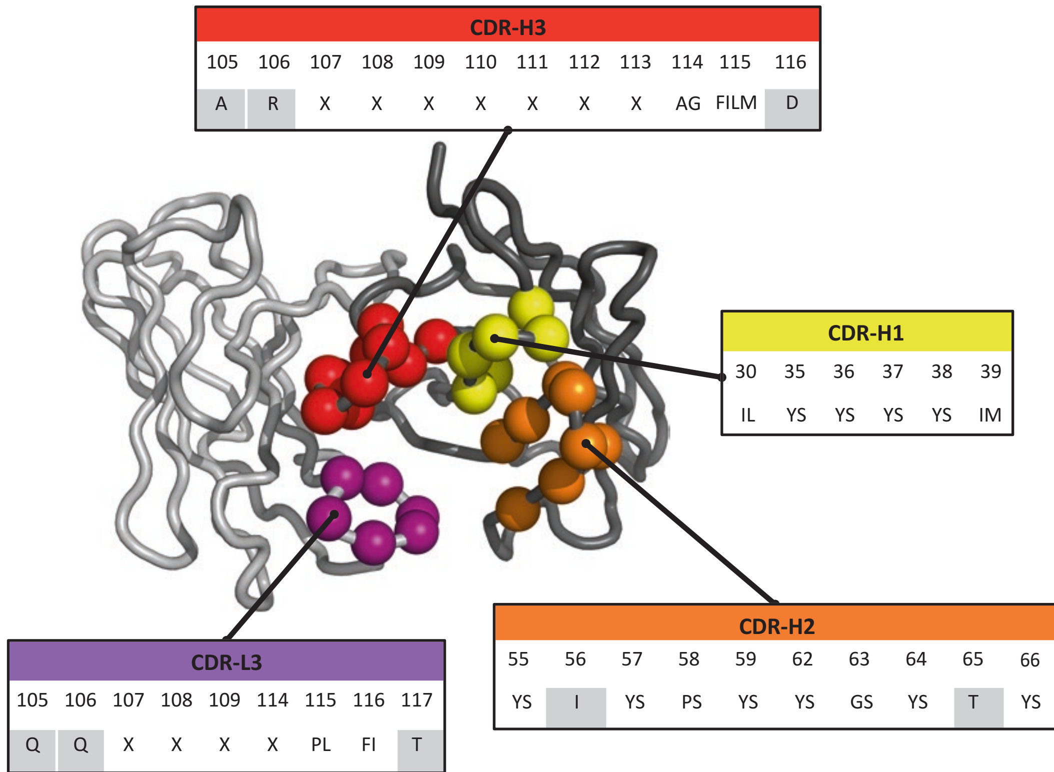

Fig. 5.2 Design of synthetic Fab Library F. The backbones of the heavy and light chains are shown as dark or light gray tubes, respectively. Diversified CDR positions are shown as spheres colored as follows: CDR-H1 (yellow), CDR-H2 (orange), CDR-H3 (red), CDR-L3 (purple). The figure was redrawn from Persson et al. [122] using PDB entry 1FVC [30] as a template. CDR positions shaded in gray were not varied and X indicates a mixture of nine amino acids (Y, S, G, A, F, W, H, P or V in a molar ratio of 5:4:4:2:1:1:1:1:1). Replacing the positions denoted by X with 1–17 or 3–7 degenerate codons, respectively, varied the lengths of CDR-H3 and CDR-L3

body library design using a minimalist design as a starting point [34]. Designs that include limited diversity in buried non-paratope positions and bias CDR diversity in favor of Tyr/Ser/Gly augmented with small quantities of other residues can further enhance functionality by allowing higher conformational flexibility and shape complementarity of CDR loops, respectively [34]. A current highly successful Fab library referred to as “Library F” (Fig. 5.2) is based on a very stable and well-expressed human Fab scaffold and contains restricted diversity in four of the six CDRs. Randomization in CDR-L3 and -H3 is biased towards Tyr, Ser and Gly with smaller amounts of other amino acids whereas CDR-H1 and -H2 contain binary diversity dominated by Tyr/Ser [122]. The amino acid distribution applied in CDR-L3 and CDR-H3 was designed to favor residues that are commonly found at natural protein-protein interfaces [82]. The trinucleotide phosphoramidite method, or trinucleotide-directed mutagenesis (TRIM), was applied to tailor the amino acid diversity. It uses a pre-synthesized set of trinucleotide codons for the synthesis of diversified CDRs, which is a significant technological development over simple degenerate codon synthesis. The gene encoding the heavy chain of the Fab is fused to gene III and the light chain is co-expressed using a phagemid system [42]. The Fabs assemble in the periplasm of *E. coli* and are subsequently displayed in a bivalent form on the phage coat [91]). Library F has been successfully used for high-throughput antibody generation [68, 112]. Moreover, this approach can generate antibodies with properties that cannot be achieved by immunization such as

recognition of specific protein conformations [41, 117, 128] and targeting of neo-epitopes on protein complexes [87]. Interestingly, cases where CDR-L3, which has equivalent chemical diversity as CDR-H3 in Library F, dominates antigen binding have also been found, which demonstrates that synthetic antibody function is not constrained by the same rules as natural antibodies [1, 122]. In summary, the synthetic approach has now evolved beyond simple mimicry of natural antibody repertoires and offers access to antibody specificities that would be unattainable using conventional methods [12].

In the second part of this chapter, we highlight case studies where synthetic antibody libraries combined with phage display technology have been employed to develop monoclonal antibodies targeting bacterial and viral pathogens, with an emphasis on antibody development using the fully synthetic Library F. In some cases, the properties of the generated antibodies could not be easily obtained with traditional immunization approaches or from libraries based on natural immune repertories. With the advance of synthetic antibody engineering, this *in vitro* antibody discovery approach will complement the current toolset of anti-infective antibody discovery, will open up new avenues for anti-infective antibody development, and will further our understanding of disease pathogenesis and vaccine-induced immunity.

5.2 Case Studies

5.2.1 Human Immunodeficiency Virus Type 1 (HIV-1)

HIV-1 is an enveloped retrovirus that was identified to be the causative agent of Acquired ImmunoDeficiency Syndrome (AIDS). HIV-1 infection requires fusion between the viral and host cell membranes, which is facilitated by the virus envelope glycoproteins gp120 and gp41. These two proteins form hexameric spikes at the viral surface, with each spike composed of three monomers of membrane-anchored gp41 associated with free gp120 [88, 119] (Fig. 5.3a). Viral entry is mediated by specific interaction of gp120 with the cell surface receptor CD4 and the chemokine co-receptors CCR5 or CXCR4 on CD4+ T cells and macrophages. Following co-receptor binding, gp41 undergoes a conformational change that results in insertion of the fusion peptide into the cell membrane, creating a hairpin loop intermediate that finally forms a highly stable six-helix bundle that facilitates membrane fusion [88, 119]. The two glycoproteins gp120 and gp41 represent the principal targets for the humoral response. In addition, CD4 and co-receptors CCR5 and CXCR4 are possible targets for therapeutic interventions. Phage display technology has been intensively used for decades to explore the epitope landscape recognized by HIV-1-specific antibodies, and thereby provided valuable insights about immunodominant and neutralizing epitopes and early vaccine strategies. Current development of mAb therapies against HIV-1 is heavily dependent on single B cell sorting or reverse transcriptase PCR techniques and the screening of *in vitro*-activated B cells [23].

Synthetic antibody approaches have been applied to existing anti-HIV-1 antibodies in order to increase their binding affinity and potency, as well as to broaden strain reactivity. For instance, a "CDR walking" strategy was adopted to improve a human anti-gp120 antibody directed to the CD4-binding site [6]. Residues in the CDR-H1 of the Fab were randomized for isolation of improved CDR-H1 variants, and next, the same approach was applied to the CDR-H3 using newly identified CDR-H1 variants as templates. The best clone exhibited eightfold improvement in affinity. Virus neutralization studies with laboratory isolates demonstrated a 54-fold improvement for the highest affinity clone. Moreover, neutralization studies with primary clinical isolates indicate that the improved Fab gained a broader neutralizing profile than the parent. By using a similar approach, this group improved the affinity of the same parental clone 420-fold for HIV-1 gp120 [159]. In addition to improving existing antibodies, various synthetic antibody libraries have been utilized to identify HIV-1 inhibitors binding to viral targets as well as host

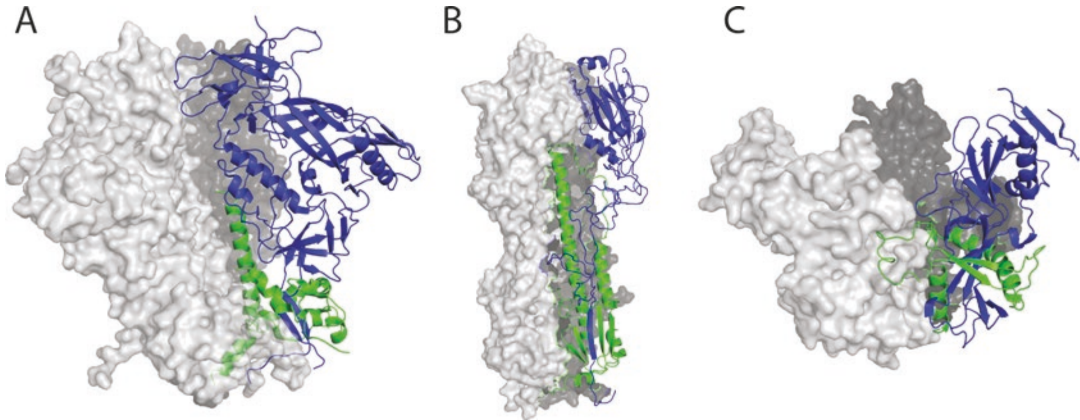

Fig. 5.3 Pre-fusion structure representation of (**a**) HIV-1 gp120-gp41 (PDB entry: 5I8H) [83], (**b**) Influenza virus HA1-HA2 (PDB entry: 4HMG) [151] and (**c**) EBOV GP1-GP2 spike (PDB entry: 3CSY) [93] ectodomains. The homotrimeric structures are depicted with one monomer shown as a colored cartoon and the other two shown as gray surfaces. The two subunits in cartoon representation are colored as following: (**a**) gp120 (blue) and gp41 (green), (**b**) HA1 (blue) and HA2 (green), (**c**) GP1 (blue) and GP2 (green). In EBOV GP, the bulky mucin-like domains that shield GP1 are not shown

receptors. By using a designed protein (5-helix) that mimics the conformation of the fusion intermediate, the Lai group identified highly specific antibodies from a minimalist phage display Fab library with binary diversity (Tyr/Ser) at selected combining sites [101]. The identified Fabs were highly specific for the HIV-1 epitope and comparable in affinity to a known scFv fragment derived from a natural antibody repertoire that targets the same region, demonstrating that minimalist synthetic antibody libraries have the potential to develop antibodies targeting HIV-1. Moreover, neutralizing human Fabs targeting gp41 derived from the synthetic HuCAL Gold library [76] have been described [61]. Some of these synthetic antibodies have been affinity matured [60] and structurally characterized [58, 59], showcasing that neutralizing antibodies with similar sequence signature and targeting epitope to those isolated from naïve or immune human antibody libraries could be obtained from synthetic antibody libraries, and more importantly, could be optimized through synthetic antibody engineering. Variable and constant domain antibody libraries have also generated promising anti-HIV binders using natural [21] or synthetic [52, 157] CDR diversity. Owing to their smaller sizes, these domain antibodies may target hidden conserved epitopes or spatially restricted regions that are not accessible to larger antibodies, therefore exhibiting unique application in viral neutralization.

5.2.2 Influenza Virus

Pandemic influenza remains a threat to global health given the absence of a universal vaccine and the emergence of new strains due to its high antigenic variability and rapid antigenic drift. Influenza viruses are a family of RNA viruses that cause respiratory tract infection in vertebrates, including birds, humans and other mammals. Influenza A viruses, the type most closely associated with human infections, are classified based on structural and antigenic characteristics of the two major viral glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [40]. The HA molecule (Fig. 5.3b) mediates attachment to sialic acid on the host cell surface and subsequent fusion of the viral and host membranes. The NA molecule facilitates the release of newly budding viral particles from the host cell by cleaving sialic acid residues from host and viral proteins [40, 138]. As is the case with HIV-1 envelope glycoproteins, both HA and NA proteins display a high

degree of antigenic diversity and are prone to evolve mutations to evade host immune surveillance. However, the HA stem domain, which is involved in the pH-dependent endosomal membrane fusion, is a more conserved region in the viral glycoprotein [11, 23].

Similar to anti-HIV-1 mAb discovery, attempts to discover broadly neutralizing mAbs against influenza have been focused on single B cell sorting and *in vitro*-activated B cell screening [25, 38]. Given the genetic diversity and rapid evolution of escape species, antibody therapy targeting the most conserved HA stem epitopes is a promising strategy for development of broad-spectrum protection against influenza viruses. Phage display technology has been applied to discover neutralizing mAbs from naïve- and immune antibody libraries constructed from infected and vaccinated donors [73, 140, 142]. Broadly neutralizing mAbs isolated from these libraries generally target the conserved stem domain and execute their neutralizing function by inhibiting endosomal membrane fusion. Moreover, the IGHV1–69 germline immunoglobulin gene (Fig. 5.4) is frequently used for influenza HA stem targeting broadly neutralizing antibodies [120, 140].

Synthetic antibody strategies have also been applied to explore specific immunoglobulin signatures associated with broad neutralization [3]. For example, a semi-synthetic library heavily biased towards the germline IGHV1–69 sequence was designed and used in phage display selection. The results revealed that hetero-subtypic binding and neutralizing activity of IGHV1–69 based mAbs is conveyed by a critical amino acid triad consisting of a pair of anchor residues in CDR-H2 and a properly positioned CDR-H3 Tyr, and as few as two V-segment substitutions, one of which is localized in CDR-H2 [3]. In a more recent study, CDR sequence preference in the heavy chain of a broadly neutralizing antibody (F10) was systematically investigated [145]. F10 is derived from IGHV1–69 and also targets the conserved HA stem epitope. In this study, one

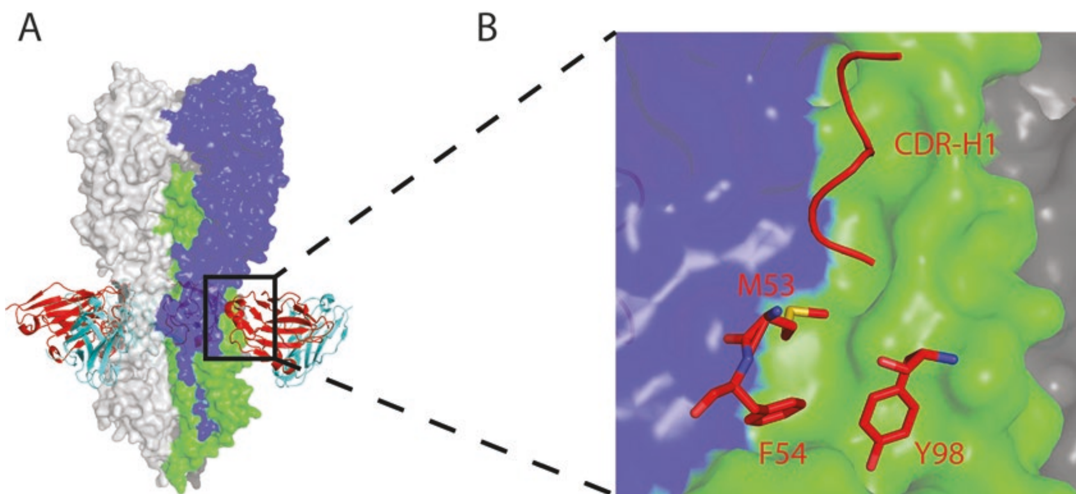

Fig. 5.4 Structural basis of germline IGHV1–69 encoded broadly neutralizing Abs against influenza A viruses. (**a**) Structure of the H5 homotrimer bound to scFv F10 (PDB entry: 3FKU) [140]. The H5 homotrimer is shown in surface-shaded view and color-coded as in Fig. 5.3. The F10 scFvs are shown as cartoons with VHs colored in red and VLs colored in cyan. (**b**) Close-up of the H5 binding interface showing H5 trimer as surface-shaded view, with the presence of signature paratope residues from F10. F10 binds to the highly conserved membrane-proximal patch on the HA stem region mainly through the germline-encoded Met53 (or Ile53 in other Abs), Phe54 in CDR-H2 and Tyr98 in CDR-H3. CDR-H1 normally interacts with a membrane-distal patch of the HA stem region through diverse configurations among IGHV1-69 encoded broadly neutralizing Abs against influenza A viruses, and in the case of F10, CDR-H1 adopts a noncanonical conformation that contacts HA with extensive intermolecular hydrogen bonding and hydrophobic interactions

library for each of the three heavy chain CDRs was constructed by completely randomizing certain CDR residues. The three libraries were screened against HA antigens from two influenza isolates. The results were used to guide the design of a fourth synthetic antibody library, from which clones with up to sevenfold higher neutralizing potency compared with the parent F10 were identified. These studies, aided by synthetic antibody approaches, help further our understanding of the structural requirements that enable a precursor IGHV1–69 germline based Ab to become a potent HA stem-directed cross-neutralizing antibody as well as the potential to identify HA stem domain targeting antibodies with higher neutralizing potency.

It has been suggested that the IGHV1–69 germline has evolved to intercept viruses in their acute phase in an SOS response before the immune system gets time to mount a more effective response by somatic hypermutation [97, 98]. Interestingly, the versatility of the human antibody repertoire is demonstrated by the identification of antibodies to the same epitope that use a different heavy chain gene (VH3-30) using phage display selection from a Fab library derived from a healthy donor [156]. Remarkably, computational protein design has been used to generate synthetic proteins *de novo* that bind to this conserved epitope [37], which have subsequently been optimized using insights from next-generation sequencing [153] and have been shown to provide *in vivo* protection in mice independently of a host immune response [78].

5.2.3 Bacterial Toxins

Although monoclonal antibodies have had limited success in treating bacterial diseases, promising outcomes have been reported in treatment of bacterial toxin-mediated diseases. Bacterial toxins are a class of highly conserved virulence factors, which play an essential role in pathogenicity. Toxin neutralization provides the potential to intervene in the pathology of the disease, alleviate host symptoms caused by infection, and may allow the host immune system to clear the bacterial infection more effectively [8]. Indeed, this strategy underlies the USFDA approval of raxibacumab (Abthrax™; 2012) and bezlotoxumab (Zinplava™; 2016), which target anthrax toxin and *Clostridium difficile* toxin B, respectively. An anticipated advantage of this approach is that it is unlikely to lead to resistance, since the antigen is separated from the toxin-producing organism, and shows that antibody therapy can effectively and safely ward off infection by targeting a bacterial toxin [110].

Recently, our synthetic Fab Library F was applied to develop mAbs targeting bacterial toxins and virulence factors. By selection against the receptor-binding component of *Clostridium difficile* transferase (CDT), a binary toxin also produced by this bacterium, a panel of synthetic antibodies was identified and demonstrated to functionally neutralize CDT cytotoxicity in cell-based assays [155]. In addition, Library F was utilized to identify neutralizing antibodies against staphyloccoccal enterotoxin B (SEB), a potent bacterial superantigen that may induce massive release of inflammatory cytokines leading to toxic shock and multiorgan failure [72]. Through synthetic antibody engineering, antibodies with sub-nanomolar antigen binding affinity were developed from initial clones, and the lead candidates demonstrated full protective efficacy from lethal SEB challenge in a mouse model. Promising synthetic antibodies to the same antigen have also been isolated [89] from the HuCAL library [76]. Moreover, Library F was recently used to isolate antibodies that neutralize Shiga Stx2 toxin *in vitro* [103].

5.2.4 Ebola Viruses

Ebola virus (EBOV) and Sudan virus (SUDV) are the most pathogenic species among the ebolaviruses, a major genre of the enveloped negative-sense RNA viruses of the filovirus family that cause severe hemorrhagic fever (Lee and Saphire [32, 85, 94, 95, 108]). The most recent 2014 Ebola virus outbreak in Western Africa highlights the urgent need for effective pre- and post-exposure treatments for ebolavirus infec-

tions. Unfortunately, there is currently no approved treatment or vaccine for these infections [109]. There are quite a few mono-specific therapies or vaccines at different stages of development, including post-exposure vaccines [33, 44, 45, 31, 43], small molecule inhibitors [26, 149], siRNA-based therapeutics [46, 47], and mAbs [132, 154]. Notably, an experimental ebolavirus-specific mAb cocktail, Zmapp [126], was used compassionately in several patients during the 2014 outbreak, demonstrating that antibody therapy holds significant promise and that the synergistic effect conferred by targeting more than one epitope may be the key to success [111, 126]. Subsequent studies have demonstrated that use of two antibodies [125] or only one [24] may be sufficient and that novel approaches using bispecific antibodies offer a promising alternative strategy [150].

Viral attachment to host cells is mediated by a glycoprotein termed GP, which is the only protein expressed on the envelope surface [131] (Fig. 5.3c). The mature filovirus GP is a trimer of three disulfide-linked GP1-GP2 heterodimers, generated by endoproteolytic cleavage of the precursor polypeptide by furin during virus assembly ([93]; Lee and Saphire [94, 95, 28]. GP1 is responsible for viral adhesion to host cells, whereas GP2 mediates fusion of the viral membrane with cellular endosomal membranes during cell entry. GP1 contains a head domain harboring a receptor-binding region, which is capped by a glycan and a heavily glycosylated mucin-like domain [93]; the base of GP1 interacts extensively with GP2 and clamps it in its prefusion conformation. The mucin-like domains and glycan caps are cleaved from the viral surface GP by host cathepsins in the endosome once filoviruses enter cells [13, 18]. This cleavage renders GP accessible for Niemann Pick C1 (NPC1) receptor binding [15, 26]. After enzymatic cleavage and receptor binding, the GP2 subunit disengages from its GP1 clamp and undergoes a series of irreversible conformational changes to form a six-helix bundle that drives the fusion of virus and host membranes [63, 105, 152].

Although encouraging progress has been made recently, the precise timing of endosomal proteolytic cleavage and concomitant exact structural changes in GP remain to be further investigated. Reagents targeting fusion intermediates of the EBOV GP will not only be greatly helpful to dissect GP intermediates in the fusion pathway, but also for potential use as immunotherapeutics or diagnostics. Natural human Ebola virus antibodies are a limited source for antibodies targeting proteolytically cleaved GP as the epitopes on the cleaved GP are normally inaccessible to the host immune system. In contrast, synthetic antibody libraries are more suitable since the repertoire is unbiased and *in vitro* display enables selection pressure directly against target epitopes inaccessible during immunization. In this regard, we and others have made encouraging attempts in developing mAbs targeting viral epitopes from synthetic antibody libraries. By using uncleaved (GP_{UNCL}) and proteolytically cleaved (GP_{CL}) forms of GP from EBOV as antigens, we successfully identified novel mAbs capable of distinguishing between GP_{UNCL} and GP_{CL} [79]. Two Fabs identified from Library F bound selectively to GP_{UNCL} or GP_{CL}, respectively. Neutralization assays with GP-containing pseudotyped viruses indicated that these antibodies inhibited GP_{CL} or GP_{UNCL}-mediated viral entry with specificity that matched their recognition profiles. This indicates that epitopes on GP_{CL} could be targeted for viral neutralization by antibodies, and these antibodies may also be useful tools in dissecting intermediates of EBOV entry. Hence, this study clearly demonstrates that highly specific functional antibodies, which have orthogonal recognition and neutralization profiles for distinct viral membrane fusion intermediates, could be obtained through *in vitro* synthetic antibody engineering. This illustrates a clear advantage of recombinant antibody development against viral targets for which limited sources of human antibodies exist in nature.

In addition to developing antibodies targeting EBOV fusion intermediates using *de novo* selection from synthetic antibody libraries, this technology can be used for humanization of non-human antibodies, which may evoke immune responses leading to increasing rates of antibody clearance and ultimately hampered efficacy if

administrated in humans [2, 130]. We recently applied structure-guided synthetic antibody engineering to humanize a SUDV-specific antibody 16F6 [20]. 16F6 is a murine Ab identified by mouse immunization [28], which limits its therapeutic utility. In search of an appropriate antibody template for 16F6 humanization, we found that 16F6 and YADS1, a humanized vascular endothelial growth factor (VEGF)-specific synthetic Ab derived from the common VH3-23 germline gene [36], have high sequence homology in the framework regions and strong structural homology of framework segments leading into the CDR loops. This analysis suggested that the YADS1 scaffold might be suitable for incorporation of 16F6-like recognition to generate anti-SUDV antibodies bearing a human framework. We then designed and constructed a 16F6 humanization library based on a chimeric template where CDR segments from 16F6 were grafted onto the YADS1 scaffold with tailored mutations introduced in selected framework positions. The 16F6 humanization library was screened against soluble GP_{SUDV} protein and a total of 17 unique clones were identified. These antibodies were produced as IgG1 molecules and their neutralizing efficacy was tested in a pseudotype virus infection model. The most potent candidates demonstrated efficacy at levels on par with murine 16F6 and had no activity against EBOV, which indicates that they maintained the specificity profile of the murine 16F6 [20]. Two clones, E10 and F4, were further shown to potently inhibit authentic SUDV and confer protection in mice from lethal SUDV challenge at similar levels as murine 16F6. These antibodies represent promising immunotherapeutic candidates for treatment of SUDV infection.

5.2.5 Engineering of Antibodies Recognizing PML-Specific Mutants in Polyomavirus JC (JCV)

Polyomavirus JC (JCV) is a highly prevalent human pathogen believed to be the causative agent of progressive multifocal leukoencephalopathy (PML), a rare but frequently fatal brain disease that afflicts a small fraction of the infected population with compromised immune system, including HIV patients and transplantation recipients on immunosuppressive therapy [54, 77, 123]. No specific therapy is available for PML, and the primary treatment relies on reconstitution of the patient's own immune response [75, 141]. Currently, interest focuses on developing diagnostic tools that could identify patients with higher risk of PML, thus reducing the risks associated with immunomodulatory therapy [146]. Although the usefulness of such diagnostics is under debate, some progress has been made so far [53, 84, 129].

Recent studies on the major capsid viral protein 1 (VP1), the key component of JCV pathogenesis, provide valuable insights into potential PML-specific biomarkers that may be essential for risk stratification of PML development [115, 116, 141]. A comprehensive analysis of JCV VP1 sequences isolated from both PML patients and healthy individuals indicated that a subset of PML-specific mutations in JCV VP1 sequences, such as leucine to phenylalanine mutation at position 55 (L55F) and S269F, might favor PML onset [141]. Therefore, early diagnosis of these PML-specific mutations may help identifying patients at high risk of PML. Monoclonal antibodies are powerful reagents in analysis of the viral protein mutant, owing to their exquisite epitope specificity. However, mutant specific mAbs with high specificity and affinity are difficult to obtain through traditional animal immunization and hybridoma technology, as mAbs cannot be directed to desired epitopes particularly in the extreme case of a single point mutation. However, *in vitro* selection can direct selection pressure to the conformational epitope centered at the mutated residues. Moreover, phage display-based antibody engineering can be readily applied to improve initial antibodies with desired properties. In searching for reagents recognizing PML-specific mutations potentially useful in diagnostics, we recently developed antibodies that specifically target the L55F mutation on JCV VP1 through phage selection and synthetic antibody engineering [19]. From synthetic antibody

Library F, antibody fragment GC058 with preferential recognition of L55F mutant over the wild type VP1 protein was initially isolated. Homolog-scanning combinatorial mutagenesis was applied to GC058 to analyze the functional contributions of individual side chains of selected CDR loops to antigen recognition. Based on the results from homolog-scanning analysis, a next generation library was constructed for specificity optimization, which yielded antibodies with much higher specificity against the L55F mutant compared with the parental GC058 [19]. This iterative process of developing antibodies specific for mutant proteins showcases the great potential of synthetic antibody engineering, which enables precise control of targeting desired epitopes.

5.3 Summary and Future Outlook

The majority of anti-infective therapeutics on the market and in development are small molecules. However, there is now a nascent pipeline of biological agents in development [70]. Antibody therapy is a promising strategy to meet current challenges of increasing multidrug resistance, inability to treat immunocompromised patients, risk of bioterrorism and new emerging diseases. Interest in antibody therapy for infectious disease has experienced resurgence and technological developments in the manufacturing of therapeutic antibodies will hopefully reduce the production cost and enable broader utilization. However, the transition from traditional broad spectrum targeting to precision medicine requires a deeper understanding of bacterial targets and pathogenesis as well as humoral antibody responses. Since the first in-depth characterization of an antibody library by deep sequencing [51], next-generation sequencing now enables identification of high frequency native VH:VL pairs in immunized subjects [48, 69, 92]. This strategy was recently applied to identify and express antibodies targeting ebolaviruses [148]. Insights from deep sequencing will likely play a central role in future efforts to design targeted synthetic antibody libraries and vaccines. Moreover, sequencing of many microbial genomes has yielded several new potential targets. However, limited biochemical knowledge makes it difficult to develop novel therapies. As an example of the complexity of infectious disease, the recent Zika virus epidemic took place in Dengue virus endemic areas where the population has acquired antibodies to this related virus. Recent studies indicate that pre-existing dengue antibodies may cross-react with Zika and thereby enhance Zika infection. It has been hypothesized that Zika virus entry through Fcγ receptors on macrophages and monocytes, facilitated by cross-reactive non-neutralizing antibodies, is responsible for this phenomenon [29].

We believe that synthetic antibody repertoires hold particular promise to provide novel insights in infectious disease and generate novel antibodies that cannot be produced by the natural immune system. Optimal implementation of synthetic antibody phage technology requires creation of large, high quality, highly functional libraries followed by functional screening. Binding sites and frameworks can be precisely tailored and the entire process can be performed in a controlled fashion. Synthetic strategies can utilize stable, highly expressed, fully human frameworks and design principles that are easily converted between formats, which will hopefully translate into higher success in the drug discovery process. Moreover, focused synthetic libraries can be designed to target a specific type of epitope by using variable regions encoding combinations of canonical structures that resemble the structural features of antibodies that bind the desired class of ligands. For example, synthetic antibody libraries specialized in peptide binding [22], hapten binding [121], carobohydrate binding [134] or binding to phosphorylated amino acids [80] have been generated. Another interesting development of synthetic antibody libraries is incorporation of an expanded genetic code in the directed evolution of proteins with specific properties. For instance, a synthetic scFv library with unnatural amino acid diversity in CDR-H3 was used to select a binder to gp120 that contained a sulfotyrosine [100]. Additional exciting progress includes phenotypic screens using cells harboring antibody libraries [158] and continuous *in vitro* evolution systems [4].

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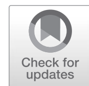

6 Monoclonal Antibodies and Antibody Like Fragments Derived from Immunised Phage Display Libraries

Obinna Ubah and Soumya Palliyil

Abstract

Morbidity and mortality associated with infectious diseases are always on the rise, especially in poorer countries and in the aging population. The inevitable, but unpredictable emergence of new infectious diseases has become a global threat. HIV/AIDS, severe acute respiratory syndrome (SARS), and the more recent H1N1 influenza are only a few of the numerous examples of emerging infectious diseases in the modern era. However despite advances in diagnostics, therapeutics and vaccines, there is need for more specific, efficacious, cost-effective and less toxic treatment and preventive drugs. In this chapter, we discuss a powerful combinatorial technology in association with animal immunisation that is capable of generating biologic drugs with high affinity, efficacy and limited off-site toxicity, and diagnostic tools with great precision. Although time consuming, immunisation still remains the preferred route for the isolation of high-affinity antibodies and antibody-like fragments. Phage display is a molecular diversity technology that allows the presentation of large peptide and protein libraries on the surface of filamentous phage. The selection of binding fragments from phage display libraries has proven significant for routine isolation of invaluable peptides, antibodies, and antibody-like domains for diagnostic and therapeutic applications. Here we highlight the many benefits of combining immunisation with phage display in combating infectious diseases, and how our knowledge of antibody engineering has played a crucial role in fully exploiting these platforms in generating therapeutic and diagnostic biologics towards antigenic targets of infectious organisms.

Keywords

Monoclonal antibodies · Phage display · Immunisation · Combinatorial technology · Infectious diseases · Diagnostic · Therapeutic

6.1 Introduction

The number of monoclonal antibody (mAb) based drugs developed by biopharmaceutical companies is at an all-time high with more and more novel anti-infective antibodies gaining regulatory approval. The emergence of multi-drug resistance has reinforced the need to develop novel anti-infective approaches. Unlike conventional antibiotics,

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monoclonal antibodies exhibit high target specificity and possess the ability to recruit immune system components for effective pathogen removal. Modes of action include: specific binding and neutralisation of microbial toxins and virulence factors, opsonising and marking pathogens for cell-death by directing phagocytic cells to the site of infection, antibody mediated bacterial agglutination and clearance, complement activation and direct bacterial lysis [77]. Host effector functions mediated through interactions with the Fc region of mAb drugs makes them highly effective in treating immunocompromised patients that are unable to generate their own immune response to fight diseases [90]. Certain monoclonal antibodies exhibit synergistic or additive effects with conventional antibiotics providing an attractive therapeutic strategy for treating infections caused by multidrug resistant organisms [1]. This chapter discusses the advantages and disadvantages of using immunised phage display libraries constructed from mammalian sources for generating monoclonal antibodies against infectious disease targets. Several groups have successfully developed monoclonal antibodies specific for bacterial, fungal and viral antigens with potential applications in the detection, diagnosis and treatment of infectious diseases which are reviewed in this chapter.

6.2 Monoclonal Antibody Generation Technologies

Originally mAbs were isolated using hybridoma technology, and required the fusion of mouse lymphocyte and myeloma cells to generate specific murine antibodies that were often highly immunogenic if used in a therapeutic setting. A limited number of human mAbs were also developed by using human B lymphocytes from naturally infected patients but this proved a technically challenging and generally unreliable approach [48]. Subsequent advances in antibody engineering removed or resolved many of these technology “road-blocks” facilitating the generation of chimeric, humanised or deimmunised mAbs and bispecific antibodies with increased potency and reduced immunogenicity. One of the major breakthroughs was the invention of phage display which revolutionised the field of antibody engineering with its robust, easy to use and highly versatile combinatorial display platform. Its successful applications include: generation of antibodies with unique functions from immune and non-immune sources, *de novo* isolation of high affinity binders from non-immune or synthetic sources and the *in vitro* affinity maturation of antibodies [43]. Phage based selection could be summarised as the display of antibodies (proteins or peptides) on the surface of bacteriophage by fusing the antibody gene to one of the phage coat proteins and selection based on the antigen binding of individual clones. Phage antibody libraries are constructed by PCR based cloning of VH and VL repertoires by random pairing into a phage or phagemid vector system and display on the surface of bacteriophage. It has been used widely in the antibody engineering as a technique to mimic B cells, which are self-replicating packaged systems containing antibody genes that encode the antibody displayed on its surface (linked genotype and phenotype) [108].

6.3 Phage Display Antibody Libraries

Phage display libraries can be constructed using antibody variable (v) genes isolated from IgM mRNA of non-immunised human donor B cells derived from diverse lymphoid sources such as peripheral blood lymphocytes (PBLs), spleen cells, tonsils, bone marrow or from non-immunised animal B cells (naïve antibody libraries). IgG mRNA from PBLs or spleen cells of immunised animals or human patients are used to build immunised libraries. A third class called synthetic and semi-synthetic antibody libraries are constructed using repertoires of rearranged V genes from gene segments using polymerase chain reaction (PCR) and introducing variation into their CDR regions using custom degenerate primers encoding for diversity and length [108].

Immunised libraries are generally constructed using antibody VH and VL genes amplified from

the mRNA of B cell pools from immunised animals or human donors. Post immunisation, the antibody secreting B cells possess a higher percentage of antigen specific heavy and light chain gene transcripts. These B cells would have undergone affinity maturation and isotype switching in germinal centres before entering into the peripheral circulation. Typically antibodies isolated from immunised libraries will have a high affinity and tighter specificity for the immunogens used [8] and can be achieved even from relatively small library diversity [108].

Unlike hybridoma technology, phage display provides researchers with more possibilities to streamlining their monoclonal antibody generation process. The main improvement is the replacement of cell culture screening steps using hybridomas. Instead of screening hundreds or thousands of hybridoma monoclonals to find positive clones, phage display, with carefully designed panning strategies, can screen billions of clones and allow the enrichment of small subsets of binders with desirable characteristics that can be further screened to identify individual binders. Antibodies recognising specific antigenic conformations and epitopes can be easily isolated by introducing selective or subtractive panning steps during the selection process. It has also been reported that using phage display high specificity antibodies can be isolated from immunised sources which would be missed by traditional immunological methods [100]. A key feature of this system is the linkage of genotype (phagemid vector) and phenotype (phage coat protein- antibody fragment) which allows immediate access to the corresponding gene sequences of selected antibodies facilitating simple subcloning into various antibody formats based on required downstream applications (Fig. 6.1). Over the last 20 years phage display technologies have been successfully used for the development of many therapeutic antibody candidates and approved drugs and with the relaxation of commercial restrictions more and more products are now entering the diagnostic and research markets as well [9].

The drawbacks of animal immunisation for the construction of phage display libraries include the time period required for the completion of such a process and the requirement to construct separate libraries for each antigen. However, by combining the power of immunisation with phage display, several high affinity monoclonal antibodies against "difficult" antigenic targets have been isolated from relative small antibody libraries and where traditional approaches have failed [33, 98]. Generating antibodies against self or toxic antigens is limited if immunisation is to be employed. In particular, human autoantigens are highly conserved amongst most routinely used laboratory mammals such as mice or rat. Therefore immunisation of non-mammals such as sharks and chickens, which are phylogenetically distant from humans, has been successfully employed for generating an immune response and antibodies and/or antibody like binders (VNAR) to epitopes conserved in mammalian species [42]. Chickens are phylogenetically distant from humans and therefore very successful in generating an immune response to mammalian proteins which are highly conserved [34], while sharks are even more distant from humans, diverging from a common ancestor approximately 450 million years ago [6, 37, 53].

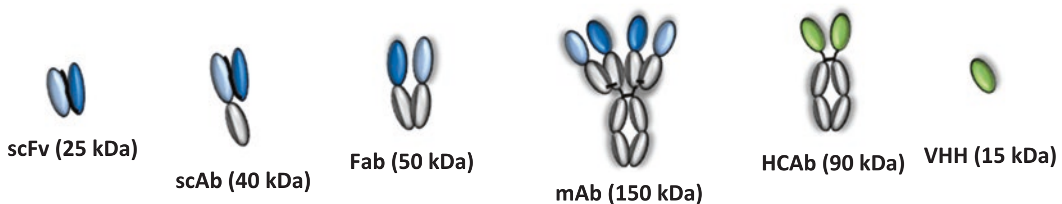

Fig. 6.1 Various antibody formats described in this chapter and their molecular mass

6.4 Anti-infective mAbs from Human Immune Libraries

Phage display libraries constructed from immune sera or bone marrow cells collected from infected patients have been used to isolate neutralising antibodies against viral infections. Human monoclonal antibodies that can selectively recognise different strains of influenza virus, Ebola virus, HIV, Herpes Simplex virus, rabies, and hepatitis B have been successfully isolated from immune human phage display libraries that have the potential for both diagnosis and therapy [13, 14, 54, 66, 76, 89, 111, 112]. Human monoclonal antibodies from patients which showed protective effects in animal model studies have also been selected against bacterial targets such as anthrax toxins [107], botulinum neurotoxin [4] and the ABC transporter of methicillin resistant *Staphylococcus aureus* [12]. In the case of the West Nile Virus neutralising mAbs were only isolated from the B cell population of convalescent human patients with large phage display libraries constructed from uninfected donors delivering nothing of consequence [105]. Conventional methods of antibody generation such as hybridoma and Epstein-Barr virus transformation are limited in their abilities to evaluate human monoclonal antibodies from different patients at various stages of their clinical course. Technologies like phage display provide a powerful tool that allows pooling of large number of patient immune B cell populations and the selecting of antibodies with distinct specificities and inhibitory activities during different stages of infection [13]. The ability to improve affinities and broaden specificities post selection is a major attribute of recombinant antibodies. Using recombinant DNA and protein engineering approaches the pharmacokinetic and pharmacodynamics properties of these molecules can be improved including half-life extension and reduced immunogenicity. For the neutralisation of toxins and viruses, it is often advantageous to generate multiple high affinity antibodies that recognise different epitopes on the same antigen or a variety of antigenic subtypes capable of delivering broad protection against pathogen variants. Such 'oligoclonal' antibodies were reported to show strong synergistic activity in neutralising botulinum neurotoxin (BoNT) assays [74]. A list of recombinant antibodies generated from phage display libraries constructed using the immune antibody gene repertoires of human patients is given in Table 6.1.

6.5 Antibacterial Phage Antibodies

Several research groups have successfully demonstrated the potential of monoclonal antibodies, fragments and single domain antibodies for the prevention and treatment of bacterial infections in animal models. However their translation into the clinic has been somewhat slow. A small number of mAb drugs under regulatory review include Obiltoxaximab, for the treatment and prevention of inhalational anthrax and Bezlotoxumab, which targets *Clostridium difficile* enterotoxin B, developed for the prevention of recurrent *C. difficile* infection [83]. With the ever increasing numbers of antibiotic resistant bacterial strains, the need to develop antibody based drugs with novel modes of killing has never been greater especially if their mode of action limits the development of resistance. Antibodies developed against bacterial targets fall into two main categories: (i) antibodies that target the bacterial cell surface directly or (ii) those that act indirectly by neutralising bacterial toxins or virulence factors and relying on the host immune system for effective pathogen clearance. Monoclonal antibodies and fragments developed against various Gram−ve and Gram+ve bacterial targets using animal immunisation and phage display based selection are summarised below.

Table 6.1 Anti-infective recombinant antibodies (fragments) generated from phage display libraries constructed using immune antibody gene repertoire of human patients

Pathogen	B cell source	Nature of mAbs selected	References
Influenza virus	Paediatrician vaccinated against influenza	Neutralising antibodies to 12 H3N2 strains of influence virus	Okada et al. [76]
Ebola virus	Bone marrow cells and peripheral blood monocytes from convalescent human donors	Recognises an immunodominant epitope on nucleoprotein which is conserved in all four subtypes of Ebola virus	Meissner et al. [66]
HIV	Bone marrow from an asymptomatic HIV+ve individual	High affinity monoclonal antibodies against gp120 of type 1 HIV	Burton et al. [13]
HIV	Bone marrow from long term nonprogressor HIV+ve patients whose sera showed high levels of HIV-1 neutralisation.	Broad HIV neutralising monoclonal antibodies binding to gp120 and gp140 which inhibited envelop mediated viral entry into the host	Zhang et al. [112]
Herpes simplex virus	Bone marrow from an asymptomatic HIV+ve individual with serum titre for HSV-1 and HSV-2	Fab fragments binding to glycoprotein B and D that are able to neutralise HSV-1 and HSV-2 and strongly reduced virus infectivity	Burton et al. [13] and Sanna et al. [89]
Rabies virus	Blood from vaccinated donors	High affinity neutralizing antibodies directed against antigenic site III of the viral glycoprotein that can be used as an antibody cocktail in rabies post-exposure prophylaxis.	Kramer et al. [54]
Hepatitis B	Lymphocytes from human volunteers vaccinated with recombinant HBsAg	High affinity Fab fragments binding to Hepatitis B surface antigen (HBsAg)	Zebedee et al. [111]
West Nile Virus	B cell population of two convalescent patients	Two strongly neutralising mAbs that inhibited WNV infection *in vitro* and *in vivo*	Vogt et al. [105]
Bacillus anthracis – anthrax toxin	Bone marrow or blood from patients vaccinated against anthrax	Neutralising subnanomolar affinity Fabs that bind to protective antigen (PA) 63, and inhibit lethal factor (LF). Fabs showed protective effect in a rat model of anthrax intoxication	Wild et al. [107]
Clostridium species – botulinum neurotoxin	Blood from a human volunteer immunized with pentavalent botulinum toxoid (A–E).	Neutralising antibodies against the immunodominant epitopes of botulinum neurotoxin	Amersdorfer et al. [4]
Methicillin resistant *Staphylococcus aureus*	Blood from patients with septicaemia caused by methicillin resistant *S. aureus*	Monoclonal antibodies against ABC transporter of *S. aureus* which showed protective effect in a mouse model of infection	Burnie et al. [12]

6.5.1 Gram Negative Bacterial Targets

6.5.1.1 Blocking Quorum Sensing in *Pseudomonas aeruginosa*

Infection control using high affinity monoclonal antibodies specifically targeting the quorum sensing (QS) molecules of *Pseudomonas aeruginosa* has been reported from our laboratory [78]. A number of Gram negative bacteria, including pathogens like *P. aeruginosa*, utilise homoserine lactones (HSLs) as QS signalling compounds and engage in cell-to-cell communication to coordinate their behaviour. As QS takes a central role in *P. aeruginosa* infection by regulating the expression of extracellular virulence factors (and also

biofilm formation), immuno-modulation of the HSL molecules by monoclonal antibodies (mAbs) can be used as a novel approach to prevent *P. aeruginosa* infections. Sheep immunisation was utilised to develop antibodies with high affinity and sensitivity towards HSL compounds. A mixture of three HSLs-N-acyl-C12-HSL, 3-oxo-C12-HSL and 3-OH-C12-HSL with different subgroups at the third carbon position were conjugated to the carrier protein Thyroglobulin (TG) and used as immunogen to enhance the chance of eliciting an antibody response in sheep. Using PBLs from immunised sheep as starting material, V_H-V_λ and V_H-V_κ anti-HSL phage display libraries were constructed in a scFv format. The panning strategy was designed to drive selection towards the enrichment of high sensitivity and cross-reactivity clones. Lead clones were reformatted into sheep-mouse chimeric IgGs and had picomolar sensitivities (IC_{50} values as determined by competition ELISA) for 3-oxo-C12-HSL which is the central QS compound in *P. aeruginosa*. These values are quite impressive, considering the chemical nature of these lipid-like compounds (average molecular weight 300 Da) which possess only a small head like structure and lack critical antigenic features such as aromaticity or charge. Modelling of these sensitive anti-HSL antibodies indicated that the level of sensitivity observed was achieved through the generation of a deep and negatively charged binding pocket [2].

Sheep immunisation was chosen for this HSL application as this approach has previously been shown to generate high affinity antibodies against haptenic targets [17] and sheep polyclonal antibodies have been used as specific high affinity immunologic probes for analytical and clinical purposes for many years [62]. In contrast to humans and mice, sheep B cell lymphopoiesis occurs predominantly in the ileal Peyer's patches (IPP) and V(D)J recombination creates limited diversity in the sheep Ig repertoire due to a very few gene segments participating in the rearrangement process. This characteristic feature makes sheep library construction simpler, as the entire heavy and light chain gene repertoire can be amplified using a small number of primers. Much of the antibody diversity is achieved through antigen-independent post-rearrangement somatic hypermutation, which diversifies all CDRs as compared to the sole variability of just CDR3 in humans and mice [17, 62, 78, 84]. Comparison of chicken and sheep responses by hyperimmunisation with the same immunogen revealed the ability of the sheep immune system to produce higher specificity antibodies than chicken [109]. The overall antibody titre was also much higher in sheep and might be due to the longer half-life (~15 days) of sheep immunoglobulins when compared to 35 h in chicken [106, 109]. It is also noteworthy that the sensitivities of the sheep polyclonal/mAb antibodies described here were far superior to the published mouse monoclonal antibody specific for the same HSL antigens [50].

The protective effects of the sheep derived, anti-quorum sensing antibodies were demonstrated *in vivo*. In a slow killing model of the nematode worm *Caenorhabditis elegans* the significant increase in survival rate in the presence of HSL mAbs is similar to the defective slow killing observed in *lasR* mutant strains [99]. Furthermore, in a non-neutropenic lung model of mice infected with *P. aeruginosa* PA058, HSL mAb monotherapy demonstrated significant efficacy, prolonging survival by up to 83%. Since no significant reduction in bacterial counts was observed in the lungs of infected mice, it is proposed that HSL specific antibodies protect mice possibly through antibody mediated scavenging of HSL compounds. This mode of action does not necessarily affecting bacterial numbers but probably prevents a switch to a more pathogenic phenotype [78].

It has been widely accepted that antibodies with sub-nanomolar affinity exhibit improved therapeutic efficacy, if the targets to be bound are present in low concentrations. The above study demonstrates the power of animal immunisation and phage display based selection strategies to

isolate high affinity monoclonal antibodies towards non-antigenic targets which inherently lack properties like aromaticity and charge.

6.5.1.2 Bacterial Toxins

ADP-ribosylating enzymes such as cholera, pertussis, diphtheria toxins and *Escherichia coli* heat-labile (LT) toxins are important virulence factors for a number of extracellular bacterial pathogens. Pathogenesis is driven by the secretion of potent toxins that utilise ADP-ribosylation as the catalytic mechanism underlying their action. ADP-ribosylating toxins comprise a large family, and all produce disease by altering key metabolic processes after transfer of an ADP-ribose moiety from NAD to specific host-cell target proteins [25, 60]. It was not until the early 2000 that the ADP-ribosylating enzyme was implicated in intracellular pathogenesis. It was shown that *Salmonella* strains were capable of invading epithelial cells and localising in macrophages during infection [60, 61]. The *Salmonella* virulence plasmid factor B (spvB) virulence gene of *Salmonella* is required for human macrophage cytotoxicity *in vitro* and for enhancing intracellular bacterial proliferation during infection. Lesnick *et al.* provided evidence that spvB encodes an ADP-ribosylating enzyme that uses actin as a substrate and depolymerises actin filaments when expressed in CHO cells [61]. A spvB blocking camel VHH single domain antibody (sdAb) capable of blocking spvB enzymatic activity at a 1:1 molar ratio was isolated from an immune phage display library generated from an spvB immunised llama [3, 68]. As an intracellular protein, spvB is inaccessible to conventional antibodies, and small molecule inhibitors of spvB are fraught with potential side effects resulting from the indiscriminate inhibition of endogenous mammalian ADP-ribosyltransferases (ARTs) [11, 51, 63]. The VHH sdAb when expressed as an intrabody, effectively protected cells from the cytotoxic activity of a translocation-competent chimeric C21N-C/spvB toxin, and transfected cells were also protected against cytoskeletal alterations induced by wild-type spvB-expressing strains of *Salmonella* [3]. This provides evidence to support the development of these sdAbs as therapeutic and experimental tools to block mammalian and toxin ARTs.

6.5.1.3 Targeting Bacterial Surface Antigens

Helicobacter pylori is a gram negative pathogenic bacteria that colonises the human stomach and can cause gastritis, gastric and duodenal ulcers and cancer. The surface proteins in *H. pylori* mediate several host-pathogen interactions and hence are attractive targets for antimicrobial therapy and vaccination. An antibody phage display library in a scFv format was constructed by hyperimmunising mice with *H. pylori* total cell lysate and a monoclonal antibody fragment recognising the outer membrane protein HopQ was isolated by performing biopanning on whole cells [88]. In another study a mAb generated through mice immunisation and hybridoma technology was engineered using phage-display as a scFv fragment and showed high affinity binding to an *H. pylori* surface antigen. This phage displayed scFv antibody was shown to inhibit the growth of six different *H. pylori* strains and offered significant protection in a mouse model of infection. The authors argued that genetically engineered bacteriophage could be used as alternatives to conventional antibiotics in the treatment of bacterial infections [15].

Spleen samples from mice immunised with gamma inactivated *Brucella melitensis* strain 16 M bacteria was used to construct a phage display library and isolate monoclonal antibody fragments that specifically recognise Brucella species. Brucella can cause long term debilitating illness in humans, can be spread as aerosols and survive extended periods outside their host. The attributes could make Brucella species possible biological warfare agents. Since many Brucella species share their immunodominant lipopolysaccharide (LPS) antigen with the closely related *Yersinia* species, a specific LPS antibody that can distinguish between these two bacteria is central for rapid detection and diagnosis of *Brucella* infection. Specific washing steps were included during bio panning of an immunised *B. melitensis* library to eliminate phage

that might be cross-reactive with strains expressing the same dominant LPS epitope on *Yersinia*. The resultant monoclonal antibody fragments was specific for the antigen and not cross-reactive towards *Yersinia* species [41].

6.5.2 Gram Positive Bacteria Targets

Botulinum neurotoxins (BoNTs), regarded as one of the most toxic substances on earth, are secreted by *Clostridium botulinum* and some other species of *Clostridium.* BoNT/A which is the most potent among seven serotypes exerts its toxicity by the cleavage of SNAP-25 (synaptosomal-associated protein), mediated by its light chain (BoNT/A-L). This proteolysis causes blockage of nerve impulses and causes flaccid paralysis, including that of respiratory muscles resulting in death [16]. Immunisation of macaques (*Macaca fascicularis*) and construction of hyper immune phage display library resulted in the isolation of nanomolar sensitivity antibody fragments to the light chain fragment of BoNT/A that are capable of inhibiting BoNT/A endopeptidase activity *in vitro.* The variable heavy and light chains of selected clones are highly similar to human germline sequences which predicts good tolerance for clinical use. Since immunoglobulin genes of non-human primates are very similar to human Abs, the differences in the conserved framework regions of macaque and human IgGs are no greater than those between human IgGs from different individuals [16].

Sub nanomolar affinity 'nearly human' Fab fragments against tetanus toxoid antigen were isolated from a small phage display Fab library constructed using the immune antibody repertoire of *Macaca fascicularis* [18]. Similarly high affinity neutralising antibody fragments against the protective antigen (PA) of anthrax toxin was isolated from a Macaca immunised phage display library. These Fab fragments were shown to bind to a particular region of PA that interacts with cell receptor thereby blocking its binding [56]. A single chain Fv phage display library was constructed from a cynomolgus macaque (*Macaca fascicularis*) immunised with Lethal Factor (LF) of anthtax toxin. Resultant high affinity scFv fragments showed efficient inhibition of anthrax toxin *in vitro* and *in vivo* [80]. In cases where anthrax vaccination is not practical or antibiotic therapy is ineffective, passive immunisation with anthrax neutralising antibodies can be an effective method of treatment.

Similar to macaques, chimpanzee immunoglobulins are very close to human antibodies and they should be well tolerated *in vivo* in human therapy [31, 92]. Lymphocytes from the bone marrow cells of two chimpanzees immunised with anthrax toxin PA, LF and Edema factor (EF) were used to construct scFv phage display libraries and neutralising antibodies were isolated against PA and LF proteins. For passive immunotherapy, these fragments were converted into bivalent full length immunoglobulins which showed strong neutralising activity against the cytotoxicity of anthrax toxin *in vitro*. These high affinity (picomolar range) mAbs demonstrated efficient protection in animals from anthrax toxin challenge *in vivo*, most likely by blocking binding of PA to the cell receptor which suggest their use in the emergency prophylaxis and treatment of anthrax [22, 23].

The nature of the antibody–antigen interaction allows for the development of molecules that can target bacterial antigens with a high degree of affinity and specificity. Target specificity of mAbs can be can be utilised to deliver antimicrobial compounds more effectively [97]. In addition, by choosing a suitable antibody Fc portion, host effector functions can be mediated through the recruitment of elements of the host immune system such as macrophages, NK cells and complement to sites of bacterial infection and accelerating clearance of the infection. Despite having a large number of candidates with promising efficacies in preclinical animal models of infection, anti-infective antibodies reaching clinical development and finally into the markets are somewhat slow. In most cases *in vivo* models can be limited in their ability to predict efficacy in humans, particularly due to differences in the pharmacokinetics of the molecules between humans and model animal species [110]. Geng

et al. reported that a large percentage of antibodies for anti-infective indications are ended in the early stage of discovery making them a high risk category in drug development [35]. Challenges in clinical efficacy and pharmacokinetics can be overcome by choosing appropriate format for the indication. For example, IgG Fc region interacts with the neonatal Fc receptor (FcRn) which is important for IgG recycling and protection from degradation, contributing towards unique pharmacokinetic properties of the molecule and extending the serum half-life to about 21 days for most IgG subclasses [86].

6.5.2.1 Single Domain Camel Antibodies to Fight Gram Positive Infections

The variable region (VHH) of Heavy chain only antibodies (HCAbs) found in *Camelidae* species possess an unusually long complementarity determining region 3 (CDR3) which can form finger-like extensions to penetrate into grooves on the surface of antigens that are usually inaccessible to conventional antibodies. *Staphylococcus aureus* produces several adhesion factors and exotoxins such as hemolysins α, β, γ, δ and Panton–Valentine leukocidin (PVL) which are important virulence determinants. *S. aureus* is a leading cause of endophtalmitis which is associated with a poor visual outcome. The expression of PVLs and leucotoxins especially LukS-PV and LukF-PV induce the activation and permeabilisation of target cells and result in cell lysis. Transgenic mice harbouring llama/human hybrid Ig heavy chain locus were immunized with recombinant LukS-PV and LukF-PV proteins. Using phage display technology high affinity VHHs were isolated and reformatted into bivalent and tetravalent camel heavy chain antibodies. Anti LukS-PV and LukF-PV HCAbs were able to inhibit PVL associated disease pathology in a non-infectious model of rabbit intravitreous PVL, when administered prophylactically. The HCAbs completely reduced inflammation in the eyes of the treatment group without any apparent damage to vision or behaviour. This shows the possibility of administering toxin neutralising antibodies in combination with an intravitreal antimicrobial strategy for post-surgery endophthalmitis [58].

Camel immunisation using *S. aureus* exoproteins and successful generation of anti β- hemolysin single domain antibodies was reported by Jangra and Singh [49]. In a neutralisation assay, their lead VHH clone completely inhibited five hemolytic units of the toxin *in vitro* and resisted thermal denaturation up to 99 °C. The CDR3 loop of VHHs is longer than the average IgG and linked with CDR1 by an interloop disulphide bond which renders additional stability to the protein. In some cases the long CDR3 loop enables the formation of a convex paratope that can access deep enzymatic clefts and cavities on the surface of an antigen [71]. The active sites of enzymes such as β- hemolysin are mostly situated in their largest cleft. The long CDR3 loop of anti β- hemolysin clone is believed to bind this site and able to neutralise enzyme activity by blocking access to the Hlb antigen [49].

VHH fragments specifically binding to tetanus toxoid 1 antigen were generated using camel immunisation and phage display technology. Two VHH fragments recognising two different epitopes of the antigen successfully neutralised tetanus toxin *in vivo* in studies conducted in mice [71, 72]. Another example where VHHs can inhibit bacterial infection by increasing the bacterial sensitivity to β-lactam antibiotics has been reported [24]. High affinity β-lactamase inhibiting VHHs were generated from immunised dromedary phage display libraries primed towards the antigens TEM-1 and BcII-lactamases, representatives of class A and class B-lactamases, respectively. In the presence of ampicillin, specific VHH fragments inhibited the growth of *E. coli* cells expressing a fusion protein of TEM-1 β-lactamase on their outer surface, whereas the cells were able to grown in higher concentrations of ampicillin (>150 μg/ml) when no VHH fragments were added [24].

The authors claim that VHHs are less immunogenic than larger murine antibodies as they show a high sequence similarity to human VH families 3 and 4. VHH nanobody dosed at 150 ng/kg capable of neutralising 3xLD_{50} of BoNT/E in challenged mice was generated from

Table 6.2 Summary of monoclonal antibodies, engineered fragments and single domain antibodies isolated from animal immunised phage display libraries against various bacterial targets

Target antigen	Immunised animal source	Application	Affinity-kD M	References
Pseudomonas aeruginosa Quorum sensing compounds	Sheep	Treatment of *P. aeruginosa* infections	ND	Palliyil et al. [78]
Helicobacter pylori surface protein	Mice	*Treating H. pylori* infection	ND	Cao et al. [15]
Helicobacter pylori outer membrane protein HopQ	Mice	Further characterisation of *H. pyroli* surface antigens	ND	Sabarth et al. [88]
Brucella melitensis	Mice	Rapid and specific detection of Brucella infections	ND	Hayhurst et al. [41]
Staphylococcus aureus LukF-PV protein	Transgenic mice harbouring llama/human hybrid Ig heavy chain locus	Treating *S. aureus* endophthalmitis	1.06e-9	Laventie et al. [58]
Staphylococcus aureus LukS-PV	Transgenic mice harbouring llama/human hybrid Ig heavy chain locus	Treating *S. aureus* endophthalmitis	3.18e-11	Laventie et al. [58]
S. aureus β- hemolysin	Indian desert camel	Developing immunosensor-based diagnostic test for detection of β- hemolysin secreting *S. aureus* isolates.	ND	Jangra and Singh [49]
Tetanus toxin	Llama	Treatment of infection or acute intoxication cause by tetanus toxin	ND	Muyldermans and Wyns [72]
Clostridium botulinum Botulinum neurotoxins (BoNTs)	Macaques (*Macaca fascicularis*)	Treatment of botulism by inhibiting BoNT/A endopeptidase activity	1.52e-9	Chahboun et al. [16]
Tetanus toxoid	Macaques (*Macaca fascicularis*)	Treatment of infection or acute intoxication caused by tetanus toxin	4e-10	Chassagne et al. [18]
Anthrax toxin PA	Macaques (*Macaca fascicularis*)	Prophylaxis and treatment of anthrax	3.4e-9	Laffly et al. [56]
Anthrax toxin LF	Macaques (*Macaca fascicularis*)	Prophylaxis and treatment of anthrax	1.02e-9	Pelat et al. [80]
Anthrax toxin PA	Chimpanzee	Prophylaxis and treatment of anthrax	4e-11	Chen et al. [20, 21]
Anthrax toxin LF	Chimpanzee	Prophylaxis and treatment of anthrax	0.69e-9	Chen et al. [22, 23]
Anthrax toxin EF	Chimpanzee	Prophylaxis and treatment of anthrax	0.12–0.5e-9	Chen et al. [22, 23]

an immune phage display library. A one-year-old male dromedary (*Camelus dromedaries*) was immunised with purified recombinant BoNT/E protein [5]. Another high affinity VHH inhibitor of BoNT/A protease activity was isolated from phage display libraries derived from B cells obtained from the immunised alpaca (*Lama pacos*) [101].

One of the main advantage of using animal immunised phage display libraries for generating antibodies against bacterial antigens is the easy isolation of high affinity neutralising antibodies without the need for further affinity maturation. Table 6.2 summarises monoclonal antibodies and antibody fragments against various bacterial targets and their affinities to target antigens.

6.6 Anti-fungal Antibodies

The eukaryotic nature of fungal pathogens presents an almost insurmountable problem when developing anti-fungal drugs. Multiple drug resistance associated with existing chemical antifungal drugs highlight the need for new, more effective antifungal therapies. Antibody based antifungal treatments can provide a much needed alternative to the near-exhausted chemical based strategies. Due to their high specificity and target selectivity, antifungal antibodies can be successfully employed for targeted killing of fungal pathogens without harming host cells. Using hybridoma technology several mAbs were generated against various antigenic components of fungal pathogens *Cryptooccus neoformans*, *Candida albicans*, *Aspergillus fumigatus*, *Pneumocystis jerovecii* and *Histoplasma capsulatum* [19, 36, 40, 70, 82, 85, 87]. The murine monoclonal antibody (mAb 18B7) generated through hybridoma technology directed against the capsular polysaccharide of *C. neoformans* mediated protection in murine model of infection and was shown safe to use in human patients in a Phase I dose escalation study [57]. In addition to the classical antibody mediated mechanisms of phagocytosis, complement activation and recruitment of inflammatory cells, mAb 18B7 was shown to increase the stiffness of the capsule by polysaccharide cross linkage which impaired yeast budding by trapping newly emerging buds inside the parental capsule [26].

Opportunistic invasive fungal pathogens cause over 2 million life-threatening infections per year worldwide with mortality ranging from 20–95% [10]. The most common cause of invasive fungal infections in the intensive care unit are *Candida* spp., and in the USA and Western Europe, the incidence of *Candida* infections is second only to *Staphylococcus aureus* (including MRSA) [104]. Recent data indicate that the incidence of candidaemia continues to rise unchecked by current clinical practice or drug regimens [73]. Candida mannan specific monoclonal antibodies have been generated through sheep immunisation in our laboratory. A single chain antibody phage display library was constructed using the immune repertoire of a sheep hyperimmunised with *Candida albicans* hyphal cell wall preparation. This antibody library, one of the very first and largest against Candida cell wall antigens, has been successfully used to isolate high affinity and specificity binders to Candida α-mannan, which are typically poorly immunogenic glycans present on the fungal cell surface. Analysis via SEM of polyclonal sera derived from immunised sheep confirmed that the library contained a varied collection of antibodies with specificity to both fungal cell wall carbohydrates (mannans and glucans) as well as a number of important cell wall proteins (Patent WO2014174293 (A1)).

HM-1 killer toxin protein is secreted by most yeasts to stop the growth of other competing strains by inhibiting the transmembrane enzyme β glucan synthase involved in their cell wall synthesis. A neutralising monoclonal antibody generated against HM-1 toxin (nmAb-HM-1) was used to immunise mice and isolate HM-1 anti-idiotypic antibodies from a scFv phage display library constructed using mice spleenocytes. Anti-idiotypic scFv fragments that inhibited β-1,3-glucan synthase activity were isolated from this library and showed *in vitro* cell killing in four pathogenic species of Candida including *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. glabrata*. The MIC values of these scFvs were in the range 1.56–12.5 μg/ml [93]. Biopanning using *Aspergillus fumigatus* membrane fraction (AMF) and employing competitive panning elution methods, scFv clones which showed *in vitro* antifungal activity against *A. fumigatus* was isolated from the same library [55]. The authors argue the potential of these scFvs as candidates for developing as antifungal drugs by either engineering as scFv-drug conjugates or reformatting into whole *IgG for* complement-mediated and antibody-dependent cytotoxic pathways [93].

A number of human antibody fragments recognising *C. albicans* cell surface antigens were isolated from human phage display libraries [38, 113] which is beyond the scope of this review. The authors have demonstrated antibody mediated opsonisation of C. *albicans* cells, phagocytosis and killing of the fungus by mouse macrophages and activation of the mouse complement cascade in *in vivo* models [113]. Combination immunotherapy is another attrac-

tive therapeutic strategy where anti-mannan mAbs were shown to have protective effect. In combination immunotherapy a chemical antifungal drug is administered along with an antibody. For example, mAb B6.1 is synergistic with amphotericin B [39] and augments the therapeutic effect of fluconazole in a mouse model of disseminated candidiasis [59]. These examples demonstrate the potential for antifungal mAbs in clinical settings and the use of drug combinations as a powerful strategy to enhance antifungal efficacy and abolish drug resistance.

6.7 Phage Antibodies Against Viral Antigens

The majority of anti-infective monoclonal antibodies developed in the 1990s were antiviral drugs with a main focus on Human Immunodeficiency Virus (HIV) neutralisers. Using mice immunisations and sera from infected humans, several mAbs were developed against the HIV Type-1 envelop with only a few of them broadly neutralising across all HIV-1 subtypes. All these neutralising mAbs were generated as a result of human infection and antibody responses against a range of epitopes such as gp120, gp41 or various epitopes on gp120 which are exposed after CD4 binding [64]. Forsman *et al*. published the generation of several high affinity and cross-reactive VHHs neutralising HIV-1 primary isolates belonging to subtype B and C. Llamas were immunised with recombinant gp120 belonging to subtype B and the resultant phage display libraries were panned using recombinant gp120 from subtypes A, B and C. A competitive elution strategy using sCD4 was employed during panning to isolate clones which were shown to inhibit the binding of sCD4 to HIV-1 gp120 and gp140 from selected subtypes. It is proposed that the VHH domains inhibit HIV- 1 infection by interacting with gp120 prior to its engagement with CD4, which makes them potential HIV-1 inhibitors [32]. Subsequently, a family specific phage display library was constructed using PBLS from the same llama immunised with recombinant gp120 derived from HIV-1 CN54 and tailored using degenerate primers based on the nucleotide sequences of the CDR3-FR4 region of the positive HIV-1 neutralising VHHs described above. Resultant picomolar affinity VHHs were shown to neutralise a broad range of HIV-1 virus belonging to subtypes B and C [52].

Another set of nanobodies that recognise the chemokine receptor CXCR4 was isolated by llama immunisation and phage display. CXCR4 belong to GPCR family where therapeutic targeting using conventional antibodies has proved unsuccessful probably because traditional antibodies struggle to access the cryptic and buried antigenic sites that make up the ligand binding pockets on the receptor surface. CXCR4 plays an important role in stem cell physiology, tissue repair, inflammation, metastatic spread of cancer and also serves as a co-entry receptor for HIV. Highly potent nanobodies generated using whole cell immunisation (CXCR4-expressing HEK293T cells), phage display library construction and biopanning behaved as competitive CXCR4 antagonists by totally blocking ligand CXCL12 binding and inhibiting chemotaxis and HIV-1 entry [47].

Two monoclonal antibodies specifically recognising the ORF-2 capsid protein of Hepatitis E virus were isolated from a cDNA library constructed using the lymphocytes isolated from a chimpanzee bone marrow [91]. This chimpanzee was experimentally infected with all five recognised Hepatitis virus – Hepatitis A to E. Phage displayed biopanning using recombinant HEV ORF2 proteins from human HEV strain (Pakistani strain SAR-55) and a swine HEV strain (U.S. strain Meng) generated two unique mAbs with nanomolar binding affinities as determined by BIAcore analysis. The γ1 heavy chains of anti-HEV mAbs had the most sequence identity with the human VH3 family of germ line segments (89.4% and 88.5% overall identity) and the k light-chain sequences exhibited the most identity with the human Vk1 family of germ line segments. These mAbs were able to neutralise SAR-55 strain of HEV and offer protection from HEV infection in rhesus monkeys.

The authors have highlighted the significance of using chimpanzee as a donor for antigen

primed antibody repertoire including the possibility of infecting them with viral pathogens of human relevance and reduced immunogenicity when used in human immunotherapy. Earlier studies have reported that human antibodies are recognized as self by the chimpanzee immune system thereby generating little immunogenicity compared to other primates and the half-life of a human mAb is equivalent to the estimated half-life of IgG in humans [75].

Schofield and colleagues also reported the generation of four monoclonal antibodies against Hepatitis A virus capsid from the same chimpanzee bone marrow derived cDNA library using inactivated whole HAV capsid from strain HM-175 for biopanning [92]. All four mAbs neutralized the strain HM-175 and two of the four MAbs also neutralized the divergent AGM-27 strain. However the actual mechanism of neutralisation remain unclear as the authors noticed that the Fab fragments are not inhibiting virus attachment to cells via soluble simian cell receptor for HAV, HAVCR1. No further developments for these monoclonal Fab fragments are available in the public domain.

Generating VHHs from immunised llama libraries to Hepatitis B surface antigen (HBsAg) and Hepatitis B core antigen (HBcAg) were explored as an approach to inhibit HBV replication thereby tackling viral infection [94, 95]. The resultant VHH fragments were expressed as intrabodies to target the antigens which exert their functions in different compartments of the host cell (cytoplasm, ER and nucleus). The intrabodies were shown to supress the secretion of HBsAg and HBcAg *in vitro* and inhibition of viral release in a mouse model [94].

Other VHH fragments developed using animal immunisation and phage display technology include those binding to Rotavirus gp6, H5 hemagglutinin to reduce viral replication in H5N1 influenza virus, VHH recognising the tail of infectious phage in Lactococcus bacteria. The list with relevant publications is summarised in Table 6.3. Also a comprehensive list of antibodies raised against viral targets using non-immune libraries was presented in a recent review publication [102].

There is currently no effective treatment for respiratory syncytical virus (RSV) lower respiratory tract infection. RSV is a major worldwide cause of morbidity and mortality in infants and young children, immunocompromised patients and the elderly. Treatments remain largely supportive and RSV-specific options for prophylaxis are limited to palivizumab [65, 67]. ALX-0171 (Ablynx) is a trivalent nanobody in phase II clinical trials for the treatment of RSV infections. It is one of the promising candidate biologic drugs in development for the prevention and/or treatment of RSV infections (see Mazur et al. [65] for a full review). ALX-0171 was originally isolated from a llama derived immune library as a monovalent domain prior to reformatting into a multivalent construct using genetic fusion. The VHH domain demonstrated high neutralising potency against RSV-A and RSV-B, and demonstrated superiority over palivizumab in blocking RSV replication [28].

6.8 Anti-parasite Antibodies

Camel VHHs or nanobodies have also been developed to treat diseases such as trypanosomiasis (African sleeping sickness) caused by parasites from the Trypanosoma genus. *Trypanosoma brucei* has evolved a range of sophisticated immune evasion mechanisms, which include repeatedly generating variations of the immunogenic variant-specific surface glycoprotein (VSG), efficient internalisation of antibody-VSG complexes from the surface by endocytosis and proteolysis of the immunoglobulin [7]. Due to the dense packaging of VSG protein on the surface of Trypanosoma, conventional antibodies are unable to access the more conserved VSG epitopes. High affinity nanobody clones directed towards isotype-specific and conserved epitope on the VSG protein coat, were isolated from an immunised camelid phage display VHH library. These nanobodies were found to be trypanolytic both *in vitro* and *in vivo*, which shows the potential of high affinity monovalent fragments to exert a cytotoxic effect on parasites even in the

Table 6.3 Summary of monoclonal antibodies, engineered fragments and single domain antibodies isolated from animal immunised phage display libraries against various viral targets

Virus	Target antigen	Immunised animal source	Clinical/future clinical application	Affinity-kD	References
HIV-1	HIV-1 Capsid protein p24	Mice	Diagnosis, treatment and Prophylaxis of HIV infection	2 nM	Mohammadzadeh et al. [69]
SARS coronavirus	SARS CoV	Blood of a convalescent SARS patient	Prophylaxis of SARS CoV infections in humans	105 nM	Duan et al. [30]
HIV-1	Gp120	Llama	HIV-1 entry inhibitor	0.097 nM	Forsman et al. [32]
	CXCR4	Llama	Antiretroviral, inhibition of CXCR4-using viruses in HIV infected individuals	0.35 nM	Jähnichen et al. [47]
Hepatitis E	SAR-55 ORF2 protein	Chimpanzee	Prophylaxis against Hepatitis E virus infections in human	1.7–5.4 nM	Schofield et al. [91]
Hepatitis B	HBcAg	Llama	Antiviral inhibition of Hepatitis B virus replication	ND	Serruys et al. [94]
Rotavirus	Strain RRV, Serotype G3	Llama	Treatment of rotavirus induced diarrhoea	ND	Pant et al. [79] and Van der Vaart et al. [103]
Influenza A	H5N1 hemagglutinin	Llama	Inhibition of influenza A virus replication	0.45–0.64 nM	Hultberg et al. [45] and Ibañez et al. [46]
Ebolavirus	Ebola-Zaire glycoprotein	Cynomolgus macaque	Potential diagnostic for Ebolavirus infection	ND	Druar et al. [29]
Western equine encephalitis virus (WEEV)	B-PL-Inactivated WEEV	Cynomolgus macaque	Pre- and post-exposure treatment of WEEV infections	ND	Hülseweh et al. [44]
Hantavirus	Nucleoprotein	Llama	Early diagnosis of hantavirus pulmonary syndrome	3.3–197 nM	Pereira et al. [81]
Vaccinia, Variola virus	Vaccinia B5 envelope protein	Chimpanzee	Prevention and treatment of vaccinia virus-induced complications of vaccination against small pox; immunoprophylaxis and immunotherapy of smallpox	0.2–0.7 nM	Chen et al. [20, 21]
Yellow fever virus (YFV)	Domain II of envelope protein	Human	Neutralisation of YFV	ND	Daffis et al. [27]
Respiratory syncytial virus (RSV)	Inactivated RSV-A	Llama	RSV neutralising nanobodies, for treatment of RSV infections	0.1 nM	Detalle et al. [28]

absence of Fc domain. The authors have demonstrated the modes of action of these domains which appear to include: rapid arrest of cell mobility, blockage of endocytosis, flagellar swelling, collapse of mitochondrial membrane potential and ATP exhaustion [96].

6.9 Conclusion

Monoclonal antibodies and novel smaller antigen binding scaffolds presents an attractive option for the development of new therapies and molecular drug targets against a wide range of human diseases and also in diagnostics due to their specific-

ity and flexibility. In this book chapter, we have been able to present the robustness of combining immunisation and phage display technology in raising antibodies specific to antigens relevant in a number of infectious diseases of public health interest, and also those antigens classified as bioweapons. Although the limitations associated with animal immunisation, especially those relating to ethical issues surrounding animal welfare and the need to immunise for every antigen of interest may limit access to animal immunisation in the future. However it is the opinion of the authors that the debate regarding animal use for research purposes should be substantially assessed on the basis of a risk-benefit analysis and suitability.

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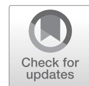

7 Anti-bacterial Monoclonal Antibodies

Eszter Nagy, Gábor Nagy, Christine A. Power, Adriana Badarau, and Valéria Szijártó

Abstract

The failing efficacy of antibiotics and the high mortality rate among high-risk patients calls for new treatment modalities for bacterial infections. Due to the vastly divergent pathogenesis of human pathogens, each microbe requires a tailored approach. The main modes of action of anti-bacterial antibodies are virulence factor neutralization, complement-mediated bacterial lysis and enhancement of opsonophagocytic uptake and killing (OPK). Gram-positive bacteria cannot be lysed by complement and their pathogenesis often involves secreted toxins, therefore typically toxin-neutralization and OPK activity are required to prevent and ameliorate disease. In fact, the success stories in terms of approved products, in the anti-bacterial mAb field are based on toxin neutralization (*Bacillus anthracis, Clostridium difficile*). In contrast, Gram-negative bacteria are vulnerable to antibody-dependent complement-mediated lysis, while their pathogenesis rarely relies on secreted exotoxins, and involves the pro-inflammatory endotoxin (lipopolysaccharide). Given the complexity of bacterial pathogenesis, antibody therapeutics are expected to be most efficient upon targeting more than one virulence factor and/or combining different modes of action. The improved understanding of bacterial pathogenesis combined with the versatility and maturity of antibody discovery technologies available today are pivotal for the design of novel anti-bacterial therapeutics. The intensified research generating promising proof-of-concept data, and the increasing number of clinical programs with anti-bacterial mAbs, indicate that the field is ready to fulfill its promise in the coming years.

Keywords

Microbial pathogenesis · Antibody targets · Toxin neutralization · Bactericidal antibodies · Passive immunization

7.1 Introduction

Humoral immunity is the cornerstone of host defense against infectious diseases. The protective role of antibodies in the fight against bacteria, particularly extracellular pathogens, is well established. In spite of this, there are only three monoclonal antibody (mAb) based anti-bacterial reagents approved for human use [27].

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Historically, the prophylactic and therapeutic potential of serum therapy was recognized in the late nineteenth century when von Behring and Kitasato used hyper-immune sera from horses immunized with toxoids from *Corynebacterium diptheriae* and *Clostridium tetani,* to treat patients with diphtheria and tetanus [148]. We know now that the efficacy of the treatment was due to neutralization of toxins by specific antibodies present in the horse serum, although at the time the agents responsible for the success of the treatment had not been characterized. The benefits of serum therapy outweighed the side effects of serum sickness. So by the early twentieth century, serum therapy was being used for the treatment of both viral and bacterial diseases [17]. The clinical benefit of serum therapy was demonstrated not only for toxin mediated bacterial infections, such as diphtheria and anthrax, but also for diseases caused by *Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae* type b and *Neisseria meningitidis*.

Following the introduction of antibiotics in the 1940s, and vaccines against childhood diseases, including tetanus, diphtheria and pertussis, serum therapies were largely abandoned. Antibiotics were easier to manufacture, could be more easily formulated in preparations that had consistent activity, and had less toxicity. They have been hugely successful in the fight against bacterial infections over the past 70 years.

However, the widespread emergence of antibiotic resistant bacteria is on the verge of becoming a major public health threat. The appearance of drug-resistant bacteria quickly follows the introduction of a new antibiotic. Unfortunately, the rate at which new antibiotics are being discovered has slowed dramatically in the past several decades such that antibiotic resistance is now developing faster than the identification of new therapies. Furthermore, many large pharmaceutical companies no longer have strong research and development programs for short-term infections, so the antibiotic pipeline is drying up [21].

Importantly, the majority of bacterial infections are still caused by antibiotic susceptible pathogens. Mortality in hospitalized high-risk patients is unacceptably high even with appropriate antibiotic therapy. Antibiotics do not address virulence and inflammation that is exacerbated upon bacterial lysis, mainly due to released exo- and endotoxins that maintain damaging inflammatory responses even after no viable bacteria are present.

Alternative approaches are now needed to combat bacterial infections. In a recent pipeline portfolio review of alternatives to antibiotics [23] the development of pathogen-specific monoclonal antibodies is considered to be the approach, which is most likely to have an immediate clinical impact.

In spite of the great success of prophylactic active immunization that has changed human demographics and saved more lives than any other medical and healthcare interventions, there is still a need for passive immunization approaches. Even the highly efficacious existing vaccines are suboptimal in certain patient populations e.g. in the elderly and immunocompromised individuals. Certain infections such as nosocomial infections, affect a subpopulation, thus are not amenable to population-wide immunization, and protection is typically needed immediately.

Monoclonal antibodies have a history of safe use, a strong scientific basis, and a high degree of technical feasibility. Passive immunization can be used prophylactically to prevent infection, or therapeutically as an adjunct to antibiotics, to treat infections, while at the same time harnessing the power of the immune system. There are many examples in preclinical studies where antibodies have been shown to act synergistically with antibiotics providing levels of protection significantly greater than seen with antibiotics or the antibody alone [29, 55, 114].

In both prophylactic and therapeutic modes, protection is achieved immediately and is long lasting, depending on the half-life of the antibody. In addition, because of their high target specificity for a single species of bacteria, antibodies do not disrupt the microbiome. Perturbations of the microbiome caused by the use of broad spectrum antibiotics has been

implicated in the increased incidence of many diseases, including diabetes, obesity, autism and immune disturbances [90].

The vast majority of mAbs in clinical development today are human or humanized. Advances in methodologies for humanization mean that a high degree of homology to human germline sequences can be achieved. Therefore immunogenicity is not a major concern anymore unlike with the first mAbs that were murine or chimeric. Additionally, in most cases, only a single administration of the antibody will be required, whereas for other diseases, mAbs are administered repeatedly over periods ranging from weeks to months (for cancer) to years (for autoimmune diseases).

In this review we have given a broad overview of bacterial pathogenesis mechanisms with the objective of describing the key targets for anti-bacterial therapies. We also discuss the relevance of different antibody isotypes and formats for generating fit-for-purpose antibodies. We provide a survey of on-going research with anti-bacterial antibodies, citing examples of antibodies against a variety of bacterial targets, and with different mechanisms of action, and detail the approaches currently in clinical development, or approved for human use. Finally, we look at how we can use the information gained from past and present clinical trials, together with recent advances in antibody discovery and engineering technologies, to develop next generation anti-bacterial agents.

7.2 Antibody Target Selection in the Light of Bacterial Pathogenesis

7.2.1 Overview

From a teleological point of view, bacteria aim to regenerate themselves by exploiting the host's resources without ultimately killing it, which would also lead to the death of the bacterial population within the host. Commensal bacteria successfully colonize mucosal sites without causing obvious harm to their hosts (and sometimes even provide benefit). The complex role and contribution of the microbiome to human health has recently started to be recognized. From these "overpopulated" microbial communities, however, some bacteria have evolved strategies to spread to new niches, e.g. by facilitating the spread from one host to another or by invading sterile body compartments. This leads to mucosal infections (with bacterial shedding, associated with a higher likelihood of transmission) or invasive infections. In extreme cases, infections may cause the death of the host, however this is a rather "unintentional" consequence from the bacterial perspective. From the human perspective, bacteria using these strategies for dissemination are considered pathogens.

Bacterial pathogenesis is mediated by traits recognized as virulence factors. Certain virulence mechanisms are broadly conserved within bacterial genera, others are species-specific, or even exclusively present in virulent strains of a given species (often acquired by horizontal gene transfer). Certain panels of virulence factors determine the sites and type of infection, e.g. in the case of the commensal bacterium *Escherichia coli,* certain variants have evolved to cause either intestinal or extra-intestinal infections [61]. Even among intestinal pathogenic *E. coli*, different "pathotypes" are differentiated based on the type of disease they cause, and the dominating virulence mechanisms (e.g. enterotoxigenic, enterohemorrhagic *E. coli*).

In general, two different bacterial pathomechanisms can be distinguished: (i) toxin-mediated and (ii) invasive bacterial infections. Many pathogens, nevertheless, utilize a combination of these two mechanisms.

7.2.2 Toxin-Mediated Pathogenesis

In the case of true toxin-mediated diseases, bacteria residing at mucosal sites secrete exotoxins that act locally or target host cells at distant sites. Depending on the toxin type, a single species may cause different types of disease, e.g. *S. aureus* may elicit diarrhea, skin infections, or a systemic infection through production of enterotoxins, exfoliative toxin or toxic shock syndrome toxin (TSST), respectively [70].

Similarly, intestinal pathogenic *E. coli* may cause diarrhea or systemic life-threatening infections elicited by specific toxins (e.g. LT/ST or verotoxins). Importantly, these bacteria do not invade sub-mucosal sites, and lack of toxin production renders them avirulent. In contrast, invasive bacteria break through mucosal barriers and cause a local infection (e.g. pneumonia, dysentery, pyelonephritis) or disseminate through the bloodstream (causing bacteremia, meningitis, multi-organ infections). Nevertheless, invasion is often mediated and/or supported by cytolytic exotoxins. Alpha-hemolysin (Hla) of *S. aureus* and pneumolysin (Ply) of *Streptococcus pneumoniae* lyse epithelial and endothelial cells, thereby disrupting the physical barriers and hence pave the way for invasive infections. Other toxins, e.g. *S. aureus* bi-component leukocidins or *S. pyogenes* streptolysins, intoxicate white blood cells and hence contribute to immune evasion and indirectly to bacterial dissemination.

Many Gram-negative bacteria – besides being capable of secreting exotoxins – have a more intimate way of manipulating/intoxicating eukaryotic host cells. Through a syringe-like apparatus, called the 'type three secretion system' (T3SS), that spans both the bacterial and target cell membrane, bacterial 'effector molecules' are injected directly into the host cell in order to elicit engulfment of bacteria (e.g. *Shigella and Salmonella* spp.); inhibit phagocytosis (*Yersinia* spp.), induce necrosis/apoptosis (*Salmonella*, *Shigella, Pseudomonas*, and enteropathogenic *E. coli*); express ligands for bacterial adherence (enteropathogenic/hemorrhagic *E. coli*) or allow an intracellular life cycle (*Chlamydia, Salmonella and Shigella*). Secreted enzymes typical for several Gram-positive pathogens can also contribute to invasion through degradation of tissues and weakening immune functions.

7.2.3 Invasive Bacterial Pathogenesis

Invasive bacteria need additional virulence factors, besides exotoxins and injected effector molecules, in order to survive in different body compartments where nutrients are typically very limited, and where innate immune mechanisms pose a constant threat. The vague term "bacterial fitness" involves all mechanisms that enable rapid replication, by utilization of available energy sources in a given *milieu*. Free iron is severely limited in the body, consequently, most invasive pathogens have evolved strategies to scavenge iron from host iron-transporting or storage complexes such as hemin, lactoferrin, and hemoglobin. Certain pathogens utilize alternative metabolic pathways adapted for the micro-environment in the host, for example enzymes that liberate host-specific energy sources (e.g. sialidases) and thus contribute to *in vivo* fitness and virulence.

Another important patho-mechanism is the ability to withstand innate immune mechanisms, for instance complement-mediated killing or phagocytosis. Gram-positive bacteria exhibit intrinsic resistance to complement-mediated lysis due to their thick cell wall. Additionally, several Gram-positive pathogens secrete proteins that contribute to the degradation or inhibition of complement factors [73]. Gram-negative pathogens apply different strategies to mask their outer membranes from the different complement pathways. Lipopolysaccharides (LPS) and certain types of polysaccharide capsules are able to confer complement resistance by inhibiting the insertion of the membrane attack complex (MAC) into the outer membrane, or by sequestering complement factors away from the bacterial membrane. Additionally or alternatively, several outer membrane proteins have been described to counteract the complement cascade by different mechanisms [12]. Resisting opsonophagocytic killing is equally important for Gram-positive and Gram-negative pathogens. Theoretically, three distinct strategies can be distinguished: (i) killing phagocytes (e.g. with exotoxins or T3SS effectors); (ii) avoiding phagocytosis; or (iii) survival in phagocytes. The protective role of carbohydrate capsules and exo-polysaccharides has long been known to contribute to resistance to phagocytosis. Most invasive Gram-positive and Gram-negative pathogens are encapsulated, and mutation of the genes encoding capsule

expression renders them avirulent. Alternatively, several pathogens have evolved strategies to survive or even multiply within professional or non-professional phagocytes [118]. These pathogens mediate their uptake by phagocytes *via* surface proteins (in the case of e.g., *Listeria* or *Yersinia*) or T3SS effector molecules (e.g., *Shigella, Salmonella*).

7.2.4 Antibody Targets

Based on the different patho-mechanisms outlined above, the molecular targets of anti-bacterial mAbs can be classified into two major classes. Antibodies against secreted bacterial components aim to neutralize exotoxins or other secreted virulence factors, while those targeting the bacterial surface are typically expected to induce bactericidal activity by different means (see Sect. 7.4 for details).

Anti-toxin antibodies have traditionally been considered a success story since the introduction of serum therapy 100 years ago. In fact, in strictly toxin-mediated diseases such as tetanus, diphtheria, and botulism, neutralization of the corresponding exotoxins effectively prevents disease or even cures, if applied in a timely manner. Moreover, since the antibodies pose no evolutionary pressure on bacteria, escape mutants are not expected to emerge. In cases where pathogenesis is driven by several exotoxins, cross-neutralizing mAbs or combinations of mAbs with different specificities would be desirable. Although anti-toxin mAbs alone are expected to interfere with toxin-mediated disease, it is very likely that passive immunization will be performed simultaneously with antibiotic therapy in order to reduce the burden of bacteria producing the toxins. Apart from the obvious additive effects due to the complimentary actions of mAbs and antibiotics, further synergy is expected due to the increased expression and/or release of toxins described for various bacteria upon antibiotic treatment [68, 112, 126].

The second class of anti-bacterial mAbs target surface structures of invasive bacteria. In this case, selection of the optimal targets is a much greater challenge. The molecular targets need to fulfil two major criteria: (i) be accessible on the surface and at the same time, (ii) show structural and sequence conservation among the majority of strains to be targeted. Surface proteins are often shielded by highly variable carbohydrate structures such as capsular antigens, exo-polysaccharides, or smooth LPS. While these carbohydrate antigens are readily accessible and are generally considered protective antigens, they have evolved great variability in order to evade the adaptive immune response. Therefore, mAbs with subspecies specificity or combinations thereof, may be of increasing interest, especially when supported by strong epidemiological data. For instance, although multiple serotypes of *Neisseria meningitides* and *Haemophilus influenzae* exist, only a few serotypes are associated with the majority of cases. Similarly, although *Enterobacteriaceae* are known to have evolved a huge number of different serotypes, multi-drug resistant variants are often associated with successful clonal lineages, so called "high-risk clones" [86, 144] that warrant the need for developing serotype/clone-specific mAbs.

Another approach is to select protein antigens that extend across the carbohydrate shield and are less prone to structural variability due to their sophisticated and/or important function. Examples are the fimbrial adhesins, invasins, T3SS components, or bacterial uptake receptors for various compounds, especially when indispensable for the pathogenesis, which may also represent valid mAb targets.

A systematic overview of potential target antigens is summarized in Fig. 7.1, while specific examples will be discussed later in Sect. 7.4.

7.3 Antibody Formats Applied to Anti-bacterial mAbs

7.3.1 Full Immunoglobulins

7.3.1.1 IgG

Functional (protective) antibody responses vary depending on the nature of the pathogen and target antigen, and will often be skewed with respect

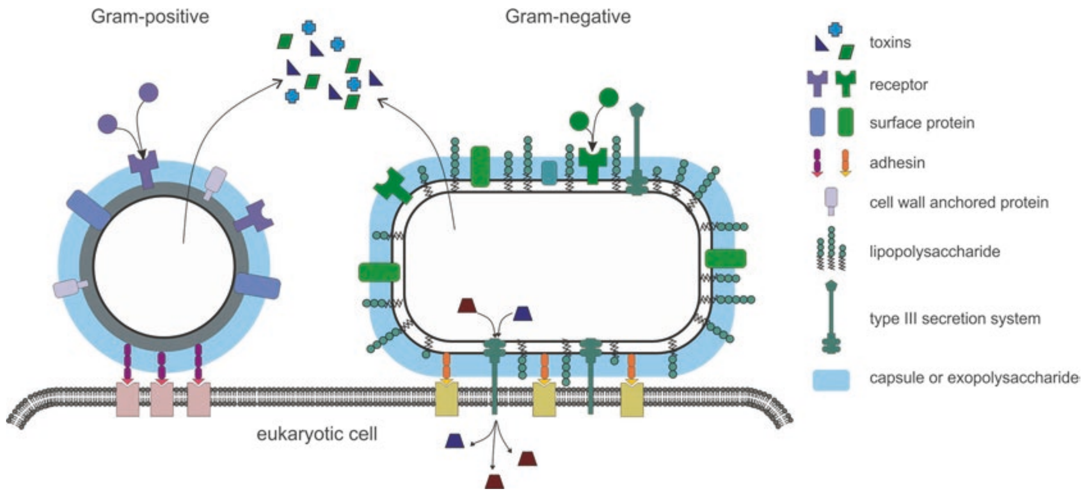

Fig. 7.1 Overview of potential bacterial target antigens

to isotype and subclass, and have also been shown to vary with age, gender and immune status of individuals. Nevertheless, the majority of antibodies against bacterial antigens that are currently on the market or in clinical development use a human IgG1 backbone with a kappa light chain. The predominance of kappa light chain antibodies is mainly historical and arises from the fact that many of the antibodies in clinical development are humanized antibodies originally discovered in mice, where the ratio of kappa to lambda light chain usage is about 95% vs. 5%. In humans the ratio is closer to 60:40 [2].

The preference for IgG1 on the other hand is mostly due to the fact that this isotype is the most abundant isotype present in human serum, it is highly stable, has a longer half-life (~21 days) than other isotypes, is well expressed in mammalian cell systems used for manufacturing, and is amenable to generic purification processes using Protein A. Most importantly, the Fc domain of IgG1 mediates potent effector functions critical for efficient pathogen clearance including complement fixation, complement dependent cytotoxicity (CDC) and opsonophagocytic killing (OPK).

Although there are a couple of IgG2 and IgG4 antibodies in the clinic for certain disease indications, to our knowledge, none are in development as anti-bacterials even though the IgG2 subclass has been shown to predominate in responses to bacterial polysaccharides *in vivo*. There are also no IgG3 isotypes in development. This may primarily be due to attributes such as the increased likelihood of proteolysis due to the extended hinge region, the many different IgG3 allotypes [11] that exist across populations, and its shorter half-life (~8 days), notwithstanding the potential manufacturability issue that it cannot be purified on Protein A, the mainstay of downstream bioprocessing.

7.3.1.2 IgM

IgM is a highly effective isotype for complement-mediated killing and complement-dependent phagocytosis of infectious bacteria. Even though IgMs have low affinity against their targets, high avidity due to their pentameric nature renders them able to elicit effective complement activation. Polysaccharides (including LPS) are T-cell-independent antigens, and antibodies induced in response to them are mostly of the IgM isotype. However manufacturing issues such as aggregate formation have hampered clinical development of IgMs. Currently there is only one IgM mAb in clinical trials – Aerumab™ (Aridis Pharmaceuticals) directed against *P. aeruginosa* LPS serotype O11.

7.3.1.3 Half-Life Extension Modifications

Our knowledge of the key residues involved in Fc gamma receptor interactions, and complement and neonatal Fc receptor binding, has increased dramatically over the last decade such that the next generation of antibodies have been engineered to have superior functional activity and extended serum half-life [80]. Although, antibodies of the IgG1 isotype have long serum half-life (about 3 weeks), certain indications may require the presence of anti-bacterial therapeutics for longer periods.

The interaction of IgG with the neonatal Fc receptor (FcRn) plays a critical role in determining the serum half-life of circulating antibodies. Antibodies are taken up by endothelial cells by pinocytosis, and bind to FcRn in an acidic endosomal compartment (pH 6.0). The FcRn then recycles the IgG back to the cell surface where it is released back into the circulation at the basic pH of blood (pH 7.4) and thereby avoids undergoing lysosomal degradation. The residues responsible for binding of IgG to FcRn have been elucidated [141] and an area rich in histidine residues between the CH2 and CH3 domains is thought to act as a kind of pH sensor [85]. Several groups have identified key residues that enhance the FcRn binding affinity and have been shown to result in a concomitant increased serum half-life in transgenic rodents, monkeys and humans [113, 152]. For example, a triple mutation in the Fc domain of human IgG1, the so called 'YTE' mutation (M252Y, S254T, T265E), was shown to enhance FcRn binding tenfold in an anti-RSV antibody (Motavizumab-YTE) resulting in a serum half-life extension 2–4 fold longer than in an antibody containing a native IgG1 Fc domain. This technology has been applied to two anti-bacterial mAbs currently in clinical development, MEDI4893 and MEDI3902 for *S. aureus* and *P. aeruginosa* infections, respectively. The obvious advantage of such half-life extension is that a single administration would offer long-acting efficacy.

7.3.2 Antibody Fragments

The development of antibody fragments in the field of infectious diseases has lagged behind their development for other therapeutic areas, mainly due to the need for specific antibody effector functions mediated through the Fc portion, for efficient pathogen killing and clearance, in addition to target neutralization. One of the main advantages of small antibody fragments is greater tissue penetrability. In manufacturing, the smaller size of fragments may permit cheaper, faster production in microbial systems, although proof of this assertion has not yet been described in the literature. However, the lack of an Fc domain impacts severely on serum half-life unless specific counter measures are taken.

Two antibody fragments have been tested in clinical trials. The first, KB001-A (Kalobios), is a pegylated Fab derived from a 'humaneered' mouse mAb that was being developed for the treatment of *P. aeruginosa* infection in cystic fibrosis patients [89]. Because KB001-A lacks the IgG Fc region, it does not activate immune cells which could potentially exacerbate inflammation. PEGylation of the Fab extends serum half-life and also protects against inactivation in the lung. Proof of concept studies in mice demonstrated that functional activity did not require the Fc portion. The second, a single chain antibody fragment (scFv) produced in *E. coli* that binds to the GrfA ABC transporter of *S. aureus* (Aurograb from Neutec), was developed and tested preclinically and clinically against *S. aureus* infections. This molecule was discontinued in 2008 after the Phase 2 trial failed to show efficacy. The same scFv technology was applied to an anti-Candida product candidate, Mycograb that was considered efficacious clinically, however, there were concerns about the manufacturing process (high molecular weight aggregates, the refolding of the molecule and the levels of host cell proteins) and so further development was discontinued by Novartis after acquisition of Neutec [31].

7.3.3 Bispecific mAbs

Natural IgG antibodies express two identical antigen-binding domains, and are therefore mono-specific, and bivalent. In contrast, bispecific molecules possess two different antigen-binding domains, each with different target specificity or targeting different epitopes (biparatopic) on the same molecule. Over 40 different bi-specific antibody formats have been described in the literature [34]. This staggering number has largely arisen from the need to develop proprietary technologies in order to avoid the high milestone and royalty burden of in-licensing others' technology. The majority of formats, however, have yet to be validated in the clinic. Furthermore many bispecific antibody formats may not be amenable to the generic two- or three-step antibody purification processes that are widely used in the industry.

Bispecific antibodies have already been in clinical development for many years for diseases like cancer and in autoimmunity, as it is becoming increasingly evident that neutralization of a single target may be insufficient for multifactorial diseases including bacterial infections [71]. At the time of writing we are aware of only one bispecific antibody in clinical development for bacterial infection – MEDI3902 (Medimmune), which targets two distinct antigens on *P. aeruginosa*, namely PcrV and Psl. Three different bispecific mAb configurations were tested before selecting the BiS4αPA format (see Sect. 7.4.5.5), which had enhanced cytoprotective activity by inactivating PcrV, without compromising Psl-dependent OPK activity, as clinical candidate [140]. The bispecific format was shown to be more efficacious than a combination of the two parental mAbs against the individual targets. This is explained by increased binding to PcrV through the Psl component that is abundant and thought to concentrate the bispecific antibody close to the bacterial surface where the much less abundant PcrV molecule is located. This example highlights the importance not only of the choice of the targets for a bispecific but also the configuration – one size may not fit all.

7.3.4 Engineered Multi-specific or Designed Cross-Reactive mAbs

Multi-specific mAbs are those targeting unrelated antigens, by using either different binding sites/modules to target different antigens (analogous to bispecific mAbs) or by using a single binding site which can exist in different conformations or involve different residues in the interaction with the antigens. Given the complex patho-mechanisms of bacterial pathogens, the advantage of simultaneous inhibition of several virulence mechanisms is obvious.

An example of the former is the IgG centyrin (mAbtyrin) developed by Janssen Biotech against *S. aureus*, which is an IgG – centyrin fusion protein. Centyrins are novel scaffolds based on the human fibronectin III domain. In mAbtyrin, the mAb portion targets bacterial surface adhesins, while the centyrin, located at the C-terminus of either the light or heavy chain of the antibody, targets up to two staphylococcal leukotoxins: LukGH (LukAB), LukE or LukD [133]. It was shown that both the adhesin binding and LukGH neutralization functions of this fusion protein were required for inhibiting PMN cytotoxicity of an *S. aureus* culture supernatant in an *ex vivo* PMN infection model. This was taken by the authors to suggest the importance of intracellular neutralization of the LukGH toxin.

Another example of a multi-specific mAb, in this case one that uses a single antigen binding site to interact with two different antigen epitopes, is a mAb targeting two subtypes of the botulinum toxin (BoNT), i.e. A1 and A2 [66]. Starting with a scFv that binds the BoNT/A1 subtype with high (pM) affinity and BoNT/A2 subtype with low (nM) affinity, using yeast display and co-selection, the affinity for BoNT/A2 was increased by over three orders of magnitude, without compromising the affinity for subtype A1. Structural and thermodynamic studies have shown that the interactions of the cross-reactive antibody with subtype A2 occurs *via* residues that have limited or no contact with subtype A1. This work shows how an antibody can be

engineered to bind two different antigens despite structural differences in the antigen-antibody interface and may provide a general strategy for tuning antibody specificity and cross-reactivity.

Not truly multi-specific, but multi-target mAbs that are cross-reactive with several virulence factors are also considered for bacterial diseases. Quadruple toxin cross-reactive antibodies recognizing four different serotypes of botulinum toxin that share as low as 36% overall amino acid identity have been reported [39]. These antibodies, derived from B cells of immunized humans and mice, were optimized for high affinity and broad binding specificity using yeast-displayed scFv libraries. They targeted an epitope that is well conserved among the different toxin types, and important for toxin function [39].

Multi-target antibodies cross-reactive with five different pore-forming toxins of *S. aureus*, namely alpha-hemolysin (Hla) and four different bi-component leukocidins, that share only ~26% overall amino acid sequence identity, were reported by our group at Arsanis [2]. Similar to the botulinum toxin cross-neutralizing antibodies, these mAbs recognize a micro-domain that is highly conserved among the targeted toxins and is involved in pore formation. High affinity mAbs that neutralized Hla were first selected from human IgG1 libraries displayed on yeast using Hla as capture antigen, followed by the selection of mAbs that showed binding to S- or F-components of the five bi-component toxins produced by *S. aureus*, HlgAB, HlgCB, LukED, LukSF and LukGH. These mAbs were further optimized for higher affinity in two successive selection cycles (light chain shuffling and targeted mutagenesis of CDR1 and CDR2 of the heavy chain) using decreasing concentrations of Hla. A final affinity maturation cycle involved error-prone PCR of the variable region of the heavy or light chain and alternating the different F-component molecules (LukD, LukF and HlgB) and Hla in successive selection rounds. Several antibodies were identified that inhibited cytotoxic activity of all of the corresponding toxin molecules except LukGH, which does not contain the conserved epitope recognized by the cross-reactive mAbs [114]. One of these multi-targeting mAbs, ASN-1, was selected as clinical lead (see Sect. 7.4.5.5) for use in combination with ASN-2, targeting LukGH [9].

7.3.5 Antibody Drug Conjugates

Antibody drug conjugates (ADC) combine the specificity of an antibody with the activity of a cytotoxic drug – the so called 'magic bullet' concept. The cytotoxic prodrug is inactive and is usually attached to the antibody *via* a chemical linker with labile bonds. At the antibody target site, the linker is cleaved through the action of specific enzymes or proteases expressed at the disease site, releasing the active drug which can then exert its cytotoxic effects. Over 50 ADC are reported to be in clinical development [120], but none are for bacterial infections.

Recently the development of a novel antibody-antibiotic conjugate or AAC that targets intracellular *S. aureus* was reported [77]. It is known that *S. aureus* is able to invade and survive in mammalian cells, notably phagocytic cells that are responsible for pathogen clearance. Incomplete clearance or killing of phagocytosed bacteria can lead to their dissemination away from the initial site of infection and cause infections such as osteomyelitis, rhinosinusitis and endocarditis etc. Lehar and colleagues have developed a novel AAC that is activated specifically in cells that have taken up *S. aureus*. The AAC consists of an anti-*S. aureus* antibody (THIOMAB™) which targets cell wall teichoic acids, that has been engineered to contain unpaired cysteines, that allow the covalent linkage of an antibiotic *via* a cathepsin cleavable linker. The linker is only cleaved in the proteolytic environment of the phagolysosome. The antibiotic used was a derivative of rifamycin known as a rifalogue, which has high bactericidal activity even at low pH. When conjugated to the antibody, rifalogue is unable to kill bacteria, but is fully active after cleavage with cathepsin B *in vitro*. The authors showed that in a mouse model of MRSA bacteremia, a single dose of the AAC administered after infection was able to clear the bacterial infection, whereas treatment with vancomycin, the current

standard-of-care, or the un-conjugated antibody, failed. These results show that arming antibodies with unique bactericidal antibiotics has potential as a promising new therapy. This AAC strategy may also be useful for the delivery of potent antibiotic candidates that have failed in clinical trials due to poor pharmacokinetic profiles or host toxicity. The THIOMAB™ antibiotic conjugate RG7861 (Roche/Genentech) is currently being evaluated in Phase I clinical trials for serious infections caused *by S. aureus.*

7.3.6 Nanobodies

A class of antibodies known as nanobodies has potential for the treatment of infectious diseases. Nanobodies are small approx. 15 kDa single VHH domain antibodies that were discovered in the early 1990s in camelids [50]. They contain a single antigen binding domain, are more stable than conventional mAbs, and can easily be produced in *E. coli*. Part of their attractiveness is that nanobodies against different antigens can be covalently linked to each other to create bispecific or even multi-specific formats, whilst linking to nanobodies specific for albumin can confer a serum half-life comparable to conventional antibodies. Furthermore, development of protease resistant nanobodies may render them suitable for oral administration. A review of nanobodies that are currently in pre-clinical development for a variety of diseases including bacterial infections, e.g. caused by enterotoxigenic- and Shiga-toxin-producing *E. coli*, *P. aeruginosa* and *A. baumanii* can be found in Steeland et al. [124].

7.3.7 Antibody Combinations

One of the major challenges in developing mAbs against a bacterial pathogen is to obtain efficacious/potent activity with a single mAb. It is mainly due to the complex pathogenesis and/or insufficient conservation of the antibody target in a broad range of strains. Therefore antibody combinations are considered, and shown to be necessary and beneficial in multiple cases.

The potential benefit of combining two antibodies for the treatment of hemolytic uremic syndrome (HUS) (Shigamab™, see Sect. 7.4.5.8) is justified because two different toxins, Shiga toxin 1 and 2, are involved in causing severe disease [87]. Another example is the two-antibody combination, ASN100 (see Sect. 7.4.5.4) being developed for the prophylaxis and treatment of severe *S. aureus* infections. It is a combination of two toxin-neutralizing mAbs, the multi-target ASN-1, and ASN-2, which targets the highly polymorphic leukocidin, LukGH. Together these two mAbs neutralize six cytotoxins involved in the complex pathogenesis of *S. aureus*. Another combination, an anti-Hla and an anti-ClfA mAb was shown to improve strain coverage in protection studies in mouse *S. aureus* bacteremia models, arguing for the benefit of simultaneous targeting of multiple virulence factors in *S. aureus* infections [132].

An advantage of antibody combinations over bispecifics is that antibody combinations can be tested relatively easily *in vitro* and *in vivo* to identify synergistic combinations. This is not the case for bispecifics, where the antigen binding domains need to be cloned into the desired format in different configurations in order to find combinations which retain full activity comparable to the individual components. The two specificities on a bispecific antibody also cannot be titered independently. In cases where the two targets differ greatly in abundance or are differentially located (secreted vs. surface expressed), availability of mAbs for binding both targets can be compromised. So while proof of concept studies testing combinations of individual antibodies against distinct targets or even against different epitopes on the same target are critical for selection of efficacious combinations, this does not guarantee success when antigen binding domains are reformatted as a bispecific.

As antibody combinations require parallel production of two or more antibodies, manufacturing costs (cost of goods) and complexity may be significantly higher than for a single mAb or a

bispecific mAb, using current technologies. However, the discovery process for bispecifics is typically longer, and there may also be manufacturability challenges (e.g. stability, solubility). The development of combinations of more than two antibodies may be driven by the balance between manufacturing costs and the expected advantage in efficacy. It is also unclear though, how many mAbs in a cocktail would entail a switch to oligoclonal or polyclonal expression systems. Symphogen have developed a mAb mixture approach with which it is possible to simultaneously express antibodies against multiple distinct targets [111]. In partnership with Meiji, they were reported to be developing a mixture of 5–10 mAbs targeting *P. aeruginosa*, although no development beyond preclinical stage has been reported since 2013. Nevertheless, the regulatory path forward to obtain approval for combinations of more than two or three antibodies is not clear.

At present, the main development hurdle for antibody combinations is the potential requirement for multi-arm clinical trials to test the efficacy of the individual components. A recent example was the development of actoxumab and bezlotoxumab (ZINPLAVA™) against *C. difficile* toxins A and B, respectively, which was originally intended as a combination therapy for the treatment of recurrent *C. difficile* infection. Phase 3 efficacy trials involved testing the combination and each mAb alone in order to demonstrate superiority of the combination. However the results showed that the mAb against toxin A did not contribute to the protection conferred by the combination thus only anti-toxin B mAb was approved (see Sect. 7.4.4.3).

7.4 Modes of Action of Anti-bacterial Antibodies

7.4.1 Anti-virulence Mechanisms

Targeting bacterial virulence has emerged as a way of counteracting the ever-spreading antibiotic resistance, to limit collateral damage and support the host's innate and adaptive immune defense mechanisms. Blocking virulence mechanisms offers the advantage of not putting pressure on microbial growth directly, and thus avoiding the strong selective pressure for resistance to develop. Although anti-toxin therapies have long been recognized as highly effective, more recently other virulence factors have also been assessed as potential antibody targets, such as the type III secretion system, adhesins and pili, and outer membrane transporters. These antigens are located on the bacterial surface (unlike exotoxins) and hence mAbs targeting them may not only elicit neutralization (blocking of function), but also trigger bactericidal effects (Fig. 7.2).

7.4.1.1 Exotoxin Neutralization

The pathogenesis of *C. difficile*, the main causative agent of hospital acquired antibiotic-associated colitis, is driven by its two toxins, enterotoxin A (TcdA) and cytotoxin B (TcdB) [135]. A combination of two neutralizing human mAbs against TcdA and TcdB has been tested in Phase 2 clinical trials for *C. difficile* infections and was shown to significantly decrease reoccurrence of *C. difficile* infections in the presence of antibiotics [81]. However, in efficacy studies in piglets, the anti-TcdA mAbs not only proved inefficient, but also appeared to induce more severe disease, while anti-TcdB mAbs were highly protective against systemic illness [125]. The deleterious effects observed with anti-TcdA mAbs in piglets could not be rationalized (e.g. toxin enhancing capacity) [125], and were at odds with the observation in rodents, where both anti-TcdA and TcdB were shown to be protective [65, 79] thereby emphasizing the importance of using multiple, well validated animal models [16]. Furthermore, clinical efficacy data could not demonstrate the benefit of the anti-TcdA mAb (see Sect. 7.4.4.3).

The Shiga toxins (Stxs) produced by enterohemorrhagic *E. coli* (EHEC) are major virulence factors responsible for the development of HUS, and act intracellularly by inhibiting protein synthesis in host cells following pinocytosis. After colonizing the intestinal epithelium, EHEC releases Shiga toxins into the bloodstream, which are then transported to various target organs,

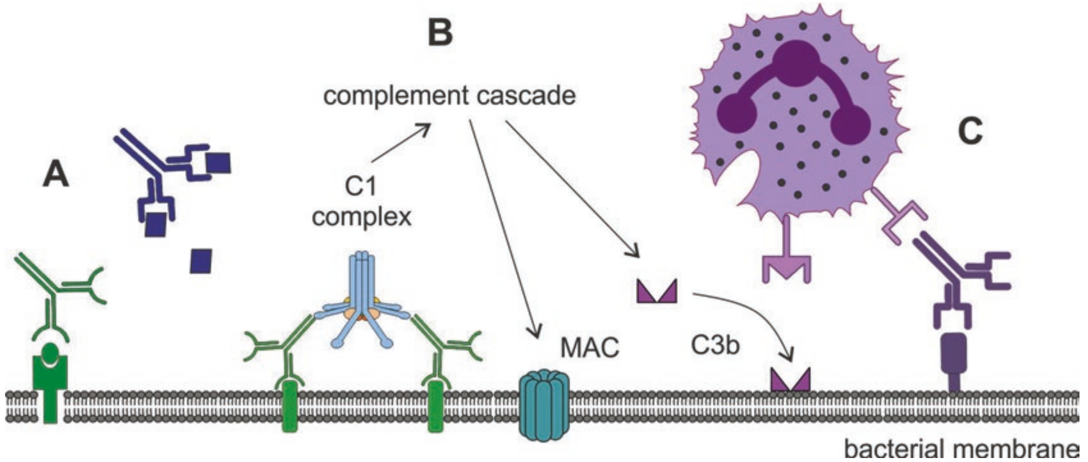

Fig. 7.2 Modes of action of anti-bacterial mAbs. Monoclonal antibodies can neutralize bacterial toxins or surface associated virulence factors (**A**). Upon binding to surface antigens, antibodies can activate the complement cascade and induce killing of bacteria through insertion of the membrane attack complex (MAC) (**B**) or opsonize bacteria for phagocytosis (**C**)

primarily the kidneys, which are rich in receptors for the toxin. The receptor for Shiga toxins is a glycosphingolipid receptor, globotriaosylceramide (Gb3) [100]. Two major Stx classes are known, Stx1 and Stx2, each being composed of one A, and five B subunits (AB5 type toxins), which are involved in enzymatic activity and cell targeting, respectively [87]. Stx2 has been found to be up to 400 times more toxic than Stx1 and is associated with the vast majority (>90%) of STEC-HUS cases reported. Several antibodies against both domains of Stx1 and Stx2 have been characterized for inhibition of toxin binding to the cellular receptor, intracellular trafficking, and enzymatic activity *in vitro* and in mouse models [87]. Clinical efficacy studies are being planned with such antibodies (see Sect. 5.2.8).

Another toxin-producing pathogen is *Bordetella pertussis*, the causative agent of whooping cough. The pertussis toxin (PTx) is a critical virulence factor, essential for full bacterial pathogenicity, including inhibition of the innate immune response and induction of leukocytosis [97]. Two anti-PTx antibodies, one of which competitively inhibits binding to the cellular receptor, and the other which alters PTx intracellular trafficking, are highly protective alone, but also show synergistic activity in aerosol and intra-cerebral murine models [97]. When administered therapeutically to baboons, the combination of the two antibodies leads to an increase in white blood cell count and accelerated bacterial clearance rates [97].

A special class of toxins, are the beta-barrel pore-forming toxins (PFTs), produced mainly by Gram-positive bacteria. Although they have diverse pore architectures, they are able to lyse host cells using the same mechanism, which involves host cell binding, oligomerization, and beta-barrel pore-formation [24].

One subclass of PFTs are the cholesterol dependent cytolysins (CDCs), present in *S. pneumoniae* (pneumolysin), *S. pyogenes* (streptolysin O), *L. monocytogenes* (lysteriolysin O), *C. perfringens* (perfringolysin) and *S. intermedius* (intermedilysin), pathogens involved in a wide range of diseases, ranging from pneumonia, meningitis, and gastroenteritis to gas gangrene [18]. These toxins bind to a common receptor, cholesterol, and are known to form large pores (250–300 Å diameter comprised of 35–50 monomers) on host cell membranes. They share between 40 and 60% sequence homology. Detoxified pneumolysin was shown to induce neutralizing antibodies in mice, and active vaccination significantly protected mice against lethal pneumococcal infection [117]. Three mAbs against pneumolysin showed a significant decrease in

bacterial lung colonization and lower frequencies of bacteremia and tissue injury following prophylactic administration to mice infected intranasally with *S. pneumoniae* [40]. The effects of the mAbs were synergistic [40], indicating that targeting multiple epitopes may be beneficial for the inhibition of cytolytic function. Interestingly, one of these mAbs, Ply-5, which recognizes a peptide in the cell binding region of pneumolysin, also binds to other members of the CDC toxin family [59] showing how a common ligand binding site can be potentially exploited as an epitope for cross-reactive antibodies.

Other examples of pore forming toxins are alpha-hemolysin (Hla) and the bi-component leukocidins produced by *S. aureus.* Alpha-hemolysin targets human epithelial and endothelial cells and has a well-established role in *S. aureus* pathogenesis, especially in pneumonia. Several active and passive immunization approaches have been pursued to inactivate this toxin. Three anti-Hla specific mAbs have been described: 243-4/AR-301 [115], LC10 [54, 121] and LTM14 [35, 36]. 243-4/AR-301 and LC10 have already entered clinical trials for safety/efficacy (see Sect. 7.4.5.2) [150]. LC10 and LTM14 both target the cell binding domain of Hla, but have non-overlapping epitopes: LTM14 directly competes with the host cell protein receptor (ADAM-10) binding [35], and LC10 has a dual mode of action: inhibition of cell binding and oligomerization [102]. Based on competition studies, 243-4/AR-301 shares an epitope with LTM14 [114]. All three mAbs have very tight binding to the Hla toxin (equilibrium dissociation constants, K_Ds, in the pM range).

The bi-component toxins of *S. aureus*: LukSF-PV, LukED, HlgAB, HlgCB and LukGH, require the association of equal amounts of an S- and F-component to form functional pores, and target phagocytic cells *via* recognition of important immune molecules (complement and chemokine receptors) and thus contribute to immune evasion [30, 136]. Active vaccination approaches have been proposed for LukSF (NCT01011335) [62]. Since these five leukocidins play redundant roles, all have to be blocked to avoid lysis of phagocytes and enable the host to eliminate *S. aureus* at infection sites. An additional difficulty is related to the LukGH toxin that is unique among the leukocidins in many respects, as it forms a stable dimer of S- and F-components before cell binding, and is expressed as clade variants by different *S. aureus* isolates [10]. To address this complexity, Arsanis discovered two mAbs able to neutralize all six toxins. A broadly cross-reactive toxin neutralizing antibody ASN-1 (Hla-F#5), was selected based on its potency against Hla and four of the five bi-component toxins, LukSF-PV, HlgAB, HlgCB and LukED [28, 114] (see Sect. 7.3.4). Despite the low sequence homology between the toxins, there is a conserved three-dimensional epitope found in Hla and three F-components (LukF-PV, HlgB, and LukD) that is targeted by ASN-1. The region of the toxins harboring this epitope is essential as it is involved in cell binding. Importantly, it is conserved between the four different toxins in virtually all sequence variants identified in sequenced genomes (approximately 7000) in publicly available databases [10]. This highlights that broadly-cross-neutralizing mAbs can take advantage of conserved functional features of the antigens that are less likely to be refuted by escape mutants or allelic variation. Discovery of LukGH neutralizing antibodies benefited from the identification of its complex formation in solution, and relied on binding to the most divergent sequence variants [8, 9]. ASN-2, the most potent LukGH mAb with the broadest neutralization activity was selected as a clinical lead [9] to be used in combination with ASN-1 (see Sect. 7.4.5.4).

Toxin neutralizing antibodies of particular interest are those directed against bioterrorism agents, the anthrax and botulinum toxins. Anthrax is caused by breathing in spores of the *Bacillus anthracis*, which, when inhaled, replicate and produce a potentially lethal, three-component toxin comprising a protective antigen (PA), lethal factor (LF) and edema factor (EF). This is an A-B type toxin, where PA is the cell binding, or B subunit, and LF and EF are enzymes (zinc metalloprotease and adenylate cyclase, respectively) or A subunits, which combine with the former to make the edema toxin and lethal toxin,

respectively [20]. Given its central role in anthrax pathophysiology, the protective antigen has been the main target for anti-anthrax vaccination approaches. Several anti-PA mAbs, with different modes of action ranging from inhibition of receptor binding to disruption of pre-formed PA heptamers, are currently in clinical development [20] and two have already been approved for clinical use (see Sect. 7.4.4.1 and 7.4.4.2). Even though less studied, the protective properties of anti-LF mAbs have also been investigated, particularly in combination with anti-PA mAbs [108]. It has been shown that despite the fact that all of the anti-LF mAbs recognize the antigen, and some are even cross-reactive with PA and EF, not all neutralize the toxin. It is not the avidity and binding capacity that determine toxin neutralization efficiency, but rather the nature of the epitope. Moreover, *in vivo*, none of the anti-LF mAbs were protective alone, as opposed to combinations of two anti-LF mAbs or two anti-LF mAbs and one anti-PA mAb, which also showed synergy *in vitro* [108]. Another interesting finding of the study was that toxin neutralization is partly dependent on Fc receptors *in vitro*, in agreement with a previous study which reported Fc receptor dependent protection against *B. anthracis* in mice with an anti-PA mAb [1].

The botulinum neurotoxin (BoNT) produced by *Clostridium botulinum* is one of the most poisonous substances known to man, and also one of the most serious bioterrorism threats. BoNTs are three domain proteins containing binding, translocation and catalytic (zinc endopeptidase) domains [38]. There are eight serotypes of BoNT [26], which differ from each other by 34–64% at the amino acid level, and five of these cause naturally occurring human botulism (A, B, E, F and H) [11, 74]. Early work on mAbs against BoNT/A showed that single mAbs were ineffective and toxin neutralization *in vitro* and *in vivo* required a combination of two to three mAbs targeting non-overlapping epitopes on the cell binding domain, which interacts with host cells *via* at least two different sites [98]. Later on, a high affinity (pM) mAb against both BoNT subtypes A1 and A2 was engineered [38] (see Sect. 7.3.4 for details). Interestingly, different residues on the mAb are involved in the interaction with the two BoNT/A subtypes, so this cross-reactivity does not take advantage of conserved epitopes. More recently however, targeting a conserved epitope at the tip of the translocation domain allowed cross-reactive and cross-neutralizing mAbs across different serotypes (A, B, E and F) to be identified [39]. Clinical efforts have concentrated on the neutralization of BoNT/A, and a three mAb cocktail (XOMA 3AB) targeting four BoNT/A subtypes (A1, A2, A3 and A4) has recently completed Phase 1 testing in human volunteers [95].

7.4.1.2 Endotoxin Neutralization

Endotoxin (lipid A) is part of the lipopolysaccharide (LPS) that forms the outer leaflet of the outer membrane in most Gram-negative bacteria. While lipid A is embedded in the outer membrane, the polysaccharide portion of LPS (LPS-core and the O-antigen side chain) are on the surface. Lipid A is exposed when LPS is shed, which is facilitated upon bacterial lysis. In Gram-negative sepsis, the main contributor to septic shock syndrome is the LPS released by bacteria, which binds to the MD2-TLR4 complex *via* the lipid A moiety, thereby initiating the shock cascade, at concentrations as low as 10 pg/ml [104]. As lipid A is well-conserved among the clinically relevant Gram-negative bacteria, several passive immunization approaches were tested as adjunct therapies for Gram-negative sepsis in the early 1990s [25]. Three mAbs (human or murine) were tested in humans: Nebacumab (Centoxin or HA-1A, Centocor), edabacomab (Xomen-E5, XOMA) and T88 (Chiron). All three were IgMs and demonstrated low affinity and poly-reactive characteristics. They also raised safety concerns [22] and failed due to lack of efficacy.

The best-characterized LPS neutralizing antibody is WN1 222-5 ([92, 93]), which is a cross-reactive murine IgG against a large panel of pathogenic *E. coli*, *Salmonella, Shigella* and *Citrobacter* strains, due to targeting a conserved epitope in the inner core region of LPS [92, 93]. In addition, the antibody displays one of the highest affinities reported for an anti-carbohydrate antibody (K_D of ~30 nM in the monovalent state). The unusually high affinity is explained, based

on the 3-D crystal structure of the complex between WN1 Fab and the core region of *E. coli* R2 LPS, by the involvement of seven sugar residues and 13 H-bonds [43]. The monovalent affinities for carbohydrates are generally much lower than for protein antigens (low μM to high nM K_D range for carbohydrates versus low nM to pM for proteins), however this is compensated for by a strong avidity effect due to the high abundance, and typically higher accessibility of the carbohydrate antigens on the bacterial surface. This is demonstrated by the functional activity of the WN1 222-5 mAb *in vitro* through neutralization of LPS-induced release of clinically relevant cytokines and chemokines, and *in vivo,* by blockade of endotoxin-induced pyrogenicity in rabbits, and lethality in galactosamine-sensitized mice [25].

Antibodies directed against lipid A and/or the inner core are expected to directly neutralize binding of endotoxin to its innate receptors. Interestingly, a mAb against the O-antigen portion of LPS was also shown to elicit neutralization of the endotoxin effect of specific LPS molecules both *in vitro* and *in vivo* [130]. The efficacy of LPS neutralization exceeded that of polymyxin B (a lipid A-binding antibiotic) by several orders of magnitude. Additionally, *in vivo* studies confirmed the advantage of removing LPS molecules from the circulation by Fc dependent mechanisms (immune complexes). Obviously, endotoxin neutralization by such mAbs is type specific, but the narrow spectrum of such prospective therapeutics could be overcome by suitable diagnostic tools.

7.4.1.3 Inhibition of the Type III Secretion System

The type III secretion system (T3SS) has been described in many Gram-negative pathogens as a prominent virulence factor (see Sect. 7.2 for details). The structural proteins of T3SS, particularly orthologues of the needle tip proteins, were shown to be useful antibody targets. Such antibodies interfere with the formation of the translocon pore in the membranes of host cells [46].

EspA is a needle tip protein of the *E. coli* T3SS, and anti-EspA antibodies were shown to interfere with effector translocation, completely blocking cytoskeletal changes within the host cell, without affecting cell adhesion [72]. In Shigella, anti-IpaD mAbs were shown to prevent translocation insertion, hemolysis of erythrocytes, and Shigella entry into epithelial cells *in vitro* [32].

In *Yersinia pestis* the orthologous needle tip antigen, LcrV is highly protective in both active and passive immunization in various animal models of disease, including bubonic and pneumonic plague [123]. An LcrV-specific mAb was shown to function by two independent mechanisms: an Fc-independent neutralization of the T3SS and an Fc-dependent opsonic activity (see below). These two activities were dissected by using antibody fragments and Fc mutants with inactivated effector functions [58]. PcrV in *P. aeruginosa* also represents an attractive target. KaloBios tested an anti-PcrV PEGylated Fab, KB001-A in a Phase 2 trial. The lack of Fc allowed neutralizing potency only, with the advantage of minimizing inflammation in the targeted cystic fibrosis patients. Medimmune developed another antibody against PcrV that provided superior protection over the KB001-A parent IgG, Mab 166 [140]. The function of the Medimmune antibody relies on neutralizing cytotoxic activity of the T3SS and was later combined into a bispecific mAb that affords greater neutralizing and additional opsonic activity (see below).

7.4.1.4 Inhibition of Adhesion

Adhesins are bacterial surface proteins that mediate the physical interaction between the pathogen and the host, and are ubiquitous in bacterial pathogens. They are typically required for colonization, therefore represent attractive antigens for prophylactic vaccines. Depending on the infection site, pathogens may display different types of adhesins. Only a few examples of mAbs considered for adhesion targeting have been reported.

In uropathogenic *E. coli*, FimH is a lectin-like protein, incorporated into the tip of type 1 fimbriae, binding terminally exposed mannose, and is critical for virulence [60]. An antibody with a

new mode of action against FimH has been described, which binds adjacent to the ligand binding pocket, a so-called parasteric (next to ligand) inhibitor [69]. This antibody is very potent in blocking bacterial adhesion and is also able to reverse microbial attachment, dissolving surface-adherent biofilms and protecting mice from urinary bladder infection [69]. The principle of parasteric inhibition is appealing because the antibody is effective in the presence of high concentrations of endogenous ligand, and can trigger reversal of ligand binding.

In *S. aureus*, clumping factor A (ClfA) was one of the first surface proteins to be identified as a vaccine antigen for prophylactic vaccination, and is currently being tested in clinical trials as part of a multicomponent *S. aureus* vaccine [3, 4]. There was a debate recently as to whether ClfA is indeed a suitable vaccine candidate, given that it lacks efficacy in certain animal models of *S. aureus* infections, and in some cases of either active or passive immunization, even enhancement of bacteremia was observed [78].

In another Gram-positive pathogen, *Enterococcus faecalis*, a mAb to the major pilus component, EbpC, diminished biofilm formation and prevented the establishment of a rat endocarditis infection [107]. In addition, the labelled mAb proved to be specific in imaging the established enterococcal infection, providing an example of how the same agent can be used in both diagnostic and therapeutic applications [107].

Therapeutic antibodies do not necessarily need to act directly at the level of adhesin-receptor interaction and, particularly in the case of chronic infections (or colonization), beneficial effects can be brought about by the immunomodulatory properties of the mAbs. Potential mechanisms of antibody-mediated immunomodulation include masking or exposure of dominant versus cryptic epitopes, alterations in proteolysis, antigen processing and antigen presentation [13, 41]. This has been well exemplified by mAbs against the P1 adhesin of *Streptococcus mutans*, the predominant etiologic agent of dental caries, which mediates adherence to salivary agglutinin [57]. It was shown that mAbs that target a certain P1 epitope, although unable to inhibit adherence directly and being non-opsonic, are able to induce serum antibodies in the treated host, most likely via immune complexes formed by the mAb and antigen, that strongly inhibit adherence [57].

7.4.1.5 Inhibition of Metal Transport

Metal bioavailability in the human host varies significantly in different tissue types and also during infection, therefore pathogenic bacteria have evolved to produce a variety of metal transporters to facilitate metal uptake, and in some cases, metal excretion. Many of these transporters are recognized as virulence factors [63]. The role of iron in bacterial pathogenesis has long been recognized, but recently, the importance of other metal ion transporters, such as those for zinc and manganese has become evident [19, 145]. The extracellular entities of these transporters are potential vaccines and antibody targets. However, mainly vaccine clinical trials have been reported to date.

Antibodies targeting outer membrane proteins involved in iron acquisition by *Acinetobacter baumanii* were shown to block siderophore-mediated iron uptake besides providing bactericidal effects (see below) [42].

The heme binding protein IsdB, one of several outer membrane proteins involved in iron acquisition in *S. aureus* [51], when used as vaccine, showed protection in several murine models of *S. aureus* infection, but failed to demonstrate efficacy in preventing *S. aureus* infections in cardiothoracic surgery populations [3, 4]. However, mAbs against IsdB, which showed *in vivo* protection were demonstrated to be active in OPK, but did not affect heme binding [103].

Another *S. aureus* metal transporter, the Mn transporter C (the surface lipoprotein component of MntABC) involved in nutrient acquisition, phagosome survival and resistance to oxidative stress, is being tested as part of a multicomponent *S. aureus* vaccine, which also includes capsular polysaccharides and ClfA [3], and is currently in Phase 2b clinical trials. Interference mapping analysis of mAbs generated against MntC, revealed that the antibodies belonged to three non-competing groups or bins [4]. Fine epitope mapping of mAbs from each group showed that

one of the mAbs acts by preventing Mn-binding to the metal ion site in MntC, while the other two interfere with Mn transfer across the bacterial membrane by disrupting the MntC interaction with the downstream permease MntB [48]. Both modes of action lead to manganese starvation of the bacterium, which in turn reduces the activity of the manganese dependent superoxide dismutase, and increases the susceptibility of the pathogen to the oxidative stress encountered after opsonophagocytosis by neutrophils [48].

In the Gram-negative bacterium *Nesseria meningitidis*, an outer membrane zinc and possibly iron importer, ZnuD, was shown to induce bactericidal antibodies in mice and anti-ZnuD antibodies have also been detected in human sera [127]. Based on these observations, combined with the high ZnuD sequence conservation (>90% in over 500 strains) and an exposed predicted zinc binding loop (loop 3), ZnuD has been considered as a candidate for a universal *Neisseria meningitidis* vaccine [56]. Recently, a detailed structural study revealed that ZnuD samples different conformations during substrate binding, and it is proposed that the flexible conformation of the metal binding loops prevents the immune system from directly binding and inhibiting the functional loop 3 site [14]. This study aids the structure-based design of ZnuD derivatives with improved vaccine efficacy, but at the same time suggests potential therapeutic applications for mAbs targeting a certain conformational epitope of the protein.

7.4.2 Bactericidal Mechanisms

In order to elicit bactericidal activity, antibodies must bind to the surface of the bacteria. Therefore, an important criterion to be addressed at the stage of molecular target selection, is the surface exposure and abundance of the antigen.

In the vast majority of cases, antibodies cannot induce bacteriolysis directly, and require the co-operation of phagocytic cells and/or complement. Upon binding to the bacterial surface, antibodies recruit complement components, either as opsonins for enhanced phagocytosis (C3b) or initiators of the complement cascade (C1q, mannan-binding proteins) resulting in the assembly of the membrane attack complex (MAC) in the bacterial membrane. While Gram-positive bacteria are inherently protected from MAC-mediated lysis due to their thick peptidoglycan cell wall, Gram-negative bacteria are susceptible, unless they evolve mechanisms to prevent this. In the case of Gram-negative bacteria, antibody-dependent complement-mediated killing as well as opsonization may occur simultaneously. Dissection of the contribution of these individual modes of action for protection is challenging, especially in animal models. Blockade of phagocytic receptors or specific small-molecule inhibitors of phagocytosis not affecting the complement system are good candidates for such assays. Interestingly, phagocytotic uptake does not necessarily lead to bacterial killing. Besides facultative intracellular bacteria, more and more bacterial pathogens, traditionally considered as extracellular bacteria, are reported to be able to survive inside phagocytic cells and actually use this strategy for hiding and/or spreading within the host. *S. aureus* [37], *K. pneumoniae* [15] and extraintestinal pathogenic *E. coli* [96] have all been reported to withstand intra-phagocytic killing.

An important consideration regarding the bactericidal mode of action is the clinical indication, as certain patient populations may not be able to fully benefit from such therapeutic mAbs. These include immunocompromized patients with neutropenia, or premature infants [119] and patients with severe liver disease [142] due to low complement activity.

7.4.2.1 Antibody-Dependent Complement Mediated Killing

Surface bound antibodies recruit C1q and initiate the activation of the classical complement cascade ultimately leading to the formation of MAC. The initiator of the cascade, C1q has a hexameric structure, with six Fc-binding heads. At least two heads (with more being even better) need to bind to Fc portions to activate C1q. Given the pentameric structure of IgM, this antibody class is highly efficient in C1q activation,

however IgM is not the most desirable isotype due to inferior manufacturability characteritics compared to IgG. For IgG-induced activation of C1q, at least two antibody molecules are required that bind within a proximity of 30–40 nm. This emphasizes the advantage of targeting antigens that are highly abundant or at least reach a high topical density. Carbohydrate antigens, although inferior in many other aspects to proteinaceous antigens (low immunogenicity, lower affinity interaction) seem to be advantageous given their polymeric nature enabling high density binding of Ig molecules. LPS O-antigen specific antibodies (both IgM and IgG) were shown to exhibit complement-mediated bactericidal activities against *Pseudomonas aeruginosa* [53], *Salmonella enterica* [83] and extraintestinal pathogenic *E. coli* [128]. Antibody-dependent serum killing was also shown for mAbs raised against iron regulated outer membrane proteins of *Acinetobacter baumanii*, however, efficient bactericidal effects were only observed for the IgM class at high concentrations against bacteria grown in iron-deprived medium [42]. mAbs against factor H binding protein (fHbp) of *N. meningitides* showed different modes of action for protection, including complement-mediated killing [137]. This activity was only apparent with the combination of two IgGs targeting non-overlapping epitopes, corroborating the need for multiple adjacent Fc portions in engaging C1q. The advantage of this mode of action for protection is the dispensability of phagocytes, i.e. the effect is expected to be retained even in the most immunocompromised patients.

7.4.2.2 Opsonophagocytotic Killing Activity

Antibodies binding to bacteria can induce/increase phagocytic uptake through two mechanisms. Following initiation of the classical complement pathway, the cleavage product C3b deposits on the bacterial surface, which can be engaged by C3b-receptors on phagocytic cells. Additionally, phagocytes express different Fc receptors that are able to recognize various isotypes of antibodies. Triggering Fc-receptors may lead to complement-independent phagocytic uptake [44]. Nevertheless, complement receptor and Fc-receptor co-ligation can produce synergistic effects that are additionally influenced by other, antibody-independent signals, e.g. from Toll-like receptors [134]. Although soluble antibodies can also be bound by Fc receptors, optimal signalling is induced by simultaneous ligand binding by adjacent Fc receptors, only. This again, necessitates high density binding of antibodies on the bacterial surface. As mentioned previously, target selection is critical given that highly conserved antigens are masked by abundant carbohydrates. On the other hand, the readily accessible carbohydrate antigens have evolved high variability. Although opsonization of bacteria with capsule or LPS O-antigen specific mAbs provides protection, it is not suitable for broad-spectrum, i.e. species-wide protection. Still, clinically important clonal lineages can be addressed by opsonizing anti-capsular and/or O-antigen specific mAbs. For instance, the majority of neonatal meningitis cases are caused by *E. coli* O18:K1, and mAbs against both the O18 antigen and the K1 antigen were shown to be protective in animal models [66, 110]. Interestingly, as the *E. coli* K1 antigen is essentially identical to the meningococcus type B capsular polysaccharide, mAbs raised against either one elicit cross-protection against these two major meningitis pathogens [66, 105].

Type-specific antibodies against O- or K-antigens of multi-drug resistant (MDR) bacteria represent a feasible approach, given that the emergence of MDR strains is often a clonal phenomenon. Panobacumab, a human IgM targeting *Pseudomonas aeruginosa* O11, which is responsible for a significant portion of nosocomial Pseudomonas infections, is being tested clinically. Other LPS O-antigen specific mAbs targeting the worldwide distributed MDR clones, *E. coli* ST131 and *K. pneumoniae* ST258, are reported [128, 129] and considered for prophylaxis and therapy by passive immunization. Interestingly, both mAbs exhibit multiple modes of action, including opsonization, bactericidal effects and LPS neutralization and provide high efficacy at very low doses in animal models [128, 130]. Similarly, an O-antigen specific murine

mAb was recently reported to be efficacious against *S. enterica* serovar Typhimurium in an animal model, with a proposed mode of action of inducing increased uptake by, and subsequent killing in macrophages [6]. Hypervirulent strains of Klebsiella associated with pyogenic liver abscesses are being targeted with mAbs raised against the capsular antigen. Such mAbs were shown to be protective through inducing opsonophagocytic killing [26]. Similarly, OPK activity and *in vivo* efficacy was proven for mAbs targeting the dominant O1 LPS antigen of *K. pneumoniae* [52, 116]. Type A and type B strains (the most virulent types) of the biodefense category A agent *Francisella tularensis*, the causative agent of tularemia, share the same O-antigen [49] and express the same carbohydrate structure as an O-antigen capsule [5]. Passive immunization with mAbs against this shared O-antigen were shown to be protective in different animal models and hold promise for further development.

Proof of concept was shown with mAbs targeting another type of carbohydrate, known as alginate, a widely distributed cell surface polysaccharide on *P. aeruginosa*. Aerucin, a human IgG1 mAb specific to alginate was reported to bind greater than 90% of clinical isolates of *P. aeruginosa* and enhance complement deposition, leading to enhanced phagocytic destruction of *P. aeruginosa* by the immune system. Aerucin has been shown to protect mice from lethal challenges with a variety of *P. aeruginosa s*trains in an acute pneumonia model [106], and against eye infections in a keratitis model [151]. The product is being developed initially as an adjunctive anti-infective to treat hospital-acquired and ventilator-associated pneumonia due to *P. aeruginosa*. A Phase 1 clinical trial (NCT02486770) in 16 volunteers has been completed.

Besides carbohydrate surface structures, protein antigens are appropriate targets for opsonizing antibodies provided that they are accessible and sufficiently conserved. Fimbriae, T3SS needles, and receptors for larger molecules may fulfil these criteria. Antibodies binding to these structures can have multiple effects, i.e. trigger opsonophagocytic killing but might also act through neutralization of the function (immune evasion, adhesion, cytotoxicity, or nutrient uptake as discussed in Sect. 7.4.1). For example, Staphylococcal Surface Protein A (SpA or Protein A) is one of the most abundant surface proteins of *S. aureus*, and is a multifunctional virulence factor that interacts with several human proteins to facilitate immune evasion [33]. Most importantly, it has been proposed that by binding to the Fc portion of immunoglobulins, SpA protects *S. aureus* from phagocytosis. On the other hand, Spa binding to VH3-type Fab regions of antibodies leads to B-cell receptor (BCR) cross-linking and thus non-antigen specific B-cell activation and immune exhaustion. *S. aureus* mutants lacking SpA are phagocytosed and killed more rapidly in blood than wild-type staphylococci and are also defective in the formation of abscess lesions in mouse models [67]. MAbs generated by hybridoma technology using mice immunized with a mutant variant of SpA that lacks Fcγ and VH3 binding were shown to enhance opsonophagocytosis and killing *in vitro* and lead to protective immunity against *S. aureus* infection [67, 131]. It is not entirely clear how neutralizing the virulence functions of SpA contributes to this protection. Although no clinical development is reported for anti-SpA mAbs, two companies have active research programs in this area, Staurus Pharma [82] and Arsanis Biosciences [94]. Another proteinaceous surface structure, MrkA, the type 3 fimbrial shaft subunit of Klebsiella was identified as a potential mAb target for opsonophagocytic killing activity. In line with their *in vitro* efficacy, MrkA-specific mAbs showed protection in animal models [139]. A protective human mAb targeting the iron acquisition protein IsdB in *S. aureus* was also shown to act through opsonophagocytic killing (but was not able to block heme uptake) [103]. Finally, cross-reactive mAbs against the pneumococcal variable surface protein PspA, which is involved in complement resistance, were reported to mediate opsonophagocytic killing [45]. These examples also highlight the importance of dissecting the exact mode of action, which may be crucial for the clinical applicability, as unlike true anti-virulence neutralizing mAbs, opsonic mAbs require retained phagocyte functions.

7.4.2.3 Direct Bactericidal Activity

Direct, i.e. complement-independent bactericidal activity has been reported for only one Gram-negative bacterium, *Borrelia burgdorferi*. Antibodies specific for the OspA and OspB surface proteins induced by infection or by active vaccination are known to induce lysis of Borrelia [99]. An Osp-specific mAb was also reported to have bactericidal activity without complement by destabilizing the outer membrane, thus increasing permeability and subsequent osmotic lysis [75]. This activity appeared to be Borrelia-specific, since *E. coli* expressing recombinant OspB was not susceptible to complement-independent lysis [76]. Therefore, direct killing by mAbs is not considered to be a general phenomenon and applies to very specific cases.

7.4.3 Anti-bacterial mAbs in the Clinic

As of 2016, three anti-bacterial mAb products have been approved for human use, all are based on neutralization of exotoxins from Gram-positive pathogens. Table 7.1 lists these antibodies and those currently being tested for efficacy in the clinic. Most of these are based on exotoxin neutralization, and/or target *S. aureus*.

7.4.4 Approved Anti-bacterial mAbs

7.4.4.1 Raxibacumab (ABthrax®)

Raxibacumab is a human mAb that targets the protective antigen (PA) component of the lethal toxin and also of the edema toxin of *Bacillus anthracis*. The antibody was discovered using human antibody phage display library technology in a joint venture between Cambridge Antibody Technology and Human Genome Sciences (acquired by GSK in 2012). The development of raxibacumab was the result of a coordinated response to a recognized public health threat and the US government's request for new medical counter-measures in the event of an anthrax attack against the civilian population. Raxibacumab was shown to be efficacious in rabbit and monkey models [88]. Its development was according to the US Food and Drug Administration (FDA) Animal Rule regulation, specifically intended for agents for which the conduct of definitive human efficacy studies is not ethical or feasible. Its safety was evaluated in 326 healthy adult volunteers treated with the recommended dose. In December 2012, raxibacumab was approved by the FDA for the treatment of inhalational anthrax in combination with appropriate anti-bacterial drugs, and for prophylaxis of inhalational anthrax when alternative therapies are not available or are not appropriate. Raxibacumab is marketed in the US by GSK, however the sole customer is the US government.

7.4.4.2 Obiltoxaximab (Anthim®)

Obiltoxaximab is another anthrax toxin neutralizing mAb targeting free PA. It was developed by Elusys Therapeutics (supported by the US Department of Health and Human Services' Biomedical Advanced Research and Development Authority) for the prevention and treatment of inhalational anthrax.

Obiltoxaximab is an affinity-enhanced, chimeric, deimmunized mAb that was generated from the murine mAb 14B7. Mutations that enhanced the affinity of 14B7 were identified using single-chain Fv (scFv) phage display technology. A chimeric antibody was then generated by fusing the enhanced versions of the murine 14B7 VH and VL genes to human CH1 and κ light chain constant regions, respectively. In addition, the murine VH and VL segments were subjected to further modification to reduce immunogenicity using BioVation's (Aberdeen, United Kingdom) deimmunisation technology, which entailed modifying canonical human T-cell stimulatory motifs to reduce immunogenic potential. Obiltoxaximab was shown to be efficacious in animal models of anthrax that served as the basis for approval, as clinical trials with intentional exposure of humans to anthrax were not feasible. In March 2016, intravenous obiltoxaximab was approved in the USA for the treatment and prophylaxis of inhalational anthrax in combination with appropriate anti-bacterial drugs [47].

Table 7.1 Monoclonal antibodies and antibody-based biologics that have been tested in clinical trials

MAb	Species	Isotype	Organism target molecule	Discovery technology	Developing company	Clinical indication	Latest development status reported	Clinical trials
Raxibacumab ABthrax®	Human	IgG1 – lambda	*Bacillus anthracis* Protective antigen (PA)	Cambridge Antibody Technology (CAT) human antibody (scFv) phage display library	GSK/Human Genome Sciences Inc.	Inhalation anthrax – treatment	marketed approved by FDA on December 14, 2012	NCT02016963 NCT02339155 NCT00639678 NCT02177721
Obiltoxaximab ANTHIM® ETI-204	Mouse/ human chimeric	IgG1 – kappa	*Bacillus anthracis* Protective antigen (PA)	Derived from murine 14B7, with affinity enhanced VH and VL genes derived from scFv. Deimmunized using Biovation's technology	Elusys Therapeutics, Inc.	Inhalation anthrax – treatment	Approved 2016	NCT01453907 NCT01952444 NCT00829582 NCT01929226 NCT01932242 NCT01932437 NCT00138411
Bezlotoxumab ZINPLAVA® MK-6072, CDB-1, MDX-1388	Human	IgG1 – kappa	*Clostridium difficile* Toxin B	HuMAb-Mouse® (Medarex)	Merck & Co.	*C.difficile* infectious diarrhea – prevention of recurrence	Phase 3 ZINPLAVA™ approved by FDA on October 18, 2016	NCT01241552 NCT01513239
Actoxumab CDA 1,CDA-1, MBL-CDA1, MDX-066, MK-3415, 3D8	Human	IgG1 – kappa	*Clostridium difficile* Toxin A	HuMAb-Mouse® (Medarex)	Merck & Co.	*C.difficile* infectious diarrhea – prevention of recurrence	Phase 3 completed. Discontinued	NCT01241552 NCT01513239

(continued)

Table 7.1 (continued)

MAb	Species	Isotype	Organism target molecule	Discovery technology	Developing company	Clinical indication	Latest development status reported	Clinical trials
Panobacumab; Aerumab 11, KBPA-101, AR101	Human	IgM – kappa	*Pseudomonas aeruginosa* LPS O antigen (O11)	Human B cells MabIgX© technology	Kenta Biotech (now acquired by Aridis)	*Pseudomonas aeruginosa* hospital-acquired pneumonia caused by serotype 011 – treatment	Phase 2/3 No development reported	NCT00851435
Tosatoxumab (Salvecin); KBSA301, AR301	Human	IgG1 – lambda	*Staphylococcus aureus* alpha toxin (alpha-hemolysin, Hla)	Human B cells	Aridis (acquired from Kenta Biotech)	*S. aureus* ventilator-associated pneumonia-treatment	Phase 2	NCT01589185
MEDI-4893	Human	IgG1 – YTE (M252Y, S254T, T265E)	*Staphylococcus aureus* alpha toxin (alpha-hemolysin, Hla)	Velocimmune (Regeneron)	MedImmune	*S. aureus* ventilator-associated pneumonia-prevention	Phase 2	NCT01769417 NCT02296320
ASN100 (combination of ASN-1 and ASN-2)	Human	IgG1 kappa multispecific	*Staphylococcus aureus* alpha-hemolysin and F-components of gamma-hemolysins (HlgAB and HlgCB), LukED and LukSF (Panton-Valentine Leukocidin); as well as LukGH	Yeast IgG display (Adimab)	Arsanis	*S. aureus* ventilator-associated pneumonia-prevention	Phase 2	NCT02940626

MEDI-3902; BiS4aPA	Human	IgG1 kappa bispecific	*Pseudomonas aeruginosa* PcrV type III secretion system (T3SS) and persistence factor Psl	Cambridge Antibody Technology (CAT) human antibody phage display and Velocimmune	MedImmune	*P. aeruginosa* pneumonia	Phase 2	NCT02696902 NCT02255760
514G3	Human		*Staphylococcus aureus* Protein A (SpA)	True Human™	Xbiotech	*S.aureus* bacteremia	Phase 1/2	NCT02357966
SAR279356; F598	Human		Several pathogens Poly-N-acetylated glucosamine (PNAG)	Human B cells	Sanofi (licensed from Alopexx)	Prevention of bacteial infections in intensive care units	Phase 1/2 trial terminated	NCT01389700
Pritoxaximab cαStx1, Shigamabs® is drug name for the combination of pritoxaximab and setoxaximab	Mouse/ human chimeric	IgG1 – kappa	Shiga-like toxin-producing *E. coli* (STEC) shiga toxin type 1, stx1, Stx1, Stx-1, Shiga-like toxin 1, SLT-1, SLT-I; B subunit	Mouse hybridoma	Thallion Pharmaceuticals (acquired by Bellus Pharmaceuticals)	STEC infection causing diarrhea and hemolytic-uremic syndrome (HUS) – treatment	completed Phase 1, Phase 2 planned	NCT01252199
Setoxaximab cαStx2, Shigamabs® is drug name for the combination of setoxaximab and pritoxaximab	Mouse/ human chimeric	IgG1 – kappa	Shiga-like toxin-producing *E. coli* (STEC) shiga toxin type 2, stx2, Stx2, Stx-2, Shiga-like toxin 2, SLT-2, SLT-II: A subunit	Mouse hybridoma	Thallion Pharmaceuticals (acquired by Bellus Pharmaceuticals)	STEC infection causing diarrhea and hemolytic-uremic syndrome (HUS) – treatment	completed Phase 1, Phase 2 planned	NCT01252199

7.4.4.3 *Bezlotoxumab* (ZINPLAVA™)

C. difficile infection (CDI) is the most prevalent cause of antibiotic-associated gastrointestinal infections in healthcare facilities in the developed world. The Centers for Disease Control and Prevention estimated that CDI caused almost 500,000 illnesses and 29,000 deaths within 1 month of the initial diagnosis in 2011. The standard therapy for CDI involves administration of the antibiotics vancomycin or metronidazole but relapse occurs in 10–20% of patients on cessation of treatment [101]. Disease symptoms are mainly caused by two exotoxins TcdA and TcdB.

A combination of two human mAbs, actoxumab (MK-3415, aka CDA1, MDX-066) and bezlotoxumab (MK-6072 aka CDB1, MDX-1388), against TcdA and TcdB, respectively, were developed by researchers at the University of Massachusetts Medical School's MassBiologics Laboratory in conjunction with Medarex (now part of Bristol Myers Squibb), using Medarex's human antibody transgenic mice technology. The antibodies were shown to be protective in multiple animal models of CDI, including systemic and local (gut) toxin challenge models, as well as in primary and recurrent models of infection in mice, hamsters and piglets [7, 125]. A systemically administered combination of actoxumab and bezlotoxumab prevented both damage to the gut wall and the inflammatory response. Mutant antibodies, with a N297Q mutation that diminished binding to Fcγ receptors, gave a similar level of protection to wild-type antibodies, demonstrating that the mechanism of protection is through direct neutralization of the toxins and does not involve host effector functions [149].

Actoxumab and bezlotoxumab were licensed to Merck & Co in 2009. Two global, Phase 3 double-blind studies – MODIFY I and MODIFY II (Monoclonal antibodies for *C. difficile* therapy) were conducted to evaluate bezlotoxumab, either alone or in combination with actoxumab compared to placebo, for the prevention of recurrent *C. difficile* infection in patients on standard-of-care antibiotics for a primary or recurrent *C. difficile* infection [143]. The MODIFY I study enrolled 1452 patients in 19 countries and the MODIFY II study enrolled 1203 patients in 17 countries. The primary endpoint for each study was evaluated through 12 weeks following study drug administration. Treatment with the combination of bezlotoxumab and actoxumab did not provide added efficacy over bezlotoxumab alone. Furthermore, actoxumab alone provided no benefit in the prevention of *C. difficile* recurrence compared with placebo. Based on these results, only bezlotoxumab (ZINPLAVA™) was selected for the marketing authorization application. ZINPLAVA™ was approved by the FDA in October, 2016 to reduce recurrence of *C. difficile* infection in adult patients receiving anti-bacterial drug treatment.

7.4.5 Anti-bacterial mAbs in Clinical Efficacy Testing

7.4.5.1 Panobacumab

Panobacumab also known as AR-101, KBPA-101, or Aerumab™, is a human pentameric IgM/κ mAb with a mouse J chain, developed by Kenta Biotech and acquired by Aridis Pharmaceuticals, that is directed against lipopolysaccharide (LPS) O-polysaccharide moiety of *Pseudomonas aeruginosa* serotype O11. Panobacumab was generated by immunizing a healthy volunteer with an octovalent O-polysaccharide-toxin A conjugate vaccine. Antigen-specific B cells were enriched from the patient's peripheral blood followed by Epstein-Barr virus (EBV) transformation with a cell culture supernatant from the B95-8 marmoset cell line. This resulted in lymphoblastoid cell lines (LCL) producing antibodies against *P. aeruginosa* LPS of serotype O11, which were then fused to the heterohybridoma cell line LA55 [53].

The LPS O11 serotype has a prevalence of approximately 22% of all *P. aeruginosa* hospital-acquired infections worldwide. The mechanism of action of Panobacumab is thought to be through enhanced complement deposition on *P. aeruginosa*, leading to complement-dependent bacterial

killing. The antibody is intended to be a first line adjunctive therapy to complement standard-of-care antibiotics for patients with severe *P. aeruginosa* pneumonia in hospital settings.

Panobacumab has completed Phase 1/2a in hospital-acquired pneumonia and ventilator-associated pneumonia (VAP) patients. The Phase 2a preliminary proof-of-concept study evaluated this mAb in 18 very high risk HAP and VAP patients as an adjunctive therapy to antibiotics. The results showed that AR-101 met the primary safety endpoints, accelerated clinical cure of pneumonia, and protected patients from mortality as compared to the standard-of-care antibiotics alone group [109]. Panobacumab has been granted orphan drug designation in the US and EU and, according to the Aridis website, a multinational Phase 2b double blinded placebo controlled clinical study in HAP/VAP patients is being planned.

7.4.5.2 Tosatoxumab

Tosatoxumab, also known as Salvecin™ and AR-301, is a fully human IgG1 mAb specifically targeting *S. aureus* Hla. This antibody, formerly known as KBSA301 (Kenta Biotech, acquired by Aridis Pharmaceuticals in 2013) was discovered by screening B-cell lymphocytes of a patient with confirmed *S. aureus* infection and developed using the MabIgX platform [138]. AR-301 blocks the assembly of Hla subunits, repressing functional toxin pore formation leading to protection of host cells from Hla-dependent destruction. Its mode of action is independent of the antibiotic resistance profile of *S. aureus*, hence it is active against infections caused by both MRSA and MSSA. Tosataxumab is being tested as an adjunctive therapy to standard-of-care antibiotics in HAP and VAP patients in a double-blind, placebo controlled Phase 2a clinical trial. Tosataxumab was granted Fast Track designation by the FDA in September 2015.

7.4.5.3 MEDI4893

MEDI4893 is a neutralizing human IgG1 mAb developed by Medimmune/AstraZeneca against *S. aureus* Hla. It is an engineered version of mAb LC10, discovered using Regeneron's proprietary Velocimmune® human antibody transgenic mouse technology. It has an extended serum half-life through insertion of the YTE mutations in the Fc domain. MEDI4893 recognizes a novel epitope in the so-called "rim" domain of Hla and exerts its neutralizing effect through a dual mechanism of sterically blocking the binding of Hla to its receptor and preventing it from adopting a lytic heptameric trans-membrane conformation. In preclinical studies, prophylaxis with MEDI4893* (a version without YTE, as YTE is ineffective in mice) resulted in reduced bacterial numbers and limited damage to the lung epithelium, accompanied by dose-dependent increases in survival rates relative to an IgG1 isotype control [54, 55]. In adjunctive treatment, MEDI4893* increased survival in mice which were treated with sub-therapeutic doses of the antibiotics linezolid or vancomycin plus a sub-therapeutic dose of MEDI4893* over mice which received antibody or antibiotics alone [55]. Similarly, combination of MEDI4893* with relevant antibiotics showed improved protection in a murine dermonecrosis model [52]. MEDI4893 is currently being tested in a Phase 2 clinical trial (with the acronym SAATELLITE) with approximately 460 patients, for the prevention of *S. aureus* ventilator-associated pneumonia in patients not receiving antibiotics that are effective against *S. aureus*. MEDI4893 received FDA fast track status in 2014.

7.4.5.4 ASN100

ASN100 is a therapeutic antibody combination being developed by Arsanis, Inc. for severe *S. aureus* infections, the first clinical target being prevention of pneumonia in mechanically ventilated patients. It is currently the only mAb product in development against *S. aureus* that targets multiple virulence factors simultaneously. ASN100 is a combination of two fully human mAbs, ASN-1 and ASN-2 that together neutralize six clinically important *S. aureus* cytotoxins that inflict damage to lung epithelial cells and inhibit an effective immune response. ASN-1 neutralizes Hla and four of the five leukocidins: HlgAB, HlgCB, LukSF (also known as Panton-Valentine leukocidin or PVL), and LukED [114].

ASN-2 neutralizes the fifth leukocidin, LukGH also known as LukAB [9]. Both antibodies are IgG1/kappa isotype and were discovered using Adimab's proprietary human antibody yeast display platform.

ASN-1 displays superior protective efficacy against severe *S. aureus* pneumonia in an established rabbit model when compared with a single-toxin neutralizing mAb against Hla. The data also show that the rabbit pneumonia model is more clinically relevant than traditional murine models, as mice are relatively insensitive to the effects of *S. aureus* leukocidins, while rabbits more closely mimic human sensitivity [28]. ASN100 has been shown to synergize with antibiotics in murine therapeutic pneumonia models [114]. A Phase 1 safety and pharmacokinetic trial of ASN100 initiated in 2015 has been completed, and the Phase 2 clinical trial was initiated in December 2016 for the prevention of *S. aureus* pneumonia in mechanically ventilated patients who are heavily colonized by *S. aureus*, irrespective of whether they receive antibiotics or not. ASN100 was granted Fast Track designation by the FDA in November 2016.

7.4.5.5 MEDI3902

MEDI3902 is a multi-mechanistic bivalent, bispecific antibody that targets *P. aeruginosa* antigens Psl and PcrV, being developed by Medimmune/AstraZeneca for the prophylaxis or adjunctive treatment of *P. aeruginosa* infections. It is the first bispecific antibody to enter clinical testing against a bacterial pathogen. An anti-PcrV mAb, V2L2-MD (IgG1) was used as the scaffold, and an anti-Psl scFv was inserted in the upper hinge region of the anti-PcrV mAb creating a unique bispecific configuration with an intermediate interparatopic distance between antigen binding sites against the two targets. This bispecific mAb showed opsonophagocytic killing activity, inhibited cell attachment and cytotoxicity *in vitro*. This mAb is also reported to have potent serotype-independent activity against diverse strain types, including MDR strains, in multiple animal infection models in both prophylactic and therapeutic regimens, and shows synergy *in vivo* in adjunctive therapy with multiple antibiotic classes [29]. A Phase 1 trial in healthy volunteers for the prevention of nosocomial pneumonia was completed in April 2015 (NCT02255760). A Phase 2, proof-of-concept safety and efficacy study of MEDI3902 was initiated in March 2016 with patients at high risk of developing *P. aeruginosa* pneumonia (EVADE: Effort to Prevent Nosocomial Pneumonia caused by *Pseudomonas aeruginosa* in Mechanically Ventilated Subjects).

7.4.5.6 514G3

514G3 was developed from a healthy human donor with natural antibodies effective against *S. aureus,* using XBiotech's 'True Human' therapeutic antibody platform. The mAb targets Protein A/SpA and the suggested mechanism of action is neutralization of this virulence factor and induction of OPK [122]. In October 2015, 514G3 received Fast Track designation for MRSA infections in USA. A Phase1/2 clinical study was completed measuring the safety and efficacy of 514G3 in subjects hospitalized with bacteremia due to *S. aureus.* Patients enrolled in the Phase 2 part of the study (initiated in March 2016) received 514G3 plus standard-of-care antibiotics or placebo plus antibiotics. Initial data reported in a press release indicated trend for efficacy [146].

7.4.5.7 SAR279356

Several species of bacteria share a common surface polysaccharide, poly-β-1,6-N-acetylglucosamine (PNAG). Natural antibodies to PNAG are present in normal human sera, but are not protective. It was shown that a deacetylated form of PNAG (dPNAG) elicited antibodies with opsonophagocytic killing activity in mice [84]. Human mAbs that bound to both dPNAG and PNAG were subsequently generated by human hybridoma technology using B cells isolated from a patient who had recovered 3 years earlier from *S. aureus* bacteremia. One of the antibodies F598, originally secreted as an IgG2, was reformatted as an IgG1 and was shown to enhance complement deposition thus supporting opsonophagocytic activity, and protected mice against lethality caused by PNAG-positive *S. aureus* strains [64].

Alopexx Pharmaceuticals obtained the rights for all antibodies developed against dPNAG by Gerald Pier and colleagues including F598. The mAb F598 is intended as an alternative to antibiotics for the prevention and treatment of serious bacterial infections such as *S. aureus*, *S. epidermidis*, *E. coli*, *and Y. pestis*. A Phase 1 trial of F598 was performed by Alopexx in 2010 on healthy adult volunteers for safety and pharmacokinetic analysis. Functional activity of the antibody, as measured by opsonophagocytic assays, was seen at all doses up to day 50, the last time point in the study, suggesting that a single administration of F598 could provide protection for a prolonged period of time. In 2010 Sanofi-Aventis exercised its option to acquire an exclusive and worldwide license of Alopexx's F598, now called SAR279356. However, in December 2013, Sanofi terminated a Phase 2 trial of SAR279356 for patients on mechanical ventilation in intensive care units, apparently because of difficulties with patient recruitment. No information regarding further clinical testing is available.

7.4.5.8 Pritoxaximab/Setoxaximab

Pritoxaximab and setoxaximab are mouse/human chimeric IgG1/kappa antibodies that target Shiga Toxin-1 (Stx1) and Shiga Toxin-2 (Stx2). The combination of pritoxaximab and setoxaximab, (acquired from Thallion Pharmaceuticals by Bellus Health) is called Shigamab™, and isbeing developed for the treatment of HUS caused by Shiga toxin-producing *E. coli* (STEC), a rare disease that is characterized by haemolytic anemia and renal failure. It can lead to acute dialysis, and in certain cases chronic kidney disease and death, primarily in children. STEC infections are generally food-borne. Following ingestion of STEC, the bacteria rapidly multiply in the intestine causing colitis leading to profuse and eventually bloody diarrhea. The neutralizing mAbs specifically eliminate their respective Shiga toxin targets from the circulation.

The safety and pharmacokinetics of Shigamab™ have been evaluated in four Phase 1 clinical studies involving 35 adult healthy volunteers as well as in one Phase 2 clinical study in 45 STEC-infected children. In these studies, Shigamab™ was found to be safe and well-tolerated. According to the company's website, Bellus Health has recently completed an additional pre-clinical study in a HUS baboon model, in which Shigamab™ was shown to rescue the animals against a lethal dose of toxin when administered up to 48 h post-intoxication. A Phase 2 study protocol is currently being designed for the assessment of the efficacy of Shigamab™ in the treatment of STEC-HUS.

7.5 Concluding Remarks, Future Perspectives

Monoclonal antibodies have great potential for the treatment of infectious diseases. They show exquisite specificity against their targets, are able to recruit additional host immune system components to fight infection, and unlike vaccines, are fast acting – conferring near-immediate immunity following administration – and can be administered to all populations, young or old, regardless of immune status. Monoclonal antibodies targeting bacteria are expected to be generally free from adverse reactions, due to the lack of cross-reactivity with host antigens. Their pathogen specificity and precision targeting is the basis of not inducing resistance in other bacteria present in the microbiome. Nevertheless, despite the clear role antibodies play in the defense against many infections it is surprising that of the 63 currently (as of 30th of May 2017) approved therapeutic antibodies there are only 4 that target infectious disease agents, namely Pavilizumab (Synagis®) against RSV, Raxibacumab (ABThrax®) and Obiltoxaximab (Anthim®) against *B. anthracis* and most recently approved, Bezlotoxumab (ZINPLAVA™) against *C. difficile*.

The main reason why mAbs have not played a more prominent role as anti-bacterial therapeutics in the field of infectious diseases is the lack of or few recent precedents for successful development (approval) based on Phase 3 efficacy trials. The high profile failures of LPS mAbs against Gram-negative sepsis, and of the multiple approaches against *S. aureus*, discouraged

targeting bacterial infections. In hindsight, there are plausible explanations for these failures. The LPS mAbs tested previously had several attributes that likely contributed to lack of efficacy. Lipid A is buried in the bacterial membrane and is accessible for antibody binding only upon bacterial lysis. Depending on the concentration of free LPS and the level of LPS-binding proteins in plasma, LPS forms micelles with the hydrophobic lipid A head facing inside, and therefore, is not accessible for mAbs. All three failed LPS mAb programs used IgM, which – besides being difficult to manufacture – had low affinity. High affinity binding is likely to be required to inhibit TLR4 signaling, i.e. endotoxin neutralization. In addition, the clinical development strategy targeting therapy of sepsis, instead of prevention, is also questionable. Septicemia is a process with different stages and severity, and once triggered by LPS, it is maintained by the cytokine storm evoked, even in the absence of LPS. Selecting lipid A as mAb target was most likely influenced by the notion that anti-bacterial mAbs that are not broad-spectrum (pan-Gram-negative approach) are not commercially viable. Abandoning unrealistic clinical aims and designing feasible clinical studies are crucial for the future success of anti-bacterial mAbs. Failed mAb programs targeting *S. aureus* infections also have a lot in common: all targeted a single virulence factor/molecular structure on the bacterial surface, and aimed to enhance opsonophagocytic uptake and killing. However it is now clear that secreted exotoxins are central to *S. aureus* pathogenesis. Differential species specificity of these toxins in animal models hindered the recognition of their important role. Among these are the leukocidins that target phagocytic cells for lysis. Neutrophils and macrophages are the cornerstones of defense against *S. aureus*, and these are the cell types that could be exploited by opsonophagocytic mAbs. Importantly, opsonophagocytic anti-*S. aureus* antibodies are abundant in serum, thus providing an excess of such antibodies might seem to be futile, especially without protecting the effector cells, the phagocytes. Thus, due to the complexity and redundancy in *S. aureus* pathogenesis, it is doubtful that targeting one single virulence factor would be sufficient for efficacy.

Based on these and other examples, we can also conclude that it is highly unlikely that efficacious anti-bacterial mAbs can be cross-species specific. Instead they should be considered rather as narrow spectrum, precision biologics. This approach requires appropriate (companion) diagnostic tools for the rapid identification of patients infected with the specific pathogen.

Historically, a major concern regarding the use of mAbs for infectious diseases is the perceived expensive manufacturing process and high cost of goods (COGs), especially compared to antibiotics and vaccines. The COGs are mainly driven by the potency of the mAbs and thus the required dose. In recent years anti-bacterial mAb developments have become more sophisticated and rationally designed based on the growing knowledge on pathogenesis. Combined with improved mAb technologies, higher affinity and more potent mAbs can be generated resulting in considerably lower effective doses. Moreover, with improvements in mAb manufacturing processes, attractive COGs can be achieved. A recent review suggesting that manufacturing costs as low as $35–100/g mAb are attainable [147] translates to a $28–80/dose at 10 mg/kg dosing in humans (based on an average 80 kg body weight). Meanwhile the price of newly approved antibiotics has risen, exemplified by the price for a full course of treatment with the recently approved antibiotic, Avycaz® (ceftazidime and avibactam) that costs between $7500 and $15,000 [91]. Furthermore it is likely that for most anti-bacterial mAbs, only a single dose would suffice.

An important aspect, however, is the clear differentiation in expectations from anti-bacterial mAbs and antibiotics. Considering anti-bacterial mAbs only as an alternative to antibiotics is a highly simplified view. In the still rare cases of pan-resistant infections, the use of bactericidal mAbs alone is certainly justified. However, anti-bacterial mAbs offer great potential in bacterial infections when mortality is high, in spite of appropriate antibiotics. This is certainly the case with nosocomial infections affecting vulnerable

populations. Moreover, hospital-associated infections are often caused by drug resistant pathogens, where effective therapy is delivered with delay, and pathology is more severe. Taming the virulence of pathogens by antibodies (toxin and other virulence factor neutralization) is expected to support host immunity, and reduce mortality in combination with antibiotics in therapeutic settings.

An unexploited opportunity for anti-bacterial mAbs is in the realm of prophylaxis and/or preemptive therapy for high-risk individuals. For these applications it is especially important to demonstrate high efficacy and pharmacoeconomic benefit. The very favorable safety profile of mAbs is recognized, especially when targeting foreign (non-human) antigens. Finally, as anti-bacterial mAbs are pathogen specific, no harmful effect on the microbiome or induction of resistance in the colonizing bacterial flora is expected. All of these attributes make them attractive for broader use and are in line with antibiotic stewardship efforts.

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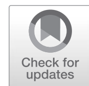

Anti-parasitic Antibodies from Phage Display

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Abstract

Parasite infections affect billions of people and their domesticated animals worldwide, and remain as a significant cause of morbidity and mortality, but such diseases are still neglected in endemic countries. Therapeutic interventions consisted mostly of drugs, which are highly toxic and may lead to resistance. The immunopathology of parasites is very complex due to their multistage life cycles and long lifetime involving several hosts, leading many times to chronic infections and sometimes to death, by compromising nutritional status, affecting cognitive processes, and inducing severe tissue reactions. Vaccination is a challenge, and immunotherapy is completely disregarded because of their complex interactions with hosts and vectors. This review will bring concepts of immunological aspects for some important parasitic infections, and present the most recent phage display-derived antibodies or peptidomimetics for parasite targets. This chapter will also discuss the future perspectives of such potential anti-infective immunobiologicals for parasitic diseases.

Keywords

Antibody · Phage display · Immunopathology · Malaria · Leishmaniasis · Toxoplasmosis · Helminthiasis · Schistosomiasis · Parasitic diseases

8.1 Introduction

Although parasitic infections are highly prevalent worldwide, they are considered neglected diseases, for which diagnosis and treatment are still a challenge, especially due to the wide spectrum of parasites, multistage life cycles, multiple hosts with very broad interactions, and very large variability. Novel diagnostics and therapeutic strategies used in other chronic diseases are rarely employed in parasitic infections. The develop-

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ment of antibodies targeting parasitic infections has not been explored as therapeutic interventions due to the complexity of parasites' life stages coupled with their immunomodulatory actions in the hosts (Stadecker [64, 101, 116]), which are many times associated with evasion mechanisms against cytotoxic effects of the immune response [82]. However, the lack of interest of the pharmaceutical industry for supporting active research and development programs on parasitic infections is one of the major explanations for this unexplored field. Therefore, most of scientific advancement in this area is financed largely by philanthropic and government-funded agencies [64]. Furthermore, treatment of parasitic infections rely primarily on public sanitation programs, but diseases are still highly prevalent in developing countries, and drug treatments do not offer protection against rapid re-infection [6]. Understanding how parasitic infections modulate host immune responses that lead to pathological responses and evasion are key elements and targets for antibody development, a strategy that may be used to activate the immune surveillance and parasite killing. However, the host immune response that mediates protection is dependent on the particular parasite [6], and such strategy must be parasite specific.

While helminths are extracellular parasites that release a spectrum of mediators to dampen host immunity, including exosome-like vesicles and proteins that act on host cell receptors and intracellular signaling [19], several protozoans grow within host cells. Some protozoan parasites are restricted to some host cells (*Leishmania* sp. in macrophages), others grow and divide into several types of host cells (*Plasmodium* sp., *Toxoplasma gondii, Trypanosoma cruzi*). This intracellular location may be a protection mechanism for parasites against harmful effects of humoral or cellular responses [96], and such evasion mechanism is further complicated by the fact that parasites present significant antigenic variations [55, 117], which are not known or fully mapped, probably because part of this genetic variability may be generated by epigenetic mechanisms as observed for malaria [95].

Antibodies used to control parasitic infections are yet to be explored, and most of the antibodies generated by Phage Display (PD) to date are used for diagnosis. In general, strategies to obtain antibodies by PD are based on immune libraries. Briefly, microplates are coated with the target antigen, which is bound by adsorption to the plates by non-covalent electrostatic or Van-der-Waals interactions. After coating, plates are blocked to avoid unwanted binding, and then washed with a mild stringent solution containing Tween-20 to remove unbounded antigen molecules. Then, the antibody library is added and incubated under gentle agitation to improve the chances of binding between specific phages and the antigen. Unbound phages are then removed by washing steps. Bound phages with strong affinity that were kept bound to the antigen are eluted by competition with positive polyclonal antibodies or by reducing the pH to 2. Eluted phages are then transfected into *E. coli* and amplified by culture, titrated and used in another round of selection, resulting in single chain fragment variable (scFv) clones with strong affinities. After two or three rounds of selection, histidine-tagged antibodies are amplified by culture and purified by immobilized-metal (Ni) affinity chromatography. The primary screening of selected antibodies is performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blotting and/or enzyme linked immunosorbent assay (ELISA) in order to evaluate and validate their capacity to recognize the target antigens. Validated antibody clones are then sequenced, characterized by bioinformatics, and used for additional assays [38, 41].

Immune libraries have two major advantages, a selection highly biased towards V-genes that encode antibodies against the immunogen, and many of the genes will encode affinity-matured antibodies, increasing the number of high affinity antibodies [41]. The absence of control over the immune response leads to antibodies with great variability in a specific library (comprising a population of 10^{10} clones), which makes possible selection of antibodies against different antigenic targets. Among the many pitfalls of selected antibodies, the lack of stability continues to be a

major challenge, besides some functional limitations, such as inadequate pharmacokinetics, tissue accessibility and impaired interactions with the immune system [14]. The interactions among host immunity, intestinal microbiota and parasites biology result in a complex ecosystem, where all components play relevant roles in modulating each other, and specific antibodies for one parasitic stage may not be significant to another stage either for therapeutic or diagnostic purposes.

This chapter will explore some important immunobiological aspects of each disease in order to propose targets for future diagnostics and immunotherapeutic approaches, and will also discuss how current and future combinatorial antibodies may function in the complexity of parasite-host interactions.

8.2 Malaria

According to the World Health Organization (WHO), 214 million malaria cases with 438,000 deaths were estimated worldwide in 2015, and about 3.2 billion people remain at risk of malaria. Malaria diagnosis must be promptly ascertained to prevent spread, associated morbidities, and death. Malaria diagnosis can be difficult because many patients are asymptomatic, but remained as important carriers; therefore, malaria infection does not mean illness.

The gold standard diagnostic test for confirmation is a Giemsa-stained blood smear observed under the microscope, but antigen detection assays by rapid immunochromatographic tests may be considered as substitutes of the microscopic analysis, although their performances in the field, improved accuracy and lowering costs are still important issues [115]. Among other analytical tests, the polymerase chain reaction (PCR) determines the species of the malaria parasite, which is not available in all healthcare settings; serology detects antibodies and determines only whether a person was exposed, but does not indicate active infection; and drug resistance tests must also be employed due to the extensive parasite variability, but such tests are only predictive [12]. It is mandatory that new diagnostic tests and markers must be developed to break such important diagnostic hurdles in order to reduce malaria morbidity, drug resistance and associated economic losses. Therefore, new anti-malarial drugs or vaccines must also be complemented with improved diagnostic tools [115]. For diagnostic purposes PD has been used to obtain two antibody clones (D2 and F9) that were successfully isolated and tested against the recombinant PfHRP2 and native proteins [56].

Currently, most antimalarial drugs target the erythrocytic stage of malaria infection that causes symptomatic illness, but treatment relies on three broad categories of drugs; quinine derivatives that basically prevent hemozoin crystals formation within erythrocytes, antifolate compounds, and artemisinin derivatives, and treatment regimens are based on combination of drugs [7]. Such drugs are highly toxic although tolerable, and emergence and spread of parasite resistance is not fully understood, because resistance can be developed to any antimalarial drug. Important factors such as drug pressure, drug half-life, parasite biomass, and transmission intensity are considered key contributors for the drug resistance [104].

Due to increase in insecticide resistance of mosquitoes, parasite resistance to antimalarial drugs and the incidence of the disease, malaria is being intensively studied, and several antigens are under tests in different phases of clinical trials [16, 45]. Although several candidate antigens are being tested only the pre-erythrocytic vaccine RTS, S has completed a Phase III clinical trial to prevent *P. falciparum* malaria in children [45]. However, a meta-analysis research focused on this candidate vaccine has demonstrated its insufficient efficacy [65].

A computational stability design to enhance thermal stability of a *Plasmodium falciparum* vaccine candidate has been developed using the reticulocyte-binding protein homolog 5 (PfRH5), which is essential for erythrocyte invasion [11]. Interestingly, this strategy may contribute to the development of vaccine immunogens against several other diseases. PfUIS3, a protein expressed in sporozoites and liver-stages, was

also evaluated as a vaccine candidate in mice models administered together with ME-TRAP, another pre-erythrocytic vaccine candidate, which have reached 100% sterile protection [62]. BK-SE36, a blood-stage candidate formulated with a genetically-modified protein (serine repeat antigen-5) and alum, passed Phase I trial in malaria-naive Japanese adults, reducing infection by 72% [81].

Studies have been driven to the development of malaria transmission-blocking vaccine candidates, such as the pf48-45, which plays a crucial role on male fertility [84], the Pf230 related to macrogametocytes fertilization by microgametocytes [29], and the Pf25 that is expressed on the surface of zygote and ookinetes. A recent study evaluated the usefulness of Pfs25 in a phase I trial, and with different carrier proteins and adjuvants to enhance responses. Results indicated that proteins and adjuvants augmented the humoral response, and demonstrated its potential application as a transmission-blocking vaccine candidate [86, 103].

Considering immunotherapy-driven studies, the first evidence that this could be an effective mean of controlling malaria was demonstrated by an FcγRI-directed therapy, which was able to mediate a potent anti-malarial immunity in mice model. Antibodies derived by immune antibody PD libraries from malaria-exposed donors were transformed into fully functional human antibodies IgG1, which targeted the merozoite surface protein 1 – p19 carboxyl terminal region ($MSP1_{19}$). The engineered IgG1s recognized parasites in infected erythrocytes and triggered potent human FcγRI-mediated protection against malaria [70]. Interestingly, the target $MSP1_{19}$ antigen was 100% detected in the four species; *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. This protein is also considered a vaccine candidate for both *P. falciparum* and *P. vivax* with a well-characterized immune response [74].

The best candidates to develop new diagnostic and therapeutic tools by PD may be the glycosylphosphatidylinositol-anchored proteins (GPI-APs), which are localized on merozoite membranes or apical organelles with possible invasion roles [36] GPIs are synthesized by all eukaryotes, but differ across organisms in sugar and lipid composition. Despite their ubiquitous presence, GPIs are present in extremely low concentrations in plasma membranes of most organisms, except in the parasitic protozoa, in which they are very abundant [51, 69]. This protein are interesting therapeutic targets, and antibodies against PvGAMA fragments (*P.vivax* GPI-anchored micronemal antigen) that binds to human erythrocytes, regardless of Duffy antigen status, inhibited PvGAMA binding to erythrocytes in a dose-dependent manner [17]. The generation of highly specific antibody fragments against pGPI is a great opportunity for the PD technology, which also includes the development of peptides that may mimic the carbohydrate-phosphoinositol functional core for vaccine development. A similar strategy has been applied for the *Plasmodium yoelii* adhesion proteins, the apical membrane antigen 1 (AMA1) and the 235-kDa rhoptry proteins (P235). After demonstration of a successful passive immunization with monoclonal antibodies (MAbs), 45B1 and 25.77, against AMA1 and P235, respectively, protecting against lethal infection with *P. yoelii* YM, PD constrained peptides were selected against these MAbs, and both peptides have shown to be protective when used to immunize against challenged infections [77].

Another possible target to PD technology to better understand malaria and develop new tools against this disease is *P.vivax* Duffy binding protein (DBP), which may also be considered a future perspective for malaria therapeutics. Selected scFv clones that reacted with DPB were able to inhibit erythrocyte binding to this protein, and these neutralizing scFvs may be valuable for both passive and active immunization strategies for malaria control [54], although *in vivo* challenges were not performed.

Although, there are no immunotherapeutic approaches against malaria, some strategies can be envisioned. One of the first strategies to protect humans against malaria is by using scFv antibodies that bind and neutralize important *Plasmodium* proteins, such as the merozoite surface protein 1 (MSP1) and its C-terminal product (MSP1–19), the apical membrane antigen 1

(AMA1), and the merozoite surface protein 3 (MSP3), which prepare them for destruction by immune cells. Such strategy using PD has proven to be effective against AMA1 [58], and one PD-derived peptide ligand was found to be a potent inhibitor of the invasion of *P. falciparum* merozoites into human erythrocytes [53, 58]. Other targets for the development of neutralyzing scFv antibodies are the sporozoite GAPDH glyceraldehyde 3-phosphate dehydrogenase, which interacts with CD68 on the Kupffer cell surface during invasion [13]. Tryptophan-rich antigens that also interact with host molecules are crucial for parasite survival [4]. Anti-MSP-142 antibodies have inhibited merozoite invasion *in vitro*, and the MSP-142 protein has elicited protective response in experimental models [28].

Therefore, the use of antibodies against malaria as therapeutic approach is possible and must be pursued either as fully engineered antibodies, antibody fragments or as antibody peptidomimetics. However, the identification of novel monoclonal antibodies derived from PD with protective functions maybe more useful as targets for random peptide selection by PD to mimic non-protein antigens towards future vaccine development.

8.3 Leishmaniasis

In 2014, more than 90% of new cases reported by WHO occurred in six countries: Brazil, Ethiopia, India, Somalia, South Sudan, and Sudan. Visceral leishmaniasis (VL) diagnosis is routinely performed by the gold standard microscopic analyses of parasites in tissue biopsies (bone marrow, lymph node, liver and spleen), and confirmatory detection and species identification is further performed by PCR due to its higher sensitivity [78, 99], but this test remains restricted to referral hospitals and research centers. Conventional serological tests, although technically easier and cost effective, cannot distinguish active disease from asymptomatic VL or after cure [15, 89]; however, new mimotopes derived from PD selections against polyclonal antibodies have been revealed as very powerful antigens for serodiagnosis that could discriminate the active disease [20–22].

Recently, detection of *Leishmania infantum/donovani* antigens by enzyme-linked immunosorbent assay (ELISA) was developed, including iron superoxide dismutase 1 (Li-isd1), tryparedoxin 1 (Li-trx1), and nuclear transport factor 2 (Li-ntf2), which may become an important tool not only for active disease diagnosis, but also to monitor therapeutic efficacy of VL [2]. These markers also open new opportunities for PD selections of antibody fragments or peptidomimetics against multiple *Leishmania* antigens, aiming to improve VL diagnosis, by generating new sensor platforms with specific antigens as shown elsewhere [39].

Currently, leishmaniasis treatment is performed with rational chemotherapy and vector control, since no vaccine is available. However, the drugs used to control the disease are highly toxic, expensive, with limited diversity, and may cause severe adverse reactions [1]. The effectiveness is also an important issue, because excess use may lead to drug resistance [100], and besides, the survival mechanism of *Leishmania* sp. mediated by glycosylinositolphospholipid (GIPL), the most abundant promastigote surface molecule is also a problem.

Initially, killed or live attenuated *Leishmania* parasites were tested in the first generation vaccines, but this strategy was abandoned due to the risks involved, which has led to second generation vaccines based on purified, native synthetic or recombinant antigens [76]. Some antigens from different leishmanial stages have been suggested as potential vaccine candidates for visceral leishmaniasis, such as the A2 antigen and cysteine proteinases (CP) expressed in amastigote, surface antigen-2 (PSA-2), Kinetoplastid membrane protein-11 (KMP-11) and the nucleoside hydrolase 36 kDa (NH36) expressed in promastigotes [26]. Although no vaccine for leishmaniasis is available, efforts have been made to test protein candidates, and PD has been used that led to the selection of two phage-fused peptides that mimic *Leishmania infantum* antigens, which were tested as vaccine candidates and pre-

sented significant reduction on the parasitic burden [21].

Another good candidate parasite target for diagnostics and vaccine development through PD is the GIPL, which may prevent the expression of both classes of major histocompatibility complex (MHC) molecules within the macrophage, inhibiting antigen presentation, and repressing the microbicidal activity of macrophages. The degree of MHC suppression depends on both the duration of infection and the parasitic load [80]. Therefore, GIPL may become an important target for immunotherapy using PD selection strategies in order to improve the immune response of infected individuals. This hypothesis is supported by results using fragment antigen binding (Fab) monoclonal antibody against GIPL, in which Fab was pre-incubated with parasites, and macrophage infectivity was reduced by 80% of promastigotes and 30% of amastigotes [102]. These authors have also shown that *L. amazonensis* does not have GIPL, so infections with this parasite species would not benefit from this immunotherapeutic approach. Fucose-mannose ligand (FML) is another important protein expressed on the surface of the parasite, which strongly inhibits the *in vitro* internalization of macrophages by both promastigotes and amastigotes [75, 76]. This protein can also be a target on PD for the development of neutralyzing scFv antibodies.

Other 50 possible protein targets corresponding to up-regulated genes in the infective stages of the parasite have been reported and can be tested as candidates for vaccine development [5], which can also be used for PD development of diagnostic antigens, and as possible imunotherapeutic targets.

Several proteins with important role on parasites' life cycle secreted or present on the tegument can be possible targets for PD to obtain important molecules with diagnostic, therapeutic or vaccine candidate characteristics. However, currently the first and only immunotherapeutic approach used for leishmaniasis is similar to the one used for malaria, which searched peptidomimetics of antibody ligands against *L. major* metacyclic promastigotes parasites using PD. This strategy revealed that a peptide (P2) interacted with the major surface protease gp63, and inhibited *in vitro L. major* growth kinetics, and reduced cutaneous lesions in BALB/c mice [90]. This promising immunotherapeutic molecule may also be used in the near future in combination with chemotherapeutic regimens.

8.4 Chagas Disease

It is estimated that eight million people are infected with *Trypanosoma cruzi* worldwide. In Latin America, Chagas disease is one of the biggest public health problems, causing more than 10,000 deaths per year. In the last decades due to immigration, infection has been increasingly detected in non-endemic areas such as in the United States. An important characteristic of this disease is that the parasite can be transmitted through blood transfusion and tissue transplantation, and up to 30% of chronically infected people develop cardiac alterations [114].

Gold standard diagnosis is achieved through trypomastigotes visualization on Giemsa stained blood smear, but in the chronic phase once the immune response to *T. cruzi* is established, parasitological diagnosis is difficult due to the low parasitic burden. To overcome this challenge, serology using crude antigens for detection of antibodies against *T. cruzi* has been developed; however, cross-reactivity with *Leishmania* sp. exists. In addition, sensitivity of current tests is lower than generally reported, leading to potential underdiagnosis. This is a particular concern when these tests are used as a screening for blood bank samples [3]. Another drawback of serology is its inability to distinguish between acute and chronic infections, or cured versus active cases, or to detect immunocompromised individuals that do not develop an appropriate humoral immunological response. Besides that, reproducibility is highly compromised when using crude antigens from different antigenic sources, since these antigens are composed by complex mixtures of proteins leading to difficulties in standardization, and to cross-reactive responses in serological tests. To overcome such problems, several recombinant antigens have been

developed, and recently, it was reported the TSSA2 antigen that discriminates chagasic from leishmaniasic patients [18]. Cruzipain and Tc24 antigens have also been developed to monitor patient's treatment [35], and the ribosomal protein TcP2β has been claimed as cure marker [30].

Management of Chagas disease involves parasite elimination through treatment with two drugs, benznidazole and nifurtimox, which have limited effect on parasitologic cure, especially in chronically infected patients. In this latter group, benznidazole does not significantly reduce cardiac clinical deterioration, besides that both drugs have serious and unacceptable side effects [61]. There is an urgent need for new therapeutic targets and preventative efforts.

Although no vaccine for Chagas disease is available, several efforts have been made to develop an effective tool. Recently mice were subjected to injections with DNA coding for TcG2 and TcG4 proteins, and after 2 weeks they were able to keep parasitic load two to threefold lower than unvaccinated ones [43]. Another vaccine approach has also been successfully developed by using two recombinant antigens, a 24-kDa trypomastigote excretory–secretory protein known as Tc24, and a *T. cruzi* trypomastigote surface trans-sialidase known as TSA-1 [49]. Recent data have listed 15 antigens already tested as candidates for a Chagas vaccine development, but from these only 9 diminished parasitemia and increased survival in mice, which are: live attenuated live *Trypanosoma rangeli;* recombinant amastigote surface protein 2 (rASP-2 + Alum or CpG ODN); adenovirus or vaccinia expressing trans-sialidase surface antigen (TSSA CD8+ epitope); adenovirus expressing trans-sialidase and amastigote surface protein 2 (TS and ASP-2); Sendai virus expressing amastigote surface protein and trans-sialidase (ASP-2; TSA-1, ASP-1 and ASP-2 with IL-12 and GM-CSF); amastigote surface protein alone or fused with ubiquitin (ASP-2 or UB-ASP-2); DNA + adenovirus expressing trans-sialidase and amastigote surface protein (TS and ASP-2 clone 9) (Prime-Boost) [85]. An alternative would be to develop and test a therapeutic vaccine that could be administered as an immunotherapy [27].

Besides developing vaccines, novel biomarkers for disease progression and cure for therapeutics monitoring would be key to improve Chagas control. In this context, PD can be useful to study-parasite/host interaction, develop new antigenic sources, and for discovery of new drugs and therapeutic targets. Recently, a combination of endothelial cell immortalization and PD has been used to investigate both *T. cruzi* cell entering and infection, and the role of gp85/trans-sialidase [107]. The study of these host-parasite interactions by using PD can lead to the discovery of important receptors or molecules involved on parasite's survival and the intervention on this kind of molecules can avoid parasite internalization or infection progression through the use of blocking antibodies or mimetic peptides. In an effort to differentiate serologically the different chronic forms of Chagas disease (cardiac, indeterminate and digestive) our group performed PD technique. In this occasion IgG from patients with each form of the disease were used in a strategy to select peptideomimetics with potential use in diagnosis, our results indicated the possible application to differentiate patients with the cardiac form from those with the digestive one, although more studies are necessary to better understand the role of these peptides in each form and the potential application on future diagnostic tests (not published).

Currently, there is only one report about the selection of antibodies by PD in Chagas disease, a cross-reactive nanobody (Nb392) was used to develop a highly specific antibody against the conserved paraflagellar rod protein (FR1), which may be useful to detect the *T. cruzi* antigen, and especially valuable to detect active disease, even in immunocompromised patients. Nb392 targets paraflagellar rod protein (PFR1) of *T. evansi*, *T. brucei*, *T. congolense* and *T. vivax* [79].

Important *T. cruzi* antigens and candidates for antibody development by PD must be considered in future developments aiming either immunotherapy and/or diagnosis of Chagas disease, such as: GPI anchors with immunostimulatory activities [23], the Cruzipain protein that modulates host responses by interrupting the NF-kB P65, and allowing parasite replication and survival,

and the parasite cyclophilin 19 that binds to and isomerizes the innate immune cationic antimicrobial peptide from the vector neutralizing its antiparasitic activity, consequently allowing infection [111]. Using this latter target, we can envision an immunotherapeutic application of a chimeric protein comprised of a high affinity peptidomimetic that neutralize cyclophilin 19 fused to a specific drug for direct parasite killing.

Although PD is a highly versatile technology, very few studies have aimed Chagas disease. The very restricted infection potential and the complexity of host-parasite interactions explain its neglect, replicating what has been observed for all other parasitic diseases, especially the reduced research resources.

8.5 Toxoplasmosis

Caused by an obligate intracellular protozoan named *Toxoplasma gondii*, it is generally assumed that approximately 25–30% of the world's human population is infected [73]. The disease is generally asymptomatic, but 10–20% of pregnant women infected with *T. gondii* become symptomatic. It is the most common cause of intraocular inflammation and uveitis in immunocompetent hosts, besides the chronic disease may be related to the etiology of different mental disorders [33]. The parasite may also disseminate in immunocompromised hosts leading to a severe and life-threatening disease. During pregnancy infection can cause severe damage or even death of the fetus [63].

Diagnosis is confirmed after *T. gondii* demonstration in blood, body fluids, or tissue, but techniques are invasive, difficult and time consuming. Indirect diagnosis through ELISA can also be used and differentiate acute and chronic infections, since anti-*Toxoplasma* IgM appears in the first week of the infection, and then declines in the next few months, while the presence of IgG indicates a likely past infection [72]. Avidity tests can also be performed to differentiate patients with acute infection from those with chronic infection, and higher avidity indexes indicates an old infection [87]. To improve diagnostic efficiency several recombinant antigens have been developed and tested, including granule antigens (GRA1, GRA2, GRA4, GRA5, GRA6, GRA7, and GRA8), rhoptry proteins (ROP1 and ROP2), matrix protein (MAG1), microneme proteins (MIC1, MIC2, MIC3, MIC4, and MIC5) and surface antigens SAG1, SAG2 and SAG3 [46, 60]. A combination of serological tests is usually used to differentiate present from past infections [72]. Molecular diagnosis can be performed to detect parasite's nucleic acids in biological samples, mainly used for intrauterine infections [60].

Although several researchers have been trying to develop an effective vaccine to control this parasite there is no one available, and this is due to the complexity of the parasite life cycle, which exhibits a highly antigenic polymorphism, complex immune responses, and besides the problems to produce recombinant vaccines, protection is still low even with high antibody titers [59]. The efficacy of inactivated parasites, crude, purified or recombinant antigens and DNA were already tested, and the majority of antigen candidates for vaccine development are located either on the surface of the parasite or as secreted proteins, generally essential for invasion [25].

During invasion contents of microneme, rhoptry, and dense granules are sequentially released; MIC for host cell recognition, ROP for parasitophorous vacuole formation and GRA during and after invasion to stabilize parasitophorous vacuole, making possible *Toxoplasma* development. It is believed that among those proteins present on dense granules there are least 23 GRA proteins, in which the majority have unknown function [93]. They are mainly associated with transmembrane domains or have become associated with vacuole membrane that are important for parasite development or invasion, and also with kinases related with parasite proliferation and differentiation, which can be possible candidates for PD selections to obtain antibody fragments, peptidomimetics or peptides for drug development. MIC2 is related with gliding and host cell invasion, and PD could be useful to develop a neutralizing scFv antibody, blocking these adhesins and consequently avoiding invasion.

Considering the current information about parasite/host interactions, PD could be a powerful tool for diagnostics, drug discovery and vaccine development, leading to the selection of new molecules that can avoid parasite invasion, replication within the host cell or even block substances secreted into the host cytoplasm.

8.6 Schistosomiasis

Schistosomiasis affects almost 240 million people worldwide, and more than 700 million people live in endemic areas. The burden of disease due to schistosomiasis is underestimated, and the disease leads to 200,000 deaths yearly, being the third most devastating tropical disease in the world. This parasite can affect the nervous system causing neuroschistosomiasis. Infection occurs when the larval forms present in contaminated fresh water penetrate the skin, and the presence of the specific snail host is crucial for the dissemination of the disease. There are two major forms: intestinal (*Schistosoma mansoni, S. japonicum* and *S. mekongi*) and urogenital (*S. haematobium*) and diagnosis relies on detection of the parasite on stool or urine, respectively. Parasitological diagnosis has poor sensitivity since egg output is generally low and in light infections, and in chronic cases the amount of eggs in stool is very low. Serological tests can be useful in these cases, but cross-reactions or false positive results may occur, and this procedure is not able to distinguish recent from past infections. Antibody detection by ELISA was initially based on the use of crude antigens from eggs (SEA) or adult worms (SWAP), but the low specificity has led to the use of purified antigenic fractions, which present better diagnostic parameters. Circulating antigens (anodic -CAA) and cathodic – CCA) can also be detected, but the sensitivity in endemic areas is low, although this test can be used to monitor cure after treatment. PCR is also available to detect parasitic DNA in biological samples [98], but this test is not extensively used in routine.

Regarding therapeutic strategies, although treatment with praziquantel is available, there are reported cases of parasite's resistance, and mass treatment does not prevent reinfection. This context has led to development efforts towards more efficient vaccines. Potential antigens of *S. mansoni* have been tested, such as: Sm28-GST, Sm28-TPI, paramyosin, Ir-V5, triose-phosphate isomerase, Sm23, Sm14, Sm29, Sm-p80 and SmTSP2, mostly from the tegument of the parasite [71], but the complex host-parasite interactions and evasion mechanisms of the parasite are the biggest hurdles to obtain an efficient vaccine. Only one vaccine against *S. mansoni* is under clinical trial, which is based on the recombinant fatty acid binding protein Sm14 with the GLA-SE adjuvant developed in Brazil [105]. Sm14 is expressed in the cercariae, schistosomula, adult worm, and eggs, and is located in the parasite tegument and gut [9].

Some strategies using PD were developed for schistosomiasis japonica, in which an scFv library was constructed using serum from buffalo to identify antigens from *S. japonicum* for diagnostic purposes. The scFv clones were able to significantly detect the SWAP antigen above the background level of mouse blood proteins in ELISA [48]. Buffalo lymph nodes were also used to construct an scFv library specific against whole formaldehyde-fixed and live *S. japonicum* schistosomules capable of recognizing several schistosome proteins present on a microarray. These proteins can be further evaluated for diagnostic and vaccine purposes [47]. *S. japonicum* cercariaecDNA library was also screened by the SIEA26-28 ku scFv to obtain new candidate molecules for vaccine development. The related genes, SjRPS4 and SjRPL7, were selected and tested, achieving 45% and 40% of egg reduction in mice vaccinated, respectively [34]. Another scFv clone specific to *S. japonicum* soluble immature egg antigen (SIEA) was used to deliver IL-18 to the site of hepatic fibrosis. The scFv was fused to IL-18 and the recombinant vector was used for DNA vaccination. This approach showed to be promising to decrease hepatic fibrosis in infected mice [106]. PD was also used to evaluate the host-exposed *S. mansoni* surface antigens, and an scFv library was constructed by using antibodies from schistosome resistant rats.

Selection of scFv clones was performed against fixed adult schistosomes. Five clone ligands were obtained against apical membrane extracts as targets, and their affinities were tested by ELISA, western blotting and fluorescence [97]. These data provided evidences that additional characterization of the tegument is necessary, which may be associated with both diagnosis and vaccine development.

Other important targets for PD selections and drug development for schistosomiasis may also be linked with tetraspanins (transmembrane proteins that mediate specific protein-protein interactions), fatty acid binding proteins (cytosolic proteins expressed in the basal lamella of the worm tegument), calpains (calcium-activated neutral cysteineproteases present in the tegument, and underlying musculature of adult worms), and paramyosin (myofibrillar protein present on the surface of the schistosomula tegument and in cercariae penetration glands) [71], because these proteins are critical for parasite development.

8.7 Cysticercosis

Cysticercosis is acquired by ingestion of food or water contaminated by *Taenia solium* eggs. Oncospheres are activated on stomach and released when eggs reach the intestine, and then they enter the blood stream and can lodge on muscles or other tissues, developing into metacestodes (cisticerci). These forms can also infect the central nervous system causing neurocysticercosis (NC). Neurocysticercosis is the major cause of epilepsy worldwide, and is estimated to cause more than 29% of all epilepsy cases in countries where the parasite is endemic [113]. In 2015, the WHO Foodborne disease burden Epidemiology Reference Group identified *T. solium* as the main cause of deaths from foodborne diseases, resulting in a considerable total of 2.8 million disability-adjusted life-years (DALYs). Economic losses caused by *T. solium* infection are very high, since this parasite infects pigs causing economic negative impact for pig raising farmers. In humans, NC affects mainly children and adults at productive age, causing chronic disability and consequently a huge economic burden for public health systems.

Metacestodes remain viable for several years then they degenerate releasing a lot of parasitic antigens eliciting a strong immune response, and this inflammatory process brings several symptoms, such as seizures, epilepsy, hypertension or hydrocephaly. Clinical manifestations depend on the location, number and size of metacestodes. The disease occurs mainly at Central and South America, Southeast Asia, and sub Saharan Africa; however, due to the immigration several cases have been reported in the United States and Europe [112].

Diagnosis is a challenge and confirmation can be achieved by using neuroimaging findings on Computerized Tomography or Magnetic Resonance Imaging, but these methods are expensive and are not available at the poorest endemic areas. Besides, the neuroimaging findings are non-pathognomonic and diagnosis must be made by a combination of clinical, radiologic, immunologic, and epidemiologic data [24]. Currently, neuroimaging and serology are the most reliable diagnostic tools. For serological tests (ELISA and western blotting), the cerebrospinal fluid (CSF) or serum are the most commonly used body fluids, but CSF collection is very painful and invasive.

Although there are several efforts to develop serological tests, problems related with sensitivity and cross-reactions are still present. Several antigens tested have been reported previously: crude antigens from *T. solium* or heterologous antigens from *Taenia saginata* or *T. crassiceps* (these antigens can be used due to epitope sharing), purified fractions (obtained after *T. solium* or heterologous antigens fractionation using chromatographic techniques or detergents – such as Triton X-114 or X-100), recombinant proteins (rGP50, rT24, TsRS1, Ts18 var. 1, Ts8B2, AG1V1, AG2, TSOL 18, TSOL 45-1A and 10 kDa) and synthetic peptides (HP6, Ts45W, Ts45S, TEG-1, Ts14, Ts18 var1, TSRS2 var 1) [110].

PD was already used to generate a *T. solium* genomic DNA library that was used to select

clones anti-*T. crassiceps* in hyperimmune sera. Two clones were further tested for immunoreactivity with CSF and plasma from NC patients in an attempt to develop new immunodiagnostic or immunotherapeutic tools [37]; however, the assays did not reach good diagnostic parameters, reaching a positivity of only 52.9% in CSF samples confirmed by neuroimaging. Another research strategy used a random peptide phage display library (PhD-12) to screen IgY from chickens immunized with total saline extract from *T. solium* metacestodes, resulting in seven highly immunoreactive peptides to IgG (sera) of NC patients, and one phage clone was able to distinguish active from inactive NC [66]. Similarly, the same library was used in a selection strategy against IgG from patients with NC, and a subtractive step against other parasitic diseases and negative individuals were performed to obtain peptides with potential use in NC diagnosis. Ten (10) phage clones were obtained with excellent diagnostic parameters in ELISA tests [92]. Human antibodies were used for peptide screening against the Scolex protein antigen from *Cysticercus cellulosae* (SPACc) using PD and one specific clone was obtained anti-SPACc IgGs [44]. In an attempt to develop a vaccine based on a phage-displayed epitope, the antibody was grafted into Ig heavy-chain CDRs, and successfully used as imunogen for murine cysticercosis [68]. Phage-displayed random library (Ph.D.-12) was also used to select mimotopes against a monoclonal antibody specific to TSOL18, and selected mimotopes were successfully used to detect antibodies in pigs vaccinated with TSOL18 [42].

Interestingly, a unique strategy to obtain highly specific scFv antibodies was used by selecting clones against highly antigenic phage mimotopes that were previously obtained [92], and these scFv clones were used to capture native antigens from *T. solium* metacestodes. The captured antigens for NC diagnosis showed excellent diagnostic parameters [91], and may become potential immunogens.

Currently efforts are driven to the development of a vaccine against porcine cysticercosis as a control measure to stop taeniasis transmission to humans, consequently eliminating the source of cysticercosis. Several recombinant antigens have been tested [50, 52] and one of these trials used recombinant antigens expressed on the surface of the M13 bacteriophage [67].

In the overall, very few targets have been identified as useful for parasite control; however, some proteins are important to be investigated, such as calreticulin that is present on the tegument and sub-tegument, and involved in embryogenesis, ovogenesis and spermiogenesis processes, and the oncosphere antigens, which have shown some promising results [109], and both may used as target for PD selections and vaccine development.

8.8 Strongyloidiasis

Strongyloidiasis is a soil-transmitted helminthiasis considered one of the neglected tropical diseases listed by the World Health Organization, and is estimated to affect at least 370 million people [8]. The exact prevalence of strongyloidiasis is not known because in many tropical and subtropical countries *S. stercoralis* can infect up to 60% of the population. More than 1.5 billion people, or 24% of the world's population, are infected with soil-transmitted helminth infections that are widely distributed in tropical and subtropical areas. In the parasite's life cycle there is larvae migration through the lungs and gastrointestinal tract. The disease is asymptomatic in immunocompetent hosts, but due to the *Strongyloides* ability of autoinfection, it may lead to hyperinfection and dissemination in immunocompromised hosts, and the disease may become fatal. The disease is underestimated due to the absence of symptoms and the low sensitivity of the standard diagnostic tests. Gold standard diagnosis is achieved through larvae visualization during stool examination, but this procedure lacks sensitivity due to the irregular larval output. To overcome this fact, serological tests have been developed by using crude or fractionated antigens from *S. stercoralis, S. venezuelensis* or *S. ratti* [10]. Serology has problems with low sensitivity in immunocompromised hosts, specificity

is highly affected due to the occurrence of cross-reactions with related helminthic species, such as hookworms (*Ascaris lumbricoides* and *Enterobius vermicularis*), and cannot differentiate active from chronic infections.

Besides the lack of epidemiological data and the diagnostic challenges, reports on vaccine development are also scarce. Few vaccination protocols have been tested, which include the deoxycholate-soluble affinity-purified L3 antigens (SS-IR), a *S. stercoralis* surface-specific antigen, the Na + –K + ATPase DNA, the *S. ratti* Hsp60, and the monoclonal antibody anti-srHSP60 IgM, but no vaccine is available for strongyloidiasis [108].

Recently, the PD technology was used to discover new targets for strongyloidiasis diagnosis and for vaccine development. A Ph.D.-C7C phage display peptide library was used to select *S. stercoralis* mimotopes in a biopanning procedure using purified IgG from patients with strongyloidiasis with a subtractive strategy using IgG from patients with other parasitic diseases and negative controls, and five selected clones showed excellent diagnostic parameters [31]. Two of the best peptides were then synthetized and tested again for IgG detection in serum from patients with strongyloidiasis, and showed to be a promising diagnostic tool [32]. Interestingly, scFv clones were also selected from a combinatorial library against total proteins of *S. venezuelensis*, and a monoclonal antibody specific to the heat shock protein 60 (Hsp60) of *Strongyloides* sp. was obtained and tested to detect serum immune complexes in samples from individuals with strongyloidiasis, resulting in very high diagnostic parameters [57]. These targets can be potentially used in vaccine protocols.

Although several efforts have been attempted, this is the most neglected parasite among the ones listed in this review, because little is known about its structure and interactions with the host, but PD may be a promising tool to solve these problems, and new targets may arise for diagnosis, immunotherapy and vaccines.

8.9 Future Perspectives and Considerations

Interesting therapeutic approaches have suggested that new strategies can be developed by better understanding specific immunological responses during disease onset and progression. A naturally occurring antibody fragment derived from the constant region of IgMs in human serum represented by a phosphorylated peptide (K40H) was synthesized and successfully applied against HIV and *Candida albicans* infection, suggesting that such antibody fragments may have an innate role against infectious agents [83]. This finding is further supported by an evasion mechanism found in *S. mansoni*, in which the parasite inhibits IgE from binding to CD23 and FcεRI, IgE receptors, and may be considered a protective mechanism from allergic reactions [40], since acquired immunity against helminths and allergic sensitization are both characterized by high IgE antibody levels [94]. Therefore, short peptides mimicking antibody fragments open a research path for Phage Display developments, which may lead to highly specific antibody-like peptides that are more stable, easy to synthesize, and present similar or improved function, as shown elsewhere [53, 58, 88, 90].

Briefly, this review brings the most recent advances using antibodies and peptidomimetics selected by PD to control parasite infections with theranostic applications, and brings to light the scarcity of reports in this field, indicating that this is a deprived research area due to its non-commercial appealing. However, the identification of targets for diagnostic, therapeutic and vaccine purposes has been functionally recognized in most of the diseases, and suggestions for improvements have been made. We believe that these neglected diseases still require special care and approaches to control them, and probably public institutions will fill out this specific demand for high-end research applications and improvements.

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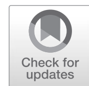

9 Therapeutic Antibodies for Biodefense

Arnaud Avril

Abstract

Diseases can be caused naturally by biological agents such as bacteria, viruses and toxins (natural risk). However, such biological agents can be intentionally disseminated in the environment by a State (military context) or terrorists to cause diseases in a population or livestock, to destabilize a nation by creating a climate of terror, destabilizing the economy and undermining institutions. Biological agents can be classified according to the severity of illness they cause, its mortality and how easily the agent can be spread. The Centers for Diseases Control and Prevention (CDC) classify biological agents in three categories (A, B and C); Category A consists of the six pathogens most suitable for use as bioweapons (*Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, botulinum neurotoxins, smallpox and viral hemorrhagic fevers). Antibodies represent a perfect biomedical countermeasure as they present both prophylactic and therapeutic properties, act fast and are highly specific to the target. This review focuses on the main biological agents that could be used as bioweapons, the history of biowarfare and antibodies that have been developed to neutralize these agents.

Keywords

Bioterrorism · Antibodies · Prophylaxis · Therapeutic · Biodefense · Bioweapon · Biowarfare agent · Toxin · Virus · CBRN

9.1 Introduction

9.1.1 Definitions

Various definitions of biological agent, biological weapon (bioweapon) and bioterrorism are proposed by international organizations (World Health Organization, Centers for Diseases Control and Prevention) and States.

A **biological agent** could be defined as (i) a micro-organism (natural or genetically engineered) (ii) a cell culture (iii) a human endoparasite (iv) a toxin that can induce a reaction, allergy or poisoning in a host (human or not) [64].

A **biowarfare agent** could be defined as a biological agent that is conducive to the development of a bioweapon. In the context of terrorism, the term **biothreat agent** could also be used.

A **bioweapon** could be defined as an intentionally harmful combination of one or more biological agents with a vector (bomb, rocket, nebulizer, animal, etc.). A bioweapon could be

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used directly to incapacitate or kill a single person or population, or indirectly to destabilize a nation by targeting livestock and agriculture [67].

A **danger** could be defined as any kind of damage that could affect a system (e.g. human, country, institution…).

A **threat** could be defined as a factor increasing the risk.

A **risk** could be defined as the probability of exposition of a system to a danger. The risk is increase by the threat.

Biological risk could be defined as the risk of intentional utilization of a bioweapon to harm human beings—directly or not—by means of a **threat** (e.g. State, terrorist). The notion of risk is directly connected to the notion of **vulnerability** (e.g. absence of control of the food and water supply, absence of ways of detecting biological agents, high urban population density, etc.). Once a risk is identified it may be possible to **neutralize** it (e.g. human smallpox was eradicated by mass immunization).

The **vulnerability** could be defined as the possibility for any system to be damaged by a danger.

Countermeasures are tools or devices used to prevent or to mitigate the impact of the danger (Fig. 9.1).

9.1.2 Classification

Several classification systems for biowarfare agents have been proposed by States and international organizations (World Health Organization, Centers for Disease Control and Prevention, etc.). Certain biowarfare agents are considered as a priority for biodefense in almost of these classifications but, there are some differences between the various systems because there is no consensus on selection criteria and some threats are specific to certain countries or geographical areas.

In 1949, the U.S. military microbiologist Theodor Rosebury proposed a list of ten criteria to take into account when weighing up the potential of any biological agents for biowarfare (in order of importance): [20, 102]

- Infectivity: the biological agent should be highly infective;
- Casualty effectiveness: the biological agent should be virulent and able to cause fatal or incapacitating disease in a short time frame;
- Availability: the biological agent should be easy and cheap to produce in bulk;
- Resistance: the biological agent should be able to persist in the environment (heat, ultraviolet, sunlight-radiation, disinfectant, etc.) for long enough to infect the target population. If a biological agent is not naturally resistant enough, it might be possible to engineer it;
- Mean of transmission: the biological agent should be easy to disseminate in the environment (ideally by nebulization);
- Epidemicity: depending on the desired outcome, the biological agent could be highly contagious or not. If a biological agent is highly contagious, the risk is that the aggressor could also be contaminated ("boomerang effect");

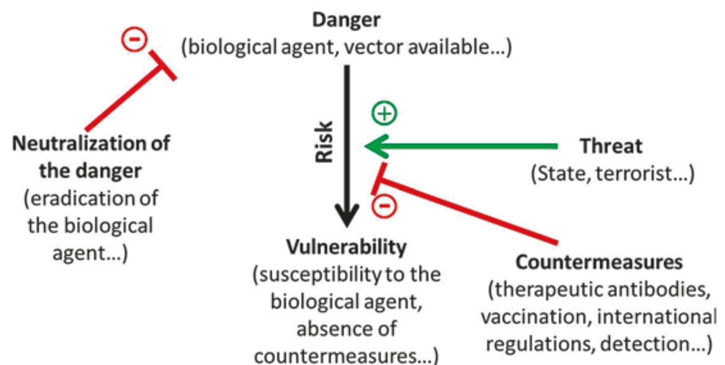

Fig. 9.1 Relationship between risk, threat and vulnerability
Green arrow: positive effect on the system.
Red arrows: negative effect on the system

– Specific immunization: the target population should not be naturally immunized against the agent or have access to a vaccine (although the aggressor should have a vaccine). Any bacterium should be resistant to conventional antibiotics;
– Therapy: the aggressor should have access to protections against the biological agent (preventive or curative treatment, individual protection equipment, etc.) to prevent a boomerang effect. No therapy should be available for the target population;
– Detection: defenders will find it more difficult to respond if it is difficult to detect—diagnose—the biological agent or the source of contamination,;
– Retroactivity: the capacity of a bioweapon to backfire on the aggressor will affect whether a nation decides to use it or not.

If bulk production of the biological agent is cheap, more countries will be able to develop the bioweapon.

The ten criteria of Theodor Rosebury were defined for the identification of a biological agent for military purposes but they are as relevant to bioterrorism, although other criteria also come into play in this context:

– The biological agent should be easily available—illegally bought, stolen or found in nature—to terrorists.
– Production of the biological agent should be easy and cheap with minimal resources;
– Resultant mortality or degree of incapacity should be high;
– Treatments should not be available, even for the terrorists (kamikazes);
– The delay between contamination and the onset of symptoms could be long, to complicate investigation and identification of the source of contamination;
– The choice of biological agent should take into account the terror that will be elicited in the population. The resulting panic would destabilize the economy and the normal function of the State.

The main classification system used for biological agents of interest for biodefense is that of the Centers for Disease Control and Prevention (CDC) (Tables 9.1 and 9.2) [22]. Two main criteria were used for this classification, namely ease of spread of the biological agent and the severity/lethality of the illness caused. Category A contains the six biological agents of major concern for biodefense; Category B lists 12 agents that are considered of secondary potential; Category C agents are those that are considered as emerging-threats for biodefense.

9.1.3 History of Bioterrorism

9.1.3.1 Use of Biological Agents in the Military Context

Bioweapons could be far more deadly than chemical or nuclear weapons, e.g. it has been estimated that 10 g of anthrax could kill as many people as a metric ton of sarin [113]. In the last century, more than 500 million people died of naturally spread infectious diseases but tens of thousands also died as a result of the deliberate release of a biological agent (Table 9.3). Paradoxically, at the same time as humankind developed drugs and systems to cure sick people, it developed bioweapons in parallel. This could be because the knowledge required to develop drugs and vaccines against pathogens can be misused to develop bioweapons. Establishing exactly when bioweapons have been used is difficult because of lack of information and the absence of formal evidence. Similarly, estimating mortality following a biological attack is complicated because it can be difficult to differentiate deaths due to a natural epidemic from those directly resulting from the attack.

The first utilization of a bioweapon seems to go back to 1350 BCE when Hittites (an ancient people who lived in Anatolia) left sick animals in villages they had plundered to infect the local population. Recent analyses reveal that the animals had died of tularemia which is in CDC Category A [123].

Table 9.1 CDC Class A, B and C biological agents

	Category A	Category B	Category C
Properties	Easily spread or transmission from person to person	Moderately easy to spread	Easily available
	High mortality rates	Induce moderate illness rates and low mortality rates	Easy to produce and spread
	Major impact for public health	Require specific enhancements of CDC's laboratory capacity and enhanced disease monitoring	Potential for high morbidity and mortality rates and major health impact
	Cause public panic and social disruption		
	Require special action for public health preparedness		
Bacteria	Anthrax (*Bacillus anthracis*)	Brucellosis (*Brucella* species)	Some multidrug-resistant bacteria (e.g. *Mycobacterium tuberculosis*)
	Plague (*Yersinia pestis*)	Epsilon toxin of *Clostridium perfringens*	
	Tularemia (*Francisella tularensis*)	Food safety threats (e.g. Salmonella species, *Escherichia coli* O157:H7, *Shigella dysenteriae*…)	
		Water safety threats (e.g. *Vibrio cholerae*…)	
		Glanders (*Burkholderia mallei*)	
		Melioidosis (*Burkholderia pseudomallei*)	
		Psittacosis (*Chlamydia psittaci*)	
		Q fever (*Coxiella burnetii*)	
		Typhus fever (*Rickettsia prowazekii*)	
Virus	Smallpox (*Variola major*)	Viral encephalitis caused by alphaviruses (e.g. Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis…)	Viral emerging infectious diseases (e.g. Nipah virus, hantavirus…)
	Viral hemorrhagic fevers: filoviruses (Ebola, Marburg…) and arenaviruses (Lassa, Machupo…)		
Parasite and fungi		*Cryptosporidium parvum* (water safety threat)	
Toxines	Botulism (Clostridium botulinum toxins)	Ricin toxin (*Ricinus communis*)	
		Staphylococcal enterotoxin B	

The A and B lists are exhaustive; Category C is an open list

More information is available about an attack on the seaside city of Kaffa (currently Feodossiia, Crimean peninsula) in 1346 AD. The Mongols were besieging the city and catapulted the corpses of plague victims over the city's fortifications [62, 15, 133]. This attack had major physical and psychological impact on the citizens, leading to the town's evacuation by sea. This diaspora resulted in a five-year plague epidemic that killed about a third of the population of Europe. The direct impact of the use of plague as a bioweapon is difficult to quantify because the disease might have spread naturally as a result of wartime unhygienic conditions in Kaffa.

The first use of a virus as a bioweapon was documented in 1763 in mail sent by Henri Bourquet, a British mercenary, to Jeffrey Amherst, the British military governor of the New-France. After the defeat of the French in the Seven Year War (1754–1760), strict conditions were imposed on Amerindians who had fought with the French [2, 82]. Consequently, the Amerindians rebelled

Table 9.2 Main characteristics of the biological agents that are most likely to be used as bioweapons

Biological agent	CDC category	Infectious dose	Incubation period	Interhuman transmission	Case fatality rate in absence of treatment	Prophylaxis	Treatment (approved or IND)	Serotherapy (approved or IND)
Bacillus anthracis	A	8000–10,000 spores	2–6 days	No	10% (cutaneous anthrax) to ~100% (inhalational anthrax)	Vaccine. Antibiotics.	Antibiotics: ciprofloxacin or doxycycline plus one or two antibiotics effective against anthrax (e.g. meropenem) plus a protein synthesis inhibitor (e.g. linezolid).	Yes (monoclonals): Raxibacumab and Obiltoxaximab
Yersinia pestis	A	100–500 bacteria	1–6 days	Infectious (pulmonary plague)	50–60% or ~100% (pulmonary plague)	Antibiotics.	Antibiotics: streptomycin, gentamicin, doxycycline, ciprofloxacin and/or chloramphenicol.	No
Francisella tularensis	A	10–50 bacteria	1–10 days	No	35–65% (pulmonary tularemia)	Vaccine (IND).	Antibiotherapy: streptomycin or gentamicin.	No
Variola viruses	A	10–100 virions	7–17 days	Very infectious	1% (variola minor) – 50% (variola major)	Vaccine.	Vaccination. Chemotherapy: cidofovir (IND), ST-246 (IND) or CMX001 (IND).	Yes (polyclonal): Vaccinia immune globulin (IND)
Viral hemorrhagic fevers	A	1–10 virions	4–21 days	Very infectious	High to moderate (depend on biological agent)	1 vaccine (against yellow fever). Chemotherapy: ribavirin may be effective against *Bunyaviridae* and *Arenaviridae* (IND).	Intensive supportive care. Chemotherapy: Ribavirin (IND).	Yes: Zmapp (oligoclonal) against Ebola virus (IND). Convalescent plasma for some hemorrhagic fevers (IND).
Botulinum toxins	A	LD_{50}: 1 ng.kg^{-1} (IV) to 1 μg.kg^{-1} (I)	2 h – 3 days	No	~60%	Vaccine.	Serotherapy. Antibiotics (for wound botulism). Supportive therapy.	Yes (polyclonal): BabyBIG and BAT.
Ricin	B	LD_{50}: 1 (by injection) to 25 mg.kg^{-1} (by inhalation)	3–8 h	No	2–8%	Two IND vaccines. Mask is the best protection to prevent inhalation.	Supportive therapy.	No

(continued)

Table 9.2 (continued)

Biological agent	CDC category	Infectious dose	Incubation period	Interhuman transmission	Case fatality rate in absence of treatment	Prophylaxis	Treatment (approved or IND)	Serotherapy (approved or IND)
Staphylococcus Enterotoxin B	B	Incapaciting dose: 0,4 µg. kg^{-1}	3–12 h	No	<1% but ~80% of contaminated would be incapacited for 1–2 weeks.	No	Supportive therapy.	No
Coxiella burnetii	B	1–10 bacteria	10–40 days	No	<3%	Vaccination. Antibiotics.	Antibiotics: tetracycline, doxycycline or hydroxychloroquine.	No

The case fatality rate assumes the absence of any relevant treatment or prophylaxis (vaccination.., etc.). In the event of massive contamination, the fatality case rate would depend upon the time required for diagnosis and the possibility that it would be impossible to provide effective treatment for all victims. Fatality case rate would depend upon the route of contamination and the general state of health of the target population. Here, prophylaxis is described as urgent prophylaxis (if contamination is suspected) or long-term prophylaxis (vaccination). IND: Investigational New Drug. IV: intravenous. I: ingestion

Table 9.3 Historical uses of biological agents in biowarfare

Year	Examples of utilization of biological agents as a biowarfare agent.
~1350 B.C.	Hittites leaves animals contaminated by tularemia in the villages that they had plundered.
Fourth century B.C.	According to Greek historian Herodotus, Scythian archers infect their arrows by dipping them into decomposing cadavers.
1155	Emperor Barbarossa poisons water wells with human bodies.
1346	Mongols catapult bodies of plague victims over the fortifications of Kaffa (Feodossiia, Crimean Peninsula).
1422	The Prince Zygmunt Korybutovic hurled corpses of plague-stricken soldiers, dead cows and excrement during the siege of Karlstejn (currently in Czech republic).
1495	Spanish mix wine with blood of leprosy patients to sell to their French foe (Naples, Italy).
1650	The Polish general Kazimierz Siemienowicz fire with hollow artillery spheres filled with the with the saliva of rabid dogs.
1763	British troops give smallpox-infected blankets to the Amerindians.
1797	Napoleon floods the plains around Mantua, Italy, to enhance the spread of malaria.
1785	Tunisians threw plague-infected cothing into the Chistian-held city of La Calle (currently in Algeria).
1863	Confederates sell clothing from yellow fever and smallpox patients to Union troops, USA.

This table lists some examples of reported use of biowarfare agents. The real impact of these aggressions is unclear because it is difficult to distinguish between natural and hostile contamination

against the British and won several battles. To stop the rebellion, in 1763, the British were supposed to have distributed blankets carrying the smallpox virus (obtained from the military hospitals) to "*extirpate this execrable race*". Here again, it is difficult to know whether such blankets were really given to the Amerindians and, if they were, how many Amerindians died from smallpox. Nevertheless, these communications are one of the first pieces of historical evidence of bioweapon used in a military context.

During the nineteenth century, scientists like Louis Pasteur, Robert Koch and Alexandre Yersin founded the science of microbiology with the discovery of antisepsis, pasteurization and vaccination as well as the identification of the pathogens that cause anthrax, tuberculosis and plague. While this work certainly improved medical practice, it could also be diverted for hostile purposes.

In World War I, a chemical weapon (mustard gas) was used for the first time in Ypres (France) by the Germans, leading to a defeat of the French. Following this attack, several countries set up national programs to develop chemical and biological weapons for either defensive or offensive applications, despite it being almost impossible to differentiate between the two. A number of State-sponsored program for bioweapon development are known. The Soviet program, called Biopreparat, was a major one. This program started in 1919 and ended in 1991 with a budget estimated at a billion dollars a year [14]. Eight biological agents (smallpox, plague, tularemia, glanders-like, equine encephalitis, anthrax, Q-fever and Marburg virus) were militarized and tons of biological agents were produced. Several biological agents were genetically-engineered to increase their pathogenicity and resistance to existing treatments. The danger of such programs is illustrated by the accident that occurred in Sverdlovsk in 1979 in an anthrax spore production facility for the Ministry of Defense's Scientific Research Institute of Microbiology. After a maintenance operation, some back-up particle filters in the air conditioning system were not replaced and anthrax spores were nebulized through the facilities and spread in the environment, leading to a major outbreak (66 deaths from inhalational anthrax). Following this accident, mean lethal dose (hLD_{50}) for inhalational anthrax in human beings was estimated at between 8000 and 10,000 spores [75, 103]. Another major State-sponsored program was started in Japan in 1925 with about 3000 employees. The main center was based in Manchuria and was called unit 731 (officially "Army Epidemic Prevention Research Laboratory"). Unit 731 produced several tons of pathogens causing cholera,

smallpox, botulism, bubonic plague, anthrax, tularemia and venereal disease. Thousands of Chinese prisoners were exposed to these pathogens and left untreated in order to study the effects of the induced diseases [12, 42, 100]. Some of these pathogens were also disseminated in China, either directly by inoculating reservoirs and wells or indirectly using vectors such as fleas or bombs; it has been estimated that more than 580,000 people may have died [11, 55]. An example of how difficult it is to control bioweapons in a military setting comes from 1942 when bioweapons being used against the Chinese ended up killing more than 1700 Japanese soldiers [134]. Following this event, experiments in nature were stopped and Unit 731 was disbanded after the invasion of Manchuria by the Soviet army. The British program focused on anthrax provides an example of large-scale bioweapon environmental testing. In 1942, *Bacillus anthracis* spores were disseminated in the Gruinard Island using N-bombs. The Island was subsequently quarantined until it was thoroughly decontaminated with formaldehyde in 1990. More recently in Iraq, a program of bioweapon development was initiated in 1985. This program was carried out in factories disguised as chicken-feed plants. Equipment like desiccators and fermenters as well as biological strains were legally bought from Western companies. Specifically, 24 pathogens, including the biological agents of botulism, anthrax, gangrene and brucellosis were purchased from the American Type Culture Center. The program was only stopped in 1991, after the invasion of Iraq by American troops in the Gulf War. In their report, United Nations inspectors estimated that 8500 liters of anthrax, 19,000 liters of botulinum toxin and 2200 liters of aflatoxin had been produced and introduced into explosive systems for dispersion, e.g. 157 R-400 bombs and 25 *Scud* missiles were filled with 3 different biological agents [47, 125, 139].

9.1.3.2 Protocols Restricting the Utilization of Bioweapons

Since the beginning of the twentieth century, the international community has tried to limit the proliferation of bioweapons. The Geneva Protocol of June 14, 1925 was the first protocol that limited the use of bioweapons in war. Two major limitations of this protocol were: although the use of bioweapons was restricted, possessing and developing them was not; and no organization was created to enforce the Protocol.

A convention on the prohibition of the development, production and stockpiling of bacteriological (biological) and toxin weapons and on their destruction (also referred as the "Biological Weapons Convention") was opened for signature on April 10, 1972 and entered into force on March 26, 1975 after ratification by 22 governments [1]. This Convention was the first multilateral disarmament treaty banning an entire category of weapons, as States party to the Convention undertook "*never in any circumstances to develop, produce, stockpile or otherwise acquire or retain microbial or other biological agents, or toxins whatever their origin or method of production, of types and in quantities that have no justification for prophylactic, protective or other peaceful purposes […] weapons, equipment or means of delivery designed to use such agents or toxins for hostile purposes or in armed conflict*.". Today only 155 out of the 171 governments that signed the convention have ratified it. A major limitation is that no penalty was foreseen in case of violation of the convention. To overcome the limitations of this protocol, since 1985, the "Australia Group", an informal group, seeks to ensure that international exports do not contribute to the development of chemical or biological weapons, through the harmonization of export controls among the 41 States-Parties. Coordination of all national export regulations helps Australia Group members to fulfil their obligations under the Biological Weapons Convention to the fullest extent possible. The Australia Group defined a list of biological agents that could be used for the development of bioweapons and for which exports must be regulated [7].

Following the end of the Cold War and the rise of terrorism, the United Nations Security Council unanimously adopted on April 28, 2004 Resolution 1540(2004) which affirms that the proliferation of nuclear, chemical and biological

weapons and their means of delivery constitutes a threat to international peace and security. The resolution obliges *inter alia* States to refrain from supporting, by any means, non-State actors from developing, acquiring, manufacturing, possessing, transporting, transferring or using nuclear, chemical or biological weapons and their delivery systems. On June 29, 2012 the Security Council adopted the resolution 2055(2012), which enlarged the group of experts supporting the work of the 1540 committee to nine experts.

9.1.3.3 Use of Biological Agents in Bioterrorism and Assassination

Currently, bioweapons are unlikely to be used in a military context because of the regulations and for practical reasons, *i.e.* bioweapon development and use is forbidden by the United Nations and it is difficult to control the spread of the biological agent. Specifically, the risk of contaminating your own troops, population and environment is high which is generally unacceptable to an aggressor, even one who has not signed any non-proliferation convention. Currently, the main threat is the use of pathogens by terrorists, that would have major psychological impact in addition to mortality [31].

Several historical instances of proven or suspected use of biological agents for terrorism or assassination have been reported (Table 9.4). Biological agents may be easier for terrorists to obtain than many chemicals or radioactive substances, e.g. ricin—not even classified in CDC Category A—is considered by many as having great potential in bioterrorism. Despite low toxicity compared to other toxins (LD_{50} 1 mg.kg^{-1} compared to 1 µg.mL^{-1} for botulinum neurotoxin by ingestion), ricin can be relatively easily purified from the seeds of *Ricinus communis*, a ubiquitous plant (commonly found in parks throughout the world). This toxin has been used for several assassinations such as that of Georgi Markov in London by the KGB using a spiked umbrella that could be used to fire a ricin-containing sphere [87]. In September 2001, letters containing anthrax spores were sent to American government officials. In 2008, the FBI investigations

Table 9.4 Examples of use of biological agents for terrorism and assassination

Date	Examples of utilization of biological agents in the bioterrorist or assassination context	Impact
27th of May 1942	Jan Kubis, a Czech member of the resistance used grenade coated with botulinum toxins to killed the Nazi general Reinhard Heydrich	Success: Reinhard Heydrich seems to died from botulism
1978	KGB killed Georgi Markov in London with a system dissimulated in an umbrella and injecting spheres containing rich	Success: death of Georgi MARKOV
1984	Rajneesh cult contaminated salad bars with Salmonella typhimurium is Dalles (USA)	Success: 751 persons contaminated and 45 hospitalized
April 1990	Aum Shinrikyö sect tried to spray what they thought was botulinum toxinin Tokyo, Yokohama, Yokosuka and Narita with nebulizer placed in trucks	Failed: they didn't succeed to isolate Clostridium botulinum from the soil
June 1993	Aum Shinrikyö sect tried to disseminate anthrax spores from trucks	Failed: nebulizer filter were obstructed
Summer 1993	Aum Shinrikyö sect tried to disseminate anthrax spores in Tokyo from a roof	Failed: utilization of the non-virulent 34F2 strain
Autumn 2001	Letters containing anthrax spore were sent to American officials (probably sent by the researcher Bruce Ivins)	Success: 5 deaths over 11 persons contaminated
April 2013	3 letters containing ricine were sent to the president of the USA and to American officials	Failed: no contamination
2016	DAESH planned to used anthrax in a mall in Nairoby.	Failed: attack thwarted by Kenyan police.

This table lists some examples of reported-uses (proven or suspected) of biological agents for terrorism or assassination [10, 15, 30, 57, 79, 87, 118]

identify Bruce Ivins, a researcher from the Fort Detrick research center, as the main suspect in this attack. Ivins could have stolen spores of *Bacillus anthracis* from the laboratory and, being immunized, he could safely handle the material. This example underlines how easy it is, even for a single man, to develop a bioweapon and use it successfully, if sufficiently competent and determined.

9.2 Antibodies for Biodefense

9.2.1 Antibodies As "Magic Bullets" to Fight Bioweapons

Antibodies are perfect tools to fight bioweapons. Since the end of the nineteenth century, antibodies have been shown to be effective in the treatment of a number of diseases as well as in diagnosis. Although they were supplanted in the twentieth century by antibiotics and other drugs in the treatment of infectious diseases they are still widely used to treat diseases caused by toxins (such as venom). Moreover, multidrug-resistant bacteria have spread naturally and in the context of biowarfare agents, biological agents could have been selected or engineered for their resistance to existing treatments. The development of antibodies is strategic as they can be used when other specific therapies are not available or ineffective and since they can act synergistically with other drugs. One of the main advantages of antibodies compared to other treatment modalities is their specificity of action, which decreases the risk of toxicity and adverse reactions. As antibodies are well-tolerated and their half-life can be as long as 21 days (or even longer if their Fc portion has been appropriately engineered), they are ideal for prophylaxis, e.g. for soldiers who might be exposed to bioweapons on the battlefield. When a possibility of biowarfare is suspected, the medical corps could administer the antibodies before the attack, affording weeks of powerful protection. If contamination with a biowarfare agent is detected in a soldier (military context) or a civilian (bioterrorist context), antibodies could also be administered prophylactically to everyone in the area. Such use is possible because antibodies protect so quickly, unlike other forms of prophylaxis such as vaccination, which generally requires several spaced out doses to elicit full protection. In the context of biodefense, global vaccination is generally not feasible if the biological agent is not encountered in the nature (such as smallpox). In some case, such as botulism, global vaccination is ethically questionable, as it would prevent the therapeutic use of botulinum toxin (which is used to treat more than 25 diseases as well as in plastic surgery).

Modern recombinant antibodies are very safe and can be administrated at high doses to elicit strong protection; this is important because deliberate contamination might involve higher concentrations of pathogen than natural exposure. Nevertheless, there are some limitations to the uses of antibodies. Firstly, if several injections of the same antibody formulation are administered (such as in a context of long-term prophylaxis or prolonged hospitalization), the body can produce its own antibodies against the exogenous antibody (referred as anti-drug antibodies) leading to their neutralization. However, this problem would only apply to prophylactic use, as for therapy a limited number of doses is involved and repeated contamination with the same agent is unlikely. A second limitation is that antibodies have to neutralize the biological agent before it enters its target cell. Considering this limitation, antibodies should be administered as soon as possible after contamination that could be difficult following mass contamination because medical facilities would be saturated and antibody doses might have to be reserved for the medical staff. However, even administered several hours or days after contamination, antibodies could be effective, e.g. following contamination by a virus or a bacterium, antibodies could neutralize any toxins or virions produced *in vivo*. Efforts are also being made to develop transbodies that can pass through the cell membranes and intrabodies that are directly synthesized inside the cell [70, 119]. In the context of biodefense, transbodies would neutralize a biological agent after cell penetration, thereby prolonging the therapeutic win-

dow would be essential in a context of mass contamination. Compared to the production costs of small chemical inhibitors, those of recombinant antibodies are high. However, production costs might be compensated for high success rates during clinical development and the pre-ordering of doses by States for strategic stockpiling would guarantee a fast return for companies developing antibodies for biodefense.

Another advantage of antibodies is that they could also be used for the development of diagnostic tools such as lateral or vertical flow immunoassay on a strip or column. This specific aspects of antibodies will be only presented briefly in this paragraph because it could be the topic of a separate review. Immunoassays are highly specific and sensitive which is ideal for rapid, preliminary detection of a biological agent in different sample types (blood, feces, water, food…). Such tests are generally less sensitive than other methods such as PCR or *in vivo* mortality assay but, as they are portable and fast, they are useful for preliminary screening of potentially contaminated people and samples, even in places where there is no direct access to biomedical or hospital facilities. Moreover, toxins cannot be detected by PCR and the methods necessary such as mass spectrometry are time-consuming, expensive and not always available. Immunodiagnostic tests are generally available for first responder personnel and troops on the battlefield. The Ebola crisis in Africa highlighted the need for easy, rapid and cheap diagnostic tests to detect biological agents in countries where medical facilities are limited. An immunoassay test, called eZYSCREEN developed for Ebola virus takes less than 15 min and just requires a few microliters of blood or serum. In 2014, this was tested in Guinea (Galais et al., Bulletin de la société de pathologie exotique, in press). Other antibody-based diagnostic tools have been developed for a number of biowarfare agents [6, 23, 44, 58, 96] and antibodies for diagnosis—unlike those intended for therapy—do not need to target a neutralizing epitope. In the development of therapeutic antibodies, most of the antibodies isolated specifically interact with the antigen in the (sub-)nanomolar range, but do not neutralize it. Developing immunodiagnostic assays based on such antibodies is a good way of profiting from all the time and money invested.

Considering the advantages of antibodies vis-a-vis managing a biological crisis, several States have drawn up strategies for biowarfare prevention and large-scale treatment. Specifically, some have stockpiled medical countermeasures such as antibodies (in solution or lyophilized). Section 9.2.3 will present monoclonal, polyclonal, oligoclonal and recombinant antibodies currently on the market or in clinical development, directed against the main biowarfare agents. If no such antibody is referred to in the literature, antibodies or antibody fragments at an early stage of development will be presented.

9.2.2 Mechanism of Action of Antibodies for Biodefense

CDC Category A groups together the six biological agents of major concern for biodefense. These are viruses, bacteria and toxins, and all of them could be efficiently neutralized by antibodies acting through similar mechanisms. This paragraph will present briefly the pre- and post- cell-attachment mechanisms neutralization.

Toxins may be the easiest biological agent to neutralize with an antibody. In natural infections, toxins are generally ingested (e.g. food-borne botulism or ricin intoxication), inhaled (e.g. anthrax) or directly produced by the bacteria in the body (e.g. wound botulism). In the context of biodefense, direct production of toxin in the body is unlikely; the toxin would be introduced via the food or water supply chain, or by inhalation. In this case, antibiotics would be ineffective and therapy would be based on supportive therapy or the administration of specific inhibitors, if any are licensed. In this situation, antibodies could rapidly neutralize the toxin's activity *in vivo* (Fig. 9.2). Toxin-dependent toxicity is generally based on two main steps: toxin binding and internalization into the target cell, and then catalytic activity (toxins referred as "A-B toxins"). Antibodies can neutralize such toxins by blocking any of these steps if they target a neutralizing

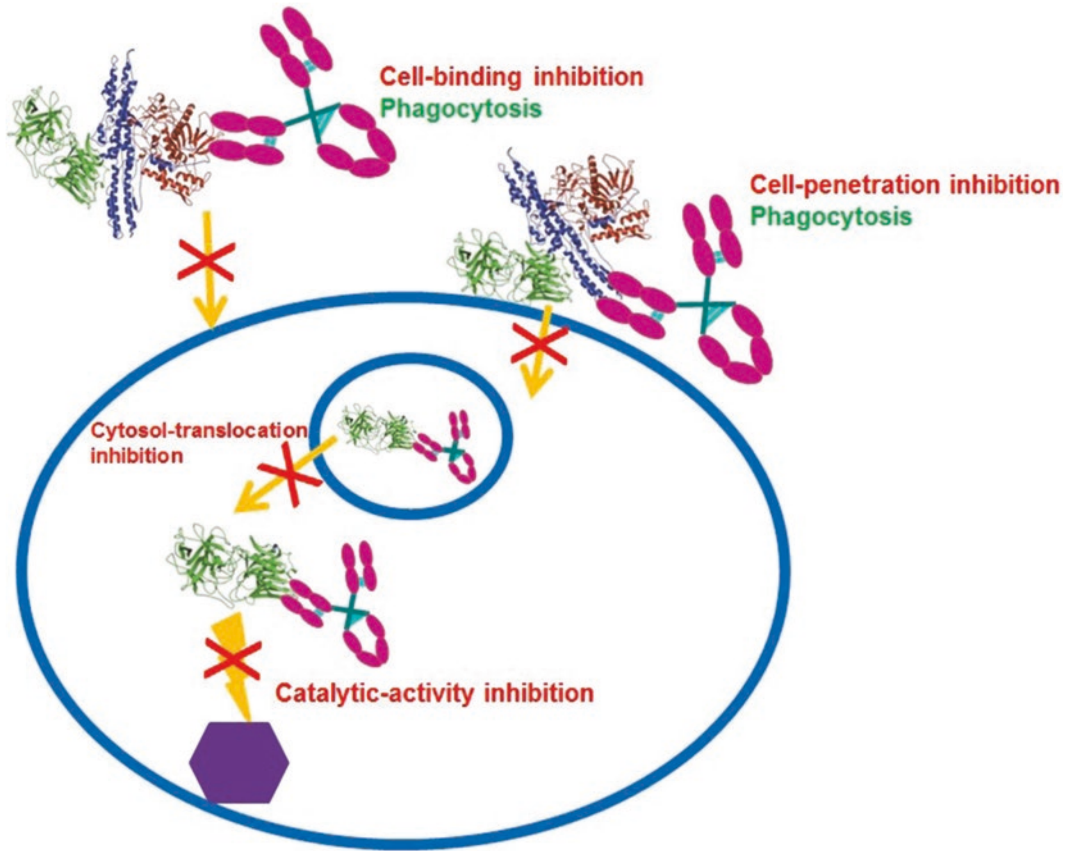

Fig. 9.2 Mechanisms involved in antibody-mediated toxin-neutralization
Proportions between different sizes are not respected in this schematic representation. Toxin actions inhibited are represented in red and antibody effector functions are represented in green

epitope. As toxins may act at very low concentration (e.g. the LD_{50} of botulinum neurotoxin A is 1 ng.kg^{-1}), the probability that a therapeutic antibody will bind a toxin in the bloodstream is relatively low. Therefore, it is very unlikely that an antibody will bind two toxins simultaneously (only one of the two binding domains will bind a toxin). Additionally, in contrast to viruses or bacteria, there is no significant repeat motif in toxins and avidity effects will not be involved in toxin-neutralization. Considering these specificities, antibodies must bind to the toxin with a very high affinity (nano- or pico-molar) to preclude dissociation of the antibody-toxin complex. If the antibody epitope is localized in the binding domain of the toxin, the antibody would bind the toxin before it interacts with the cells. If the epitope is based in the internalization domain, the antibody could bind the toxin in the bloodstream or possibly after it has bound to the cell. Antibodies can also bind the translocation domain and inhibit passage of the toxin from endocytic vesicle to cytosol. If the epitope is based on the catalytic domain, the antibody could act indirectly by steric hindrance to prevent binding or internalization, or it could act directly by blocking the toxin's catalytic activity. If the antibody-toxin complex is formed in the general circulation, it will be eliminated by immune cells recruited by the Fc domain of the antibody. A risk is that antibodies could also bind to the toxin on the cell-surface, and induce antibody-dependent cellular toxicity (ADCC) that could finally kill the cell. One limitation of antibodies is that they must be

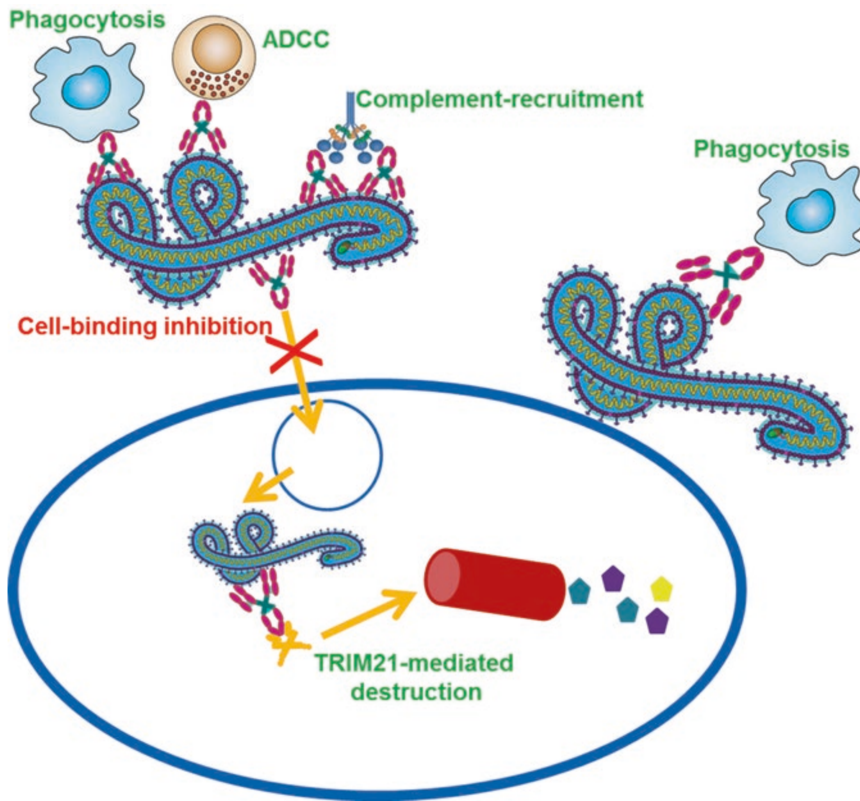

Fig. 9.3 Mechanisms involved in antibody-mediated virus or bacterium neutralization
Antibodies are involved in adcc, phagocytosis, complement recruitment, direct neutralization and trim21 recruitment. Virus/bacteria actions that are inhibited are represented in red and antibody effector functions are represented in green. Proportions between different sizes are not respected in this schematic representation. Ebola schematic representation: Dr. Ian M Mackay, virologydownunder.blogspot.com.au. Yellow: trim21. Red cylinder: proteasome

administered as soon as possible, before toxin-internalization, because unengineered antibodies alone cannot get internalized into the cell to bind its target. Once internalized, an intracellular toxin will persist until it is broken down or the cell dies. In the case of botulism, the toxin does not kill the cell but with the half-life of the botulinum neurotoxin A (BoNT/A) being of the order of months, its effects could persist for a very long time [124]. In the case of contamination with bacteria, antibodies will be able to neutralize any toxin produced *in vivo* in this case antibiotics and antibodies would act synergistically, both neutralizing the activity of the toxin and killing the bacterium.

In the context of bacteria (such as *Francisella tularensis*) or viruses (such as Ebola virus), the main mechanism of neutralization is based on ADCC and phagocytosis (Fig. 9.3) [60]. Viruses and bacteria are larger than toxins and their cell-surface is generally composed of one or more proteins present in multiple copies (often hundreds or thousands of copies) which are usually densely packed together. In this case, different antibodies could target a single virus or bacterium and both antibody binding-domains can bind their target. With such avidity effects, even if one of the two antibody binding-domains dissociates from its epitope, the antibody still remains linked to the biological agent through its second binding-domain and ultimately the free binding domain will be able to rebind. The binding of multiple antibodies on the biological agent's surface will result in efficient recruitment

of complement, phagocytes and cytotoxic cells that will ultimately result in the destruction of the pathogen. Such destruction could be also facilitated by the formation of large immune complexes that diffuse slowly in the organism and that actively recruit the immune-system cells. Nevertheless, fewer cells may end up being infected than by a monodisperse suspension of virions, but those that do may attain a higher multiplicity of infection. If the epitope is directly based on the bacteria/virus cell-binding domain or near this domain (steric hindrance), it could prevent the binding and will lead to the elimination of the pathogen by the immune system. The antibody could also target the pathogen after the binding on the cell surface and indirectly block endocytosis by steric hindrance. Such, antibodies that bind the pathogen post cell-attachment may also act partly by competition with receptors. In this case, higher concentrations of antibodies are generally required because of the valency of the virus-receptor interactions and the strengthening of initial attachment. Although previously thought to mediate protection solely in the extracellular environment, recent research has revealed that antibody-mediated protection also extends to the cytosolic compartment of cells. Indeed, internalized antibody-virion or antibody-bacteria complexes could recruit a cytosolic Fc receptor called TRIM21 (tripartite motif containing 21) that mediates antibody-dependent intracellular neutralization (ADIN) [37, 73]. TRIM21 is a ubiquitously expressed E3 ubiquitin ligase that binds IgG, IgA and IgM with subnanomolar affinity and that directs the pathogen to the proteasome for degradation and that lead to the synthesis and secretion of pro-inflammatory cytokines and chemokines. It is interesting to note that sometime the direct neutralization of virus could be complicated, in part for the neutralization of virus that can be transmitted directly from cell to cell via a virological synapse. In this case, antibodies that interfere with the formation of the synapse by binding to cellular structures and antibodies that counter virion formation or release might block this mode of transfer and indirectly contribute to the neutralization of the pathogen.

9.2.3 Antibodies Against Biowarfare Agents

9.2.3.1 Antibodies Against Anthrax

Anthrax is a disease historically caused by the toxins secreted by the Gram-positive, spore-forming, bacterium *Bacillus anthracis*, but it was recently described that *Bacillus cereus* biovar *anthracis* can also cause anthrax [19]. Distinction is made between four forms of anthrax according to the route of entry: cutaneous, pulmonary, digestive and injection. Pulmonary anthrax (inhalational anthrax) is the main threat for biodefense because spores can be nebulized and the fatality rate is ~100% when left untreated, and up to 88% if treated (45%% following the attacks of 2001 with contaminated letters) [52, 54]. *B. anthracis* contains two plasmids coding for the virulence factors, in particular the toxin subunits. The lethal toxin (LT) is composed of the protective antigen (PA) and the lethal factor (LF); the edema toxin (ET) is composed of PA and the edema factor (EF). Targeting PA and LF with antibodies is a widely-used strategy to stop the pathogenic process. As PA is shared between ET and LT, the majority of the antibodies developed against anthrax target this subunit. New-Zealand rabbits and non-human primates are reported by the US Food and Drug Administration (FDA) as the best model for the evaluation of anti-anthrax antibodies [36, 74]. Following the Sverdlovsk accident, the hLD_{50} was estimated between 8000 and 10,000 spores [75, 103]. Human anthrax prophylaxis is based in the USA on vaccination with BioThrax® (Emergent BioDefense Corporation, Michigan) of the population with high risk of exposure (soldiers, etc.). This vaccine is produced from culture filtrates of a toxigenic, but avirulent, non-encapsulated mutant V770-NP1-R of the *B. anthracis* Vollum strain and is composed chiefly of PA with small amounts of LF and EF that may vary from batch to batch. Post-exposure treatment of inhalation anthrax is based on 60-day course of antibiotics and serotherapy [18]. To complete the pharmacopeia, development programs have mainly focused on developing drugs to prolong the therapeutic window and decrease treatment time; significant efforts have

been made to develop recombinant antibodies against anthrax (Table 9.5).

Raxibacumab (ABthrax®) was the first recombinant antibody to get FDA approval (in 2012) for the prevention and the treatment of inhalational anthrax. This fully human antibody was isolated by phage-display panning of a Cambridge Antibody Technology human antibodies library. Raxibacumab binds PA with an affinity of 2.78 nM [78]. For therapeutic purposes, Raxibacumab should be administered intravenously at 40 $mg.kg^{-1}$ in adults or 80 $mg.kg^{-1}$ in children ≤15 kg, after diphenhydramine premedication. As it is not ethical to contaminate healthy volunteers with anthrax and there are not enough natural cases of inhalational anthrax, the effectiveness of Raxibacumab was assessed in New Zealand white rabbits and cynomolgus macaque models, and safety was assessed in healthy volunteers. Raxibacumab prophylactically administered at 5, 10 or 20 $mg.kg^{-1}$ subcutaneously 2 days prior, or concurrently at 40 $mg.kg^{-1}$ intravenously to rabbits (n = 12/group) challenged with 100LD_{50} aerosolized Ames spores afforded survival rates of 40%, 83%, 83% and 100% respectively, compared to 0% with placebo. The 40 $mg.kg^{-1}$ dose prophylactically administered subcutaneously to non-human primates-NHP- (n = 10) 2 days prior to a challenge with 100 LD_{50} aerosolized Ames spores was 90% protective. Treatment was also evaluated in rabbits and macaques exposed to 200 LD_{50} of aerosolized Ames spores. A 40 $mg.kg^{-1}$ single-bolus of Raxibacumab was administered intravenously to rabbits and to macaques after the detection of PA subunit in the serum or following a 1.1 °C rise in temperature. Raxibacumab post-exposure treatment provided 44% (n = 18) and 64% (n = 14) survival, respectively [78]. The safety of Raxibacumab has been evaluated in three clinical trials, in 326 healthy subjects treated with one or two doses of 40 $mg.kg^{-1}$ of Raxibacumab or of a placebo, alone or in combination with ciprofloxacin. During the clinical studies, only four subjects (1.2%) had their infusion of Raxibacumab discontinued for adverse reactions. Anti-drug antibodies were not detected in any Raxibacumab-treated human subjects during the clinical studies.

Obiltoxaximab Anthim®, is a chimeric (mouse/human) antibody isolated in 1988, but that was further engineered to increase its affinity and to decrease its immunogenicity [124]. After affinity-enhancement, it binds PA with an affinity of 0.33 nM. Obiltoxaximab tolerance-improvement was realized with the DeImmunisation® technology that localized and removed T-cell epitopes through the combined use of immunological and molecular biology techniques. Obiltoxaximab effectiveness was assessed in the rabbit model. A single 10 mg (~4 $mg.kg^{-1}$) dose of Obiltoxaximab administered intravenously 30–45 min prior to an exposure of 163 or 286 LD_{50} aerosolized Ames spores, provided 100 and 88% protection, respectively. For treatment assessment, rabbits were exposed to 172 LD_{50} of aerosolized Ames spores. Twenty-four or 36 h post-infection a single bolus of 10 mg of Obiltoxaximab was administered intravenously and 80% or 50% survival rates were observed, respectively. For therapeutic purposes, Obiltoxaximab should be administered at 16 $mg.kg^{-1}$ in adults or 32 $mg.kg^{-1}$ in children ≤15 kg, by intravenous route, after diphenhydramine premedication. Obiltoxaximab should only be used for prophylaxis when its benefit for prevention of inhalational anthrax outweighs the risk of hypersensitivity and anaphylaxis. The safety of Obiltoxaximab has been evaluated in 3 clinical trials, in 320 healthy subjects treated with one or two doses of 16 $mg.kg^{-1}$ of Obiltoxaximab or of a placebo, alone or in combination with ciprofloxacin. During the clinical studies, only eight subjects (2.5%) had their infusion of Obiltoxaximab discontinued for adverse reactions. Eight subjects who received at least one dose of Obiltoxaximab were positive for anti-drug antibodies, with titers ranging from 1:20 to 1:320, underlining a potential immunogenicity despite deimmunization.

Anthrivig® (anthrax immunoglobulin intravenous, AIGIV) is a polyclonal preparation derived from the plasma of humans immunized with BioThrax® vaccine with a high titer for PA, currently in clinical phase 3 [81]. Anthrivig® effectiveness was assessed in the rabbit and macaque model. For therapeutic evaluation, rabbits

Table 9.5 Anti-anthrax antibodies

INN (International Nonproprietary Name)	Proprietary name	Company	Species	Format	Target	Development Technology	Clinical indication	Administration	Development status	Approval
Raxibacumab	ABthrax®	GlaxoSmithKline (Brentford UK)/ Human Genome Sciences Inc. (HGSI) (Rockville MD USA)	*Homo sapiens*	IgG1 – lambda	PA	Human antibody phage display naïve library	Anthrax inhalation (treatment)	Intravenous	M	FDA: December 14, 2012
Obiltoxaximab	ANTHIM®	Elusys Therapeutics, Inc. (Pine Brook NJ USA)	Chimeric (Mus musculus/ human)	IgG1 – kappa	PA	Hybridoma then deimmunization	Anthrax inhalation (prevention and treatment)	Intravenous	M	FDA: March 18, 2016
	Anthrivig™	Emergent BioSolutions Inc./ BARDA/NIAID	*Homo sapiens*	Polyclonal	PA (at least)	Isolated from the plasma collected from healthy volunteers who had been immunized with BioThrax.	Anthrax inhalation (treatment)	Intravenous	Phase 3	
	Valortim®	PharmAthene/ Bristol-Myers Squibb (formerly Medarex)	*Homo sapiens*	IgG1 – kappa	PA	Transgenic mouse (HuMab mouse)	Anthrax inhalation (prevention and treatment)	Intravenous and intramuscular	Phase 1	
	Thravixa™	Emergent BioSolutions Inc.	*Homo sapiens*	IgG1 – kappa	PA	Isolated from the plasma collected from a healthy volunteer who had been immunized with BioThrax.	Anthrax toxemia (treatment)	Intravenous	Phase 1	

This table list the antibodies that are currently on the market or in clinical trial. Data were compiled from the scientific literature and from the IMGT/mab database [63]

(n = 18/group) were challenged with 204 LD_{50} (±47 LD_{50}) aerosolized Ames spores provided. Anthrivig® was administered at 14.2 or 21.3 mg.kg^{-1} (doses corresponding to anti-PA immunoglobulin) 12 or 24 h post-challenge. Anthrivig® administered 12 h after challenge provided 89% and 100% survival rate, respectively. Twenty-four hours after challenge, Anthrivig® provided 39%% survival, regardless of the dose administered. In the macaque model, animals were challenged with 281 LD_{50} (±51 LD_{50}) and Anthrivig® was administered only after ELISA-detection of PA in the macaque serum. Up to 33%% survival rate was observed, when macaque were treated with an intravenous dose of 21.3 mg.kg^{-1}, compared to 0% in the animal group treated with Gamunex. Regardless of the dose of Anthrivig® administered and the survival outcome, all macaques showed a decrease in circulating PA levels following treatment. The safety and the pharmacokinetic of Anthrivig® were assessed in 125 healthy volunteers, with 3 doses of Anthrivig®. Pharmacokinetic data suggests that a human dose of 7 mg.kg^{-1} is similar to a dose of 21 mg.kg^{-1} in the animal models. No major issue was observed during the safety assay.

Valortim® (MDX-1303), is a fully-human anti-PA IgG1 isolated from transgenic mice, currently in phase 1 [101, 129]. Treatment was evaluated in rabbits and macaques exposed to 200 LD_{50} aerosolized Ames spores. In the rabbit model (n = 10), two intravenous doses of 1 mg.kg^{-1} of Valortim® given 1 h and 3 days post-infection, respectively, provided 90% survival. Later treatment still provided 89% (n = 9) protection, but with two doses of 10 mg.kg^{-1} given twice at 24 h and 120 h post-challenge. Treatment of NHPs (n = 6) demonstrated complete protection after a single intramuscular injection of 1 mg.kg^{-1} Valortim® given 1 h post-challenge. A pharmacokinetic and safety assay was realized in 46 healthy volunteers (43 of them completed the study and 3 stopped it). A single intravenous dose ranging from 0.3 to 20 mg.kg^{-1} or a single intramuscular dose of 100 mg.kg^{-1} was administered. Sixteen volunteers had a grade 1 adverse reaction, but no volunteers had grade 2–4. No volunteers developed anti-drug antibodies [101].

Thravixa® (AVP-21D9), is a fully human IgG1 isolated from humans immunized with BioThrax® by hybridoma technology and immortalized with Epstein Barr Virus lymphocyte. It binds PA with an affinity of 82 pM [105]. Protection and treatment by Thravixa® was evaluated using both New-Zealand white and Dutch Belted Dwarf rabbits, yielding equivalent results. An antibody dose of 10 mg.kg^{-1} administered subcutaneously concurrently with an aerosol or intranasal challenge with 100 LD_{50} of Ames spores (n = 12/group) was fully protective [91, 92]. Regarding post-exposure treatment, subcutaneous administration of Thravixa® (2 mg.kg^{-1}) to rabbits at 0, 24 or 36 h following an aerosol challenge by 102 LD_{50} Ames spores respectively provided 100%, 66% and 33% protection. New-Zealand white rabbits were challenged with an aerosol of ~200 LD_{50} and treated 24–38 h after a significant temperature increase or after PA detection in serum. In rabbits, 92% survival was observed with an intravenous dose of 5 mg.kg^{-1} (versus 0% with a placebo). Similarly, 48 cynomolgus macaques where challenged after PA detection in serum. A 70%% survival-rate was observed with an intravenous dose of 5 mg.kg^{-1} (versus 0% with a placebo). Safety and pharmacokinetic was assessed in 50 healthy volunteers; 40 of them received an intravenous antibody dose of 0.3, 1.3 or 10 mg.kg^{-1} and 10 received a placebo. No severe adverse reaction was observed after infusion [69].

Other antibodies are also in early stage of development. Antibody 35PA83 targeting PA was isolated from a NHP with recombinant PA and humanized to increase its tolerance. It is protective in the New-Zealand white rabbit model and it is in preclinical development with a phase 1 trial due to start soon [89]. Similarly, antibody 2LF protects rabbit from anthrax challenge and it is one of the only isolated antibodies that targets LF and cross-reacts with EF [120]. Such antibodies would be useful if the PA component were naturally or intentionally mutated.

9.2.3.2 Antibodies Against Plague

Plague is a natural infectious disease caused by the gram-negative bacterium *Yersinia pestis*. Distinction is made between three different types of plague namely bubonic, septicemic and pneumonic plague. Bubonic plague is the most common natural type of epidemic plague and it arises when domestic rodent populations and their fleas become infected. Pneumonic plague is of particular interest in biodefense because of its extreme lethality (~100% if untreated), its ability to be transmitted via aerosol and its high infectivity. Pneumonic plague may be contracted by inhaling infectious droplets or develop from untreated bubonic or septicemic plague that spreads to the lungs. Plague is sensitive to antibiotics but they must be administered prophylactically or within 24 h of the onset of the symptoms (which is not always possible in the context of biodefense or a major outbreak). The treatment of choice is streptomycin or gentamicin (doxycycline or ciprofloxacin as an alternative) and supportive therapy. No vaccine or specific inhibitor is yet licensed and strains resistant to antibiotics have been detected [132]. In 1946, killed whole-cell vaccines were developed to protect soldiers and health workers that might be exposed to *Y. pestis* [135]. These vaccines demonstrated protection against bubonic disease but side effects were severe, repeated inoculations were necessary and no protection was observed against pneumonic plague. The live attenuated *Y. pestis* strain EV76, which lacks the pigmentation (pgm) locus required for iron acquisition has been shown to elicit high antibody titers and protect against both bubonic and pneumonic plague when used as a vaccine, albeit with mixed results [88, 121]. *Y. pestis* virulence factors have been identified and represent potential targets for antibodies. Among virulence antigens, F1 is a dominant capsular antigen with antiphagocytic activity, the low-calcium response V antigen (LcrV) is a component of the Type III Secretion System (T3SS) which is essential for pathogenesis, Psa (also referred as Ph6) is involved in resisting phagocytosis and complement-mediated killing, and Survival protein A (SurA) is a chaperone essential for virulence [26, 88, 112]. These proteins are targets of interest for recombinant subunit vaccines and therapeutic antibodies. Evidence of antibody efficacy against *Y. pestis* was provided by two subunit vaccines composed of recombinant F1 and LcrV antigens (one in Phase 2a) that induced an antibody response against both antigens and *in vivo* protection in animal models [26, 136]. However, some studies have shown that some vaccinated NHP succumb to challenge despite possessing high-titer F1/LcrV-specific antibody [110]. It was also demonstrated that passive transfer of specific antibody protects susceptible rodents against pneumonic plague. [110].

The murine monoclonal anti-LcrV antibody mAb 7.3 binds LcrV with an affinity of 80 pM and potently protects mice from death during challenge assays [98]. MAb 7.3 directly neutralizes Yop-dependent cytotoxicity and promotes opsonophagocytosis in macrophages infected with *Y. pestis in vitro* [53]. All mice (n = 10) immunized intraperitoneally with a dose of 35 μg of mAb 7.3 4 h before an aerosol challenge with 88 LD_{50} of *Y. pestis* strain GB, survived. In therapy, survival rates of 80%% and 60%% were observed when mAb 7.3 was administered 24 and 48 h after the challenge, respectively [48]. This protection was described as TNFα and IFNγ-dependent [65].

The m252 anti-F1 antibody, was isolated from a naive human phage-displayed Fab library and expressed as full-length IgG [137]. Despite its isolation from a naïve library, m252 binds F1 with a sub-nanomolar affinity. Protection assays were realized in the mice bubonic plague model. A dose of 500 μg of m252 administered intraperitoneally 24 h before a subcutaneous challenge with ~25–40 LD_{50} of *Y. pestis* CO92 strain increased the mean time-to death from 7 to 13 days and one out of 6 mice survived. When this antibody was administered prophylactically concurrently with two other anti-LcrV antibodies (500 μg each), the mean time-to death increased from 7 to 14 days and 5 out of 6 mice survived, emphasizing a synergistic effect of the oligoclonal mixture. Interestingly, a single administration of m252 48 h post infection provides 100%% survival. These antibodies were not characterized in a pneumonic plague model.

Considering biothreat and the natural cases of plague, the development of anti-plague antibodies is a priority. Such antibodies will be useful for prophylaxis and the specific treatment of plague.

9.2.3.3 Antibodies Against Tularemia

Francisella tularensis, the causative agent of tularemia (also known as rabbit fever and deer fly fever), is a gram-negative, intracellular, non-mobile, coccobacillus, naturally found in the Northern hemisphere. Natural human contamination occurs after skin contact with tissues or body fluids of infected animals of from bites of infected arthropods. In the biodefense context, infection could also be transmitted by aerosol or ingestion of contaminated-food or water. Type A tularemia is caused by *F. tularensis* subspecies *tularensis* and it is the form that is the most virulent and the most likely to be weaponized. The infective dose in humans is extremely low: 10 bacteria when injected subcutaneously and 25 when given as an aerosol [85]. The live vaccine strain (LVS) was developed from *F. tularensis* subspecies *holartica*, which causes Type B tularemia.

There is no consensus on which animal model should be used to evaluate tularemia treatment [85]. Historically, grivet monkeys infected with the SCHU S4 type A strain have been used to study the pathogenesis of tularemia and evaluate vaccines. Rhesus monkeys were also used for aerosol challenges with a strain of the subspecies *holarctica*. Mice have generally been used as an experimental model of tularemia for infection with LVS vaccine or with SCHU S4 strain. It was suggested that mice infected with SCHU S4 strain should be capable of satisfying the FDA animal rule [28]. Recent studies suggest that rabbits are a good model of human pneumonic tularemia when exposed to aerosols containing a virulent, type A strain, SCHU S4 [114]. A two-week course of doxycycline or ciprofloxacin should be effective in prophylaxis and early administration of streptomycin or gentamicin is very effective in therapy. No natural resistance in *F. tularensis* to antibiotics used for clinical therapy has been demonstrated for aminoglycosides, tetracyclines, chloramphenicol and quinolones, but erythromycin resistance is prevalent in Europe. New antibiotics such as ketolides, glycylcyclines, linezolid and new fluoroquinolones are currently under evaluation for therapeutic purposes [17]. Streptomycin- and tetracycline-resistant strains of *F. tularensis* were developed for well-intentioned experimental purposes, but such works could be also a proof of concept for bioterrorists [17, 85]. The LVS vaccine is available as an Investigational New Drug (IND, clinical development phase 2). A one-dose should be administered by scarification, but it was recently shown that LVS not elicit complete protection against lethal challenge with a virulent type A *Francisella* strain [32, 106, 116]. This vaccine fully protects mice against an intradermal but not aerosol challenge with 1000 LD_{50} of SCHU S4 strain. The absence of protection against aerosol contamination is a breach in biodefense. A recent study showed that respiratory and oral vaccination improves protection conferred by the LVS against pneumonic tularemia in the rabbit model, compared to the protection conferred by scarification [114].

Currently, there is no antibody on the market or in clinical development but efficacy has been demonstrated [109]. One explanation could be that the Schu S4 strain completely abolishes the inflammatory responses that are required for efficient antibody-mediated bacterial clearance [59]. However, evidence of antibody efficiency in prophylaxis and treatment has been obtained with the passive administration of immune serum. The administration of immune serum 24 h post infection and every 3 days thereafter protected 90%% of the mice against an intranasal challenge with LVS [59]. First, immune-serum administration 48 h post-infection protected 25%% of the mice and increased the mean time-to death from 9.5 to 13.5 days. A study showed that passive transfer of antibodies directed against the membrane protein fraction (MFP) of *F. tularensis* Schu S4 to mice infected with the same strain resulted in complete protection when combined with gentamicin treatment [117].

Ab63 is a monoclonal IgG3(κ) obtained after the immunization of mice with LVS, with an affinity of 0.75 nM for the *O*-antigen of *F. tularensis* lipopolysaccharide LPS [66].

Administration of an intraperitoneal dose of 200 µg of Ab63 2 h after an intranasal challenge with 24 CFU of Schu S4 did not protect mice from death but slightly increased the mean time-to death.

Antibodies can completely protect against LVS (type B strain) but not against the more virulent Schu S4 (type A) strain, possibly due to different virulence mechanisms between strains. Developing antibodies that neutralize type A and type B strains before naturally antibiotic-resistant strains emerge is a priority for the prophylaxis and treatment of tularemia.

9.2.3.4 Antibodies Against Smallpox

Smallpox is caused by at least two species of *Variola virus*: *Variola major* which causes 10–30%% mortality (95% if untreated) and *Variola minor* that cause less than 1%% mortality. In 1958, the World Health Organization started a global vaccination campaign because humans are the only reservoir of the *Variola virus*; smallpox was officially declared eradicated on May 8, 1980. All vaccines are based on *Vaccinia virus*, another orthopoxvirus that inducea cross-protection against *Variola virus*; *Vaccinia virus* and *Variola virus* share 150 open reading frames and have an overall identity of 90%%. Several live strains ($VACV_{NYCBH}$, EM-63 and Lister) were used for the vaccination but these can induce adverse reactions (~250 cases for 1000,000 vaccinations) that can be fatal (60 deaths for 100,000,000 vaccinations) and historical vaccine production methods are incompatible with current quality rules for medicinal products [104]. In a context where smallpox is no longer found in nature, the balance between the benefits and risks of vaccination is against continued mass immunization. Vaccination was wound down in the years following eradication, and now only those at a high risk of exposure were vaccinated with a second-generation vaccine (such as ACAM2000®, FDA-approved in 2007) although strategic stockpiles of vaccine were accumulated [32, 80]. Following eradication, *Variola virus* stocks were destroyed, and now only two stocks are being kept in BSL4-secure facilities (in Atlanta, USA and in Koltsovo, Russia) for research purposes. However, in the context of the Cold War, we cannot know that all *Variola virus* stocks were indeed destroyed and natural resurgence of the virus may be possible, particularly due to the ice melting in countries were corpses were buried. Currently, only a few individuals are immunized, and the immunological status of people that were vaccinated is undetermined. A study on 680 patients with smallpox estimated that the mortality rate was 52% among those who had never been vaccinated, 1.7% among those who had been vaccinated within 10 years, and 11% among those who had been vaccinated more than 20 years ago [38, 68]. Considering the vulnerability of the population, the high aerosol infectivity (a single virion can cause smallpox) and the relative ease of large-scale production, smallpox is still considered as a major potential biowarfare agent. In the event of a smallpox outbreak in the USA, the CDC would immediately implement the Smallpox Response Plan. As there is currently no FDA-approved specific and effective treatment for smallpox, the plan largely seeks to contain the outbreak. Several bioterrorism scenarios were considered by the CDC. Depending on how many people get infected at the outset, the total number of people contaminated before final containment could vary from 4.200 (10–100 people initially contaminated) to several millions (thousands of people initially contaminated). In this last case smallpox will become endemic and several years would be required to bring the disease under control [43].

Three compounds (cidofovir, ST-246® and CMX-001®) that inhibit smallpox virus replication *in vitro* and in animal models have IND status for emergency smallpox-treatment, but none is yet FDA-approved (clinical trial phase II or phase III against other virus) [16, 84].

The efficacy of antibodies for the prophylaxis and treatment of smallpox has been demonstrated in the past. During infection, the virus exists in two antigenically distinct forms, namely intracellular mature virions (IMV) that are involved in person to person contamination, and extracellular enveloped virions (EMV) that are involved in cell to cell contamination. The membrane pro-

teins of each virion are potential antibody targets and ideally both types of virion would be targeted at the same time because a single antibody may be not sufficient to induce full protection [72]. The role of complement in antibody-dependent virus elimination has been demonstrated [13, 40, 71]. The Vaccinia Immune Globulin (VIG) is a human-derived polyclonal mixture that is approved for the specific treatment of smallpox and of complications resulting from vaccination. Studies on rhesus macaques demonstrated the prophylactic efficacy of VIG against a challenge with monkeypox [34, 72]. Unfortunately, this product is of human-origin and only available in very limited quantities in the USA. Moreover its efficacy is uncertain due to interbatch variability in potency and a lack of understanding of the molecular determinants of protection. In 1941 a study demonstrated that prophylactic administration of serum from convalescents is fully protective ([34, 72]).

Several monoclonal antibodies that cross-neutralize different orthopoxvirus (vaccine, smallpox, cowpox and monkeypox) were isolated by hybridoma technology from peripheral blood mononuclear cells (PBMCs) from a donor who had recovered from a naturally occurring monkeypox virus infection or from otherwise healthy subjects previously immunized with one of three different vaccine formulations [40]. After an initial screening against recombinant antigens (A21, A27, A28, A33, B5, D8, F9, J5, H2, H3, L1, L5, A33 and B5) or inactivated-virus lysate (monkeypox, cowpox and vaccine), 89 hybridomas were identified as reactive. 66% of the monoclonal antibodies that interact with a vaccine-antigen also interact with the orthologue *Variola*-antigen. 54%% of the hybridomas secreted antibodies that neutralized at least one orthopoxvirus (25%% neutralized simultaneously the vaccine, cowpox and monkeypox), with a maximum of neutralization effect (E_{max} > 50%%) at 100 µg.mL^{-1} or lower. IC_{50} values of individual mAbs ranged from ~0.02 to 100 µg.mL^{-1}, and E_{max} values varied from 50% (the designated cut-off threshold to identify potent neutralizing clones) to 99.5%. Interestingly, all anti-B5 antibodies failed to cross-neutralize monkeypox despite anti-B5 antibodies are the main antibodies involved in vaccine neutralization. A mixture of antibodies, containing diverse specificities with high neutralizing (low IC_{50} and high E_{max} values) and cross-neutralizing activities to both IMV and EEV, was designed. MIX4 contained single neutralizing monoclonal antibodies directed to A27, L1, B5, and A33. C57BL/6 mice were intranasally challenged with 10^5 PFU of VACV-WR. One day before, concurrently, or 1, 2 or 3 days after challenge, mice received a single dose of Mix4. Administration of Mix4 up to 1 day post-infection completely protected mice from death and weight-loss. Administration 2 or 3 days after challenge completely protected mice from death but failed to protect mice from weight-loss. Mix4 induced a better protection compared to VIG.

Recombinant and monoclonal antibodies directed against different orthopoxvirus-antigen (such as B5, A33, A27 or L1) were also previously isolated [13, 24, 25, 56, 111]. Many of the antibodies isolated were only characterized against vaccine, monkeypox or cowpox, but only a few of them were characterized against smallpox.

9.2.3.5 Antibodies Against Viral Hemorrhagic Fever

Viral hemorrhagic fevers (VHF) are a heterogeneous group that causes illnesses characterized by fever and bleeding diathesis. They are caused by four virus families: *Filoviridae*, *Arenaviridae*, *Flaviviridae* and *Bunyaviridae*, but this section will focus on *Filoviridae*. The *Filoviridae* include the Ebola and Marburg viruses. The *Arenaviridae* include the etiologic agents of Lassa, Junin, Machupo and Sabia hemorrhagic fevers. The *Flaviviridae* include dengue, yellow fever and two viruses in the tick-borne encephalitis group that cause VHF: Omsk VHF and Kyasanur Forest disease virus. The *Bunyaviridae* include the members of the *Hantavirus* genus that cause hemorrhagic fever with renal syndrome, the Congo-Crimean VHF from the *Nairovirus* genus, the Rift Valley Fever virus from the *Phlebovirus* genus. Many VHF agents are highly infectious, can be nebulized, and their fatality rate can be greater than 90%% in the absence of early, effec-

tive treatment [86]. Specifically, Ebola, Marburg, Lassa and Machupo viruses are the main threat with respect to both biodefense and natural outbreaks. Following its emergence in December 2013, the recent Zaire Ebola virus (EBOV) outbreak in West Africa has spread and persisted for more than 2 years and killed more than 11,000 individuals, making it the largest EBOV epidemic in both scale and geographical extent to date. During this outbreak, the USA, Spain, Norway and France for the first-time decided to repatriate any of their medical personnel who contracted the infection in Africa in order to provide them efficient treatment in secure, domestic medical facilities. Notwithstanding the precautions taken during repatriation and treatment, media coverage contributed to panic in the populations of the countries that repatriated health care workers [45, 51, 107]. This Ebola epidemic was useful with respect to evaluating the ability of Western countries to contain an epidemic by means of specific restriction measures such as the medical checking of passengers coming from West Africa at airports. It was also an opportunity for France to evaluate its capacity to deploy a military hospital directly in Guinea to treat infected health care workers. The outbreak furthermore allowed several countries to test their reaction capabilities that could be similarly deployed in response to a bioterrorist attack involving VHF or other infectious biowarfare agents. Finally, this outbreak afforded an opportunity to test antiviral-drugs and antibodies in clinical development. The development of anti-VHF drugs is complicated by the fact that many of these viruses have to be manipulated in a BSL4 facility.

A consensus on the appropriate animal models of diseases caused by VHF has not yet been reached due to symptom discrepancies between models and humans. Rhesus and cynomolgus macaques are representative models of filovirus infection as they exhibit remarkably similar symptoms to those observed in humans. However, practical and ethical problems limit the experimental use of non-human primate models. Furthermore, there are no inbred, genetically manipulated strains of NHP. Rodent models such as mouse, guinea pig, and hamster, have also been developed but these require adaptation of the virus to produce lethal disease and do not mirror all symptoms of human filovirus infection [35, 83].

In the case of Ebola, several antiviral drugs have been isolated but only three of them (AVI-6002, AVI 7537 and TKM 130803) completed clinical phase 1 and they are specific to the Ebola Zaire strain [21].

Serum from convalescents has been administered with some success to several patients, but only a few results have been published [27, 126]. In a nonrandomized, comparative study, 99 patients from Guinea with confirmed Ebola infection received two consecutive transfusions of 200–250 ml of ABO-compatible convalescent plasma, with each unit of plasma obtained from a separate convalescent donor. The transfusions were initiated on the day of diagnosis or up to 2 days later. No serious adverse reactions associated with the use of convalescent plasma were observed. When compared with 418 patients who had been treated at the same center during the previous 5 months, no significant improvement in survival was observed in the group that was given exogenous serum [126].

ZMapp™ is an oligoclonal mixture composed of monoclonal antibodies, that is currently under clinical phase 2 and that received the orphan drug designation by the FDA on August 25, 2014. It was developed by Mapp Biopharmaceutical Inc., LeafBio Inc. and Dreyfus Inc., starting from two mixtures composed of three monoclonal antibodies each: MB-003 that was developed by the U.S. Army Medical Research Institute of Infectious Disease (USAMRIID) and that is composed of three humanized or human-mouse chimeric antibodies (c13C6, h13F6 and c6D8) and ZMAb that was developed by the Public Health Agency of Canada's National Microbiology (NML) and that is composed of three murine monoclonal antibodies (m1H3, m2G4 and m4G7). ZMapp™ formulation is composed of c13C6 and from c2G4 and c4G7 that were chimerized (human-mouse) [95]. ZMApp™ antibodies binds to Ebola virus glycoprotein ectodomain [122]. A three dose adminis-

tration of ZMapp™ (50 mg.kg^{-1}) up to 5 days post-infection fully-protected non-human primates (n = 6) intramuscularly challenged with 628 PFU of EBOV-K (vs. 0% survival for the animal that received an irrelevant antibody). *In vitro* ZMapp™ cross-neutralized EBOV-G. ZMapp was evaluated in humans in Guinea in 2015 in a randomized study [94]. ZMapp was administered simultaneously to the standard treatment used in Guinea (that specifically includes oral favipiravir) and results were compared to those in a group of patients only administered with the standard treatment. Thirteen of the 35 patients who received the standard treatment died (37%) compared with only 8 of the 36 patients that additionally received the ZMapp (22%). Although the estimated effect of ZMapp appeared to be beneficial, the result did not meet the pre-specified statistical threshold for efficacy. The observed posterior probability that ZMapp plus the current standard of care was superior to the current standard of care alone was 91.2%, falling short of the pre-specified threshold of 97.5%.

Development of pan-Ebolavirus therapy is a priority for the public health. However, development of such antibodies is complex due to antigenic differences between the five ebolavirus species. 6D6 is a mouse monoclonal antibody isolated by hybridoma technology after the immunization of mice with EBOV virus-like particles. BALB/c mice were intraperitoneally challenged with 1000 PFU of mouse-adapted EBOV and 100 µg of 6D6 was intraperitoneally administered 24 h post-infection [39]. The treated animals survived without clinical symptoms, whereas untreated mice succumbed to infection within 9 days. Treatment with 6D6 24 h after infection delayed the onset of the disease caused by these ebolaviruses and significantly reduced the weight loss in this immunocompromised mouse strain. All 6D6-treated mice survived the EBOV infection. C57BL/6 mice were challenged wild-type EBOV and SUDV. Administration of 6D6 24 h post-infection, fully protected mice from death, delayed the onset of the disease caused by these ebolaviruses and significantly reduced the weight loss. Epitope-mapping study revealed that 6D6 targets an epitope that is shared between all known ebolavirus species. More precisely, the epitope was localized in the IFL region of the GP2. These results revealed that 6D6 represents a potential candidate for drug development and that antibodies targeting the same epitopic region should cross-neutralize Ebola viruses.

Following the last Ebola epidemic, efforts were made to develop anti-Ebola drugs. However, efforts should also be made for the development of drugs against the other VHF agents that are also of major concern in public health and biodefense. Antibodies against other VHF have already been isolated but none of them are in clinical development and they are not reviewed here.

9.2.3.6 Antibodies Against Botulinum Neurotoxins

Botulism is a rare, life-threatening disease caused by botulinum neurotoxins (BoNT), secreted by the spore-forming bacterium, *Clostridium botulinum*. Seven BoNTs serotypes (A to G) have been described but serotypes A, B and E are responsible for most cases of natural human poisoning [61]. Between each serotype, amino acid sequences share between 34% and 64%% identity, and subtypes have been identified [49, 61]. Botulinum neurotoxin A (BoNT/A) is the most toxic substance known with a human 50%% lethal dose (LD_{50}) estimated at 1 ng.kg^{-1} (intravenous and subcutaneous routes), 10 ng.kg^{-1} (pulmonary route) or 1 µg.mL^{-1} (oral route) [4, 41, 61]. BoNTs are type A-B heterodimeric molecules composed of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC). The heavy chain is successively implicated in toxin binding on motor neurons, internalization by dual-receptor-mediated endocytosis and translocation of the light chain into the cytosol. The light chain is a zinc endopeptidase which cleaves a protein of the complex implicated in the exocytosis of acetylcholine. Both chains are potential targets for antibodies. Toxin activity induces flaccid muscular paralysis that progressively spreads to involve all muscles, leading to respiratory distress and eventually death in absence of treatment. Three forms of naturally occurring botulism are described:

foodborne, intestinal and wound botulism. However, BoNTs could also be nebulized and produced a clinical picture very similar to food poisoning.

No specific chemical inhibitor of BoNTs activity is available on the market. A pentavalent vaccine against botulism neurotoxins A to E has been developed and granted IND status but this vaccine is only given to individuals at high risk of exposure. In fact, BoNTs have been introduced as a safe and effective treatment for a wide range of disorders associated with involuntary muscle contractions and spasm disorders, and these ever-increasing medical indications preclude mass immunization against botulism [3, 131].

Infant botulism cases are treated by injections of a human-derived botulinum immunoglobulin preparation (BabyBIG®, approved by the FDA in December 9, 2003), which is well tolerated but only available in very limited quantities and very expensive [5]. Only 31% of BabyBIG®-treated patients experienced a treatment-emergent adverse reaction compared with 45% of placebo-treated patients, and there was no evidence of anaphylaxis in any patient enrolled in the study [108]. Efficacy was evaluated in 122 patients with laboratory-confirmed infant botulism (caused by BoNT/A or B). Fifty-nine received BabyBIG® and 63 received placebo. When administered within 3 days of hospital admission, treatment with BabyBIG® shortened the mean hospital stay of all infant botulism patients by 3.1 weeks (from 5.7 to 2.6 weeks), the length of intensive care unit stay, the duration of mechanical ventilation and the duration of tube feeding.

A heptavalent (A to G) horse-derived derived serum (HBAT®, Emergent Biosolutions) is the only treatment available in USA (FDA approval, March 22, 2013) for both adult and infant botulism. This serum is composed of more than 90%% of Fab and $F(ab')_2$ and of less than 2%% full-length IgG. Despite lot-to-lot variation each single-use vial contains a minimum potency of 4500 Units (U) for BoNT/A, 3300 U for BoNT/B, 3000 U for BoNT/C, 600 U for BoNT/D, 5100 U for BoNT/E, 3000 U for BoNT/F and 600 U for BoNT/G [46]. This serum was already successfully administered in therapy to a patient with foodborne-botulism, and HBAT is under phase 4 to determine its pharmacokinetics in pediatric patients (NCT02051062) [50].

A trivalent horse-derived serum neutralizing BoNT A, B and E (GlaxoSmithKline/Novartis), a bivalent horse-derived serum neutralizing BoNT/A and B and a monovalent horse-derived serum neutralizing BoNT/E (Instituto Butantan) are also available. Both products were no longer provided by the CDC and were replaced by the HBAT® [29].

Nevertheless, due to their animal origin, these equine-derived antitoxins may be poorly tolerated and could induce serious adverse reactions, like serum sickness or anaphylactic shock. Development of human or human-like antibodies is thus required for safe and effective treatment of botulism. 3D12 is a fully human antibody, isolated from humans immunized with pentavalent botulinum toxoid. It binds BoNT/A with an affinity of 32 nM. Intraperitoneal injection of mice with $20LD_{50}$ of BoNT/A (Hall strain) pre-incubated with 50 μG of 3D12 significantly delayed mean time-to death but failed to protect mice from death. However, when a second mouse-derived antibody (C25 or S25) is injected simultaneously (25 μg of each antibody), all mice survived a challenge with 100 LD_{50}. When 3D12 is injected with C25 and S25 (16.7 μg each), all mice survived to a challenge with 5000 LD_{50} and 90%% of mice survived to a challenge with 20,000 LD_{50}. C25 and S25 monoclonal antibodies were humanized to decrease their immunogenicity and engineered to increase their affinity. These three antibodies constitute an oligoclonal mixture for efficient BoNT/A-neutralization. AntiBotABE European framework project developed a combination of five humanized recombinant-antibodies of NHP origin that protect mice from challenge with BoNT/A, B or E [8, 33, 76, 77, 97]. SEM120-IIIC1 and A1HC38 target BoNT/A light and heavy chain, respectively, BLC3 and B2.7 target BoNT/B light and heavy chain, respectively, and ELC18 targets BoNT/E heavy chain. Despite the proximity of NHP-antibodies with human antibodies the five antibodies developed were germline humanized

[9]. All antibodies either administrated alone or in pairs fully protected mice from an intraperitoneal challenge with the toxin of the relevant serotype.

9.2.3.7 Antibodies Against CDC Category B Agents: The Example of Ricin

CDC category A groups together the six agents considered as a priority for biodefense but other systems point up other biological agents as priorities. Some of them may be less fatal but they are nevertheless capable of incapacitating troops and populations, e.g. Staphylococcal Enterotoxin B (SEB) causes symptoms even when inhaled at very low doses in humans. A dose at least 100 times less than the lethal dose by the inhaled route would be sufficient to incapacitate 50%% of those exposed for 1–2 weeks [32]. Ricin is another example of a CDC category B agent that could be used as a bioweapon. Although its LD_{50} is higher than that of other toxins, it is easy to extract from Ricinus communis seeds.

Ricin is composed of a B-subunit (RTB) that binds sugars on the cell surface for cytosolic entry of an A-subunit (RTA) that inhibits the ribosome-dependent protein synthesis. It is lethal by the oral route, but 1000-fold more potent by the pulmonary and parenteral routes. Ricin has an aerosolized LD_{50} of 3–5 $\mu g.kg^{-1}$ in the mouse and 5.8–15 $\mu g.kg^{-1}$ in non-human primate models. There is no licensed prophylaxis or specific chemical compound that neutralizes ricin; the only treatment is supportive therapy. Two recombinant vaccines based on immunization with the RTA subunit are currently in phase 1 and 1 B development, namely RVEc™ (USAMRIID) and RiVax® (University of Texas Southwestern Medical Center) [93, 128, 130].

A proof of concept study demonstrated the efficacy of polyclonal antibodies for the full protection of mice exposed to an aerosol of ricin, when the antibodies are administered 20 min post-exposure. 43RCA is a recombinant antibody that binds ricin with an affinity of 40 pM. This antibody was isolated by phage-display technology starting from a NHP (*Macaca fascicularis*) immunized with the non-toxic A-chain of ricin. This scFv was then germline-humanized to decrease its tolerance. In a cell-free neutralizing-assay, 43RCA neutralized 89% of ricin activity at 40 $\mu g.mL^{-1}$, and 50% at 1.5 $\mu g.mL^{-1}$ (corresponding to a molar ratio [43RCA]/[ricin] of 12) [90]. This antibody was also protective in vivo (unpublished data) and was used for the development of an antibody-nebulizer for rapid and easy self- administration [99].

PB10 is a murin monoclonal IgG2b that binds a linear epitope on the enzymatic RTA chain and that neutralized ricin by interfering with the transport to the trans-Golgi complex net- work, presumably by shunting ricin to lysosomes for degradation [138]. PB10 was chimerized (cPB10) by grafting its variables domains on human IgG1 and K constant domains [115]. Despite an affinity of only 40 nM the IC_{50} of cPB10 is 0.03 $\mu g.mL^{-1}$. When administrated by i.p. 24 h before an i.p. challenge with 10 LD_{50} of ricin, a single dose of 5 µg of cPB10 fully protected mice from death. More interestingly for biodefense, this antibody fully-protect BALB/c mouse from death whatever a single dose of 10 $mg.kg^{-1}$ of cPB10 is administrated 24 h before, simultaneously or 4 h after an aerosol challenge with ~5 LD_{50} of ricin. cPB10 was then germline-humanized (hPB10) to increase its potential tolerance. After humanization, the PB10 variable domains overall identity increase from 70% to >90%, without affecting antibody functions [127]. After humanization, an i.p. administration of 5 or 40 µg of hPB10 24 h before a ricine challenge, fully protected mice from death after an i.p. or an intranasal challenge, respectively, with 10 LD_{50} of ricin. This antibody represents a potential lead drug for the biodefense.

Ten other antibodies were also isolated by phage-display technology starting from 2 non-human primates immunized with ricin-holotoxin or a subunit-vaccine. These antibodies bind ricin with (sub-)nanomolar affinities (5 of them had affinities bellow 1 pM) and neutralized toxin activity in a HeLa Ub-FL cell-based assay. Mice were challenged intranasally with 2 LD_{50} of ricin 6 h before an intravenous administration of 100 µg of antibody. The antibodies MH36, MH75 and MH73 induced significant protection, and

both MH1 and MH77 fully protected mice from death.

9.2.4 Outlook for the Development of Antibody for Biodefense

As reviewed above, considerable advances were done since the 2001 anthrax-crisis, for the development of antibodies against biowarfare agents, but, there are several limitations. First, the majority of the antibodies that were developed target CDCA-category agents. Despites efforts made against A-category agents, antibodies against pathogens such as *Francisella tularensis*, were not yet available. In the context of tularemia, one major issue is that while mice are extremely susceptible to tularemia, rats, rabbits, and non-human primates are relatively more resistant to infection. Perhaps most importantly, emerging evidence indicates that immunity against virulent strains of *F. tularensis* that cause disease in humans differs from protective immune responses elicited by model strains of *Francisella* [109]. A limitation to the CDC A-category is that even these biological agents are considered as the main threat for biodefense the list is not often actualized. As a consequence, efforts should be done for the development of antibodies neutralizing biological agents currently classified among the CDC B-category and CDC C-category (emerging pathogens). Indeed, pathogens of the B-category, such as *Vibrio cholerae*, the Epsilon toxin of *Clostridium perfringens*, some strains of *Salmonella* sp. or *Escherichia* sp., may be used to contaminate the food and water supply chain or the cattle to destabilize a country. CDC B-category agents, such as Enterohemorrhagic *E. coli* (EHEC) may be less lethal than A-agents, but may incapacitate many people for several days or weeks. Such incapacitation will lead to a significant destabilization of the economy or of impacted-regiments. One limitation for the development of such antibodies may be the antigenic diversity among some pathogen families which require identifying carefully the antigen that should be used for the antibody generation. The recent development of bi- or multi-specific antibody would represent a hope to overcome the difficulties related to the antigenic diversity. Indeed, with such antibodies it would be possible to target several serotypes or subtypes of biological agents with a single antibody. The clinical development of such multivalent molecule would also be cheaper than the simultaneous development of several antibodies. Indeed, the clinical development of oligoclonal antibodies requires the assessment of each antibody separately (as a part of the mixture) and the oligoclonal mixture in parallel, which is expensive. The lake of funds is the main explanation for the gap generally observed between research and (clinical-) development, emphasizing the interest for the development of engineered-antibodies. As there is only few or no natural diseases caused each year by the biowarfare agents, the pharmaceutical industry generally invest no money for the development of therapies against such diseases, and Stated should fund such development by their own. Considering the funds required, the possibility of drug-development is restricted to only some countries in the World (Table 9.6). Among these richest countries, some of them are not necessarily directly impacted by terrorism and invest no money for such development. Generally, for national security reasons, States preferred to be self-sufficient and thus to autonomously developed their (medical-) countermeasures. Nevertheless, in a geopolitical context where terrorism affects macro-regions of the World and considering the fact that pathogens can cross easily the borders, the development of medical therapies against biowarfare agents could be done in the context of multi-state collaborations (European Union, North Atlantic Treaty Organization…). For example, the European Framework projects funds research programs in strategic topics, but in the context of therapies, nor the European Framework projects nor European agency such as the European Defense Agency can pay for the clinical development. A key for a “win-win” partnership between States and pharmaceutical industry may be the funding of clinical development by pharmaceutical industries and the commitment of States to stockpile drug-doses. Considering the possibility of fast

Table 9.6 Plan and cost of (pre-) clinical development

	Preclinical development		Clinical development				
	Before market authorization						Post market
	In vitro studies	Animal *in vivo* studies	Phase I safety and dosage	Phase II efficacy and side effects		Phase III efficacy and monitoring of adverse reactions	Phase IV post-market safety and efficacy
Number of human volunteers	0	0	20–100 healthy volunteers or people with the disease/ condition.	Up to several hundred people with the disease/ condition.		300–3000 volunteers who have the disease or condition	All sick-peoples that received the drug.
Success rate	*Highly target-dependent*	*Highly target-dependent*	**70%**	**33%**		**25–30%**	**N.A.**
Primary goal	Looking for leads. *In vitro* confirmation of the efficacy of the molecule.	*In vivo* confirmation of the *in vitro* efficacy. Determination of the optimum dose at which the drug shows maximal biological activity with minimal side-effects in animals. Estimation of human effective-dose.	Testing of drug on healthy volunteers for dose-ranging. In some case (*i.e.* oncology) testing on people in therapeutic failure.	*2a* Proof of concept study. Confirmation of the pharmacological efficacy on 50–200 healthy volunteers. Short-term exposition to the drug and one-dose testing. Highly-toxic drug directly go to phase 2b.	*2b* Looking for the optimum dose at which the drug shows maximal biological activity with minimal side-effects. Determination of the best route of administration. Determination of adverse effects and potential drug-drug interaction. Study on 100–300 people with the disease/ condition.	Pivotal study. Demonstration whether or not the drug offers a treatment benefit to a specific population. Looking for potential long-term or rare side-effects.	Postmarketing surveillance. Long-term study of the efficacy. Reporting of long-term or rare side-effects.
Duration	**Up to 6 years**		Several months	Several months to 2 years		1–4 years	
			From 5 to 10 years				
Global cost	**800,000,000 $ to 2,000,000,000$**						

shipment all-over the world, it could be possible for several States to share their stock of drugs; in this case each State could stock a drug against a different pathogen. With the recent Ebola crisis in Africa, the pharmaceutical industry also began to be aware of the fact that emerging diseases of biodefense interest could also occur naturally and be profitable. Another challenge is that the therapeutic window of the antibodies is short. The development of new antibodies (or the engineering of existing antibodies) that can cross the cell-membrane (transbodies) would be interesting; for example, transbodies directed against Ebola virus VP40 protein were developed [119]. Transbodies would be helpful after a massive terrorist attack, because the saturation of medical facilities is likely and in this case some victims may be treated only some days after their contamination. Considering the saturation of the medical facilities, the development of system for easy auto-administration of antibodies is strategic. For example, in the context of a project co-funded by the French ministry of defense, a drug delivery system for efficient alveolar delivery of ricin-neutralizing antibody was developed [90, 99]. This system would be helpful for an easy auto-administration both by the patient in a hospital context or by a soldier in a warfare context.

9.3 Conclusion

Biological agents have been used in warfare and terrorism many times in the past. A major threat for biodefense is that, with recent progress in biology and biotechnology, it is now possible to engineer biological agents to make more devastating bioweapons. Currently, bioweapons are more likely to be used by terrorists than by any nation state. Antibodies are known to be effective in the treatment of many diseases, including some of the diseases that might be caused by biowarfare agents. Many antibodies that neutralize biowarfare agents have already been isolated but only a few of these are either licensed or in clinical development. Efforts should be made to develop antibodies against new targets and finance the clinical development of those that have already been isolated. Some diseases are rare and developing antibodies to treat them is not considered a priority by the pharmaceutical industries. In this context, partnerships between commercial parties and States should be initiated to fund clinical development and antibody production to ensure strategic stockpiles of antibodies that could be vital for biodefense.

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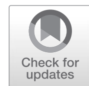

10 Engineering Antibodies for the Treatment of Infectious Diseases

Gaowei Fan and Jinming Li

Abstract

Historically, serum therapy was previously used to combat infectious pathogens. However, serum sickness and anaphylaxis limited its broad application. The advancement of antibody engineering technologies has made it feasible to generate monoclonal antibodies. There are divergent methods for antibody engineering and optimization. In this chapter, we summarized the latest developments in engineering antibodies for infectious diseases.

Keywords

Engineering antibodies · Pathogens · Treatment

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10.1 Introduction

The discovery of antibiotics has saved countless patients from pathogens. Thus, widespread availability, high efficacy and low costs make antibiotics a cornerstone of modern medicine. However, after many decades of empiric broad-spectrum antibiotics use and misuse, multiple antibiotic-resistant pathogens are emerging. These pathogens include, but not limited to, methicillin-resistant *staphylococcus aureus*, extreme drug-resistant *Mycobacterium tuberculosis* and *plasmodium falciparum* [17]. Antibiotic-resistant pathogens pose a threat to global health. Additionally, broad-spectrum antibiotics bring the risk of perturbing normal and beneficial microbiome *in vivo* [36]. The perturbations or dysbioses of normal or beneficial microbiome has been linked to a range of diseases including *Clostridium difficile* associated diarrhea, diabetes, obesity and immune defects [10]. These threats on public health worldwide are detrimental.

Clearly, there is a need to explore new weapons to combat infectious diseases. Among many alternatives, antibody-based therapy represents a major interest with the success in other diseases such as tumors (Opdivo and Keytruda), autoimmune diseases and inflammatory conditions (Remicade, Humira) [58]. With the advancement in antibody engineering, production and optimization, antibodies may hold great promise in combating infectious diseases. If successful, antibody-based therapy will provide precision weap-

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ons to destroy pathogens without comprising normal or beneficial microbiome.

Antibody-based therapy for infectious diseases is not new. As far as in the 1890s, serum immunoglobulin was used to neutralize bacterial virulence [7]. Later, serum therapy was applied to a wide range of other infectious diseases such as *meningococcal meningitis*, *pneumococcal pneumonia*, *streptococcal* scarlet fever, varicella, measles, the pandemic *Spanish influenza* [15] and Ebola virus disease as well as cytomegalovirus [14, 31]. However, adverse reactions such as serum sickness and anaphylaxis harmed the efficacy of serum therapy [9]. With the discovery of antibiotics, serum therapy in infectious pathogens quickly declined. Antibiotics therapy soon became a mainstay of bacterial infections treatment. However, the success did not last long with the emergence of antibiotic-resistant pathogens. The failure to develop new antibiotics with different mechanisms of actions again trapped patients by the super drug-resistant bacteria. Besides, for individuals with compromised immunity, antimicrobial chemotherapy provides a less effective treatment [28]. Obviously, antibiotics are ineffective at eliminating viruses. The emergence of antibiotics resistance calls for renewed efforts. Among the many options, monoclonal antibody (mAb) may be an alternative, reminiscent of the successful application of antitoxin in fighting against *meningococcal meningitis* in pre-antibiotic era. Indeed, Nebacumab, the first human therapeutic mAb reviewed by a regulatory agency, was engineered for the treatment of sepsis and gram-negative bacteremia [61]. However, the CHESS (Centocor: HA-1A Efficacy in Septic Shock) trial conducted in the USA which found non-statistically significant increase in mortality between the monoclonal anti-endotoxin antibody Nebacumab and placebo groups in patients with gram-negative bacteremia lead to the withdrawal of Nebacumab [20]. Currently, only two humanized antibodies Palivizumab and Raxibacumab are approved for infectious diseases. Many other engineering antibodies against infectious pathogens are under development [2, 5, 16, 45, 67]. In this chapter, we summarize existing technologies for antibody engineering and optimization.

10.2 Serum Therapy in the Pre-antibiotics Era

Before the discovery of antibiotics, convalescent serum was the first effective strategy to combat infectious pathogens such as *diphtheria*, *tetanus*, *hepatitis B*, *varicella*, and *cytomegalovirus* et al. [13, 14]. It was later discovered that the mechanisms behind it were predominantly due to neutralizing activity and effector functions of immunoglobulins in serum. Antibodies derived from immune sera are polyclonal in nature. However, problems such as lot-to-lot variation and the immune response against animal-derived antibodies contributed to the reduced efficacy of serum therapy. Besides, the preparation of these serum products were expensive and labor-intensive. These complications together with the discovery of antibiotics limited the application of serum therapy in infectious pathogens after 1935. Nevertheless, this early attempt opened up the possibility of antibody-based therapies in infectious diseases.

10.3 Hybridoma Technology

The year 1975 witnessed the development of monoclonal antibody by mouse hybridoma technology [40]. This innovation makes it possible to produce large quantities of antibodies with a defined specificity *in vitro*, paving the way to use therapeutic monoclonal antibodies to treat different diseases. For example, E5 (XOMA Corp, Berkeley, Calif), an IgM antibody produced by hybridoma technology, was designed to neutralize endotoxin, the lipopolysaccharide component of the outer membrane of the J5 mutant of *Echerichia coli* [29]. The inconclusive results generated by clinical trials of E5 led to the withdrawal of this anti-endotoxin mAbs [29, p. 5].

Mouse monoclonal antibodies are limited by short-half life in circulation, inability to trigger human effector functions and the generation of human anti-mouse antibodies (the HAMA response). In an attempt to reduce the immunogenicity of therapeutic antibodies, efforts were directed to produce antibodies by using antibody engineering technology. Teng et al. generated a

human monoclonal IgM antibody HA-1A by fusing human immunized B lymphocytes with heteromyeloma cells [61]. HA-1A was designed to fight against gram-negative bacteremia and shock by binding specifically to the lipid A domain of endotoxin. Clinical trials showed no overall benefit of HA-1A, but significant improvement in the survival rate in a subgroup of patients [76]. The common adverse events (hypersensitivity or allergic reactions) in children with *meningococcal* septic shock who were treated by HA-1A occurred at low rates (<2%) [26].

10.4 Approaches to Generate Humanized Antibodies

Traditional hybridoma technology generates antibodies with high immunogenicity. Such therapeutic antibodies *in vivo* invoke HAMA response. To attenuate the immunogenicity, several approaches are present to reduce non-human fragments in antibodies (Fig. 10.1).

10.4.1 "CDR Grafting" Method

Many other strategies such as recombinant DNA methods are used to attenuate the human immune response, by reducing levels of non-human fragments in mAbs. Chimeric antibodies are produced by replacing mouse constant region with the human constant region. The generated antibodies with mouse variable regions and human constant regions were perceived as less immunogenic than mouse monoclonal antibodies. Nonetheless, human anti-mouse response still exists with the use of chimeric antibodies.

To further minimize the mouse component in the antibodies, murine-derived CDR loops responsible for antigen binding were grafted into the human variable-domain framework. Caution should be noted that such replacements often change the structural relationship between CDRs and framework regions (FRs). Additionally, the change of CDR loops may influence binding properties of humanized Abs [3]. Thus, additional molecule engineering is needed to restore the binding capacity of humanized antibody [53].

"CDR grafting" method are successfully used to generate "humanized" antibodies which are able to evoke immune effector functions such as ADCC and phagocytosis more effectively. However, the murine-derived CDR loops can still evoke the immune response in patients. To minimize the anti-idiotypic response *in vivo*, several strategies were exploited.

10.4.2 Specificity-Determining Residue (SDR) Grafting

In most cases, only part of the entire CDR residues are involved in antibody-antigen complexes. These residues located in the region of high variability are designated as specificity-determining residues

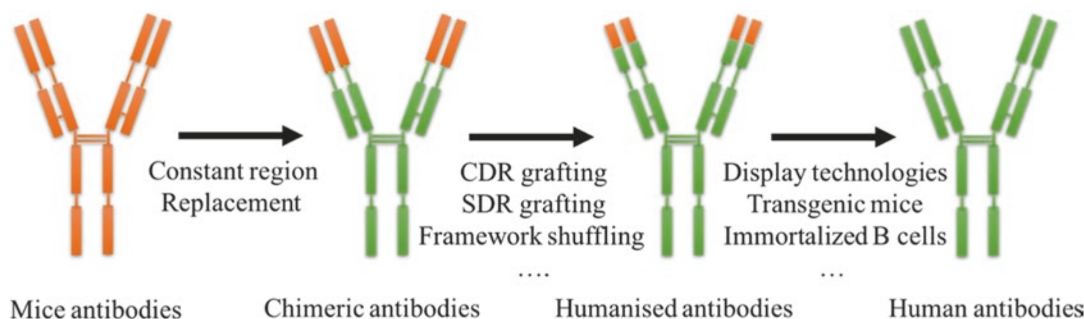

Fig. 10.1 Chimeric antibodies and humanized antibodies
The orange segments originates from murine, the green segments are from human.

(SDRs) [38]. Grafting the murine-derived SDRs onto the frameworks of human antibody generates humanized antibody with minimized immunogenicity. However, this strategy needs the identification of SDRs. Besides, the structure features of the target antibody should be preserved. Residues which are involved in VL-VH contact should also be preserved. Considerations should be taken that SDR grafting strategy usually generates antibodies with a reduced antigen-binding capacity. Thus, additional *in vitro* molecule engineering is needed to enhance antigen-binding affinity [23].

10.4.3 Framework Shuffling

"Framework shuffling" is another humanization approach by synthesizing a combinatorial library comprising CDRs of the non-human antibody fused in frame to pools of synthetic human germline frameworks. The corresponding libraries are then screened for antibodies with specific binding properties. This approach is simple, without requiring prior antibody structural knowledge, CDRs analysis or framework design [21, 22].

10.4.4 Human String Content Optimization (HSC)

Lazar et al. came up with a new paradigm termed "human string content" (HSC) for reducing immunogenic potential [43]. This approach is based on a metric of antibody humanness by comparing the non-human sequence with the human germline gene repertoire. The diversity of substitutions in a given variable heavy or light chain are scored as HSC. The targeted sequence is then humanized by maximizing its HSC.

10.4.5 Superhumanization

Superhumanization can be used to humanize mouse antibody. This method relies on comparison of CDRs. First, hypervariable loops of mice are compared with those of human. Variable sequences of human which are similar or identical to mice are then selected. Then, within the selected genes, those who share the highest homology to the mice sequence are used to construct a humanized antibody. Using this approach, antibodies with designed antigen-binding specificity and neutralizing activity can be generated [59].

10.5 Engineering Fully Human Monoclonal Antibodies

Although several humanized antibodies are approved for marketing, to some extent, humanized antibody still elicits human anti-human antibodies (HAHAs) response. For example, a patient after receiving adalimumab treatment (an effective treatment for rheumatoid arthritis) over a period of time, lost his initial response to adalimumab due to the formation of HAHAs [4]. Besides, humanization of antibodies can be labor intensive, comprising complex procedures including sequence analysis, developing engineering technology, assessing binding properties, and evaluating HAHAs response. Therefore, developing fully human antibodies is a long pursuit. Several technologies including display technologies, transgenic animals, immortalized B cells and transchromosomal calves are available to generate fully human antibody.

10.5.1 Display Technologies

In vitro display technologies are among of the most commonly used methods for fully human antibody generation. Advantages of *in vitro* display technologies are overcoming immunological tolerance and generating antibodies against defined antigens. Specifically, phage and yeast display systems are best exemplified. Other display systems such as ribosomes, bacteria and virus display are also used to display repertoires of single-chain variable antibody fragments

(scFvs), antigen-binding fragments (Fabs) or domain antibodies (Dabs) on their surface.

These techniques mainly contained three steps:

1. Antibody Library Construction

Amplify human immunoglobulin variable regions derived from human B cells.

2. Surface Display Vector Construction

The antibody library is cloned into the plasmid and transformed (transfected) in the host cell to construct surface display vector.

3. Desired Antibody Screening

Desired antibodies are isolated by panning the library against the target antigen/epitope. Antibodies that don't bind to the antigen are washed away and the binders are retained. The corresponding genes of variable regions are cloned into whole human IgG expression vectors and expressed in orthologous mammalian system to generate human mAbs.

Huse et al. first introduced a lambda phage-based system which expressed a collection of functional antibody fragments in *Escherichia coli* [32]. However, it was laborious to distribute the libraries on agar plates. Besides, this method only assessed repertoires of roughly 1×10^7, less than the natural antibody repertoire of 1×10^8 in each individual [58]. Later, progress was achieved in the area of display technologies by extending the library size and quantity. Theodore et al. produced mAbs against the West Nile virus (WNV) E protein by yeast surface display [47]. *In vitro* studies showed that one mAb could neutralize 10 different strains of WNV [47]. The animal experiment demonstrated the efficacy of the mAb against WNV by administering the mAb in a mouse model of WNV infection [47].

Different display systems may differ in antibody folding efficiency, post-translational modification and epitope accessibility which may generate antibodies with different binding properties [46]. For example, Bowley et al. compared yeast display and phage display by using the same HIV-1 immune scFv cDNA library and the same selecting antigen (HIV-1 gp120) [11]. Their results showed that yeast display system generated more full scFvs than phage display system [11]. Besides, yeast display system produced many more novel antibodies [11].

There are many display platforms, including phage display, ribosome display, yeast display et al. Among these display systems, phage display-based selections are most often preferred for the generation of fully human antibodies. Other display methods can be used as complementary methods. Display technologies have become a standard method to generate antibodies with high affinity. However, a drawback is that the antigen must be known prior, in order to select for antibodies with high affinity.

10.5.2 Transgenic Mice

Another strategy to generate human mAbs is by using human immunoglobulin transgenic mice [12]. With the development of embryonic stem cell and gene transfer methods, transgenic mice are generated to carry human immunoglobulin gene. These transgenic mice have a normal humoral immune response [34]. B cells that produce human antibodies are harvested from immunized transgenic mice, and then cloned either as hybridomas or *in vitro* combinatorial library. Antibodies obtained via transgenic mice are of good antigen-binding properties with low immunogenicity. There are three main transgenic mice platforms including XenoMouse, HuMab mouse and VelociMouse [8, 24].

However, flaws may exist when using transgenic mice method. Immune tolerance may arise when using immune antigens that are highly homologous to mouse. Thus, more immunizations or antibody screens are needed.

Transgenic mice are used to generate human antibodies against infectious pathogens including

bacteria and virus. For example, Coughlin et al. generated a human monoclonal antibody against severe acute respiratory syndrome coronavirus (SARS-CoV) using XenoMouse [18]. Paul et al. generated a Human mAb (V2L2-MD) by immunizing humanized VelocImmune mice. The generated mAb is specific for *P. aeruginosa* PcrV, and can protect murine infection models from *P. aeruginosa* challenge [66].

10.5.3 Immortalized B Cells

Memory B cells are produced after infection or vaccination, and these antigen specific B cells could persist for a lifetime. Thus, memory B cells can be used as a source of human mAbs. After immortalization by Epstein Barr Virus (EBV), the antigen-specific B cells can be cultured to secrete a large amounts of antibodies. The surface immunoglobulins of EBV-immortalized B cells can be used to select antigen-specific B cells by fluorescence-activated cell sorting (FACS). Traggiai E et al. used this method to isolate 35 human monoclonal antibodies against SARS-CoV, and found one antibody exerted effective protection *in vivo* [64]. Selection of antibodies against H5N1, HCV, HIV and CMV by EBV-immortalized B cells were also reported [55, 57, 62, 68].

Producing human antibodies by EBV-immortalized B cells was limited by the low efficiency of EBV-immortalization. This limitation is overcome with the discovery that a TLR agonist can increase the efficiency of EBV B-cell immortalization and promote cloning of immortalized B cells [64]. Now, EBV-immortalized B cells has become an interesting shortcut to generate human monoclonal antibodies by isolating memory B cells from a donor who is infected or vaccinated by infectious pathogens.

10.5.4 Transchromosomal Calves

Transchromosomal calves are recently used to produce large-amounts of human mAbs by transferring mammalian artificial chromosome vectors carrying human Ig loci to bovine primary fetal fibroblasts to produce cloned transchromosomic (Tc) calves [42]. The cloned Tc calves will contain a high rate of artificial chromosome positive cells. The Ig loci transferred in the cells undergoes rearrangement, diversification and expression. Secreted immunoglobulins in circulation will then be detected [42]. Production of Tc cattle is limited by the mitotic instability of human microchromosomes and the difference in immunophysiology between cattle and humans [42].

10.5.5 Immunospot Array Assay on a Chip (ISAAC)

Immunospot array assay on a chip (ISAAC) is a rapid and efficient method to generate antibodies from single cells [37]. This method uses a microarray chip whose surface is coated with antibodies against immunoglobulins to trap antibody-secreting cells (ACSs) [37]. ACSs are cultured on a chip for several hours. The antibodies secreted by ACSs bind to labeled antigens to form circular spots which are easily identified [37]. Then, the antigen-specific ASCs are retrieved to obtain antibody cDNA. The obtained antibody cDNA is inserted into expression vectors, and is transfected into cells for expression. The secreted antibodies can be detected by ELISA assay [37]. Jin et al. applied this system to produce human mAbs against viruses within a week, demonstrating ISAAC to be an alternative strategy for human mAbs generation [37].

10.5.6 New Technologies for Monoclonal Screening and Discovery

1. Next-generation sequencing technologies

Traditional *in vitro* monoclonal antibodies engineering technologies are laborious which depends on high-throughput screening of immortalized B cells. Reddy et al. developed a new method for antibody isolation bypassing antibody screening [50]. They used next generation

sequencing and bioinformatics analysis to analyse the variable-gene repertoires from bone marrow plasma cells. They found that the variable region of immunoglobulin derived from immunized mice became highly polarized. The high-throughput DNA sequencing enabled them to identify several abundant V_L and V_H gene sequences rapidly [50]. V_L and V_H genes with relative frequencies within the repertoire were paired and synthesized by oligonucleotide and PCR assembly. Single-chain variable fragments and full-length IgG were expressed in expression systems. Most of the antibodies produced by this method were antigen specific [50].

Next-generation sequencing is also applied to sequence single antibody-secreting B cells. DeKosky et al. used next-generation sequencing technologies to identify large numbers of V_H and V_L in a single B cell repertoire [25]. They first isolated single B cells from tetanus toxoid immunized human by depositing B cells in a high-density microwell plate. mRNA was then captured and reverse transcribed by RT-PCR. The sequence information of the transcripts was analyzed by next-generation sequencing technology [25].

2. Mass spectrometry

Wine et al. combined next-generation sequencing technology with mass spectrometry to deconvolute the polyclonal serum response after immunization [69]. They sequenced cDNA derived from desired B lymphocyte to generate a database of unique V genes by Roche 454 sequencing. Serum IgGs were pepsin-digested to obtain $F(ab)_2$. Antigen specific $F(ab)_2$ fragments were purified by standard antigen-affinity chromatography and were analyzed by bottom-up, liquid chromatography-high-resolution tandem mass spectrometry. By mapping peptides marking unique V_H CDRH3 sequences, this method can be used to identify a set of V-genes constituting the serum polyclonal responses [69].

3. Fluorescence-activated cell sorting (FACS)

Single B cells from defined subpopulations can be isolated by FACS [63]. The full-length variable region gene transcripts were obtained and amplified by RT-PCR. *In vitro* antibody were subsequently generated by eukaryotic expression system [63].

10.6 Prolong Half-Life

Once the engineered antibodies enter into the circulation, they would be eliminated by proteolysis, renal elimination and hepatic elimination, as well as neonatal Fc receptor-mediated endocytosis. To prolong serum circulation of engineered antibodies, several strategies are proposed. Extending serum half-life of therapeutic antibodies could reduce of the number of applications and the doses.

10.6.1 PEGylation

Attaching highly flexible hydrophilic molecules such as polyethylene glycol (PEG) could increase the hydrodynamic volume of engineered antibodies [27]. The increased volume thus improves the serum half-life. Careful attention should be paid that the number and size of PEG labeled may decrease the activation or binding affinity of the engineered antibodies [41]. Different coupling methods including random and site-directed approaches exist. Site-directed approach may be a better method to conjugate a single PEG chain to the antibody.

10.6.2 Fusion to Human Serum Albumin

Fusing human serum albumin to engineered antibodies provides an alternative way to extend serum half-life of antibodies. The binding site of albumin on FcRn does not alter the affinity for antigen or the affinity for FcRn. Covalent linkage of albumin can be achieved either by chemical coupling or genetic engineering methods. Albumin and IgG taken up by cells through macropinocytosis will bind to the FcRn of the early endosome. This binding will protect IgG from

degradation in the lysosomal compartment. Antibodies are then redirected to the plasma membrane and released back into the blood.

10.7 Affinity Optimization

Humanization may hamper the affinity of antibodies. Sometimes, the affinity of engineered antibodies may not satisfy actual needs. Affinity optimization is, therefore, desired. Most affinity optimization methods depend on optimizing the CDR residues [71]. Targeted or random mutagenesis is used to generate libraries of variants. Normally, libraries of individual CDRs are cloned and screened for improved binding to antigens. Yang et al. successfully generated high affinity human antibodies against human envelope glycoprotein gp120 of HIV-1 by saturation mutagenesis of CDRs [71]. The Fab fragments were displayed and selected for improved binding to the immobilized gp120 [71]. Among the CDR residues, residues in CDR3 are often concentrated for affinity optimization [52]. It is speculated that key variants in CDR3 regions play a major role in antigen affinity mainly for the reason that CDR3 regions are located in the center of the antibody combining site [52].

10.8 Fc Engineering

The therapeutic efficacy of the antibody is directly influenced by the affinity of antibodies to pathogens. However, effector functions mediated by the Fc domain also modulates the efficacy of these antibodies. Fc domain plays an important role in opsonophagocytic killing activity, toxin neutralization efficiency [1, 72]. Additionally, Fc domain is involved in mediating effector function, namely complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell phagocytosis (ADCP).

Optimizing the affinity of the Fc domain for Fc receptors is an alternative to increase the efficacy of therapeutic antibodies [56]. Several approaches including alanine scanning, site-directed mutagenesis, computational structure-based design and selection-based method are available to improve the affinity for Fc receptor [49].

Besides, the N-linked glycans in the Fc region of IgG also influence the interaction of the Fc region with its receptors. IgG1 antibodies that are deficient in fucose attached to the Asn^{297}-linked carbohydrate show improved binding affinities with FcγRIIIA receptor [54]. Several different methods can be used to modulate the fucose content on the IgGs. Different expression platforms may generate antibodies with different fucose content. For example, IgG produced by rat hybridomas technologies often has low fucose content [33]. Yeast-and plant-based systems produce antibodies with quite different glycosylation patterns [19, 44].

Antibodies of IgG isotype have a prolonged circulation through FcRn-mediated recycling. Research showed that some mutations in Fc domain may increase the affinity of Fc to its receptor at acidic pH instead of neutral pH. Incorporating such mutations in the Fc domain of IgG will extend the half-life of antibodies [75].

10.9 Immunoglobulin Isotype Selection

There are five major classes of immunoglobulin: IgG, IgA, IgD, IgE and IgM. Among these, the IgG class is the most preferred to develop therapeutic antibodies.

IgG contains four isotypes: IgG1, IgG2, IgG3 and IgG4. The main differences between IgG isotypes are located in the Fc domain. To date, most approved human mAbs are mainly of IgG1. Palivizumab (Synagis; MedImmune Inc) approved against RSV infections is a humanized IgG1 monoclonal antibody [30]. IgG1 is preferred for the reason that IgG1 can induce robust cell killing activities by activating various effector cells.

IgG2 is chosen as a therapeutic backbone when weak effector function is needed. Now, several

therapeutic IgG2 antibodies are either on market or in clinical development [51]. However, IgG2 dimers occur *in vivo* naturally. A more in-depth analysis by cleaving recombinant IgG2 suggested that Cys residues in the hinge are involved in the formation of covalent dimers. Besides, the reduced environments *in vivo* facilitate the formation of IgG2 dimers [74]. The mechanism of dimerization and the exact structure of IgG2 dimers have yet to be investigated. The report of IgG2 disulfide linkages interconverting *in vivo* may shed some light on IgG2 dimer formation [74]. One consequence of IgG2 dimerization is to enhance antibody avidity, which may play a better protective role [51]. IgG2 dimers exist at low levels (<1%) *in vivo*, and their speculative roles should not be magnified [73].

IgG3 is less chosen as a backbone of therapeutic antibodies for several reasons. First of all, IgG3 accounts for a minor component of all IgGs in humans (~10%) [51]. Secondly, IgG3 is susceptibility to proteolysis and has a short half-life of ~7 days *in vivo*. Finally, IgG3 displays extensive polymorphism, within the constant domains [35].

IgG4 can't activate the classical complement pathway effectively and has reduced effector function [65]. Besides, IgG4 antibodies exchange half-molecules *in vivo* to form undesired cross-link [70]. To abrogate half-molecule exchange of IgG4 and to eliminate the possible unseen adverse effects, Ser 228 to Pro (S228P) mutation is introduced by antibody engineers to optimize IgG4-based therapeutic antibodies. This mutation not only reduces half-molecule change to a large extent, but also extends serum half-life.

10.10 Bispecific Antibodies

Bispecific antibodies (BsAbs) have two different binding specificities, which enables them to simultaneously recognize two different mediators/pathways that exert important roles in pathogenesis [27]. This dual binding capacity will increase targeting specificity or redirect specific immune cells to pathogens or infected cells, thus enhancing pathogen elimination. Recently, bispecific antibodies BiS4αPa against *Pseudomonas aeruginosa* was generated [39]. BiS4αPa was designed to bind to PsI and PcrV. PsI is an extracellular polysaccharide which involves in immune evasion and biofilm formation. PcrV takes part in the secretion of virulence factors. Animal studies showed that binding to PsI and PcrV led to superior protective activity of BiS4αPa [39]. Now BiS4αPa is in clinical candidate for the treatment of *P. aeruginosa* [39]. Berg et al. constructed a bispecific antibody by linking one heavy/light chain pair from an antibody against CD3 on cytotoxic T cells to a heavy chain whose variable region was replaced by sequence from CD4 [6]. The constructed antibody has dual binding specificity with one arm binding to CD3 on cytotoxic T cells and the other arm which contained sequences from CD4 binding to viral envelope protein gp120 of HIV [6]. Thus, the bispecific antibody redirected cytotoxic T cells to HIV-infected cells whose surface express integral viral proteins to eliminate pathogens. Bispecific antibodies hold great promise in the elimination of HBV, bacteriophages and other pathogens [48, 60].

The formats of bispecific antibodies can be roughly divided into two categories: IgG-like molecules and non-IgG-like molecules (Fig. 10.2). Divergent approaches are used to engineer bispecific antibodies (Table 10.1). For details, please refer to Fan et al. [27].

10.11 Conclusion

In the era of antibiotic resistance, antibody-based therapy holds great promise in treating infectious pathogens. It is a holy grail to produce human antibodies with high therapeutic efficacy. The therapeutic potential of antibodies is derived from less immunogenicity, proper serum circulating time, high antigen-binding affinity, exquisite specificity and robust effector function. Advancement in antibody engineering technolo-

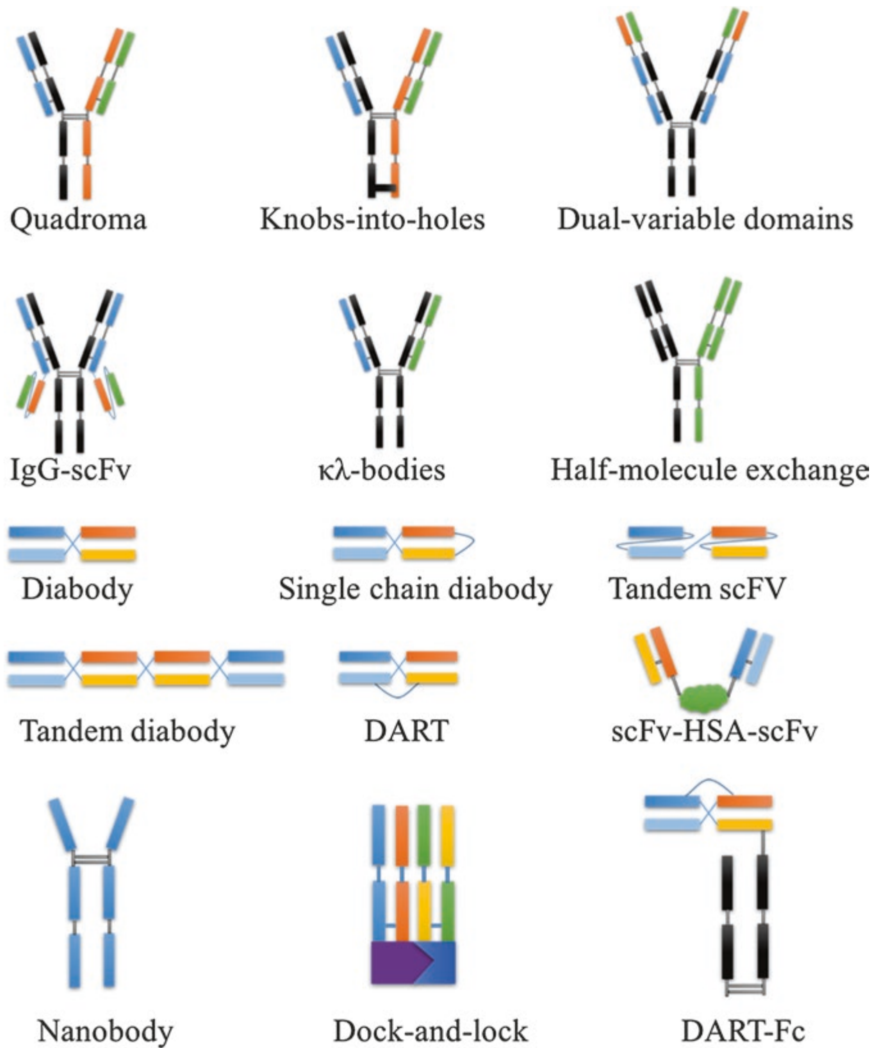

Fig. 10.2 Formats of bispecific antibodies

gies makes it possible to produce antibodies tailored to different infection pathogens. Over the past decades, the "magic bullet" has obtained fruitful success in a wide range of diseases including tumors, autoimmune diseases and inflammatory conditions. However, in the field of infections, there is a gap between preclinical researches and clinical applications. Many engineered antibodies are still making their way through preclinical/clinical tests, only few are approved for market. Here, we provided a review of technologies for antibody engineering and optimization. We are optimistic about the application of engineered antibodies to treat infectious pathogens. With the emergence of new infectious pathogens and multidrug resistant bacterial, engineered antibodies may offer another choice to combat these threats.

Table 10.1 Methods to engineer bispecific antibodies

Formats	IgG-like molecules	Non-IgG-like molecules
Methods	Quadromas	Tandem scFvs
	Knobs-into-holes	Diabody format
	Dual-variable domains Ig (DVD-Ig)	Single-chain diabodies
	IgG-single-chain Fv (IgG-scFv)	Tandem diabodies (TandAbs)
	Dual action Fab (DAF)	Dual-affinity retargeting molecules (DARTs)
	Half-molecule exchange	Nanobodies
	κλ-bodies	Dock-and-lock (DNL)
		scFvs connected to other molecules to form bispecific

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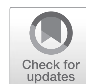

11 Computer-Aided Antibody Design: An Overview

Yee Siew Choong, Yie Vern Lee, Jia Xin Soong, Cheh Tat Law, and Yee Ying Lim

Abstract

The use of monoclonal antibody as the next generation protein therapeutics with remarkable success has surged the development of antibody engineering to design molecules for optimizing affinity, better efficacy, greater safety and therapeutic function. Therefore, computational methods have become increasingly important to generate hypotheses, interpret and guide experimental works. In this chapter, we discussed the overall antibody design by computational approches.

Keywords

In silico · Antibody design · Computational tools · Modelling · *ab initio* · Docking

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11.1 Introduction

11.1.1 Antibody as Therapeutic Drugs

The use of monoclonal antibody (mAb) for clinical purposes has reach up to 45% in the past decade [75]. The first mAb was produced in year 1975 but United States Food and Drug Administration (FDA) only approved its application for therapeutic purposes after more than 10 years [59]. MAbs were made using hybridoma technique where the antigen-stimulated B cells were immortalised by fusion to myeloma cells [117]. However, the therapeutic use of some mAbs produced from murine cells showed poor pharmacokinetics and repeated administration of mouse hybridoma-derived antibodies caused immunogenicity in human [34]. These mAbs can be recognised as foreign substances in the human body, eliciting the human anti-mouse antibody response, thus affecting the therapeutic activities [59].

Therefore, numerous engineering technologies have been developed in order to improve the safety, characteristics and properties of these therapeutic antibodies. One of the major advancements is the reduced immunogenicity observed in mouse derived mAbs [75]. Humanised and chimeric mAbs with reduced immunogenicity have

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also been widely used in earlier studies. Besides hybridoma technique, phage display technology has enabled the possibility of high-throughput generation of mAbs. It is achieved by cloning the antibody gene into a suitable phage or phagemid vector for the presentation of antibody molecules on the surface of bacteriophages. In the 1990s, phage-display and transgenic mice technology were the methods used to generate mAbs [59, 75]. There are several FDA-approved therapeutic human mAbs produced from phage display and transgenic mice technology for clinical applications [59]. About 47 therapeutic mAbs against infectious diseases, cancer and various diseases have been approved in recent years [28, 62]. Due to today's biotechnological advances and overwhelming demands, production of therapeutic mAbs is growing fast in the pharmaceutical industry, with its global sales showing a drastic sevenfold increase, from USD$4 billion in year 2001 to USD $30 billion in year 2008 [59]. In addition, the worldwide sales figure for mAbs in year 2013 also achieved nearly USD $75 billion, and the sales is predicted to hit USD $125 billion in year 2020 [28].

11.1.2 History of In Silico Antibody Design

Over the past decades, computer-aided antibody design has been progressing along with conventional experimental approaches. There are more devised methods for structure prediction, binding affinity improvement and achievement in desirable physicochemical properties of antibodies. These enhanced antibodies which are still able to retain their specificity and efficacy are thus termed as "bio-better antibodies" [9]. The rationale behind antibody design is to enhance existing antibody properties in order to increase their clinical potential when these "bio-better antibodies" are being used as therapeutics [14].

Computational design of antibodies has shown to indirectly assist the experimental validation of site-directed mutagenesis. In a study, more than 80 anti-VLA1 antibodies designed were tested on the impact of mutations on their binding affinity towards human integrin Domain I (VLA1) [22]. The VLA1 integrin is a cell surface receptor for collagen and laminin in biological pathways related to the pathogenesis of rheumatoid arthritis. The VLA1-collagen interaction was found to be responsible for immune-mediated inflammation in animal models of arthritis, colitis, nephritis, and graft versus host disease (GVHD) [10]. Therefore, anti-VLA1 antibodies against VLA1 integrin have potential therapeutic properties for inflammatory disease. The results of the study indicated that applied structure-based computational antibody design increased the binding affinity of the antibodies towards the antigen. In another study, the mutated antibodies were evaluated for the changes in electrostatic energy [61]. It was discovered that the mutant antibodies has 140-fold improvement in the binding affinity. A few years later, the relationship between high affinity antibody mutants with the altered electrostatic energy was solved whereby charged residue was found in all mutants with increased affinity [48]. Besides optimising the affinity of antibodies, the possible risk of causing an immunogenic reaction in humans has also been studied. A recent work applied molecular dynamic (MD) simulations to guide the selection of critical back-mutation sites for antibody humanisation [64]. The critical residues for back-mutation have been identified under the assumption that CDR dynamics of an antibody variant is tantamount to its wild type. Further validation of *in silico* prediction in the experiments showed the applicability of the computational method, thus highlighting its importance in antibody design.

Despite promising results by *in silico* antibody design, the success rate still much depends on the accuracy of the designed structure [22]. Therefore, efforts on refining current available antibody modelling methods are vital in the study of antibody structure and its function [1, 4, 20, 67, 122, 123]. For example, employment of machine learning approach in antibody optimisation can contribute to the understanding of anti-

bodies characteristics. It had been demonstrated that when a set of models of interest trained under defined variables, it is possible to predict the unknown functional responses in antibodies [20]. The obtained valuable information can then aid the development of potential vaccines and generate desirable antibodies.

11.2 Antibody Structure

An antibody, also known as immunoglobulin (Ig), consists of the fragment crystallisable (Fc) and fragment antigen binding (Fab) regions. The Fc region consists of two constant domains C_{H2} and C_{H3}. Each Fab region is composed of two variable domain regions, namely the variable heavy (V_H) and the variable light (V_L) chains (Fig. 11.1). There are three hypervariable loops in each variable domain region known as complementarity determining regions (CDRs), being denoted as L1, L2, L3 for V_L chain and H1, H2, H3 for V_H chain. Variability observed in CDRs arise from somatic recombination processes [91]. This process assembles an immunoglobulin domain by combining respective gene segments for light chain (V and J) and heavy chain (V, D and J) in random. Among all CDR loops, H3 loop exhibits highest diversity in its length, structure and dynamics. Owing to the role of variable domain in antigen recognition, the variable domain is often the target for engineering. Therefore, it introduces a fusion antibody known as single chain fragment variable (scFv) antibody, where its V_H and V_L domain are connected by a linker.

11.3 Ideal Antibody Design

Theoretically, an ideal antibody design involves designing an antibody from scratch using available *in silico* tools. The structure of both antigen and antibody can be predicted solely from nucleotide/protein sequence. Other than that, the characteristics of an antibody can also be studied. To grasp the concept of antibody design, the general flow of an ideal antibody design process can be referred to Fig. 11.2.

In general, protein structure predictions are performed by extracting information from existing experimental data. The query sequence is

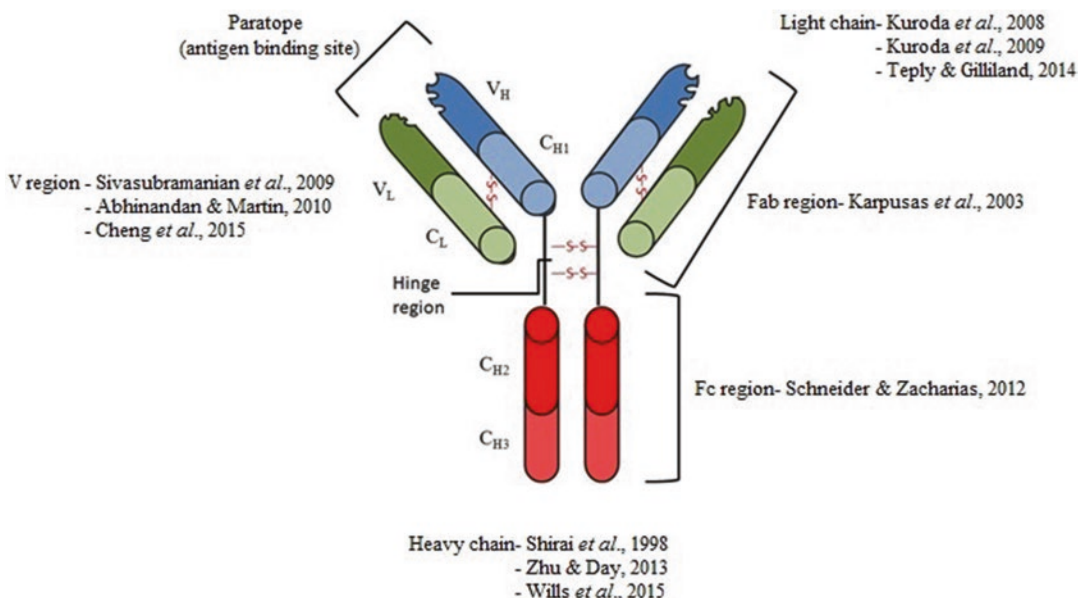

Fig. 11.1 General structure of antibody (also known as immunoglobulin; Ig) and related *in silico* studies in the past 20 years. Further information on these studies can be found in later sections of this chapter

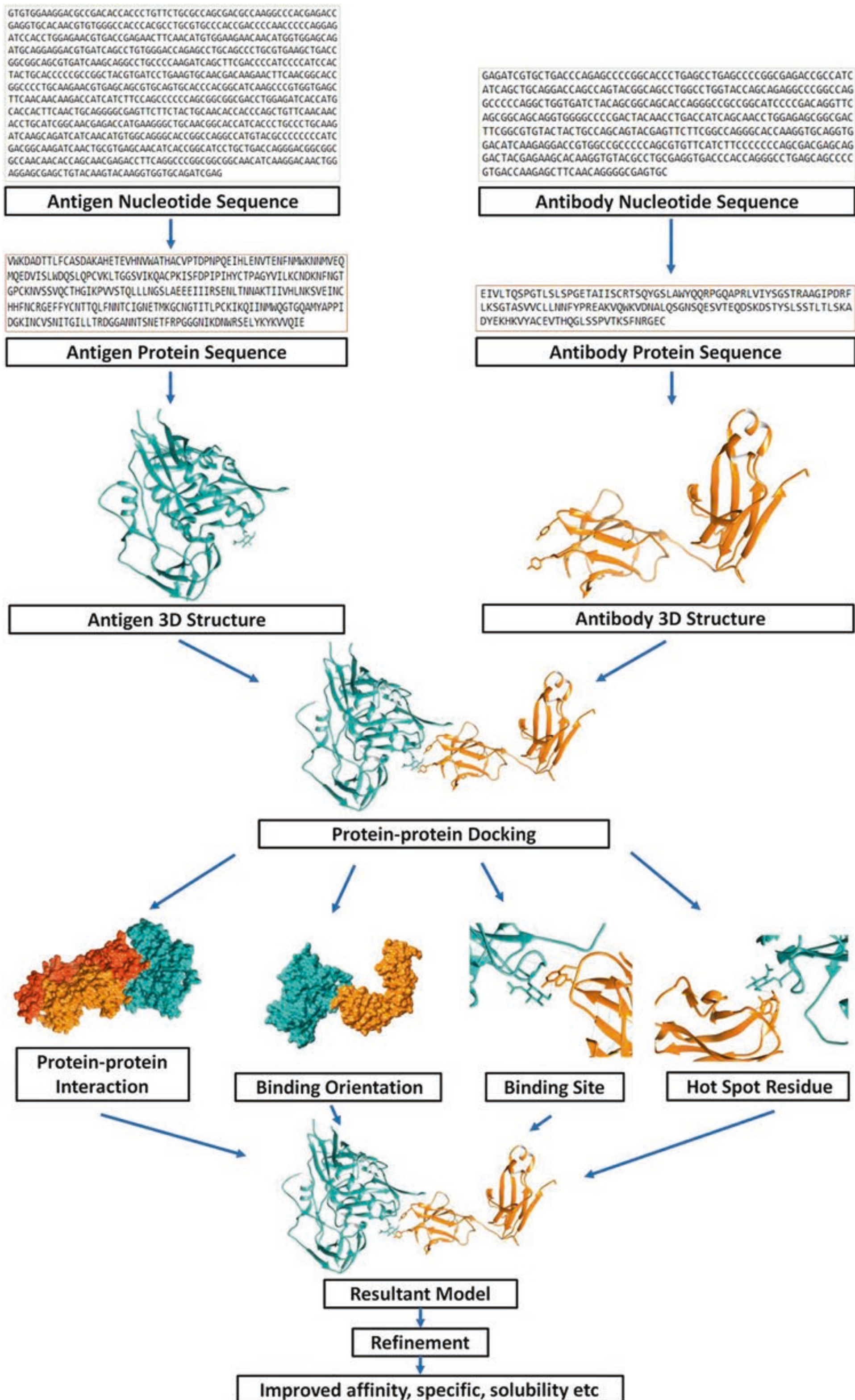

Fig. 11.2 General flow of ideal *in silico* antibody design.

modelled from its homology templates. These templates are experimentally solved structures via X-ray diffraction or nuclear magnetic resonance (NMR) with reasonable structural resolution. Template search technique such as basic local alignment search tool (BLAST) is usually employed for identifying matching templates [5]. However, problems arise when there are overwhelming hits of matching templates to the query sequence. Thus, specific criteria need to be set prior to template selection. For example, the sequence similarity, sequence identity, E-value and structure resolution. After obtaining an optimal sequence alignment between selected templates and query sequence, modelling algorithms such as rigid-body assembly [23, 37], segment matching [58], spatial restraint [31, 89] and artificial evolution [121] can be used in constructing the 3D structure of the query sequence [114]. In the case of no matching templates, *ab initio* modelling would be a preferred approach (Sect. 4.1.2).

After obtaining the theoretical models, the antigen and antibody will then be docked. A docking simulation predicts binding conformation of a receptor-acceptor complex, whereby the complex can be assembled from a protein-ligand complex or a protein-protein complex. Protein-protein docking is more challenging than a protein-ligand docking as it involves the docking of two proteins which are relatively bigger in size and with higher degrees of freedom. On the other hand, protein-ligand docking only involves docking of a smaller molecule (ligand) into the protein active site. The common problems encountered in protein-ligand docking would be the receptor flexibility, scoring function limitation and solvent effect. Similar problems are observed in protein-protein docking in an extended perspective [98]. In fact, more issues such as conformational changes during docking, structural flexibility and interface region might add further complexities in protein-protein docking [110].

After docking simulation, a series of analyses will be performed to evaluate the docked complex. These analyses are commonly: (1) the binding site analysis - epitope of antigen and paratope of antibody, (2) the binding orientation analysis - conformation of antigen-antibody complex, (3) the hotspot residue analysis - identification of important residues that contribute to the binding, and (4) the interaction analysis - the resultant formation of antigen-antibody complex, e.g. binding free energy. Those adopted approaches for analysis along with their findings (description of complexes, surface complementarity, charge complementarity, bonding interaction, mobility, accessibility, antigenicity, epitope mapping, conformational changes, and flexibility) had been discussed in great lengths by Davies et al. [26]. After characterising the antigen-antibody complex, the antibody can thus be further refined to improve its binding affinity and specificity. One of the popularly used antibody refinement approaches is hotspot residue mutagenesis.

To date, there is hardly an antibody being designed based on the aforementioned steps without hassle. This workflow could serve as a general guideline in the process of antibody design but it is inevitable that each step in the workflow might require some meticulous troubleshooting. For example, the accuracy of the predicted structure depends on the available template. Therefore, selection of a poor quality template might result in an erroneous structure prediction. Apart from that, addressing protein flexibility is often challenging in protein-protein docking. In terms of analysis and scoring, the common concerns are technical issues such as availability of the modules, softwares, and algorithms. Even though *in silico* antibody design process could be a tedious task, it is advantageous as it cuts down massive failure cost from laboratory antibody design.

11.4 Computational Tools Related to Antibody Design

11.4.1 Predictive Modelling – Structure Prediction

11.4.1.1 Comparative Modelling

Template-based modelling is the prefered choice when homologous templates are available. In general, template-based modelling starts with

suitable homologous template selection, template alignment with target, model building and built model evaluation. Of all the outlined steps, the first step of modelling which is template selection requires utmost caution. Previous work showed that quality of template structures and the selection criteria can affect the reliability of antibody model [4]. In addition, the selection between single structure or chimeric template for both chain frameworks and loops is debatable. If chimeric templates were used, V_H-V_L packing and loop grafting might give rise to another important issue such as alignment of the target sequence to templates. It is important to ensure correct alignment between the target sequence and templates in order to predict the antibody structure accurately [63].

Although CDR regions are known for their diversity, studies reported that majority of the existing CDR loop conformations have similar patterns [2, 76, 102]. These repetitions are termed canonical structures and can be identified by the length and position of key residues around the loop. Loops within canonical structures tend to assume certain conformations, which had been lengthily discussed and reclassified [76]. Prediction of Immuno Globulin Structure (PIGS) applied the concept of canonical rules in predicting antibody structures [63]. PIGS protocol can be summarised in five main steps: (a) comparing and aligning each domain framework to experimentally solved antibody domains based on homology, (b) aligning target and templates that share highest sequence identity, (c) grafting loops with matching canonical structures into predicted framework, (d) packing of two predicted domain structures utilising conserved interfacial residues and (e) modelling of side chains.

Apart from the canonical method, knowledge-based method has been demonstrated to contribute in antibody modelling [123]. In that study, matching light and heavy chain templates were selected from the same antibody structure after aligning query sequence to every antibody structure in a curated Protein Data Bank (PDB) database. The heavy and light chain frameworks of the templates with the highest resemblance to the query sequence were given precedence in template selection. Besides focusing only on query sequence and loop length, the stem residue geometry of framework templates also served as a guideline to choose potential candidates for CDR loops. Next, the loop candidates were clustered. The representative loop for each CDR was then determined by cluster size along with sequence similarity. Modelling task was initiated by building an initial framework homology model, followed by grafting of CDR loops onto the model and further refining the model. Despite attaining an average root mean square deviation (RMSD) below 1.20 Å for framework region and non-H3 CDR loops, H3 loop yielded an average RMSD of 2.91 Å. This showed that modelling an antibody structure for the variable region, in particular, is still challenging to date.

11.4.1.2 *Ab Initio* Modelling

Ab initio antibody design is similar with structure-based antibody design. Likewise, these two approaches starts with a gene sequence and is followed by structure prediction. However, not all query structures will return with templates. *Ab initio per se,* implies constructing a model from scratch in the absence of structural information. In short, the prediction of protein structure is based on primary sequence. Apart from being known as *ab initio* modelling, it is also termed as *de novo* modelling, physics-based modelling or free modelling [13, 45, 79].

Without template structures, massive computational power is required to arrange the amino acids into tertiary structure abiding by the general principle of protein folding. Therefore, *ab initio* approach is usually applicable for a small protein or a region of a large protein. In the context of an antibody, *ab initio* design normally refers to the design of CDRs, especially the H3 loop. Among six hypervariable regions in the variable domains of an antibody, H3 loop has a vast diversity, putting on a challenge to its prediction. In general, the H3 loop can be made up of 20 residues compared to the non-H3 loops with only 2–8 residues [76, 122]. When a loop has more than 12 residues, prediction becomes more

challenging due to the intrinsic flexibility of the loop [51].

An early study showed that *ab initio* method can be very reliable to predict antibody H3 loop structure, provided that the rest of its structure is accurate [122]. This would be particularly useful in antibody optimisation. Instead of relying on existing antibody loop conformations, *ab initio* method searches for the lowest energy conformation, with the assumption that the crystal structure has the minimum global free energy. Therefore, thorough sampling of conformational space is important in order to identify the lowest energy conformation. In the study of Zhu and Day [122], the reliability of the method was tested on two scenarios which involved crystal structures and non-crystal structures. In the first scenario, above 90% of H3 loop conformations were successfully predicted, whereas in the second scenario, H3 loop conformations can only be well predicted when the surrounding conformations do not undergo significant change. As a loop refinement step, other than energy-based function [122], *ab initio* method can also incorporate MD simulation or multi canonical MD [93] to search for the lowest energy structure of H3 loop. Some softwares like RossettaAntibody [94, 95] and Prime [90] are developed particularly to predict loops with low free energy using in-house minimisation algorithm and conformational sampling force field.

11.4.1.3 Threading

The concept of threading can be considered akin to comparative modelling as both approaches require structural information for fold recognition. However, the templates selected for building a model in threading are remotely related to the target sequence. In the case of low homology protein targets, threading utilises structural information from the template for prediction [118]. Despite sharing low sequence identity between target and template, threading can still perform a good target-template alignment. The concept of threading involves template library construction, target and template representation, threading algorithm and lastly template selection. Examples of protein structure prediction tools that implement the concept of threading are THREADER [46] and I-TASSER [119].

11.4.1.4 Machine Learning Approach

Machine learning approach (such as penalised logistic regression, regularised random forest, and support vector machine) trains a set of models with known quantities and specific features to predict the unknown quantities [20]. For example, predicting the packing for V_H and V_L domains of antibodies using artificial neural network (ANN) method [1]. In the study, Fv regions and information on V_H/V_L packing angles were collected. Correct packing angle of V_H and V_L domains were then predicted after identifying significant interface residues that would affect the packing angle. This prediction method could be very useful in antibody humanisation, especially in retrieving the correct binding site topography when non-human CDRs are being grafted onto a human framework region.

Amid current approaches in modelling H3 loop, the Random Forest (RF) automatic learning technique can overcome setbacks in existing approaches [67]. The RF model was trained to select template loops that is based on a few variables, namely a full sequence of antibody, canonical structures, germline families, BLOSUM40-based similarity and SASM-based similarity. Other than these variables, additional variables considered were the length of CDR loops between target and template and, the length of the matching and non-matching gaps. The built H3 structures were then ranked according to intramolecular interactions. When the model was tested on the Rosetta Antibody as benchmark, the average backbone RMSD had improved close to 1 Å. The method developed in this work achieved higher accuracy in predicting H3 structures compared to *ab initio* approach as it did not employ unreliable pseudo-energy approximate functions to identify correct predictions. In addition, its computational cost is also less expensive compared with that of *ab initio* approach.

11.4.1.5 Hybrid Methods

Every modelling approach has its own strengths and shortcomings. Thus, a combination of modelling approaches might complement each other to result in a favourable built model. A good example of such a case was presented by combining knowledge-based method with *ab initio* method in predicting H3 loop. Results showed a slight improvement in the H3 average RMSD from 2.91 Å to 2.67 Å [123]. On the other hand, canonical method coupled with Random Forest (RF) automated learning technique (discussed earlier in Sect. 4.1.4) was employed to improve accuracy in H3 loop modelling [67]. By using RF machine learning algorithm, sequence similarity and the structural related features in experimentally solved immunoglobulin structures can serve as guidelines to select the best template for H3 loop modelling. Apart from that, contact profiles of the resulting H3 models were also ranked accordingly as surrounding environment of H3 loop will influence the final structure of the loop. Therefore, RF approach can complement the canonical method which is only able to predict structure of H3 loop terminal residues next to the framework.

In light of various antibody modelling approaches, Antibody Modelling Assessment (AMA) is initiated to assess and evaluate the quality of predicted antibody structures in comparison with unpublished crystal structures [3]. To date, there are two rounds of AMA where the first one was conducted in year 2009 whereas the more recent one was held in year 2013 [4]. It functions in a similar manner as Critical Assessment of Techniques for Protein Structure Prediction (CASP). Besides providing a good platform for enthusiastic researchers to test their skills in modelling antibodies, it also contributes in improvement of current antibody modelling practices by identifying the common errors made in antibody prediction. These common errors were often associated with the choice of structure template (CDR canonical structures and V_H/V_L packing) and poor quality Protein Data Bank structures [4]. On top of that, predicting the structure of H3 loop still remains as a challenge for most of the cases. These observations thus challenge the development of new algorithms to improve antibody structure prediction.

11.4.2 Protein-Protein Interactions Analysis

11.4.2.1 Epitope Prediction

An antigen is defined as a molecule that is able to induce the immune response, whereas an epitope is a structural part of the antigen that can be recognised by the B cell receptors in the immune system [82]. In other words, the variable domains (paratope) of an antibody will bind to the epitope of an antigen. As the epitope plays an important role in determining the antigen-antibody binding site, various bioinformatics tools have been developed to identify the epitopes at the antigen.

The B-cell epitopes are categorised into two classes: linear epitopes (also known as continuous epitopes) and conformational epitopes (also referred as discontinuous epitopes) [100]. The linear epitopes are made up of a continuous string of sequentially consecutive short peptides whereas the conformational epitopes are composed of discontinuous segments which are brought together when the protein folds into its 3D structure [30, 42]. More than 90% B-cell epitopes are conformational epitopes [42]. Therefore, the prediction of conformational B-cell epitopes has greater values in epitope studies.

The approach of epitope prediction can also be further classified into structure-based epitope prediction and mimotope-based prediction. Structure-based epitope prediction by bioinformatics tools has been developed since year 1999. This method focused on the 3D structure of antigen corresponding to the geometric aspects and physical chemistry properties of the epitopes [100]. Table 11.1 lists some of the available structure- and sequence-based B cell epitopes prediction servers in recent years.

Structure-based epitopes prediction usually starts with downloading of 3D structures from PDB database [11]. After the 3D structure of an antigen is obtained, bioinformatics tools are used

Table 11.1 List of available B cell epitope prediction servers

Name	Link	References	Approach
BEPro	http://pepito.proteomics.ics.uci.edu/	[101]	Structure-based
DiscoTope	http://www.cbs.dtu.dk/services/DiscoTope/	[6]	Structure-based
ElliPro	http://tools.immuneepitope.org/tools/ElliPro/iedb_input	[84]	Structure & sequence-based
SEPPA	http://lifecenter.sgst.cn/seppa/index.php	[99]	Structure-based
Epitopia	http://epitopia.tau.ac.il/	[88]	Sequence-based
EPCES	http://sysbio.unl.edu/services/EPCES/	[60]	Structure-based

to calculate the scores or types of B-cell epitopes. One of the available servers to calculate B-cell epitope from both sequence- and structure-based is ElliPro [96]. ElliPro places the query protein sequence as the input and searches for matching protein sequence in PDB database. For unresolved protein structure, the 3D structure of the query protein is predicted by MODELLER [31]. The algorithms in ElliPro are based on Thornton's method and it differs from Thornton's by using the mass centre of residues to calculate the algorithms instead of the alpha carbon [84, 100]. The algorithms in ElliPro calculate the protusion index (PI) of the residue [84] and output a epitope score to each residue [96]. The Immune Epitope Database and Analysis Resource (IEDB) is also another available database for epitope prediction. IEDB consists of comprehensive data of epitopes of various antigens, mainly focusing on data that is related to harmful diseases and bioterrorism threats [82, 100].

On the other hand, mimotope-based prediction focuses on using the mimotopes to trace the antigen and find their epitopes. Mimotopes are peptides that have high affinity to the antibodies, by sharing similar structures and functions that mimicked the epitopes [18]. To utilise this method, phage display biopanning is required to obtain the mimotopes through rounds of screening and amplifying with a peptide library against a mAb. The peptides that bind to the mAb obtained from the biopanning are the mimotopes [100]. Table 11.2 lists some of the current bioinformatics tools for mimotope-based epitope prediction.

Mimotope-based method requires both the antigen structure and the mimotope sequence for the input data [100]. The structure of the antigen can be obtained from PDB while the mimotope sequence is acquired from phage display biopanning experiments [18]. There are biopanning databases available with the collection of mimotopes obtained from phage display experiments. These databases serve as a useful platform in mimotope-based epitope prediction, where important information such as the parameters used in the biopanning experiments, the target antigen and the library used are stored in the databases [38].

The first mimotope database was Artificially Selected Proteins/Peptides Database (ASPD) [111]. When ASPD database was first released in 2001, it has 195 data from selected experiments [100, 111]. The information that can be retrieved includes the binding target, the protein templates used in library construction, protein or peptide sequences obtained from experiments, the number of selection and amplification performed, and the occurrences of clones in the sequences [111]. This information is useful for users to repeat or to optimise similar experiments with the parameters in the database. On the other hand, the Biopanning Data Bank (BDB), rebranded and updated from MimoDB database is another database consisting of mimotope and target-unrelated peptides [38]. BDB differs from other mimotope databases by storing both the mimotopes and target-unrelated peptides from biopanning experiments. The target-unrelated peptides are the false positive and vital in the distinguishing true positive from the false positive results generated from the experiments.

Other than mimotope databases, there are also tools that predict the epitopes of an antigen based

Table 11.2 Mimotope-based epitope prediction servers in recent years

ServerServer	Link	References
ASPD	http://wwwmgs.bionet.nsc.ru/mgs/gnw/aspd	[111]
BDB	http://immunet.cn/bdb/	[38]
MimoPro	http://informatics.nenu.edu.cn/MimoPro	[18]
Pep-3D-Search	http://kyc.nenu.edu.cn/Pep3DSearch/	[44]

on mimotope analysis. For instance, the Pep-3D-Search is a method designed to predict B-cell epitopes using the antigen structure and mimotopes [44]. From the antigen structure, Pep-3D-Search creates a surface graph by identifying all the surface residues. Mimotopes are aligned with the surface graph of the query antigen. The epitope candidates are then generated through screening and clustering of the matching paths [44]. Another similar web-based tool for epitope prediction is MimoPro [18]. The approach of MimoPro is to map a mimotope set back to an antigen to search for the epitopes. It also introduces a novel parameter, known as the compactness factor (CF) to regulate the surface graph generated from overlapping patches on the antigen surface [18].

11.4.2.2 Docking Simulation

Docking simulation is an approach used to predict the preferred orientation or conformation of two molecules in achieving a stable complex, concurrently providing computed free binding energies which can be taken as reference for experimental values. In the therapeutic field, these binding molecules are often referred to as the antibody and antigen. Docking simulation is one of the key methods in designing an antibody for various applications, including for therapeutic and diagnostic purposes.

The antibody-antigen docking process involves two separate structures - the antigen and antibody. Both structures are brought together using selected algorithms [81]. Antibody-antigen docking can be performed in two steps, first by intensive calculations and sampling of the conformations of the antibody and antigen, followed by finding the best conformation through ranking of docking possesses evaluated using scoring functions [65]. The scoring function is a mathematical method to calculate the binding affinity of the antibody and the antigen, which can be expressed in an equation of binding free energy, ΔG_{Bind}:

$$\Delta G_{Bind} = G_{Complex} - G_{Antibody} - G_{Antigen} = -RT \ln\left(K_{eq}\right)$$

The equilibrium binding constant, K_{eq} can be obtained from the binding free energy where R is the perfect gas constant and T is the temperature [77]. This energy is calculated by considering the hydrophobic and electrostatic interactions, hydrogen bonds, salt bridges, some statistical and empirical factors. An important assumption in computational docking is the preferred structure always has the lowest energy as it is energetically favoured. Therefore, a favourable docking possesses are usually docking conformations with low energy [81].

In general, there are three types of docking, viz., rigid body docking, partial flexible docking and flexible docking. The algorithms and parameters used differ and is dependent on the type of docking. Rigid body docking is applied where both the receptor (i.e. antibody) and the acceptor (i.e. antigen) are kept inflexible [71]. This approach is preferred when both starting structures have highly complementary shapes [81]. One of the bioinformatics tools that use this approach is the ZDOCK server [83]. ZDOCK applies the Fast Fourier Transform algorithm to achieve effective docking search, using a 3D grid platform. The scoring is obtained by considering the electrostatics, statistical potential terms and

the shape complementarity [83]. The prediction begins with the input of the coordinates files for the receptor and acceptor, followed by selecting the blocking or contacting residues and visualising top docked models and respective scores through JMol [83]. RosettaDock is another docking tool available based on a multi-scale Monte Carlo docking algorithm [15]. It can concurrently identify preferred docking poses and optimise the positions of rigid body and side-chain conformations. The task can be performed with minimal computational power as the side-chain conformation only involves limited number of torsion angles [15, 81].

In partial flexible docking, the backbone of the receptor is held rigid while the acceptor remains flexible [71]. It is suitable for molecules that bind through induced fit mechanism, as the receptor or acceptor undergoes changes in their conformations to form a complex with minimal energy [65]. One of the docking tools that applied this concept is AutoDock [73, 86]. AutoDock applies the Monte Carlo algorithm which holds the receptor backbone rigid while allowing the acceptor flexibility, followed by using the AMBER force field [80] to obtain its scoring function. AutoDock4 [74], AutoDock Vina [108] and recently introduced AutoDockFR [87] are able to perform docking simulation by allowing partial flexibility of the receptor instead of only the acceptor. A higher docking success rate can be achieved by granting the receptor binding site flexibility (the apo conformation) which is determined by experimental [87]. This type of docking is well suited for smaller sized acceptor (i.e. drug) as a peptide or protein (i.e. antibody) would have too many flexible bonds to be handled.

FLIPDock is a docking software that incorporates flexibility for both receptor and acceptor [120]. A data structure known as the Flexibility Tree was developed and used to encode the conformational spaces between the molecules, allowing the automated docking of the acceptor into the receptor active sites [120]. Nevertheless, there is no definite approach in docking antibody and antigen. It was suggested using rigid body method for the backbone while sampling different side-chain conformations could be the preferred approach for the docking of antibody-antigen complex [81].

11.4.3 Refinement and Optimisation

Protein-protein (i.e. antigen-antibody) docking usually applies the concept of rigid-body approximation where the side chains of the protein are fixed throughout docking simulation [17]. Despite its recognition for being a fast and efficient method, majority of the docked conformations sampled seem to exhibit unfavourable side chain interactions except for complementary structures. Thus, it highlights the importance of side chains refinement to improve the accuracy in modelling the surface residues involved in protein interactions. The refinement can also ensure more accurate binding, allowing better understanding of protein-protein interactions [107]. Examples of common refinement methods are torsion space minimisation which had been incorporated in the docking software (RosettaDock) and simple minimisation algorithms such as conjugated gradient or steepest decedent [106, 115]. However, MD simulation would be preferable for the purpose of understanding the protein-protein interaction as well as studying their dynamics as it refines complexes from a theoretical model or experimentally solved structures.

11.4.3.1 Molecular Dynamics (MD) Simulation

MD simulation is a computational simulation which explores the molecular behaviour of microscopic properties in a system and the molecular conformational changes with the function of time. The exploration of simulation implements classical Newton second law of motion equation, $F = ma$, which requires the mass of a particle m and force F acting on the particle to calculate the acceleration a. Integration of the equation produces trajectory that show changes in the position and velocity of particle in the system within a given time. The equation can also be expressed as $\frac{d^2 x_i}{dt^2} = \frac{Fx_i}{m_i}$ where it describes a particle with

mass m_i at a coordinate x_i with force Fx_i that is acted on the particle. Fx_i can also be derived from the gradient of potential energy of a coordinate x_i, $Fxi = -\frac{dU}{dxi}$ where U is the potential energy.

In a relatively large system such as a biomolecule, the potential energy can be calculated using molecular mechanics (MM; also known as empirical force field or force field) method with the approximation of short-range energy terms and long-range energy terms ($E_{MM}= E_{bonded} + E_{non-bonded}$). Short-range energy terms (also known as bonded terms or intra-molecular forces) are contributed by the bond stretching energy (harmonic potential or Hooke's Law; $\sum_{bonds} \frac{k_\lambda}{2}\left[l_i - l_{i,0}\right]^2$ where k_λ is the force constant, l_i is the bond length from the reference value $l_{i,0}$), angle bending (harmonic potential or Hooke's Law; $\sum_{angles} \frac{k_\theta}{2}\left[\theta_i - \theta_{i,0}\right]^2$ where k_θ is the force constant, θ_i is the angle between atom A, B and C from the reference value $\theta_{i,0}$), dihedral/torsional energy ($\sum_{torsions} \frac{k_n}{2}\left[1+\cos\left(n\omega - \gamma\right)\right]$ where k_n is the force constant, n is the multiplicity, ω is the torsion angle, γ is the phase factor between atom A, B and C from the reference value $\theta_{i,0}$) and improper torsion energy ($\sum_{improper} k\tau \frac{k_n}{2}\left[1-\cos 2É\right]$ where $k\tau$ is the improper torsion potential and ω is the improper dihedral angle). Long-range energy terms (also known as non-bonded terms or intermolecular forces) are calculated between pairs of atom (i and j) where i and j are in different molecules or within the same molecule but separated by at least three bonds. It includes van der Waals (vdW) interactions (usually calculated by Lennard-Jones 12-6 potential; $E_{vdw} = 4\varepsilon_{ij}\left[\left(\frac{\sigma_{ij}}{r_{ij}}\right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}}\right)^{6}\right]$ where ε_{ij} is the dispersion well depth, σ_{ij} is the Lennard-Jones distance and r is the distance) and electrostatic interactions (usually calculated by Coulombic potential; $E_{electrostatic} = \frac{q_i q_j}{\varepsilon r_{ij}}$ where q_i and q_j are the atomic partial charges for atom i and atom j, ε is the dielectric constant, r_{ij} is the distance between atom i and atom j).

For a biological system, the conventional force fields includes Assisted Model Building with Energy Refinement (AMBER), Chemistry at HARvard Macromolecular Mechanics (CHARMM) and GROningen MOlecular Simulation (GROMOS). The trajectories of a system i.e. antibody-antigen complex can be computed by approximating the potential energy, velocity, and acceleration of atoms at a specific time given. In general MD simulation, the process starts with a 3D structure of antigen-antibody complex. Once the complex is well equilibrated, the production stage produces trajectory of the system relative to its time scale which can be later used to study the system dynamics and is subjected to further analysis. From the trajectory, the thermodynamic properties of the system can be determined and predicts the conformational changes of the system with the function of time. The goal of classical MD simulation serves to understand the properties of the molecular structure in microscopic interaction by measuring the thermodynamics of the system but not the chemical reaction (e.g. bond breaking/forming; proton/electron transfer). Besides, MD simulation also benefits by calculating the binding free energy of a system i.e. antibody-antigen complex. For instance, MD simulation was performed on the crystal structure of Ebola viral glycoproteins bound to antibodies found in resistant individuals [33]. Calculated energetics and RMSD terms have identified high interaction with the viral glycoprotein. This finding thus allowed the development of computational flow in general antibody design against Ebola virus.

11.4.3.2 Ensemble Docking

Ensemble docking has been developed to dock multiple antibody structures to its target antigen in a single docking run [95]. Due to uncertainties (CDR loop conformation, V_H-V_L orientation, CDR backbone and side chain conformations) in an antibody model, multiple representations of the antibody model can help to improve accuracy in docking predictions. It is also discovered that ensemble docking can accommodate conformational plasticity. Docking of an antigen simultaneously with an ensemble of antibody homology models was found to surpassed the docking of a single homology model in recovering near-native docking solutions [95]. This theory emphasises on antibody flexibility and treats the antigen as a rigid body when it is a known ligand. Subsequently, scoring function is used to scrutinise the list of predicted docking orientations and identifies the best binding pose.

To study antibody flexibility, a collection of multiple CDR-loop conformations, side-chain conformations and V_H-V_L orientations is needed to represent a flexible receptor in antibody design. There are numerous methods to obtain conformational ensembles, such as X-ray crystallography, NMR spectroscopy, clustered MD snapshots, normal mode analysis or principle component analysis of MD trajectories [16, 69]. For instance, RosettaDock uses homology modelling to allow an induce fit of the antigen to multiple CDR loop conformations of an antibody [16]. The predictions would be filtered and only the best docked models are presented. Currently, there are many docking software applicable to ensemble docking. However, there is still plenty of room for improvement of the prediction accuracy.

11.4.4 Analysis

11.4.4.1 Free Energy Calculation: MM-PBSA/ GBSA

Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) [49] and Molecular Mechanics Generalised Born Solvent Area (MM-GBSA) [21] are efficient methods to estimate protein-protein or protein-ligand binding free energy. It calculates the binding free energy of the system by estimating the free energy contributed by the macromolecular complex and its substituents [55], as shown in the following equation:

$$\Delta G_{\text{binding}} = \Delta G_{\text{water(complex)}} - \left[\Delta G_{\text{water(receptor)}} + \Delta G_{\text{water(ligand)}}\right]$$

The binding energy for each species was calculated from the absolute molecular mechanical energies (E_{MM}; discussed in Sect. 4.3.1), the solvation free energy ($G_{PB/GB}$ + $G_{non\text{-}polar}$) and the vibrational, rotational and translational entropies.

$$\Delta G_{\text{water}} = E_{\text{MM}} + \Delta G_{\text{solvation}} - T\Delta S$$

The $-T\Delta S$ represents the conformational entropy changes upon ligand binding. However, the calculation of conformational entropy change is usually omitted due to its high computational cost and its questionable prediction accuracy [40].

The efficient performance of MM-PBSA/GBSA in predicting binding free energies has led to studying antibody-antigen interactions via this method. For example, a MMPBSA study regarding binding of steroids to antiestradiol antibody had shown the binding was driven by vdW interactions whereas the specificity was contributed by electrostatic interactions [53]. The findings might be useful for further optimisations in terms of its binding affinity and specificity. Another study by Lees and colleague also exemplified the reliability of MMGBSA in predicting the impact of single amino acid substitution on the binding energy of antibody-antigen complex [56]. The critical protein interactions between broad spectrum antibodies and influenza A hemagglutinin which give rise to antigenic escape upon amino acid substitution have been successfully identified. Furthermore, good agreement between prediction and experimental results has reinforced the potential of free energy calculation studies in visualising protein interactions and its contribution to protein design.

11.4.4.2 Computational Alanine Scanning Mutagenesis

Computational alanine scanning mutagenesis approach is a common method used to identify hot spot residues that contribute to the antigen-antibody complex formation or to further improve an antibody design. During alanine scanning, alanine is substituted with a selected residue without altering the main-chain conformation or affecting steric effect. It predicts the differences of binding free energy between wild type and mutant protein complexes [57]. The combination of MM-PBSA/GBSA enables evaluation on binding free energies of wild type and its mutant in terms of the degree of increment or decrement in binding free energy. It helps in determining the importance of the side chain that contributed to overall binding free energy. In general, residues that display free binding energy difference exceeds 2 kcal/mol are known as hot-spot residues [72]. In the study of scFv anti-p17 with HIV-1 p17 epitope variants, alanine scanning was used as one of the methods to detect the hot-spot residues [109]. It was found that Met100 was the key residue contributed to binding affinity. Additional evidence from enzyme-linked immunosorbent assay (ELISA) experimental data showed that replacement of methionine with arginine/glycine exhibited an increase of electrostatic interactions and improved the binding affinity. Thus, this showed that alanine scanning can provide useful suggestion for site-directed mutagenesis that helps to improve the binding affinity.

11.4.4.3 Pairwise Decomposition

Pairwise decomposition is a technique that calculates the interaction energy between pairs of residues in a protein-protein complex. It decomposes interaction energy between pairs of residues using free energy calculations such as MM-PBSA or MM-GBSA. In the application of antibody-antigen, the CDR plays an important role in binding interactions. Therefore, the energy of the receptor (antibody) and acceptor (antigen) adapt the pairwise additive from electrostatic energy, vdW force and solvation energy to be calculated, then summed up over pairs of interacting residues [35]. A decomposition energy of more than 2 kcal/mol in a specific residue was deemed as an important residue for mutagenesis or potential residue for improvement in its binding affinity. A study showed that five residues in the CDRs of scFv anti-P17 contributed to the binding efficiency on HIV1 epitope at the matrix protein (p17), which actively participates in the HIV life cycle (Lee et al. [55]). Therefore, the identified key CDR residues can be used for further optimisation to improve the binding activities against HIV p17 epitope.

11.5 Antibody Design Approach

The approaches in antibody design can be generally classified into three categories, namely sequence-, structure- and scaffold-based approach.

11.5.1 Sequence-Based Antibody Design

Sequence-based antibody design involves antibody diversity and V(D)J recombination concept (also known as gene rearrangement of antibody during adaptive immune response). This Nobel Prize winning discovery by Tonegawa in year 1987 showed that antibody fragments such as heavy chain, light chain and other fragments are able to “mix and match” to achieve desirable binding to the antigen [104]. V (variable), D (diversity), J (joint) gene segment (also called somatic recombination) exist in the complementarity-determining region (CDRs) or framework undergoes rearrangement in nature for adaptive immune response [51]. Despite the rearrangement (somatic mutation) occurs naturally, the mutation for V(D)J arrangement are now being performed experimentally in order to improve antigen binding affinity and specificity [51]. The mutation can be rational-based, as well as random to produce the diversity of recombination, until a novel mutation is introduced. Once the mutant sequence is obtained, modelling of the antibody (for instance, the CDRs) can be performed and model design can be evaluated

accordingly. Conventionally, V(D)J recombination in laboratory is carried out using V(D)J recombinase. With computational advancement, V(D)J recombination is now aided by computational method to perform analysis tasks such as identifying the V, D, J gene segment, aligning sequence, trimming overlapping genes, introducing point mutation and n-nucleotide [113]. Therefore, potential gene arrangement can be hypothesised in order to minimise the failure counts in laboratory experiments. After obtaining the hypothesised antibody sequence, the antibody structure will only be predicted, hence sequence-based antibody design.

11.5.2 Structure-Based Antibody Design

Structure-based antibody design refers to *in silico* structure prediction together with other molecular modelling applications. The framework region of an antibody is highly conserved and can be modelled with high accuracy. However, the six CDRs would require more effort due to their high variability in sequence and structure. Of all the CDRs, CDR-H3 has the highest conformational variety due to its role in antigen recognition [51, 105]. Hence, enormous works were found focusing on CDR-H3 structure prediction [52, 92].

After the antibody structure is constructed, it is crucial to evaluate the structure to ensure the confidence level. Although there are a variety of existing online servers and tools for structural evaluation, the three main evaluation methods commonly used include backbone conformation (e.g. ERRAT) [24], 3D structure assessment (e.g. VERIFY3D) [29] and non-bonded atomic interaction (e.g. Ramachandran plot) [85]. These evaluations will score the confidence level of the predicted model compared to the experimentally solved structure. If the scores are below threshold, refinement needs to be done via approaches such as molecular docking (Sect. 4.2.2), molecular dynamics simulation (Sect. 4.3.1) and *in silico* mutagenesis (Sect. 4.4.2).

Molecular docking (Sect. 4.2.2) indirectly contributes to antibody design improvement by evaluating the reliability of the model. The reliability of an antibody model depends on its ability to bind or dock respectively to its antigen. In short, an antibody with an excellent score in the structure evaluation will not be useful if it is not reliable. Therefore, scoring function is used to predict binding energy of the docked conformations, further acknowledging the quality of predicted structure.

Another alternative to improve structure quality is via MD simulation. The fundamental concept regarded protein as a dynamic molecule that could have an ensemble of conformations. MD simulation can further refine a predicted structure and ensures the structure is close to the native structure (equilibrium) prior to analysis [32, 39]. The generation of ensemble conformations with MD simulation is able to address the flexibility of the structure. By clustering the ensemble conformations, it will result in a good dominant structure with an improved score in its structure evaluation. With these ensemble structures, ensemble docking (Sect. 4.3.2) can thus be performed.

The last approach to improve the antibody design is by introducing *in silico* mutagenesis. Unless a knowledge-based approach is applied to mutate certain residues, random mutagenesis will be performed otherwise. Computational alanine scanning (Sect. 4.4.2) replaces a residue with alanine to evaluate the binding free energy of the mutant. A list of hotspot residues will be generated after the energy evaluation. By manipulating the hotspot residues to improve the antibody quality, the objective of design is achieved [50].

11.5.3 Scaffold-Based Antibody Design

Scaffold-based antibody design is a rather new approach introduced around 2010's. It was inspired by the HIV vaccine research community to address the weak binding of HIV epitope and anti-HIV antibody [25, 78]. In the approach, an

antibody is elicited by the scaffold of antigen epitope. The known antigen epitope is screened thoroughly in PDB or any scaffold database for non-antibody binding complex to search for a scaffold protein that is able to bind to the epitope. The search can be sequence- or structure-based, depending on the certain unique sequence of the scaffold protein structural motif that is matching with the epitope. Subsequently, scaffold protein that matches the antibody will be performed to eliminate steric clashes. With the resultant scaffold protein, the antibody will undergo a series of paratope modifications in order to improve its binding affinity towards that generated scaffold. Thus, the modified antibody will have better binding towards the antigen epitope. For current therapeutic application, this approach is likely applicable to vaccine development.

11.6 Current Trends in Antibody Design: Recently Reported Success Stories

11.6.1 HCDR3 Loop

Potency and breadth of HIV antibodies had been successfully optimised by carrying out single point mutations within HCDR3 loop [116]. Mutations in HCDR3 loop had been painstakingly designed in a way that would not significantly alter the paratope surface of HCDR3 compared to wild type. Five favourable candidate antibody variants have been identified from a large pool of mutated antibodies using Rosetta software suite. Validation of these variants via neutralisation assay in the lab had shown encouraging results for two of the tested variants. A further dissection on these enhanced antibody variants suggested that increased loop thermostability might play a role in it.

11.6.2 V_H-V_L Orientation

Tandem scFv bispecific antibodies (BsAbs) were constructed in laboratory from a monovalent single chain fragment (scFv) platform that specifically target disialoganglioside (GD2) on tumor cells and CD3 on T cells [19]. A few parameters such as disulphide bonds, V_H-V_L orientation and linker length had been tested for their effects on BsAb. From the experimental results, it was discovered that additional disulphide bonds, V_H-V_L orientation with 15-residue GS linker exhibited stronger binding. To further investigate the interactions that contributed to strong binding, they employed comparative modelling to build structures of BsAbs with different designs. As a result, two key residues involved in the binding interaction had been identified and was further confirmed from docking and MD simulations. This showed that computational findings can serve as supplementary guidance on the design of BsAb for T-cell mediated therapy.

11.6.3 CDR-H3 and CDR-L3

Although CDR-H3 is crucial for affinity maturation mutations, modifications of other CDR loops have been reported to enhance the affinity of the antibody. An *in silico* approach combined with *in vitro* experiment has been carried out to optimise the affinity maturation of antibodies against gastrin [8]. After *in vitro* mutagenesis on both CDR-H3 and CDR-L3 loops, computational modelling was used to construct the two scFvs. It was followed by MD-based docking with gastrin, in order to identify the specific sites for mutagenesis in CDR-H3 and CDR-L3. It has been reported that a final 454-fold improvement was found in the antibody affinity maturation compared with the initial structure. The improvement of antibody construct unveils its potential as a candidate for therapeutic use, particularly in pancreatic and gastric cancer studies.

11.6.4 The Modelling of scFv

ScFv is a popular format because it can be expressed in bacteria such as *Escherichia coli* as a single protein and can also be expressed using

phage display method. Many studies are directed at scFv modelling to study the interactions between antibody and antigen, such as anti-amoxicillin scFv towards peniclin [68], and mouse scFv against fumonisin B1 [41]. In the fumonisin B1 antibody study, the conformation of scFv towards the antigen was predicted [41]. The results showed that more favourable binding with improved hydrogen bonding, electrostatic interaction and hydrophobic interaction with its antigen.

11.6.5 CDR Grafting for Intrinsically Disordered Proteins

CDR grafting is a structure-based antibody design technique used to improve the binding affinity and specificity of mAb CDRs towards the antigen. Besides improving the specificity and affinity towards the antigen, this technique also aims to reduce mAb immunogenicity in the human body. The CDR grafting technique has been applied to improve the antibody against intrinsically disordered proteins involved in Alzheimer's disease, Parkinson disease, and type II diabetes [97]. These intrinsically disordered proteins play important roles in biochemical processes in living organisms due to the functional diversity provided by the disordered regions. These intrinsically disordered proteins are characterised to be highly flexible and lack of secondary structure and tertiary structures, thus allowing multiple interactions with multiple biological components. Once the regulatory processes failed to correct the intrinsically disordered protein, different pathological conditions may arise.

In the aforementioned research, the sequence-based method was applied to identify the epitopes from Aβ peptide, α-synuclein and islet amyloid polypeptide (Alzheimer's & Parkinson and type II diabetes related peptides). The designed complementary peptides were then crafted in place on the CDR loops of antibody scaffold, followed by some mutations on the CDR to improve the binding affinity. The results showed that designed antibodies can bind specifically and with higher affinity to the target antigen.

11.6.6 Fc Variant

The Fc region of an antibody will bind with Fcγ receptors (FcγRs) for cellular cytotoxic effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP). Of all three types of FcγRs, not all can bind to Fc region with good affinity. A variant of IgG-Fc region has been produced from available structures of human IgG-Fc bound with FcγRs and predicted homology models [54]. In the study, variable residues at the Fc region was mutated to generate a list of Fc variants via *in silico* approach. Subsequently, Protein Design Automation (PDA) algorithm was used to evaluate energy of the variants. A total of 1000 Fc variants resulted from PDA calculation were then subjected for Sequence Prediction Algorithm (SPA) to obtain 300 sequences of Fc variant. After subsequent design with information from structure-activity relationship (SAR), more than 200 sequences of Fc variant were subjected for experimental evaluation. The *in vivo* evaluation of these Fc variants showed significant improvement in their cytotoxic effector functions. This showed that computational design is also applicable to the Fc region of an antibody.

11.7 Concluding Remarks

11.7.1 Challenges & Limitations

Instead of generating a totally new and full antibody via *in silico* approaches which can be computationally expensive, there is a growing trend towards designing only the important regions such as the CDRs, V_H-V_L orientation, CDR-H3/L3, scFv and Fc (Sect. 6). The prediction accuracy is very particular at the discovery and modelling stage whereas binding affinity optimisation focuses for the refinement stage. All these have contributed to the ultimate quality attributed to the antibody, i.e. the antibody stability. As a potential therapeutic drug, an antibody needs to be stable to overcome challenges such as high

concentration delivery into a host and long term storage [103].

In molecular modelling, the variability of CDRs regions causes antibody structure prediction task to be challenging. If the length of H3 loop exceeds its average length, the modelling process will turn out to be complicated as it does not comply to a typical loop construction benchmark [91]. Furthermore, the conformation at CDR3 region is dependent on the presence of antigen [91]. In this case, *ab initio* approach is usually applied. Despite *ab initio* design is aided by various algorithms and servers, accuracy of the prediction is still a concern. As mentioned earlier (Sect. 4.1.2), successful prediction for the H3 loop is dependent on its surrounding conformation, the presence of antigen, and templates available to detect the lowest energy conformation. Therefore, current sampling of conformational space can be explored for further optimisation.

The protein flexibility in docking simulation remains a problem to be solved due to its high degree of freedom in the structure and the limitation of computational tools [43]. Besides, it is challenging to simulate the backbone movements that occur upon the formation of the antigen-antibody complex. This is because the degrees of freedom present in the backbone loop regions, makes it difficult to effectively calculate the score of potential stable conformations [81]. In addition, the sufficient number of conformation in acceptor and receptor, i.e. antigen and antibody, that needed to be explored in a docking calculation is still a subjective question, where a standard number has not been established [70].

MD simulation explores favourable binding conformation in explicit water environment and also the dynamic of both antibody and antigen. The current computational power enables MD simulation to be performed from picoseconds to microseconds, describing the biological system at detailed atomic level. In spite of that, it does not truly resemble the biological process inherent to protein dimerisation. Close mimic of the folding process requires higher computational power. Thus, computer aided antibody design would definitely benefit a lot from advanced computational methods. Coarse-grained system [66] and new program such as GENeralized-Ensemble Simulation System (GENESIS) [47] are introduced to help understand a large biological system containing millions of atoms. Although coarse-grained MD simulation emerges as a promising solution, its accuracy pale in comparison to conventional MD simulation. In addition, obtaining a near native conformation can be challenging and costly. Another challenge is the accuracy and efficiency of the scoring function to calculate and identify the near-native conformation of antibody with antigen. The calculated binding free energy of optimised antibody need to be equal or better than the experimental binding assay, that is within factor of two (~0.4 kcal/mol) [12]. Thus, the free energy calculation still requires improvement in sampling configuration space and force field.

Antibody design approaches as mentioned earlier can be either sequence-, structure- or scaffold-based (Sect. 5). The sequence-based approach involved V(D)J recombination will produce a repertoire of antibody gene sequence awaiting analysis. Problems usually occurs during clustering of the antibody repertoire due to a lack of general standardisation for clustering as it is case dependent in its own custom-made pipeline [36]. On the other hand, structure-based approach faces challenges involving difficulty in obtaining template for modelling. In general, a complete antibody is too large for NMR spectroscopy. Hence, X-ray crystallography is till the main method to obtain the experimental conformation of antigen.

Although intensive research has been done towards *in silico* antibody design, there are still numerous challenges to be solved. These challenges will be the motivation for researchers to improve currently available computational tools in various aspects. *In silico* antibody design is useful as it saves time and cost. No doubt, *in silico* approach has proven to be able to aid in the antibody improvement which can be applied in diagnostic or therapeutic fields. Although the resultant data from computational work need to

be validated experimentally, it can still cut down massive experimental cost. However, we need to bear in mind that no matter how powerful a computer can be, the accuracy of the results can only be as accurate as the data entered into it.

11.7.2 Future Prospects

Existing computational prediction methods provide good opportunity to study antibodies that lack experimental data. Although the framework regions of antibody can be predicted accurately, precision in predicting CDRs is needed. Thus, existing structural database for the antibody such as Structural Antibody Database (SAbDab) [27] provides ensemble of experimentally derived antibody structures to be downloaded for analysis and have their structural data be compared with query sequence.

In addition, the design of antibody as therapeutic drug depends on its target antigen. Screening of antigen with the designed antibody is a great challenge. In laboratory, screening of antigen often involves high experimental cost, thus drives the popularity for *in silico* screening. The demand of antigen structure for screening purpose has brought out many antigen databases such as AntigenDB [7], Immune Epitope Database (IEDB) [112] and etc. These databases have contributed to the progress in antibody design by narrowing down the search for antigen. Even though numerous computational tools are available for antibody design, the improvement especially in terms of efficiency and reliability are still needed.

Although *in silico* approaches in antibody designing have proven to be applicable in numerous experiments, improvements in various aspects such as algorithms, scoring functions, databases and benchmarking tools are vital to be focused in future research. These improvements will solve current limitations and challenges in computational antibody designing thus making *in silico* approach a better tool in developing new therapeutic antibodies.

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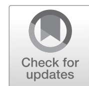

12 Using High-Throughput Sequencing to Characterize the Development of the Antibody Repertoire During Infections: A Case Study of HIV-1

Felix Breden and Corey T. Watson

Abstract

High throughput sequencing (HTS) approaches have only recently been applied to describing the antibody/B-cell repertoire in fine detail, but these data sets have already become critical to the design of vaccines and therapeutics, and monitoring of cancer immunotherapy. As a case study, we describe the potential and present limitations of HTS studies of the Ab repertoire during infection with HIV-1. Most of the present studies restrict their analyses to lineages of specific bnAbs. We discuss future initiatives to expand this type of analysis to more complete repertoires and to improve comparing and sharing of these Ab repertoire data across studies and institutions.

Keywords

Antibody repertoire · High throughput sequencing · HIV-1 · Broadly neutralizaing antibodies

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12.1 Introduction

B lymphocytes are key players in the adaptive immune response, and in concert with other cells of the immune system, are capable of directing the recognition of and response against a vast array of pathogens [43]. The critical functions of B cells are made possible by the expression of diverse proteins called immunoglobulins (IG), expressed either as membrane-bound cell surface B cell receptors (BCRs) or secreted antibodies (Abs), each comprised of two identical heavy and two identical light chains. Mirroring the diversity observed among the potential pathogens and related epitopes that could be encountered during a lifetime, the amino acid diversity of the Abs produced by an individual (i.e., the Ab repertoire) is astounding, with theoretical estimates exceeding 10^{15} unique Abs in a given repertoire [54]. The diversity observed in the naïve Ab repertoire is the consequence of unique molecular processes, beginning with combinatorial diversity that arises from the somatic "V(D)J" recombination of hundreds of germline heavy and light chain variable (V), diversity (D; heavy chain only), and joining (J) gene segments across three primary loci in the human genome, accompanied by additional junctional variation introduced via the addition of nucleotides at the junctions of V, D, and J gene segments, and the random pairing of heavy and light chains [43, 51, 63]. Additional diversity is later added to the repertoire following antigen stimulation via somatic hypermutation (SHM).

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Effectively and comprehensively capturing the diversity of the Ab repertoire has historically been a challenging endeavor. However, the recent application of high-throughput sequencing (HTS) to Ab repertoire profiling has begun to provide great insight into the dynamic nature of the Ab response [20]. With these methods, generally referred to as "RepSeq", repertoire-wide profiling of Ab variable domains (and in some cases including constant domain isotype-level information) can be conducted in depth at either the level of rearranged genomic DNA or mRNA, and can be done across millions of cells in bulk, or at the single cell level [67]. In addition, accompanying these technologies has come a surge of computational methods for analyzing such HTS data [79], allowing for complex modeling of the Ab repertoire and in depth descriptions of a range of repertoire features (e.g., IG gene segment usage; complementarity determining region (CDR) length and diversity; SHM patterns; clonal expansion). Over the past several years, these approaches have been applied in many disease and clinical contexts and across cell subsets and time points, clearly establishing that Ab repertoires can be influenced by many factors, including age, infection history, disease status, genetic background, and treatment/prophylactics [18, 21, 23, 39, 47, 50, 61, 64, 66, 77, 81]. In summary, HTS-based analyses of immune repertoires have enormous promise for understanding repertoire dynamics in immunology, vaccinology, infectious diseases, autoimmunity, and cancer biology.

In this review, we present the results of the use of HTS of antibody repertoires in the development of the adaptive immune response to HIV-1, to illustrate the importance and utility of this method. We summarize some general conclusions from such studies, but also some significant limitations, especially related to the analysis of only a limited section of the complete Ab repertoire, typically in few individuals. Finally, we review new trends in adaptive immune repertoire analysis that may provide opportunity for overcoming these limitations.

12.2 Characterizing the Development of Ab Repertoires During HIV-1 Infection

12.2.1 General Questions and Importance of HTS Repertoire Studies

Studies of the development of Ab repertoires in health and disease have been dominated by those targeting HIV-1 Abs. This is due to several circumstances, including disproportionate funding rates and the obvious immediate application of such studies to vaccine development. These studies of Ab repertoires in HIV-1 infection provide an excellent example of the power of using HTS for studying Ab repertoires, and we therefore have chosen to focus on this set of studies in our review.

At least two related questions have substantially motivated the studies of Ab repertoires during HIV-1 infection. The first question concentrates on the characteristics of anti-HIV-1 broadly neutralizing Abs (bnAbs), which an effective HIV-1 vaccine would presumably have to elicit in naïve individuals. Much has been made of the observation that the sequences of most anti-HIV-1 bnAbs are highly somatically mutated, encode long (≥20 aa) heavy chain CDR3 (CDR-H3) sequences, and are poly- or auto-reactive ([19, 40]; but see [10]). This observation led some researchers to suggest that one explanation for the rarity of bnAbs against HIV-1 is that the individual must "break tolerance" to produce these bnAbs [24]. However, all bnAbs against HIV-1 don't have these properties, and to what extent these features are required for the acquisition of neutralization breadth and potency of bnAbs remains poorly understood. The application of HTS to probe the Ab repertoire opened the possibility of describing the characteristics of bnAbs against HIV-1 in fine detail, and this remains one of the strongest motivations for these studies.

The second question motivating HTS Ab repertoire studies in HIV-1 focuses on the timing or ontogeny of the development of the broadly-neutralizing response during infection. It is well established that most HIV-1-infected individuals produce strong strain-specific bnAb responses against HIV-1 envelope (Env) soon after initial infection; yet only a small percentage of infected individuals develop broad neutralization, and then only after a year or more [22, 57, 58]. The "B-cell-lineage immunogen design" strategy for anti-HIV-1 vaccine development attempts to understand the timing and action of immunogens that led to the development of anti-HIV-1 bnAbs, and then induce such bnAbs in naïve individuals [5, 25]. This "directed evolution" approach to rational immunogen design relies on identifying clonal lineages that have led to bnAbs in order to infer their common unmutated ancestors (putative naïve B cell precursor) and intermediate sequences within the Ab clonal lineages. For a given Ab lineage, once the Ab sequences of the unmutated ancestor and intermediates along the developmental lineage have been identified, it is then possible to identify epitopes to which these Abs bind. Finally, this points to possible immunogens that might be used to drive the Ab response along specific, predetermined "evolutionary" pathways. The goal is to recapitulate these lineages in naïve individuals, and increase the chance that these naïve individuals will ultimately produce bnAbs themselves. This approach has dominated HIV-1 vaccine research for several years, and explains why many of the studies concentrate on only specific lineages, targeted to known bnAbs within the broad Ab response ("targeted" lineages). This is in contrast to studies that would attempt to capture the complete Ab milieu during infection, *i.e.*, all of the dominant Ab lineages present in the repertoire. In this review we will discuss how this emphasis on studying particular targeted lineages has shaped HIV-1 Ab repertoire research, and what advantages a broader approach examining multiple lineages might provide.

12.2.2 Survey of Studies of the Ab Response During HIV-1 Infection Using HTS

In this section, we review those studies that use HTS to characterize the anti-HIV-1 Ab response. We include only those studies that report a response to HIV-1 infection, rather than that induced by vaccination. Most of the bnAbs known to target HIV-1 bind to a small set of epitopes, and we organize the studies according to these regions (summarized in Table 12.1).

12.2.2.1 CD4 Binding Site (CD4bs)

The largest set of studies using HTS to explore antibody production and development in response to HIV-1 infection are those that target the CD4 binding site (CD4bs); one of the earliest studies of this type was by Wu et al. [76]. In this study, PBMC from 3 donors from the international AIDS-vaccine initiative (IAVI) protocol G were isolated and subjected to HTS. This set included Donor 45, from whom bnAbs VRC01, VRC02 and VRC03 had been originally isolated. These sequences, especially VRC01, were subjects of intense interest, given their ability to neutralize up to 90% of HIV-1 isolates [75].

This seminal study used several filters to exclude all Abs except those related to the neutralizing mAbs VRC01, VRC02, VRC03 and VRC-PG04. First, only antibodies utilizing the IGHV1 gene family were amplified and sequenced, and only those using the *IGHV1-2* gene were further analyzed. A second filter was applied that only accepted sequences that formed a cluster of sequences defined by divergence from the germline *IGHV1-2* gene in one dimension and divergence from the template antibodies VRC01 and VRC03 in the second dimension. This is a prime example of the approach used to restrict analysis only to clusters of antibodies that are related to a "target" antibody, in contrast to analysis techniques that attempt to study the entire Ab repertoire. Finally, the authors used cross-donor phylogenetic analysis, a general

Table 12.1 Survey of studies of the Ab response during HIV-1infection using HTS, organized primarily by epitope

HIV Epitope	Samples	Age	Time course	Total individuals	Total repertories	Nucleic acid	Sequencing method	PBMC/B cell subsets	Chain/ isotypes	Lineage analysis	Link for HTS data deposition	References
CD4bs	Donors 45 & 74 IAVI Protocol G, Donor 0219 CHAVI Cohort 001	Adult	Single time point	3	3	mRNA ➔ cDNA	454-Roche	PBMC	Heavy and light chains	Cross donor phylogenetic analysis targeted to VRC01	SRA SRP006992	[76]
CD4bs	Donor CH505 CHAVI Cohort 001	Adult	Multiple time points	1	5	mRNA ➔ cDNA	454-Roche	PBMC	Heavy and light chains	Targeted to CH103 clonal lineage (CH103-CH106)	NA	[36]
CD4bs	Primary results from C38	Adult	Single time point	1	1	mRNA ➔ cDNA	454-Roche	PBMC; IgG and IgM	Heavy and light chains	Cross donor phylogenetic analysis targeted to VRC01-like antibodies	SRA SRP026397	[85]
CD4bs	NIAD 43; IAVI 23, 57, 74; RU 3	Adult	Single time point	5	5	mRNA ➔ cDNA	454-Roche	PBMC	Light chains only	Targeted to VRC01-like antibodies; gene specific amplification	SRA SRA072279	[82]
CD4bs	Donor 45 IAVI Protocol G	Adult	Multiple time points	1	10	mRNA ➔ cDNA	454-Roche	PBMC; IgG+ B Cells	Heavy and light chains	Cross donor phylogenetic analysis targeted to VRC01	SRA SRP052625	[77]
CD4bs	RU01, RU08, 44 and C38 from NIAID protocols (new repertoires)	Adult	Single time point	4	4	mRNA ➔ cDNA	454-Roche	PBMC	Heavy chains	Cross-donor phylogenetic analysis targeted to anti-CD4bs Abs	SRA SRP055520	[83]

CD4bs	Donor CH505 CHAVI001 acute HIV-1 infection cohort, source of CH103 class Abs	Adult	Multiple time points	1	15	gDNA	454-Roche	PBMC	Heavy chains	Cloanalyst analysis targeted to CH235 lineage	SRA SRP067168	[6]
	HIV-1 Hepatitis C negative individuals LP32647, LP08248 and LP23810											
CD4bs	Z258 from NIAID protocol	Adult	Single time point	1	1	mRNA ➔ cDNA	Illumina MiSeq	PBMC memory B cells	Heavy and light chains	Targeted analysis to N6 lineage	SRA: SRR4417615–SRR4417632	[30]
V1 V2	1- Donor CAP256	Adult	Multiple time points	1	6	mRNA ➔ cDNA	454-Roche/ MiSeq (paired VH/VL)	PBMC	Heavy and light chains	Intra-donor phylogenetic analysis targeted to CAP256-VRC26	SRA SRP034555 & SRP017087	[13]
V1 V2	1- Donor CAP256	Adult	Multiple time points	1	6	mRNA ➔ cDNA	454-Roche	PBMC	Heavy chains	Targeted to CAP256-VRC26	SRA SRR2126754 SRR2126755	[4]
V1 V2	1- Donor CAP256	Adult	Multiple time points	1	6	mRNA ➔ cDNA	454-Roche	PBMC	Heavy and light chains	Targeted to CAP256-VRC26	SRA SRR2126754 SRR2126755	[14]
MPER	2 - Donors N152 and IAVI 84	Adult	Single time point	2	2	mRNA ➔ cDNA	454-Roche	PBMC	Heavy and light chains	Targeted bioinformatic sieving	SRA SRP018335	[85]
MPER	1-Donor N152	Adult	Single time point	1	1	mRNA➔ cDNA	454-Roche	PBMC	Heavy and light chains	Targeted bioinformatic sieving	SRA SRP018335	[60]

(continued)

Table 12.1 (continued)

HIV Epitope	Samples	Age	Time course	Total individuals	Total repertories	Nucleic acid	Sequencing method	PBMC/B cell subsets	Chain/ isotypes	Lineage analysis	Link for HTS data deposition	References
N332 region	1 - Donor 17 IAVI Protocol G	Adult	Single time point	1	1	mRNA → cDNA	454-Roche	PBMC	Heavy and light; IgG+ memory B-cells	Gene-specific amplification targeted to PGT 121–123	NA	[59]
N332 region	Donor 17 IAVI Protocol G	Adult	Single time point	3	8	mRNA → cDNA	Ion Torrent PGM sequencing	PBMC	Heavy and light chains	Targeted to 6 PGT 121-class mAbs & full repertoire	NA	[26]
N332 region	Donor 39 IAVI Protocol G	Adult	Single time point	1	1	mRNA → cDNA	454-Roche	PBMC	Heavy and light chains	Targeted to PGT 135–137	SRA SRA055820	[84]
N332 region	Donor PC76 IAVI Protocol C	Adult	Multiple time points	1	1	mRNA → cDNA	Illumina MiSeq	PBMC or IgG+ memory B cells	Heavy and light chains	Targeted to panel of PCDN mAbs	SRA PRJNA: 304,070	[38]
Non-targeted analysis of HIV infection	HIV-1 infected patient	Adult/ neonates	Multiple time points	8 (HIV+)	Pooled Libraries	mRNA → cDNA	454-Roche	IgM and IgG from PBMC and bone marrow	Heavy chains	Complete repertoire	NA	[78]
Non-targeted analysis of HIV infection	8 HIV-1 infected patients; 3 untreated, 5 treated	Adults	Up to 8 time points	8	48	mRNA → cDNA	Illumina MiSeq	PBMC	Heavy chains	Complete repertoire	ENA: ERP000572	[27]
Non-targeted analysis of HIV infection and SLE	4 controls; 4 SLE; 4 HIV+ treated; 4 HIV+ untreated	Adults	Single time point	16	16	mRNA → cDNA	454-Roche	PBMC	Heavy chains	Complete repertoire	NA	[81]

technique used in several similar studies, to further characterize the Ab repertoire. In this instance, similar antibodies from 2 other donors were added to the filtered antibodies from the primary donor, Donor 45, and a tree was constructed from these Ab sequences. A node on the tree was identified that included all VRC01 sequences from these 3 donors, and then all Ab sequences nested within that subtree emanating from that node were used in the final analysis. From studying this final set of Abs, the authors concluded that VRC01-like antibodies from all three donors exhibited similar developmental pathways, in terms of rates of diversification and other parameters. Their functional relatedness was confirmed by the observation that complementation of these identified heavy chains with standard VRC01 light chains from different donors produced neutralization.

Liao et al. [36] also examined anti-CD4bs Abs, and produced a major advance by sequencing both the Ab response to HIV-1, as well as the HIV-1 clonal lineages that were evolving in response to the Ab repertoire. They concentrated their study on one donor, CH505, the source of the VRC01-class bnAb CH103 ("VRC01-class" refers to Abs with similar properties to the canonical VRC01). By sequencing both Ab and HIV-1 lineages, Liao and colleagues were able to infer the co-evolution of the virus with the Abs, as the virus mutated to escape control by the Abs, and the Abs mutated to bind to the changing viral strains. The reconstruction of the CH103 lineage made it possible to infer the sequence of its unmutated common ancestor, and then it was shown that this ancestral sequence was able to bind transmitted/founder HIV-1 envelope glycoprotein. Furthermore, the Ab response of lineage CH103 broadened through time, following viral diversification of the CH103 binding site. This reconstruction of the evolution of the Ab lineage leading to the production of a bnAb is critical to the B-cell-lineage immunogen design strategy of vaccine development, as described in Sect. 12.2.1.

Zhou et al. [82] applied the HTS approach to multiple donors, again only targeting lineages based on VRC01-class bnAbs. They found that VRC01-class antibodies were obtained in multiple donors, characterized by similar maturation pathways and structural solutions to binding. This supported the hypothesis that the development of bnAbs such as the VRC01 class could be elicited in a large proportion of naïve individuals through the use of the proper immunogens (*i.e.*, the B-cell-lineage immunogen design strategy).

Based on several studies, VRC01-class bnAbs have been characterized by the use of the *IGHV1–2* gene for the heavy chain and a light chain with a 5-amino acid third complementary-determining region (CDR-L3). Several other recent studies have used HTS to help identify and describe the development of this class of antibodies in other donors. For example, Zhu et al. [85] discovered several Abs in this class from donor C38. Although many of the Abs from this study did not exhibit high sequence homology to other VRC01 class Abs, when reconstituted with VRC01 light chains, most of these new VRC01-class Abs showed substantial neutralization of HIV-1 isolates. Huang et al. [30] also found VRC01 class antibodies in a previously unstudied individual, Z258, from the NIAID protocol. Strikingly, the N6 Ab from this donor, in particular, neutralized 98% of HIV-1 isolates, including several strains that were not neutralized by previously discovered VRC01-class Abs

As a final example, Gao et al. [19] used HTS to discover that the donor who produced the bnAb CH103 [36] also produced a second bnAb, CH235. HTS of HIV-1 isolates and of the Ab repertoire from multiple time points during this individual's infection revealed the coevolution of the virus and the Ab response in fine detail. The authors hypothesized that CH235 developed first, and that an escape mutation on HIV-1 in response to CH235 facilitated the development of the broadly neutralizing Ab CH103; thus, it was hypothesized that cooperation of the two Ab lineages led to broad neutralization in this donor.

This large set of studies examining broadly neutralizing anti-CD4bs Abs has led to several recent conclusions that may be important for guiding HIV-1 vaccine design. Zhou et al. [83] summarized these studies by concluding that bnAbs against CD4bs could be characterized by

either: (1) the use of particular IGHV genes, usually *IGHV1-2* or *IGHV1-46*, or (2) dominated by particular CDR-H3 sequences. Furthermore, Wu et al. [77] concluded that the development of multiple Ab lineages initially with high mutation rates was associated with the production of bnAbs against HIV-1, but that this mutation rate tended to slow during the development of the expressed anti-HIV-1 repertoire.

12.2.2.2 Variable regions V1 and V2 (V1V2)

HTS studies of the Ab repertoire have also been critical to our understanding of the development of bnAbs that bind to variable regions 1 and 2 (V1V2) of the HIV-1 envelope. One of the most comprehensive analyses of this sort is based on a set of 12 bnAbs that were isolated from donor CAP256 (enrolled with the Centre for the AIDS Programme of Research in South Africa (CAPRISA)), who was infected with a clade C virus; these 12 Abs are referred to as (CAP256-VRC26.01–12), where VRC26 refers to the Ab lineage [13]. This lineage is extraordinary in that it exhibits a 35–37 amino acid long CDR-H3. HTS sequencing of the Ab repertoire from this donor was conducted at eight time points between 15 and 206 weeks post-infection. Targeted lineage analysis of HTS from this donor, using these 12 bnAbs as targets, defined a broad Ab lineage whose unmutated ancestor was able to neutralize the virus that superinfected this individual 15 weeks after initial infection [13]. This result is similar to the inferred history of the lineage leading to the CH103 anti-CD4bs bnAbs, in that the inferred unmutated ancestor bound the HIV-1 target [36]. Inference of the unmutated common ancestor heavy chain sequence supported the hypothesis that this long CDR-H3 region was present in the original B-cell whose descendants ultimately produced these VRC26 bnAbs.

Doria-Rose et al. [14] conducted further studies of the Ab response from this same individual, donor 256. They used microneutralization and single-cell sorting to isolate 21 more bnAbs from this donor, all related to the original VRC26 lineage, referred to as VRC26.13–33. All available HTS data from the Ab repertoire from this individual were used to place the complete set of 33 VRC26 Abs within one phylogenetic lineage (in this case the targeted lineage analysis was based on CDR-H3 sequences, not full Ab sequences). The lineage defined by this targeted approach bifurcated early into two distinct lineages, one of which died out and one of which developed bnAbs. HTS of the viral component in this individual [4] showed that viral escape created multiple immunotypes, some of which were able to tolerate variability at key epitope contacts and thus contribute to this neutralization breadth. This mechanism of developing breadth within one Ab lineage suggests that viral diversification may be commonly associated with broadening of neutralization, and contrasts with the multi-lineage cooperative pathway hypothesized for the development of breadth in some anti-CD4Abs (*e.g.*, [19]).

12.2.2.3 Membrane-Proximal External Region (MPER)

HTS studies of the Ab response against the MPER have concentrated on donor N152, who was the source of one of the most broadly neutralizing anti-HIV-1bnAbs so far discovered, 10^8 [29]. The heavy chain of 10^8 shows a high rate of divergence (21%) from its inferred germline gene. Zhu et al. [85] sequenced heavy and light chains of related Abs separately, and then used a targeted analysis focused on 10^8, to obtain separate pools of heavy and light chain sequences only related to 10^8. Phylogenetic trees were then inferred separately from heavy and light chain sequences. The topologies of these phylogenetic trees were similar, allowing for heavy and light chain sequences to be paired based on these topologies. Abs identified by this topology-based pairing approach had lower auto-reactivity than was exhibited by pairs of sequences drawn without respect to the tree topology. Further sequencing from one time point of the N152 donor produced more Abs related to the 10^8 lineage and allowed for the inference of the unmutated common ancestor, which bound only weakly to the original MPER target [60]. Much of the motivation for the study of 10^8 stemmed from the fact that the inferred intermediate sequences in the

reconstructed lineage might identify potential immunogens for the induction of bnAb 10^8, following the logic of the B-cell-lineage immunogen design strategy.

12.2.2.4 N332 Glycan Supersite in V3 Loop of GP120

Several strongly neutralizing Abs have also been described that target the high mannose patch centered on glycan N332 in the V3 loop of GP120. This site is an important vaccine target, given that passive administration of Abs binding to this region have been shown to prevent infection [42] and significantly decrease the strength of an ongoing infection in non-human primates [2]. HTS was used to analyze the Ab response in donor 17 of IAVI Protocol G [59], from whom the PGT121-class bnAbs that target this region had been isolated. Within this class of Abs, they found a positive correlation between the level of SHM and the development of neutralization breadth and potency. Putative intermediates within this lineage were characterized that showed only approximately half the mutation level of PGT121-134, but were still capable of neutralizing roughly 40–80% of PGT121-134 sensitive viral isolates. Such intermediates characterized by lower SHM are attractive vaccine targets, because they may be more easily elicited by the B-cell-lineage immunogen design approach than highly mutated Abs.

He and colleagues [26] further studied the Ab repertoire from donor 17 and compared this to repertoires from two individuals that were not infected with HIV-1. The Ab repertoires were surveyed in two ways: amplified with gene specific heavy and light chain primers, matching the germline components of PG121-class Abs, and by 5′ RACE. Since 5′ RACE is not based on family- or gene-specific primers, it should produce an estimate of the complete Ab repertoire, in an unbiased manner. Much of the analysis in this study employed intra-donor phylogenetic analysis, targeted on PGT121 Abs. Results from this analysis greatly expanded the known number of Abs in this family, based on Ion-torrent PGM sequencing technology compared to the standard 454 platform. The Ab repertoire results using 5′ RACE and the bioinformatic analysis not targeted to a specific Ab family are included in the next section, which summarizes HTS studies of complete Ab repertoires during HIV-1 infection.

HTS was also used to sequence the Ab repertoire from another donor, 39, of the IAVI protocol G [84]. This individual was the source of the bnAbs PGT135-137, which also target the N332 supersite. In this study, a lineage of Abs related to PGT135-137 was identified by targeted phylogenetic analysis. This apparent lineage exhibited 15 distinct clusters of heavy chain sequences and 10 clusters of light chain sequences; sequences chosen to represent these somatic populations showed diverse neutralization characteristics.

Finally, the most recent study to use HTS data to study Ab responses against this region identified 12 somatically related bnAbs from donor PC76, 16–38 months post-infection, referred to as the PCDN lineage [38]. These Abs were not as highly mutated as previous bnAbs raised against this site, and did not include insertion–deletion variants. Again, the closer a bnAb is to its germline configuration, the better it is as a vaccine target according to the B-cell-lineage immunogen design approach, given that these Abs would be easier to elicit using a series of immunogens compared to more highly mutated Abs, which may not always follow predictable maturation pathways. Macleod et al. [38] also showed that multiple pathways led to neutralization breadth, and the authors hypothesized that early diversification followed by maturation of parallel lineages is a requirement for obtaining neutralization breadth.

12.2.2.5 Non-targeted Analysis of Ab Repertoire During HIV-1 Infection

A few studies have used HTS to examine the complete Ab repertoire during HIV-1 infection, referred to here as non-targeted approaches. These studies contrast the targeted studies in two ways: amplification of all IGHV genes (either by using primers amplifying all V genes, or 5'RACE), and the use of bioinformatic tools that examine all Abs and lineages, rather than only sequences related to a target Ab.

For example, Xiao et al. [78] used HTS to explore the Ab response in control individuals and an HIV-positive individual. They reported on V(D)J recombination frequencies, but did not perform lineage analyses. The primary findings were that the dominant IGHV family used in HIV-1+ compared to control individuals varied between IgM and IgG libraries, and from early to late infection.

The results of He et al. [26] were mentioned previously, in terms of Ab repertoires that focused on the PG121-class of Abs. They also reported on non-targeted Ab repertoires from an HIV-1+ and two control individuals. By examining the complete Ab repertoire, they observed that the dominant IGHV gene used in the repertoire from the infected individual was not the same IGHV gene associated with the PG121-class Abs. Also, one of the unbiased repertoires sequenced from a non-HIV-1 infected individual showed a strongly skewed repertoire in terms of IGHV gene usage. It is clear from this study of only a few individuals that unbiased repertoires can be highly different between individuals whether they are infected or not, consistent with what has been observed in other studies of healthy individuals (*e.g.*, [18, 9]).

Yin et al. [81] analyzed complete Ab repertoires, comparing control individuals (n = 4), systemic lupus erythematosus (SLE) patients (n = 4), and HIV-positive infected individuals either undergoing treatment (n = 4) or not (n = 4). They only analyzed the CDR-H3 region of the Ab from the IgM compartment. They observed that gene usage, CDR-H3 length distribution, and SHM did not differ between these groups. Specifically, anti-retroviral therapy (ART) did not normalize the diversity of the IgM Ab repertoire.

Hoehn et al. [28] used HTS to probe the Ab repertoire in eight HIV-positive individuals, five of whom were being treated with ART and three who were not. These repertoires were compared to Ab repertoires of six healthy patients from a previous study. These researchers applied several measures of clonality to the entire Ab repertoire, and in general observed that the HIV-1+ individuals exhibited higher clonality. There was significant variability among HIV-positive patients treated with ART in terms of the clonality of their repertoires, such that no differences were observed between treated and untreated patients. This is similar to the conclusion of Yin et al. [81], except with larger sample sizes in two of the group subsets. Across all HIV-1+ patients, there was no association between repertoire clonality and clinical variables, such as viral load or $CD4^+$ T cell count.

12.2.2.6 Summary of Results from HTS Studies of the Anti-HIV-1 Ab Repertoire

Many of the HTS studies of Ab repertoires during infection have focused on HIV-1 infection. As mentioned earlier, these have most often targeted particular bnAbs and their relatives within the Ab repertoire. The perfect vaccine candidate Ab would be able to neutralize a broad set of HIV-1 isolates, use common genes, not require an unusually long CDR-H3, and would be common in the naïve repertoire of most individuals. This bias toward HIV-1 studies of particular bnAbs reflects the desire to learn how to elicit such bnAbs in naïve individuals.

In our review of these studies, we have gathered a diverse set of conclusions regarding the conditions eliciting these bnAbs that could be vaccine candidates. These conditions include high rates of SHM [59], strong viral diversification [4, 36], unmutated common ancestors binding to the infecting HIV-1 strain [13, 36], multiple parallel Ab lineages [38], presence of competing Ab lineages [19], and early rates of viral diversification [36]. Despite these numerous correlations, there doesn't seem to be any conditions that are consistently associated with the development of anti-HIV-1 bnAbs across multiple studies. Several factors could plausibly explain such inconsistencies.

For one, these studies are characterized by large datasets, often in the 10s of millions of sequences, but sequenced from only a few individuals. In fact, several donors (*e.g.*, N152, CAP256, Donors 17 and 45 of IAVI Protocol G, and CH103; Table 12.1) have repeatedly been sampled across studies, because they are known to produce some of the most broadly neutralizing

Abs. This focus on a small number of donors makes sense, given the importance of the bnAbs that they produce, but this limits the generality of the results that we can draw from the set of studies undertaken so far. Also, as noted several times, the above patterns were observed mostly in studies where bioinformatic filters had been used to restrict the analysis to only Abs that are related to targeted Abs, usually the bnAbs that these donors are known to produce. This again restricts the scope of any conclusions that can be drawn from these studies. Finally, the bioinformatic approaches used to capture related Abs, such as intra- and inter-donor phylogenetic analysis, only capture sets of Abs that are functionally related (*e.g.*, utilization of the same IGHV and J gene, with similar CDR-H3), but there is no way to estimate how likely it is that these Abs may be related as ancestor/descendants which is the usual definition of a clonal lineage. In such circumstances, for example, conclusions on the necessity of binding patterns of these unmutated common ancestors or early bifurcation of B-cell lineages may not make sense if the B-cell lineages referred to are in fact multiple biological lineages. In the next section we discuss the utility of non-targeted approaches, and what is needed to make these larger scale comparisons possible (*i.e.*, sharing of large data sets across studies and institutions).

12.3 Future Directions to Enhance the Value of Ab Repertoire Studies

12.3.1 Germline Genes and Haplotypes

The Ab repertoire community is now placing a greater focus on understanding the origins of key inter-individual similarities and differences observed among repertoires responding to a given pathogen. The identification of common repertoire signatures in larger subsets of individuals is likely to improve strategies for the design of effective therapeutics and prophylactics. Given the importance of directing the development of the repertoires of naive individuals under the B-cell-lineage immunogen design strategy, some have questioned whether all individuals have the capacity to actually respond to a given immunogen in the same way [5]. It is certainly evident in many contexts that not all vaccines elicit equivalent responses in all individuals of the population [8]. While in most scenarios, many factors are at play (*e.g.*, an individual's infection/vaccine history and age), there is a growing appreciation for the potential influence of heritable factors on the development of both the baseline naïve and antigen-stimulated Ab repertoires [18, 21, 50, 66]. This coincides with a growing body of work describing extreme levels of genetic diversity at the IG loci among human populations, including single nucleotide variants in both the coding and non-coding portions of IG V, D, J, and C genes, as well as large structural variants that can influence the number of functional IG gene segments present in a given genome (e.g., [11, 32, 35, 41, 53, 65, 69]). Importantly, there are now many studies that have directly linked IG germline polymorphisms to differences in Ab repertoire signatures and gene usage, Ab function, and disease and clinical outcomes (e.g., [1, 15, 33, 45, 46, 52, 56, 62, 73]), together suggesting that any two individuals may not necessarily be predisposed to mount identical responses. With these examples in mind, moving forward we believe the inclusion of data on germline V, D, J and C gene variants in the study of variation in the Ab repertoire in health and disease (ideally in larger cohorts) will be critically important (see [68, 70]).

A potential role for germline variation in the development of CD4-directed neutralizing Abs has been specifically observed among repertoires in HIV+ individuals. Yacoob et al. [80] recently showed that particular alleles (namely, *02, *03, and *04) at the human *IGHV1-2* gene that encode three key amino acids serve as better germline precursors for VRC01 bnAbs, with better binding affinities, noting that this is consistent with the observation that all VRC01-class bnAbs identified to date are encoded by the germline *IGHV1-2* allele *02 [72]. A critical observation was that only 8/9 subjects examined in their study carried *02 alleles, suggesting that some individuals in

the population may have a reduced capacity to produce effective VRC01-class bnAbs [80]; this may be specifically relevant to germline targeting-based vaccine priming approaches [31]. Allelic restrictions have also been noted for *IGHV1-69*-encoded bnAbs against gp41 elicited by vaccination [74], asl well as for critical binding residues of influenza hemagglutinin stem-directed bnAbs [1, 45, 73].

12.3.2 Integration of Heavy and Light Chain Data, and B-Cell and T-Cell Repertoires

Many of the studies surveyed report data only on immunoglobulin heavy chain sequences, and sometimes only for the CDR-H3 region. However, both the heavy and light chain can contribute to Ab binding properties, and including both the heavy and light chain is necessary to most accurately determine the clonal lineages within an Ab or T-cell receptor (TCR) repertoire. In fact, several labs have developed the ability to report both the heavy and the light chain sequences for B cells and T cells. The earliest approaches involved pairing heavy and light chain sequences based on the topologies and frequencies of corresponding branches of phylogenies constructed separately from heavy and light chain sequences (*e.g.*, [85]). More recent approaches are based on emulsion techniques (*e.g.*, [12]) or other sorting techniques that isolate single cells and capture paired heavy and light chain sequences from each B cell [44, 49]. Parallel analyses of Ab/B-cell and T-cell repertoires are starting to reveal common and divergent patterns between the two compartments [3, 47], and isolation of mRNA from single cells allows relating complete-length paired heavy and light chain Ab sequences to T-cell receptor repertoires in fine detail [7].

12.3.3 Integration of HTS B-Cell Repertoire Data with Other Types of Genomic-Level Data

One of the most important future developments for HTS studies of Ab repertoires will be to integrate these data with other large scale, genomic datasets. For example, Georgiou and colleagues [34] have combined HTS sequencing of the Ab repertoire with high-resolution protein mass spectrometry of the serum antibodies (Ig-seq) in order to connect soluble Ig in blood and secretions with clonally expanded peripheral B cells [34]. This can lead to identifying Ag-specific lineages both functionally and developmentally. In addition, interesting insights have come from combining HTS of gut microbiota with Ab data [16, 37].

12.3.4 Importance of Non-targeted, Complete Ab Repertoire Studies

This review shows that most of the studies of Ab repertoires in HIV-1 infected individuals have taken a “targeted” approach, in that they only examine sequences that are related to particular target bNAbs. We contrast this to non-targeted, complete repertoire studies that attempt to estimate the number and relative expansion of all of the Ab lineages in an individual’s repertoire. Of course, it is practically impossible to completely characterize an individual’s Ab repertoire, given that it has been estimated that an individual can have as many as 10^{11}–10^{12} lymphocytes [55], many of which express unique Ab/B-cell or T-cell receptors.

The targeted approach can answer some specific questions very well, such as describing the development of a specific lineage of Abs that led to the development of a specific bnAb. However, many questions about the development of Ab

repertoires, including understanding the conditions and broader repertoire context leading to bnAbs, require a description of the complete Ab "milieu" within which a particular Ab lineage was formed (*i.e.*, the number and estimated size of each lineage). This question comes down to: what additional insights do we obtain from unbiased, non-targeted sequencing coupled with attempts to describe the complete Ab repertoire?

One example of a question that demands a complete repertoire analysis, rather than one targeted to specific pre-defined lineages, would be how often are certain lineages produced in response to HIV-1, such as those related to the important bnAb, VRC01? In one such study, Zhu et al. [85] reported 15 clusters of sequences all related to PGT135-137, and these clusters exhibited highly variable levels of neutralization. From this observation, the authors concluded that the VRC01 lineages could be produced by a large proportion of individuals. However, without comparing the frequency of this type of lineage within a broader representation of related Ab lineages, it is difficult to estimate the naturally occurring frequency of this lineage among individuals.

Also, several studies have investigated the relationship between the expansion of clones early in HIV-1 infection and the production of bnAbs. Again, one would have to have a complete description of the clonal structure of each individual to determine which individuals are characterized by clonal expansion, even of related clones, and when those clones expanded. Any comparison of V, D, or J gene usage, or the unevenness of repertoires in individuals who do or do not produce bnAbs, would also only be possible based on an unbiased view of the repertoire. Given that these signals might discriminate individuals producing or not producing bnAbs, it is obvious that we must strive to describe the entire Ab repertoire as often as possible.

Furthermore, it is worth noting that the necessity of understanding the complete Ab repertoire is not just restricted to studies of the development of anti-HIV-1 bnAbs. It is likely that the Ab repertoire will be completely different between individuals depending on many different conditions, from infection to autoimmune disease to cancer, and understanding the effects of these conditions will demand describing the complete Ab responses in many individuals. "Public" Abs, those produced by many, including presumably healthy, individuals, and using commonly expressed V, D, and J genes, would be obvious targets for most vaccine attempts. Targeted approaches to Ab repertoires demand large sequence datasets, but attempts to describe the complete Ab repertoire will demand even larger datasets, and answering the above questions will likely involve comparing and sharing these huge data sets across studies, disease states and institutions. The next section addresses some of the challenges in reporting these data to improve reproducibility and in sharing these data in order to understand what drives similarities and differences in Ab repertoires.

12.3.5 Standardizing Data Generation, Analysis and Sharing Protocols

HTS of Ab/B-cell and T-cell receptor repertoires has increased dramatically since the technique was first applied to immune receptor repertoires in 2009 [9, 17, 48, 71]. This experimental approach allows us to explore the development of the adaptive immune system in exquisite detail, and holds significant translational promise as diagnostic and prognostic tools. However, as discussed in this review, there are several limitations to the present generation and analysis of these type of data. Efforts to standardize protocols and

facilitate the sharing of data could help to answer many of these limitations.

A primary issue that arises when trying to draw conclusions based on analyses across large, complex and shared data sets is reproducibility. This largely concerns the development of standards centered around the reporting of key details related to the production and analysis of data that ensure another researcher can reproduce the data and recapitulate the analysis, and ultimately be confident that the data are of sufficient quality to be shared and compared.

Facilitating comparing and sharing of these data sets within and across labs, disease states and institutions also demands new protocols for data deposition and sharing in public repositories, as well as bioinformatic solutions to analyze these complex data. In some cases, these protocols will also need to take into account the protection of intellectual property (IP), data security, and donor confidentiality. The Adaptive Immune Receptor Repertoire (AIRR) Community is a group of immunologists, immunogeneticists, clinicians, bioinformaticians, and experts in legal, IP and security aspects of genomic data sharing, formed in 2015 to address these issues for this rapidly expanding area of immunological research. The progress and recommendations of the working groups of the AIRR Community are summarized at airr-community.org; anyone interested in joining the Community can also do so at that site.

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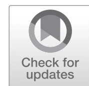

Safety and General Considerations for the Use of Antibodies in Infectious Diseases

13

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Abstract

Monocolonal antibodies are valuable potential new tools for meeting unmet needs in treating infectious dieseases and to provide alternatives and supplements to antibiotics in these times of growing resistance. Especially when considering the ability to screen for antibodies reacting to very diverse target antigens and the ability to design and engineer them to work specifically to hit and overcome their strategies, like toxins and their hiding in specific cells to evade the immuneresponse and their special features enabling killing of the infectious agents and or the cells harbouring them. Antibodies are generally very safe and adverse effects of treatments with therapeutic antibodies are usually related to exaggeration of the intended pharmacology. In this chapter general safety considerations for the use of antibodies is reviewed and the general procedures for nonclinical testing to support their clinical development. Special considerations for anti-infective mAb treatments are provided including the special features that makes nonclinical safety programs for anti-infective mAbs much more simple and restricted. However at a cost since only limited information for clinical safety and modeling can be derived from such programs. Then strategies for optimally designing antibodies are discussed including the use of combination of antibodies. Finally ways to facilitate development of more than the currently only three approved mAb based treatments are discussed with a special focus on high costs and high price and how collaboration and new strategies for development in emerging markets can be a driver for this.

Keywords

Monoclonal antibodies · Safety · Nonclinical test · Anti-infective · Immune response · Antibody engineering

13.1 Introduction

Diseases caused by infectious agent's results from invasion into and proliferation of viruses, bacteria, fungi and parasites in human organs and tissues. The management of such diseases aims both at curing the infected patient and to avoid contamination of the surroundings and direct transfer of the infections agent resulting in spread of the infection. Antibodies were used in this process as early as in 1890 where Emil A. Von Behring and Shibasaburo Kitasato introduced serotherapy in the form of anti-diphtera serum extracted from immunized horses [1]. Up to

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around 1930 the use of serum from immunized animals provided the first effective treatment options against infections with e.g. Clostridium tetani, Neisseria meningitidis and Corynebacterium diphteriae [2, 3]. These treatments were however quite expensive due to management of the animals used for extracting the serum and were associated with multiple side effects including serum sickness due to the administration of large amounts of foreign proteins. When penicillin was discovered by Fleming in 1928, and with the subsequent introduction of the much cheaper and safer antibiotics in the 1930s, serum therapy was largely abandoned. Then Millstein and Köhler in 1975 published their hybridoma technology for generation of monoclonal antibodies and sparked a new life for the use of monoclonal antibodies in research and development of new medicines. This technique allowed the production of large amounts of homogenous antibodies with defined specificity and a single Ig class and isotype [4]. This was quickly adapted for clinical use, and in the 1980s the OKT3 anti-CD3 monoclonal murine IgG2 antibody was the first one in the class approved for prevention of organ transplant rejection [5, 6]. The ability to generate monoclonal antibodies in unlimited amounts and against almost any target protein provided extremely valuable tools for detecting, locating, inhibiting, and blocking specific markers and pathways in general biological and medical research and in setting up analyses for numerous markers via enzyme-linked immunosorbent assay (ELISA) or immunohistochemistry. It also played a major role in basic research into the mechanisms of antibody action. More than 60 monoclonal antibodies are or have been approved in several different indications in oncology, transplantation, autoimmune and infectious diseases [7–9] see Table 13.1 for list of approved mAbs to date. Unfortunately, only three of these are approved for infectious diseases, Palivizumab (Synagis®) for prevention of respiratoy cyncytical virus (RSV) Raxibacumab (ABThrax®) for treatment of infections with *bacillus anthracis* and Bezlotoxumab (Zimplava®) against infections with *clostridium difficile*. Many features of mAbs have contributed to this, an important one being the high cost of manufacturing and the resulting high price a major challenge for healthcare providers worldwide. Then their high specificity which as described later from a safety point of view is very attractive but in contrast to antibiotics makes them active only against one specific strain virus or bacteria. Finally the difficulty in establishing relevant animal disease models and the resulting poor translatability of animal data to humans results in many products failing in Ph II and Ph III trials.

Fully human therapeutic antibodies are now generated using screening techniques like phage display [10, 11], single human B cell screening [12, 13] and knock-out mice with human germline B cell genes. Of these, phage display has due to the in-vitro screening some advantages compared to the in-vivo generation background of the two other screening techniques, in enabling generation of antibodies against self-antigens and against carbohydrate, lipid and lipoprotein targets and against antigens/targets not immunogenic or only active at certain conditions [11, 14].

Early therapeutic monoclonal antibodies were due to their generation by immunization of mice of mouse origin resulting in safety issues in the form of immunogenicity and anaphylactic reactions. These were followed by chimeric and humanized antibodies where the amount of mouse sequence was reduced as well as the safety issues to the fully human antibodies [15]. The safety of these are due to the general properties of antibodies, high specificity and lack of entry to and interaction with cytoplasmic proteins, very benign and adverse effects mostly related to exaggeration of the intended pharmacology. The fully human nature then further reduces the risk of immunogenicity and generation of anti-drug antibodies and the potentially related adverse effects and effects on clearance and efficacy. This is true in the human setting, however in the animal setting the fully human mAbs are considered foreign and when assessing safety and PK in toxicology studies to support the different phases of clinical development immunogenicity can be a challenge in getting sufficient exposure and pharmacology in the animals to make a proper safety assessment and interpretation of potential findings in the ani-

Table 13.1 List of approved mAbs as of May 2017

Name: antibody	Target: antibody type	Indication	Company	Approval date
1. Muronomab CD3<TB>OKT3[a]:	CD3: murine, IgG2a	Autoimmune	Johnson & Johnson	1986 (U.S.)
2. Abciximab ReoPro®	PIIb/IIIa: chimeric, IgG1, Fab	Homeostasis	Johnson & Johnson	1984 (U.S.)
3. Rituximab Rituxan®; Mabthera®	CD20: chimeric, IgG1	Cancer	Genentech	1997 (U.S.) 1998 (E.U.)
4. Raclizumab Zenapax[a]:®	CD25: humanized, IgG1	Autoimmune	Roche	1997 (U.S.) 1999 (E.U.)
5. Basiliximab Simulect®	CD25: chimeric, IgG1	Autoimmune	Novartis	1998 (U.S, E.U.)
6. Palivizumab Synagis®	RSV: humanized, IgG1	Infections	MedImmune	1998 (U.S.) 1999 (E.U.)
7. Infliximab Remicade®	TNFα: chimeric, IgG1	Autoimmune	Johnson & Johnson	1998 (U.S.) 1999 (E.U.)
8. Trastuzumab Herceptin®	HER2: humanized, IgG1	Cancer	Genentech/Roche	1998 (U.S.) 2000 (E.U.)
9. Emtuzumab ozogamicin Mylotarg[a]:®	CD33: humanized, IgG4, immunotoxin	Cancer	Wyeth/Pfizer	2000 (U.S.)
10. Alemtuzumab Campath®	CD52: humanized, IgG1	Cancer	Genzyme	2001 (U.S.) 2001(E.U.)
11. Ibritumomab tiuxetan Zevalin®	CD20: murine, IgG1, radiolabeled (yttrium 90)	Cancer	Biogen Idec	2002 (U.S.) 2004 (E.U.)
12. Adalimumab Humira®	TNFα: human, IgG1	Autoimmune	Abbott	2002 (U.S.) 2003 (E.U.)
13. Omalizumab Xolair®	IgE: humanized, IgG1	Autoimmune	Genentech/Roche	2003 (U.S.)
14. Tositumoma b-I-131 Bexxar®	CD20: murine, IgG2a, radiolabeled (iodine 131)	Cancer	Corixa/GSK	2003 (U.S.)
15. Falizumab Raptiva[a]®	CD11a: humanized, IgG1	Autoimmune	Genentech/Roche	2003 (U.S.) 2004 (E.U.)
16. Cetuximab Erbitux®	EGFR: chimeric, IgG1	Cancer	Imclone/Lilly	2004 (U.S.) 2004 (E.U.)
17. Bevacizumab Avastin®	VEGF: humanized, IgG1	Cancer	Genentech/Roche	2004 (U.S.) 2005 (E.U.)
18. Natalizumab Tysabri®	α4-Intergrin: humanized, IgG4	Autoimmune	Biogen Idec	2004 (U.S.)
19. Tocilizumab Actemra®	Anti-IL-6R: humanized, IgG1	Autoimmune	Chugai/Roche	2005 (Japan) 2010 (U.S.)

(continued)

Table 13.1 (continued)

Name: antibody	Target: antibody type	Indication	Company	Approval date
20. Panitumumab Vectibix®	EGFR: human, IgG2	Cancer	Amgen	2006 (U.S.)
21. Ranibizumab Lucentis®	VEGF: humanized IgG1 Fab	Macular degeneration	Genentech/Roche	2006 (U.S.)
22. Eculizumab Soliris®	C5: humanized IgG2/4	Blood disorders	Alexion	2007 (U.S.)
23. Certolizumab pegol Cimzia®	TNFα: humanized, pegylated Fab	Autoimmune	UCB	2008 (US)
24. Catumaxomab Removab®	Anti-EpCAM/CD3, rat IgG2b/mouse IgG2a bi-specific	Cancer: Malignant ascites	Trion pharma (infusion reactions)	2009 (EU)
25. Golimumab Simponi®	TNFα: human IgG1	Autoimmune	Johnson & Johnson	2009 (U.S., E.U., Canada)
26. Canakinumab Ilaris®	IL1b: human IgG1	Inflammatory	Novartis	2009 (U.S., E.U.)
27. Ustekinumab Stelara®	IL-12/23: human IgG1	Autoimmune	Johnson & Johnson	2008 (E.U.) 2009 (U.S.)
28. Ofatumumab Arzerra®	Anti-CD20: human IgG1	Cancer MS	Genmab	2009 (E.U.)
29. Denosumab Prolia®	RANK ligand: human IgG2	Bone loss	Amgen	2010 (U.S.)
30. Raxibacumab ABThrax®	*B. anthrasis* PA, human, IgG1	Anti-infection	GSK	2012 (U.S.)
31. Belimumab Benlysta®	BLyS: human IgG1	Autoimmune	HGS	2011 (U.S.)
32. Ipilimumab Yervoy®	CTLA-4: human IgG1	Cancer	BMS	2011 (U.S.)
33. Brentuximab vedotin Adcetris®	CD30: chimeric, IgG1, drug- conjugate	Cancer	Seattle genetics	2011 (U.S.)
34. Pertuzumab Perjeta®	Her2: humanized, IgG1	Cancer	Genentech/Roche	2012 (U.S.)
35. Ado-trastuzumab emtansine Kadcyla®	Her2: humanized, IgG1, drug-conjugate	Cancer	Genentech/Roche	2013 (U.S.)
36. Vedolizumab Entyvio®	Integrin $\alpha_4\beta_7$: humanized IgG1	Crohn's disease, ulcerative colitis	Takeda	2014 (U.S.)
37. Ramuciruumab Cyramza®	Anti-VEGFR2: human IgG1	Gastric cancer	Lilly	2014 (U.S.)
38. Obinutuzumab Gazyva®	Anti-CD20: humanized IgG1 glucoengineered	Cancer/chronic lymphocytic leukemia (CLL)	Genenteck/Roche	2014 (U.S.)

(continued)

Table 13.1 (continued)

Name: antibody	Target: antibody type	Indication	Company	Approval date
39. Situximab Sylvant®	Anti IL-6: chimeric IgG1	Castleman disease	Janssen	2014 (U.S) 2014 (E.U.)
2015				
40. Nivolumab Opdivo®	Anti PD-1: human IgG4	Melanoma	BMS (immune mediated adverse reactions, hypo−/ hyperthyroidism, embryo fetal toxicity	2014 (US) 2015 (EU)
41. Pembrolizuumab Keytruda®	Anti-PD-1: humanized, IgG4	Melanoma	Merck	2014 (U.S.)
42. Sekukinumab Cosentyx®	Anti IL-17a: human IgG1	Psoriasis	Novartis	2015 (US)
43. Dinutuximab Unituxin®	Anti glucolipid GD2, chimeric,	Cancer: High risk neuroblastoma	United Therapeutics (serious infusion reactions, neurotoxicity – Neuropathic pain and peripheral neuropathy)	March 2105 (US)
44. Alirocumab Praluent®	Anti PCSK9, human	Heterozygous familial hypercholeserolemia	Sanofi Aventis (allergic reactions)	July 2015 (US)
45. Idarucizumab Praxbind®	Anti dabigatran (Pradaxa®), humanized, Fab fragment	Reversal of anti-coagulant effects	Boehringer Ingelheim (thromboembolic risk, hypersensitivity reactions)	Oct 2015 (US)
46. Evolocumab Repatha®	Anti PCSK9, human	Hypercholesterolemia	Amgen Inc. (allergic reactions)	Aug 2015 (US)
47. Daratumumab Darzalex®	Anti-CD38, human, IgG1	Cancer: Multiple myeloma	Janssen Biotech (infusion reactions, interference with cross-matching)	Nov 2015 (US)
48. Mepolizumab Nucala®	Anti-IL-5, humanized, IgG1	Severe eosinophilic asthma	Glaxosmithkline (hypersensitivity)	Nov 2015 (US)
49. Necitumumab Portrazza®	Anti-EGFR, human, IgG1	Cancer: Squamous non-small cell lung carcinoma	Eli Lilly Co.	Nov 2015 (US)
50. Elotuzumab Empliciti®	Anti-CS1 (CD319), humanized, IgG1	Cancer: Multiple myeloma	Bristol Myers Squibb (infusion reactions, infections, second primary malignancies, hepatotoxicity)	Nov 2105 (US)
2016				
51. Obilotoxaximab Anthim®	Protective antigen of Bacillus antracis,		Elusys Therapeutics	March 2016 (US)
52. Ixekizumab Taltz®	Anti-IL-17a	Plaque psoriasis (infection, TB, hypersensitivity, IBD)	Eli Lilly	March 2016 (US)
53. Reslizumab Cinqair®	Anti-IL-5, humanized	Add-on severe asthma (anaphylaxis)	Teva	March 2016 (US)
54. Atezolizumab Tecentriq®	Anti-PDL1	Cancer (immune-related disorders)	Genentech Inc	May 2016 (US)

(continued)

Table 13.1 (continued)

Name: antibody	Target: antibody type	Indication	Company	Approval date
55. Daclizumab Zinbryta® (previously Zenapax®)	Anti CD25, antagonist, humanized,	Relapsing MS (hepatic injury and immune—Mediated disorders)	Biogen	May 2016 (US)
56. Adalimumab-Atto (Amjevita® – Biosimilar of Humira)	Anti-TNF-α	Rheumatoid arthritis (RA) juvenile idiopathic arthritis (JIA); psoriatic arthritis (PsA); ankylosing spondylitis (AS); adult Crohn's disease (CD); ulcerative colitis (UC); plaque psoriasis (Ps) (serious infections, increased risk of malignancies, heart failure, lupus –like symptoms)	Amgen Inc	Sept. 2016 (US)
57. Ustekinumab Stelara®	Anti IL-12/IL-23 (bi-specific), human, IgG1	Crohns disease, plaque psoriasis, psoriatic arthritis	Jansen biotech (serious infections)	Sept. 2016 (US)
58. Atezolizumab Tecentriq®	Anti-PD-L1, humanized,	Cancer: Urothelial carcinoma, metastatic NSCLC,	Roche/Genentech Inc. (infusion-reactions, embryofetal toxicity, pneumonitis, hepatitis, colitis, hormone gland and nervous system problems, inflammation of the eyes and severe infections)	Oct. 2016 (US)
59. Olaratumab Lartruvo®	Anti-PDGFR-α, human	Cancer (soft tissue sarcoma)	Eli Lilly (hypersensitivity, neutropenia, musculoskeletal pain)	Oct. 2016 (US)
60. Bezlotoxumab Zinplava®	Anti Clostridium difficile toxin B	Reduce recurrence of clostridium difficile infection	Merck (Heart failure)	Oct 2016 (US)
2017				
61. Ocrelizumab Ocrevus®	Anti-CD20, humanized	PPMS	Roche	March 2017(US)
62. Avelumab Bavencio®	Anti PD-L1, human (CD254) IgG1	Cancer: Merkel cell carcinoma in	Merck/Pfizer	US (March 2017)
63. Durvalumab Infinzi®	Anti PD-L1 (CD254), human IgG1	Cancer: (locally advanced or metastatic urothelial carcinoma)	Astra Zeneca	US (May 2017)

mal toxicology studies. Immunogenicity and formation of anti-drug antibodies in animal studies is therefore not considered predictive for potential immunogenicity in humans.

The first part of this chapter will address general safety issues associated with therapeutic antibodies followed by a description of the normal procedures and programs set up to do safety assessment of monoclonal antibodies as outlined in relevant regulatory guidance's.

A schematic drawing of the general structure of IgG antibodies and potential antibody derived alternative treatment modalities is provided in Fig. 13.1.

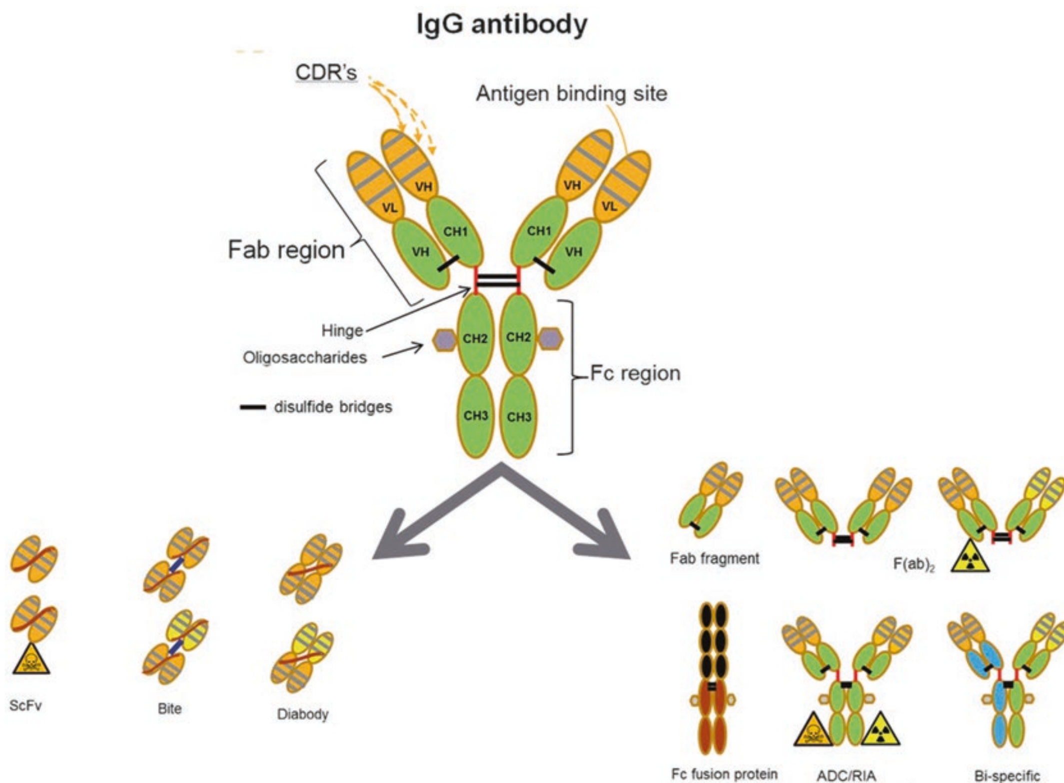

Fig. 13.1 IgG antibody and derived formats

Apart from safety, several other considerations for developing a therapeutic antibody product are central. The choice of modality – full antibody, fAb, fAb_2, ScFv or bi-specific antibodies or fAb_2 (Fig. 13.1) to provide best target access and duration of action. The choice of IgG isotype and glycosylation pattern of the Fc part is also important for avoiding or enhancing effector functions like ADCC and CDC. Furthermore, modulation of FcRn affinity to increase half-life and finally whether to use single or multiple antibodies or bispecific antibodies in the product to combine mechanisms or especially for anti-infective's to avoid resistance due to escape mutations. The last part of this chapter will review considerations and strategies in selecting the optimal product nature and characteristics with some special attention to treatments for infectious diseases.

13.2 Safety Aspects in Use of Antibodies or Antibody Based Therapies

13.2.1 General Safety of Monoclonal Antibodies (mAbs)

More than 60 monoclonal antibodies are or have been approved in several different indications in oncology, transplantation, autoimmune and infectious diseases (Table 13.1) [7–9]. As stated above, only three of these are approved for infectious diseases. Antibiotics are, despite the rising resistance challenge together with vaccines the main therapies for treatment and prevention of infectious diseases. Monoclonal antibodies (mAb's) possesses several features like high specificity for their target, benign safety profile and long half-life which give them advantages relative to traditional small molecule based therapies. mAbs usually, due to the high specificity for

their target have very little off target binding and unwanted/unexpected side effects. They don't interact directly with cytochrome P450 metabolizing enzymes and do not enter cells and interact with cytoplasmic proteins or chromosomes, resulting in mutagenesis or genetic toxicity. However although generally well tolerated mAb's are also associated with safety issues related to immune reactions like hypersensitivity, acute anaphylaxis, serum sickness and the generation of anti-drug antibodies (ADAs). These side effects are however, due to the high specificity of mAbs, usually related to exaggeration of the specific and intended pharmacology. This can be in the form of increased risk of infections, organ specific adverse effects such as cardiotoxicity or exaggerated life-threatening immune responses like the severe effects and organ failure caused by the cytokine release syndrome observed with TGN1412 in a first in human study back in March 2006 [17].

In the following sections considerations and examples of the main types of safety issues seen with Mab treatments is provided. In Table 13.1 a list of the currently approved mAbs is provided.

13.2.2 Immune Reactions

Monoclonal antibodies do, despite their general high tolerability cause immune reactions in treated subjects. This can be in the form of hypersensitivity reactions of anaphylactic (IgE mediated) or, anaphylactoid grade following infusion or as tumor lysis syndrome in cancer treatment of e.g. leukemias in relation to killing of large number of tumor cells, serum sickness or cytokine release syndrome [32]. The clinical consequences of these types of reactions range from local reactions at the injection site to acute anaphylaxis and life-threatening systemic inflammatory responses. Therapeutic mAbs might also cause formation of antibodies against themselves which can result in antibody complexes being trapped in critical locations such as glomeruli in the kidneys and cause inflammation and organ damage. Furthermore these ADAs can lead both to more rapid clearance of the antibody and shorter time of activity or total neutralization of efficacy. The latter might not be a safety-issue in itself but if treating a life-threatening disease, then absence of activity could bring the patient in a critical situation requiring alternative treatment if available.

Severity and frequency of these reactions often correlate with the amount of mouse and human sequence in the antibody but also to some degree on their target/mechanism of action. Early generation mAbs were due to their generation in mouse hybridoma cells [4], containing a mixture of human and mouse sequences being called chimeric antibodies in carrying human heavy and light constant chains and mouse variable chains (See Table 13.2). Examples of early chimeric antibodies are rituximab (Rituxan®) and infliximab (Remicade®). Subsequent generations provided first humanized antibodies where only the three highly variable complementarity determining regions (CDRs) on each of the heavy and light variable chains were still of mouse origin (e.g. trastuzumab (Herceptin®), ocrelizumab (Ocrevus®), and finally fully human antibodies no longer carrying any mouse sequences (e.g. ofatumumab (Arzerra®), secukinumab (Cosentyx®). However, even when fully human, antibodies can still arise to the idiotype of the antibodes (the variable antigen binding parts of the antibody). The first fully human antibody approved in the US was adalimumab which was developed using phage display as screening tool and transgenic mice producing the human IgGs.

To distinguish antibodies based on their degree of humanization and on their target, agreed naming conventions (WHO/PHARM s/NOM 1570; Guidelines on the use of International Nonproprietary Names (INNs) FOR PHARMACEUTICAL SUBSTANCES [18]) have been put in place (Latest list 77, 2017, for examples [19]). The degree of humanization is signified in the name by before the mab suffix, adding xi f or chimeric antibodies, zu for humanized antibodies an u, for fully human antibodies. Further naming conventions are in place to signify the target of the mAb in the name. As example insertion of les, indicate inflammatory lesion target, li indicate immune system target, ne nervous

Table 13.2 Principles for INN naming of monoclonal antibody therapies

Degree of humanization (substem)		Target	
Xi	Chimeric	ba/bac	Bacterial
Zu	Humanized	ci/c	Cardiovascular
U	Fully human	fu	Fungal
		ki/k	Interleukin
		le/les	Inflammatory lesion
		li/l	Immunomodulatory
		ne/n	Nervous system
		so/os/s	Bone
		tox/toxa	Toxin
		tu/t	Tumor
		V i/v	Viral

Mouse Chimeric

Humanized Human

system target, ci circulatory system target and ma, me and pr targets on mammary, melanoma and prostate tumors. Please see Table 13.2. Names of monoclonal antibodies normally contain first a unique identifier pre-fix, followed by a target indicator, then a suffix(substem) indicating degree of humanization and finally mab is added to indicate it is a monoclonal antibody. Examples are Palivizumab – Pali (unique prefix)-vi (viral target (RSV))-zu (humanized) and mab (monoclonal antibody) and Canakinumab – Cana(unique prefix)-ki (interleukin target (IL-1β)) – u (fully human)-mab monoclonal antibody).

Infusion reactions: Infusion reactions occur frequently following the initial dosings with mAbs and involve activation of the immune system. Some (anaphylactic) are allergic in nature and usually are mediated by immunoglobulin E (lgE). Others (anaphylactoid) are not true allergic reactions and are not mediated by lgE but usually caused by release of cytokines. Although reactions can be allergic or non-allergic, the clinical manifestations are the same and require prompt, accurate assessment and management to avoid severe and potentially fatal adverse events. Rituximab is an example of a drug with the potential for varying types of infusion reactions and the highest incidence of infusion reactions among the approved monoclonal antibodies are seen with rituximab and trastuzumab. In clinical trials, rituximab caused mild-to-moderate infusion reactions in most/all lymphoma patients, usually within 30–120 minutes of starting the first infusion [7]. The incidence declined from 77% during the first infusion to 30% with the fourth infusion and 14% with the eighth infusion. Severe infusion reactions, usually reversible with appropriate interventions and supportive care, were reported in approximately 10% of patients, but in rare cases proved fatal [7]. Severe infusion and hypersensitivity reactions including anaphylaxis, however, were also reported during the initial investigational development of the agent, although the overall incidence of these severe

events is rare (1%). With cetuximab, mild-to-moderate infusion reactions were usually associated with the first infusion and ranged in incidence from 12% (SCCHN patients to 19% (advanced colorectal cancer patients receiving single agent therapy) [7]. Overall, pooled results indicate that severe infusion reactions have occurred in 46 of 1485 patients (3%) treated in clinical trials, but were rarely fatal (1 in 1000) once the infusion was discontinued and supportive measures provided. Approximately 90% of the severe infusion reactions were associated with the first cetuximab (Erbitux®) infusion. In the case of panitumumab (Synagis®), infusion reactions occurred in approximately 4% of patients, and were severe in approximately 1% of cases. None was fatal. Finally, infusion reactions occurred in 3% of patients during the first infusion of bevacizumab, and were severe in 0.2% of cases [7, 16]. Therefore, the incidence of infusion reactions varies among monoclonal antibodies, occurring predominantly with the first infusion.

Anaphylactic reactions are usually managed by stopping infusion and administering epinephrine. For anaphylactoid reactions with mild grade fever and flu-like symptoms these can usually be managed by acetaminophen. A common practice these days during clinical trials is the use of antihistamine/corticosteroid pre-medication to avoid or minimize infusion reactions.

13.2.3 Cytokine Release Syndrome

The term cytokine release syndrome or cytokine storm, emerged from the events around first in human testing of the anti-CD28 antibody TGN1412 in 6 volunteers [17, 20]. Near fatal systemic inflammations caused by massive release of pro-inflammatory cytokines resulted in pulmonary infiltrates, lung failure, renal failure and disseminated intravascular coagulation. Treatment for up to 2 weeks in an intensive care unit was required to avoid mortalities. TGN1412 stimulated a broad range of cytokines including TNF-α, IFNγ, IL1β, IL-2 (especially in high amounts correlating with level of disease and), IL-4, IL-5, IL8, IL10, IL12 and IL-13. The TGN1412 story is a good example for pharmaceutical research and development – know you target and know expression and function of your target in the animal models used to test safety of your product compared to humans. TGN1412 targets CD28 and had in rats been demonstrated to induce upregulation of regulatory CD4 T cells. When TGN1412 was tested in monkeys there were no similar effects on T cells seen and no cytokine storm. In the subsequent investigations it was discovered that monkeys does not express CD28 on their CD4 effector memory cells and thus were not able to respond to the super-agonist stimulation of TGN1412. Human effector memory CD4 T cells however express high levels of CD28 and thus responded very strongly with the resulting cytokine storm [21]. The incidence really surprised and shocked health authorities, pharmaceutical companies and patients around the world and highlighted limitations in the programs and species used for nonclinical safety testing of new drugs aiming at modifying the immune system. A report on the incidence, issued by the Medicines Healthcare Products Regulatory Agency (MHRA) [24] and a subsequent new guidance from the European Medicines Agency [25] on conduct of first in human clinical trials with high risk therapeutics triggered efforts to establish cytokine release assay able to properly predict safety in humans [32]. Interestingly it turned out not to be an easy task to actually get human cell preparations to respond to TGN1412 and elicit the cytokine response seen in the clinical trial. Only dry or wet coating the assay plates with TGN1412 could trigger human blood cells able to elicit the release of cytokines [22]. Different labs in pharmaceutical companies and CRO's are working on and discussing format, endpoints and conditions best suited to provide a meaningful result. This include considerations on number of donors, cell preparation (whole blood or PBMC) panel of cytokines to be assessed, suitable positive and negative controls and what fold of increase of cytokine release relative to control should be considered significant in relation to

safety assessment. The results of a survey representing responses from 16 pharmaceutical companies and independent CROs [22, 23] highlighted important issues to be considered and different approaches needed when also considering different targets, target expression (on immune cells or e.g. endothelial cells) and different disease conditions where the drug would be applied. Another question is whether the data from such assays should be used for go/no-go decisions and or mainly for dose setting for toxicology studies and FIM clinical trials. The antibody merunumab (OKT-3 anti-CD3®) was shown in such an assay to stimulate similar range of cytokines and are together with TGN1412 one of the positive controls used in CRA in several labs. Several of the antibodies directed against the IL-2α receptor (CD25) like daclizumab (Zinbryta®) and basiliximab (Simulect®) (Novartis) was shown to trigger release of IL-2 [20] an important T cell activating cytokine. CRA is now an expectation from most health authorities, when doing safety assessment of antibodies (and other molecules/formats) stimulating the immune system and most pharmaceutical companies are now routinely considering these and doing these when relevant in their development programs.

13.2.4 Tumor Lysis Syndrome

Tumor lysis syndrome (TLS) is seen mostly in leukemia patients undergoing treatment and is related to the killing of large numbers of tumor cells. It is most often seen in patients with high tumor load and is not seen in patients with solid tumors. It is characterized by hyperkalemia, hypercalcemia, hyperphosphatemia, and hyperuricemia, resulting in ionic imbalance leading to renal failure cardiac arythmias, seizures and potentially death. The renal failure is caused by tubular deposition of uric acid or calcium phosphate crystals. Antibodies associated with TLS are rituximab (Rituxan®), brenituximab (Adcetris®), alemtuzumab (Campath®), ipilimumab®) (Yervoy, and obinutuzumab (Gazyva®) [7, 16].

13.2.5 Antibody Dependent Enhancement (ADE)

Antibody Dependent Enhancement is observed in several different viral infections – Dengue, HIV, Respiratory syncytical virus (RSV), Ebola and others, and is characterized by enhancing viral infections due to presence of antibodies from previous infections with another serotype, antibodies transferred to fetuses in the placenta and still present after birth. These antibodies can still bind the new viral serotype but is not neutralizing and instead of mediating killing of the virus they brings the virus in close contact with potential host cells like macrophages and dendritic cells (dengue virus [26]) and through enhancement of the fusion efficiency of the virus leads to faster and more severe infection. In case new therapeutic anti-viral antibodies were developed these could, due to their likely high serotype specificity, give rise to ADE upon later re-infection with another serotype of the same virus. It is therefore important to design such anti-infectious antibodies to have strong FcγR binding to trigger effector functions leading to killing of the virus or virally infected cells. Then, also to try to prolong the half-life of the mAb by increasing affinity of the antibody for FcRn enabling the antibody to stay longer and clear the virus [27].

13.2.6 Infections

When developing mAbs with immunomodulatory properties like B cell depleting anti-CD20 antibodies (rituximab (Rituxan®), ocrelizumab (Ocrevus®)), anti-TNFα antibodies like Adalimumab (Humira®), or Infliximab (Remicade®, or antibodies inhibiting the action of complement, gaps occur in the normal ability of the immunesystem to fight infections. This can result in increased sensitivity to bacterial and viral infections and especially sensitivity to re-activation of latent viral infections like HBV, HCV, CMV, VZV, EBV and HSV. Another viral infection s een due to re-activation following monoclonal antibody treatment is progressive

multifocal leukoencephalopathy (PML) which is a de-myelinating disease caused be re-activation of latent John Cunningham virus. This has been seen in patients treated for multiple schlerosis (MS) another demyelinating disease, with both the anti CD11a mAb efalizumab (Raptiva®), rituximab (Rituxan®) and natalizumab (Tysabri®).

Also increased incidence of bacterial infections with tuberculosis (TB) has been seen especially in patients treated with anti-TNF-α, antibodies like infliximab, adalimumab (Humira®) and certolizumab pegol (Cimzia®).

Eculizumab (Soliris®) an antibody against C5a of the complement system for treatment of paroxysmal nocturnal haemoglobinurea (PNH) has shown increased risk of infections and especially meningococcal infections. This resulted in a recommendation for meningococcal vaccination 2 weeks prior to initiation of treatment with eculizumab.

Most therapeutic immune-modulating antibodies have some degree of suspicion regarding risk for infections, but due to confounding factors like underlying disease and concomitant medication it is often difficult to directly correlate the infections to treatment. However warning against treatment in case of ongoing or chronic infections is in their labels. For antibodies used in cancer therapy the concomitant treatment with chemotherapy is most often seen to increase the risk for infections. A paradox is the actions to take when potential infections occur. A natural thing would be to stop treatment, however due to the long half-life of monoclonal antibodies in humans (approximately 21 days), dilution/elimination of the antibody and it's pharmacological effects, can only be obtained by plasmapheresis carrying its own risks. Furthermore cases of diseases like HIV and and TB a phenomenon called immune reconstitution inflammatory syndrome (IRIS) is observed where upon halting of immunosuppressive treatment the immunesystem overreacts to the infection creating worsening instead of improvement in the condition of the patient [47]. Antibodies reported in cases of IRIS are natalizumab (Tysabri®) in a localized form in the central nervous system and infliximab (Remicade® and adalimumab (Humira®) in patients infected with TB.

13.2.7 Platelet and Thrombotic Effects

Severe thrombocytopaenia has been observed in relation to treatment with both infliximab (anti TNF-α (Remicade®), efalizumab ((Raptiva®) anti CD11a) and rituximab ((Rituxan® anti CD20) antibodies with different targets and mechanisms of action used in widely different indications however the underlying mechanism(s) for the effect on platelets remains unknown [7, 16].

Treatment with alemtuzumab ((Campath®) anti-CD-52) causes months to a year of depletion of several immune cells including CD4 and CD8 T cells, NK cells, monocytes and some B cells. Alemtuzumab has had great success in treatment of e.g. MS but potentially fatal thrombocytopenia in 3% of treated patients has resulted in the need for thorough monitoring of hematological effects during treatment [7].

A problem of the opposite nature is seen with anti-CD40L antibodies where thrombocytaemia and thromboembolic complications were first observed in monkeys and later also in human studies [28] putting an end to the clinical studies of antibodies to this target.

13.2.8 Cardiotoxicity

Trastuzumab (Herceptin®) is a humanized mAb directed against human ERBB2 (also known as HER2/neu). It has been successfully used in women with ERBB2-positive metastatic breast cancer. An unexpected adverse event in women treated with trastuzumab in clinical trials was that of cardiotoxicity. The anti-tumor and cardiac muscle cell cytotoxic effects are linked through trastuzumab effects on mitochondrial outer membrane permeabilization (MoMp). Cardiac dysfunction caused by trastuzumab is

mostly an asymptomatic decrease in left ventricular ejection fraction. Cardiac myocyte proliferation and development is dependent on signalling via ERBBS-ERBB4 heterodimers, and the effects causing inhibition and death of Her2 positive cells in breast cancer also affects cardiomyocytes and negatively affects left ventricular ejection fraction. Cardiac dysfunction was observed in up to 4% of women treated with trastuzumab, with higher incidence in females taking additional anthracyclines. Supporting a role for trastuzumab in sensitization to anthracycline-induced cardiotoxicity. When trastuzumab was given alone for breast cancer, there were no cases of heart failure and no cardiotoxic effects.

Another antibody Necitumumab (Portrazza®) directed against the epidermal growth factor receptor (EGFR) carries a warning for cardiopulmonary arrest, and sudden death. Necitumumab is indicated for metastatic squamous nonsmall cell lung cancer in combination with gemcitabine and cisplatin. Cardiopulmonary arrest or sudden death occurred in 15 of 538 patients (2.8%) treated with necitumumab-gemcitabine-cisplatin compared to three of 541 (0.6%) of patients given gemcitabine and cisplatin alone [7, 16].

13.2.9 Dermatological Effects

For three antibodies directed against the epidermal growth factor receptor (EGFR) Cetuximab (Erbitux®) and Panitumumab (Vectibix®) and Necitumumab (Portrazza®), all indicated for cancer indications, skin rashes (dermatological toxicity in the form of achne like rash which can be of high grade and cover large part of the body, is a common side effect and in many cases represents dose limiting toxicity. Skin rashes are seen in about 80–90% of patients where around 10–15% are of severe grade [7]. The localization of these side effects to mucocutaneous sites and the high frequency of these reactions is thought to reflect the function of EGFR in the epidermis, hair follicle and periungual tissues. Inhibition of EGFR at these sites then results in abnormal proliferation, migration and differentiation of target cells, and consequent disruption of the integrity of the skin with, local infections the recruitment of inflammatory cells and resulting cutaneous manifestations [29].

13.2.10 Hematological Effects Cytopaenias

Hematological effects or cytopenias occur in many patients treated with immunomodulating anticancer mAbs. This includes the effects on platelets and thrombocytopenias described above, the efficient and intended depletion of B cells with the anti-CD20 antibodies rituximab (Rituxan®, ofatumumab (Afrezza®) and ocrelizumab (Ocrevus®). The elimination of the B cells is resulting from immune mediated eliminination by ADCC and CDC, however the mechanisms leading to effects on red blood cells, neutrophils, lymphocytes and platelets are however not well understood.

Boxed warnings from FDA for the risk of severe cytopenias has been given for ibritumomab tiuxetan (Zevalin®) and alemtuzumab (Campath®), while general warnings and precautions are set down for obinutuzumab (Gazyva®) (thrombocytopenia, neutropenia), ofatumumab (Afrezza®) (cytopenias), brentuximab vedotin (Adcetris®) (neutropenia), trastuzumab (Herceptin®) (neutropenia), and ado-trastuzumab emtansine (Kadycla®) (thrombocytopenia).

Other warnings/adverse events anticancer mAbs include cytopenias for catumaxomab (Removab®), brentuximab vedotin (Adcetris®), and pertuzumab (Perjeta®); lymphopenia for elotuzumab (Empliciti®); lymphopenia and leukopenia for blinatumomab (Blincyto®) (; neutropenia for rituximab Rituxan®); thrombocytopenia for daratumumab (Darzalex®); thrombocytopenia and anemia for trastuzumab (Herceptin®); and thrombocytopenia, lymphopenia, and neutropenia for dinutuximab (Unotuxin®). Autoimmune forms of thrombocytopenia and hemolytic anemia are type II hypersensitivities, and reductions in the platelet, erythrocyte, and neutrophil counts, especially in the lymphoproliferative diseases, may sometimes have an immune basis. Early and late neutropenia has been reported for rituximab

(Rituxan®). Late onset neutropenia (grade 3–4 neutropenia occurring 3–4 weeks after last treatment) is seen with an incidence of 4–23% and appears to be caused by immune mechanisms, including autoantibodies.

Also for rituximab, severe anemia has been reported at an incidence of 1.1 and 5.2% [7, 30].

13.2.11 Cancer/Carcinogenicity

Around half of the approved therapeutic antibodies are anti-cancer antibodies. These are antibodies with different mechanisms of action aiming to activate the immune system to kill tumor cells directly and/or to inhibit growth and spread of tumors. Some of these like the anti-CD20 antibodies (rituximab (Rituxan®), ofatumumab (Afrezza®), ocrelizumab (Ocrevus®) by killing of tumor cells like e.g. B cells in leukemia by coating CD20 expressing tumor cells with antibodies leading to killing of the tumor cells by activated NK cells via antibody mediated cellular cytotoxicity (ADCC), via activation of the complement system by complement dependent cytotoxicity (CDC) or a combination of these. As outlined in several of the previous sections these antibodies carry a number of safety risks like cytokine release syndrome, tumor lysis syndrome and hematological or dermatological toxicities. Other mechanisms of action employed include limiting development of the blood vessels needed for oxygen and nutrient supply to the tumor by anti-vascular endothelial growth factor (VEGF), and recently the hype around targeting of immune check-point inhibitors like PD-1 (Pembrolizumab (Keytruda®), Nivolumab (Opdivo®)) where blocking of the ligands PD1L or PL2L expressed on tumor cells inhibiting T cells from killing the tumors via binding to PD1 the surface to the T cells has provided very promising data in melanoma and non-small cell lung cancer. These have their own safety related issues like inflammation in lungs, liver, kidney and colon but which in the risk/benefit evaluation in late stage cancer treatment are acceptable. In many companies multiple combinations of PD1 inhibitors and other immunomodulating agents are being investigated. Although safety of the individual partners in these combinations is usually benign, it will be extremely interesting to follow potential added anti-tumor effects and safety issues of these combinations.

On one side of the coin are the safety issues related to activation of the immune system to kill tumor cells as described in the above sections. On the other side of the coin is the risk of developing tumors or stimulating growth or spread of tumors by antibodies aiming at treating autoimmune and inflammatory diseases like rheumatoid arthritis (RA), systemic lupus erythromatosis (SLE), inflammatory bowel syndrome (IBS), multiple sclerosis (MS) psoriasis and multiple others. In these, the aim is to inhibit or deplete parts of the immune system opening the door for growth and spread of newly arisen tumors via decrease in tumor surveillance, or increasing risk of viral infections or control of latent viral infections with e.g. Epstein Barr virus (EBV) or polyoma virus linked to oncogenicity when during lytic cycles of viral replication and infection of surrounding cells result in mutations causing uncontrolled growth and proliferation of the affected cell turning it into an oncogenic clone.

13.3 Nonclinical Safety Testing of mAbs

When developing new medicinal products, design and conduct of a nonclinical safety program needs to be done to support the different stages of clinical development and registration. This must be driven by a rational design of program and individual studies and a thorough scientific understanding of the target biology and expression of the target in humans and in the different species used for nonclinical safety testing.

The main regulatory guidance for nonclinical safety assessment of biotechnology derived pharmaceuticals which includes covers mAbs and mAb-derived products, is the International Conference on Harmonization (ICH) ICH S6 (R1) – “Preclinical Safety evaluation of Biotechnology-derived Pharmaceuticals”. This guidance covers details to be considered with respect to choice of species, dose selection,

assessment of immunogenicity and sequencing/timing of studies supporting first in human studies and chronic toxicity studies, assessment of safety pharmacology, juvenile toxicity, carcinogenicity and reproductive toxicity needed for Ph II, Ph III and applications for approval. Other regulatory guidance's providing important information for designing the nonclinical program include and ICH M3 (R2) Nonclinical Safety Studies for the conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals, ICH S5 [3] "Detection of Toxicity to Reproduction for Medicinal Products & Toxicity to Male Fertility". An overview of relevant guidance's for biopharmaceutical safety testing is provided in Table 13.3 below.

Table 13.3 Guidances relevant for nonclinical safety testing of mAbs

Guideline title	Regulatory authority	Year
ICH M3 (R2): Nonclinical safety studies for the conduct of human clinical trials with pharmaceuticals. Step 4	EMA/ FDA/ Japan	2009
ICH S6 (R1): Preclinical safety evaluation of biotechnology-derived pharmaceuticals	EMA/ FDA/ Japan	2011
Points to consider in the manufacture and testing of monoclonal antibody products for human use.	FDA	1997
ICH S5 (R3): Detection of toxicity to reproduction for medicinal products & toxicity to male fertility	EMA/ FDA/ Japan	2015
ICH S8: Immunotoxicity studies for human pharmaceuticals	EMA/ FDA/ Japan	2005
ICH S1A: Guiedline on the need for carcinogenicity studies of pharmaceruicals	EMA/ FDA	1995
ICH S9: Nonclinical evaluation for anticancer pharmaceuticals	EMA/ FDA/ Japan	2009
Guideline on strategies to identify and mitigate risks for first-in-human and early clinical trial with investigational medicinal products.	EMA	2007 Revised version 2016

As described in ICH S6 (R1), the main purpose of the nonclinical safety program is to (1). Identify an initial safe dose and subsequent dose escalation schemes in humans; (2). To identify potential target organs for toxicity and for the study whether such toxicity is reversible; and (3). To identify safety parameters for clinical monitoring.

Safety programs for mAbs in general and in accordance with the relevant guidances normally include the following parts listed below. This is however the general approach for mAb and mAb-derived treatments and due to the foreign nature of the target and lack of relevant species for anti-infective products the nonclinical safety programs for those can be quite different and abbreviated. That will be highlighted in the section following the outline below of the activities included in a more general sense for mAbs.

Activities included in general nonclinical safety programs for mAbs:

13.3.1 Selection of Relevant Species

The choice of species is a major and important part. As for small molecule drugs, nonclinical safety testing of mAbs should if feasible be done in two species a rodent and a non-rodent. However for biologics and mAbs included, the species used should be pharmacologically relevant species. This means expressing the target and for mAbs in particular the target epitope. Then the tissue distribution of the target should be similar as well as the function/biology and effects of modifying the target. Homology of target on gene and protein level between species and binding affinities are often important in this process. Regarding sequence homology monkey will often have >90% whereas rat, mouse, dog and rabbit can be in the range of 60–90%. Functional homology is most important and homology in the part determining the epitope for the antibody. For many mAbs monkey is the only relevant species and conducting the nonclinical program in one relevant species is acceptable. Supplementary studies in homologous or surrogate models or in knock out or knock in models can be considered

if it provides additional relevant information e.g. of activity in diseased states. This should be guided by a strong scientific justification given the often limited background data and questionable translatability of results from such studies, to humans. If one relevant animal species is available the generation of and use of additional models is not required.

13.3.2 Early Profiling and Short Term 2–4 Weeks Dose Range Finding Studies Informing Dose Setting for GLP Toxicology Studies

Preliminary/dose-range finding studies: The early part of a Safety programs for mAbs should, include *in vivo* and/or *in vitro* pharmacology studies to profile and confirm e.g. intended immunological properties, target binding potency/selectivity, species cross-reactivity, effector functions (ADCC and CDC assays) and for immunomodulatory antibodies where there is a cause for concern assessment of cytokine release in a suitable number of human donors. Next step will in most cases be to do preliminary PK and toxicology studies or dose range finding studies in the selected pharmacologically relevant species to gather early data supporting design of PK, immunogenicity and biomarker sampling schedule and doses and dosing frequency in the IND enabling GLP toxicology studies. These studies will often be of short duration 1–4 weeks and will usually include assessments after both single dose and multiple doses. The latter also enabling some early assessment of immunogenicity looking for changes in PK profile and PD effects from the first dose to the last dose in the study. Neutralizing immunogenicity assays are usually not developed until needed for the GLP toxicology studies or later for the human proof of concept (PoC) studies so a pharmacodynamics marker can be very helpful to assess if the generated anti-drug antibodies neutralizes the intended pharmacology. For mAbs, assays measuring degree of target saturation/target occupancy is usually also developed. This to facilitate early PK/PD modeling exercises helping in choice of dose and dosing frequency in the GLP toxicology studies.

13.3.3 Assessment of Tissue Cross-Reactivity (TXR)

Tissue cross-reactivity (TCR) studies are in vitro tissue-binding assays employing immunohistochemical (IHC) techniques conducted across 42 different human tissues from 3 individual donors (Points to consider in the manufacture and testing of Monoclonal Antibody Products for Human Use, 1997) to characterize binding of monoclonal antibodies and related antibody-like products to antigenic determinants in tissues. According to ICH S6 (R1), other technologies can be employed in place of IHC techniques to demonstrate target /binding site distribution. However TCR study with a panel of human tissues is a recommended component of the safety assessment package supporting initial clinical dosing of these products. In some cases, the clinical candidate is not a good IHC reagent and a TCR study might not be technically feasible. TCR studies can provide useful information to supplement knowledge of target distribution and can provide information on potential unexpected binding. Tissue binding per se does not indicate biological activity in vivo. In addition, binding to areas not typically accessible to the antibody in vivo (i.e., cytoplasm) is generally not relevant. Findings should be evaluated and interpreted in the context of the overall pharmacology and safety assessment data package. When there is unexpected binding in human tissues, an evaluation of selected animal tissues can provide supplemental information regarding potential correlations or lack thereof with preclinical toxicity. TCR using a full panel of animal tissues is not recommended. General limitations and issues around conduct and interpretation of tissue cross-reactivity studies have been well described and discussed by Leach et al. [31], supported by a range of case studies. The main issue with tissue cross-reactivity studies is that in preparing the tissue sections for microscopic

evaluation, the tissues are sliced through cells and membranes and enclosed organelles exposing molecules and structures that would under normal physiological conditions not be accessible to monoclonal antibodies. This results in a lot of irrelevant off-target staining and in multiple subsequent explanations for the lack of relevance of the observed binding and lack of physiological/toxicological relevance based on the lack of associated findings in the in vivo animal repeat dose toxicity studies. Many pharmaceutical companies are looking into potentially more relevant testing of binding in protein arrays e.g. the Retrogenics® array where up to 4.500 different cell-surface proteins are over-expressed on cells in a fashion and orientation supposedly more biologically relevant. Experience is being collected on the usefulness of such assays and relevance relative to the traditional tissue cross-reactivity. However, although S6 (R1) states that alternatives to TXR can be used, additional time and work is needed to gain regulatory acceptance of exchanging this type of studies for the regulatory guidance requested tissue cross-reactivity studies. Parallel assessments of TXR and protein/cell-arrays are needed and exchange and sharing of data between pharmaceutical companies to be able to present a strong scientifically supported and agreed alternative.

13.3.4 Sort Term IND-Enabling Tox Studies

Short term 4- or 13-week repeated dose toxicity studies in "two" species which in combination with the TXR study(ies) support dose setting for and IND submission for initiation of first in human clinical studies. These studies will usually also include assessments of cardiovascular, respiratory and central nervous system safetypharmacology and assessment of local tolerability at injection site. Based on the PK, immunogenicity and safety data from the preliminary studies in the preclinical phase the IND enabling GLP toxicology studies are designed. First duration of dosing and duration of dosing free recovery period for the studies are selected. The duration should be matched with the needed duration to show clinical effect in the Ph IIa proof of concept studies. In most cases at least 3 months clinical dosing in Ph IIa is needed to provide pharmacological data supporting signs of the intended effect in patients supporting further development of the product. By doing 13-week studies the desired dosing in Ph IIa is supported in the first round, and the need for doing both 4- and 13-week studies is avoided. Next the duration of recovery should be selected. Here the half-life determined in e.g. dose-range finding studies plays a major role. The relative long half-life of therapeutic antibodies in rat and monkey >2-weeks a recovery period allowing both for elimination (5 half-lives) of the antibody and recovery or at least signs of recovery of potential effects is needed. This can sometimes result in long recovery periods if full recovery is needed and shorter durations providing signs of recovery are used and accepted by regulators. Furthermore when assessing immunogenicity, most of the assays for assessing that are limited by their drug tolerance. Therefore, a need for the chosen recovery time to allow elimination of drug to levels below the drug tolerance level in the immunogenicity assay. Recovery periods in the IND enabling studies of mAbs are typically 2–6 months. When no adverse effects are expected the need for recovery groups can be considered and inclusion only to support assessment of immunogenicity is discouraged (ICH S6 (R1). Again to reduce the number of animals used, recovery is usually included only in control and high dose groups and not in low and mid dose groups.

Study lay-out: IND-enabling toxicology studies normally include a control group and 3–4 dose groups. Lowest dose is normally set at a level reflecting the anticipated human pharmacological dose. Then the high dose is according to ICH S6 (R1), set based on the following criteria – "1) a dose which provides the maximum intended pharmacological effect in the preclinical species and 2) a dose which provides an approximately 10-fold exposure multiple over the maximum exposure to be achieved in the clinic. The higher of these two doses should be chosen for the high

dose group in preclinical toxicity studies unless there is a justification for using a lower dose (e.g., maximum feasible SC/IV). Then the mid dose is set at a suitable mid-point between the high and low dose". Based on available PK/PD information and the use of modeling, the frequency of dosing is set to at least cover the frequency of dosing in clinical studies but also to ensure/enable exposure providing the desired e.g. at least tenfold relative to the exposure at the maximum expected clinical dose. A conservative approach with dosing at least 2x weekly if a 1x weekly clinical dosing frequency is anticipated can be prudent also to have flexibility to cover potential changes in clinical dosing due to need for higher exposure or in other indications requiring higher exposure. The route of administration (ROA) is usually selected IV or SC to mimic the intended human dosing. However to provide flexibility and have cover for both IV and SC dosing usually the study design include three dose groups using the intended ROA and one group using the high dose and the other IV or SC ROA. This will also provide assessment of bioavailability after SC dosing.

13.3.5 Long Term Chronic Toxicity Studies

The need for conducting longer term chronic toxicity studies does off course depend on the intended duration of treatment. For compounds intended for chronic treatment chronic toxicities should be completed. In principle these studies should also be conducted in a rodent and a non-rodent species if available. However, if the shorter term 4- or 13-week studies have shown similar effects in both species then based on a scientific justification a program using a single species for the chronic toxicity study is acceptable. In this case rat is the recommended species. As described in ICH S6 (R1), chronic toxicity studies in rodents and non-rodents of 6 months duration are considered sufficient. Chronic toxicity studies are normally conducted in sexually mature animals to allow assessment of fertility as outlined above. Then assessments of safety pharmacology, immunotoxicity/pharmacology and TDAR, as described above, can also be/are usually included for confirmation of the data from the shorter term studies and assessment of any late onset effects. As for the shorter term studies, evaluation of local tolerance is done by macroscopic and microscopic evaluations of the injection sites. Given the foreign nature of the human mAbs it is not uncommon that to see infiltration of lymphocytes and local minimal inflammatory reactions. MoA of the injected mAb (immune inhibitory or stimulating), pH and composition of the formulation can influence these reactions to the better or worse.

13.3.6 Reproductive Toxicity Testing

Reproductive toxicity testing should be done according to the ICH S5 (R2) guidance. These studies would assessment of potential effects on early embryonic development, on pre- and post-natal development and on embryo-fetal development. This either as separate studies covering pre or post-natal development or as combined so called enhanced pre and post-natal development (EPPND) studies the latter including post-natal follow up period of e.g. 6 months in monkeys. The latter usually conducted in parallel with Ph III clinical studies to be final at time of submission for registration. These studies should only be conducted in pharmacologically relevant species and preferably with the clinical candidate. For reproductive toxicity testing of mAbs, the use of relevant animal models is especially important. Both when it comes to potential placental transfer of the mAb from mother to fetus and the amount and timing of this transfer relative to expected human transfer and exposure of the fetus. mAbs have through their Fc part the ability to bind to the Fc Receptor neonate – FcRn expressed in the placenta enabling the transfer of IgG mAbs from mother to fetus [33]. In humans and monkeys there is hardly any transfer of maternal IgG in the first and second trimester but in the third trimester the transfer gradually increases and at birth the transfer results in levels of maternal Ab including the drug-mAb at simi-

lar or even higher concentrations than in the mother [34, 35]. In rodents, in contrast only a very small amount of maternal IgG is transferred over the placenta just before birth and mainly through the milk post-natally. Therefore for therapeutic mAbs the monkey is considered the most relevant species. However, the single fetus litter size makes it a very low-powered assessment and although normally 15 animals are evaluated per dose group reproductive studies in monkeys are considered mainly as hazard-identification studies. Reproductive toxicity programs can on a case-by case basis, be designed to fit the intended clinical use of the product and the pharmacological effects and potential or known risks for special phases of pregnancy or embryofetal development. In such cases separate EFD study and pre-and post-natal development studies can be conducted [36]. However for programs conducted in NHP's the most common practice is to expand this into what is termed an enhanced pre- and post-natal development study (ePPND) where animals are dosed from gestational day 20 to parturition followed by a 6 months post-natal follow up of mothers and off-spring.

13.3.7 Assessment of Potential Risk for Carcinogenicity

According to ICH S1A (Need for Carcinogenicity Studies of Pharmaceuticals), the need for this should be evaluated – This assessment is needed for products with longer than 6 months of treatment or when used chronically in a frequent intermittent way. For monoclonal antibodies the standard bioassays (1 year studies in rodents) are not considered appropriate and for antibodies only relevant in monkeys no specific animal studies assessing carcinogenicity are recommended. Carcinogenicity studies are not considered appropriate in monkeys and thus for products only active in monkeys no studies are recommended. Finally for products targeting life-threatening indications where the target population has life-expectancy of less than 2–3 years carcinogenicity studies may not be required. The most important part in this is to establish whether there based on the design of the molecule itself, the target or the intended mechanism of action and modulation of the target is a concern for carcinogenicity. Factors considered in this also includes: (I) previous demonstration of carcinogenic potential in the product class that is considered relevant to humans; (II) structure-activity relationship suggesting carcinogenic risk; (III) evidence of preneoplastic lesions in repeated dose toxicity studies; and (IV) long-term tissue retention of parent compound or metabolite(s) resulting in local tissue reactions or other pathophysiological responses (ICH S1a). The normal and recommended procedure is to make a weight of evidence based assessment including internal nonclinical and clinical data with the product, relevant literature on similar compounds, compounds with similar mechanism of action, the target itself and results from animals with target knock-out or over-expression of target. This assessment is then included in the marketing application. For submissions to FDA, via CDER there is a special executive carcinogenicity assessment committee (Exec CAC). This committee provides feed-back and concurrence both on plans for carcinogenicity studies and on the paper-based weight of evidence carcinogenicity assessments. Although assessment of carcinogenicity is not required prior to application for marketing authorization, it is recommended to submit the assessment for evaluation at least 6 months prior to the submission to allow for evaluation of and discussions with the Exec CAC to reach concurrence in good time before actual submission of the product file.

13.3.8 Specific Considerations and Challenges for Nonclinical Programs for Anti-infectious mAbs

For monoclonal antibodies and other related antibody products directed at foreign targets (i.e., bacterial, viral targets etc.), there are special challenges when conducting the nonclinical safety program due to the lack of relevant species. Therefore in the ICH S6 (R1) Guidance it is

stated that a short-term safety study of 2–4 weeks in one species (choice of species to be justified by the sponsor) can be considered. This would serve as a hazard-like assessment of any unexpected acute effects. Then, that when no relevant species exist that no additional toxicity studies, including chronic toxicity studies or reproductive toxicity studies, are appropriate or needed. In cases where special populations like children or pregnant women are indicated expectations for specific juvenile or reproductive studies can often come up. It is in these cases important to highlight that doing studies in non-relevant animal models will not provide any useful information and only a false sense of safety. Also from an ethical point of view regarding unnecessary use of animals such studies should be avoided. In some cases animal models of disease exist. However this often due to species specificity of the infective agent not in a model where the human candidate can be tested and not against the targeted human pathogen. An example of this is cytomegalovirus. For another virus RSV, there is an animal infection model, but in a very special model in cotton-rats where signs of efficacy can be assessed but no other relevant PK or safety information can be obtained. When relevant animal models of disease exist they can be used to evaluate proof of principle, and potential relevant safety assessment endpoints can be included to provide information on potential target-associated safety aspects.

One assessment which is normally included is the assessment of tissue cross-reactivity. In the absence of relevant species this can provide an assessment of potential off-target staining in human tissues. If staining is observed, TXR on tissues from rat or cyno can be included to assess if similar binding is observed in these species. If similar binding was observed, this could then be part of assessment of potential target organ effects in the subsequent short term toxicology studies in one or both of these species. When aiming to treat viruses like e.g. CMV, and assessing TXR it is worth noticing that the virus is present in most people as a silent infection and thus scattered cells in different tissues can harbor the virus and react e.g. with target expressed on infected cells. In this case a combined approach using immunohistochemistry for the assessment of binding of the antibody and in-situ hybridization for confirmation of viral infection of the cells can be used to confirm that staining is only associated with virally infected cells. As an additional activity a confirmed negative donor can be included to confirm absence of any binding in the absence of virus. If the product is intended for use in pregnant women or in children, relevant fetal or neonatal/juveninle human tissues can be included in the TXR study.

One case-example of a nonclinical safety program for an anti-infective antibody is the one conducted for palivizumab (Synagis®) (FDA pharmacology review), Synagis® is indicated for prevention of serious lower respiratory tract disease cause by syncytical virus (RSV) in pediatric patients at high risk for infection. Recommended dose of Synagis® is 15 mg/kg IM monthly during the season of RSV transmission. Synagis® is directed against the F protein on the virus important for initiation of viral entry and cell to cell fusion and is reactive against both the A and B subtypes of the virus. Synagis® was as mentioned above for many ears the only mAb approved for infectious diseases.

The nonclinical safety program conducted with Synagis®, included a first line of preclinical pharmacology studies of *in vitro* virus microneutralization studies showing inhibition of virus replication, and *in vivo* neutralization studies in cotton rats showing viral neutralization with treatment and prophylaxis of RSV challenge in lung and muscle of cotton rats. Furthermore to provide confirmation of broad coverage, neutralization of a large number of clinical isolates of RSV from different geographical locations was demonstrated. Then tissue cross-reactivity studies were conducted. First, one on the full panel of 42 human tissues from 2 adult and 1 neonatal donor. Due to some unspecific staining in a few tissues an additional study was done using a limited panel of cynomolgus monkey tissues to assess potential similar staining. After some trouble shooting to remove unspecific background staining, absence of binding was confirmed rela-

tive to infected cotton rat lung tissue serving as the positive control.

Finally 4 *in vivo* animal PK and toxicology studies were conducted. First a single dose PK study and a acute single dose toxicity study in cynomolgus monkeys. Then a single dose study in New Zealand White rabbits with SC administration, and finally a single study in Spraque Dawley rats with a 2 week observation period. No treatment related adverse effects were observed in these studies. The program outlined, supported clinical development and registration of Synagis®.

As can be appreciated from the case-example above nonclinical safety programs for anti-infectious monoclonal antibodies can be much more simple and restricted compared to the standard nonclinical program for mAbs. For Synagis®, the program was probably even more elaborate than what would have been done today with studies in three different species. Current praxis would be more like a 2–4 week study in one species. Although simple these programs also provide challenges both for setting up relevant animal infection models and then in the limited data provided for dose setting and monitoring in clinical studies.

13.3.9 Types of Assessments Not Included in Nonclinical Safety Programs for mAbs

A number of assessments normally conducted as part of nonclinical safety testing of low molecular weight drugs. These include mutagenesis or genotoxicity studies, ADME studies and studies on potential effects on ion-channels. For mutagenicity and genotoxicity, the standard phrase used in submission and other regulatory documents is that these types molecules are not considered to interact with DNA or chromosomes and therefore no studies on mutagenicity or genotoxicity were performed. For ADME studies and DDI, usually since antibodies are not metabolized by the liver and does not interact directly with CYP450 enzymes such studies are not relevant. However for some antibodies their mechanism of action results in release or inhibition of cytokines which can affect liver expression of several CYP450's. Ongoing inflammation and presence of e.g. IL6 or IL-2 or TNF-α, results in downregulation of CYP450 enzymes. There could therefore be a risk that when administering mAbs inhibiting some of these cytokines like adalimumab (Humira®) (anti-TNF-α) or toclizumab (Actemra®) (anti IL-6 receptor) then the lowering of enzymes caused by the inflammatory cytokines would be neutralized and the metabolism of concomitant drugs being metabolized by these enzymes would be faster resulting in loss of efficacy of those drugs [37, 38]. Finally for small molecules as part of the battery of safety pharmacology studies assessing potential interactions with ion-channels (sodium, potassium, calcium and hERG cannels) are investigated. However due to their size mAbs are not considered able to modulate function of these channels.

13.4 General Considerations Around Use and Development of mAbs in Particular for Infectious Diseases

As described in the first part of this chapter, mAbs or mAb derived structures are through their specificity, flexibility and safety very attractive tools for developing new therapeutics. This even more in combination with screening techniques like phage display, which allows for identifying binding peptides to a wide range of targets like toxins, lipids, carbohydrates or molecules or receptors only expressed under special conditions.

13.4.1 Antibody Subtypes and Formats

In the era of antibody-based therapies, full-size antibodies were and are still the main format and in that context mainly IgG and the vast majority of approved treatments are of the IgG1 isotype

(see Table 13.3). IgG is found in four different subtypes IgG1, IgG2, IgG3 and IgG4 with some specific characteristics (See Table 13.3). Only IgG1, IgG2 and IgG4 are used for therapeutic mAbs since they can bind to FcRn and thus have a long half-life of 21 days. IgG1, IgG2 and IgG4 differ in their binding capacity to activating FcγRs (FcγRIIIA/CD16 and FcγRIIA/CD32A) on immune effector cells, (NK cells, phagocytes), and in their ability to induce ADCC or CDC. IgG1 bind all FcγRs and fix complement and thus have the greatest potential for Fc-mediated effector functions (Table 13.3). IgG4 and IgG2 on the other hand do not bind or bind weakly to FcγRs and hence have little or no effector function [30]. Based on the different intended indication, patient population, target and mechanism of action the most suitable isotype can then be selected. For blocking a receptor on surface of cells with a wide tissue expression, IgG2 or IgG4 would be selected to avoid Fc-mediated effector functions and killing of the cells expressing the receptor and potentially triggering inflammation and cytokine release. In contrast in viral or cancer indications e.g. where killing of infected cells or tumor cells is desired, an IgG1 subtype would be selected to optimize killing by NK and cytotoxic T cells.

There is, however, work and research into variants of antibodies or new combinations of antibody-derived structures. Examples of these can be seen in Fig. 13.1 and include both bispecific full-size antibodies with antigen-binding sites with different targets capable of crosslinking targets or cells. Antibodies without Fc part – Fab or F(ab)2 fragments for blocking and when no Fc-mediated effector functions are desired, single-chain variable fragments (scFv), pairs of scFvs linked in different ways (diabodies and bites) also for blocking and targeting without Fc effector functions. Then, fusion-proteins containing, e.g., a receptor fused to an antibody Fc part, and many others not shown. Much creativity has been demonstrated in this field of research, and every imaginable variation and combination of antibody structures is being created and tested. This is done to test how these different formats and the attributes of the different parts of antibodies potentially can overcome the challenges facing the development of antibody-based therapies. These challenges include the high costs of production and finding optimal combinations of size, stability, half-life, efficacy, and very importantly, safety. These new formats also introduces new structures foreign to the immune system and thus immunogenicity is likely going to be a major factor is these developments with the potential risk of loss of efficacy, loss of exposure, potential hypersensitivity reactions, and potential cross-reactivity to endogenous receptors or immunoglobulins. As described above a lot of work was put into reducing immunogenicity and immune reactions to the animal-derived or chimeric antibodies by turning them into more and more fully human antibodies. It is therefore a paradox that with the introduction of these new formats e.g., new scaffolds with fibrinogen carrying antigen-binding sites), the risk of immunogenicity and immune reactions is being re-introduced. Time will show whether the safety and efficacy of these new formats will actually support their registration and use in patients with infectious diseases.

Antibodies also called immunoglobulins (Ig's) based on their combined structure and function in immuneresponses, are produced by B cells of the immune system. They are part of the adaptive immune response and specially designed for neutralizing and eliminating infectious agents and toxins produced by these. Antibodies are Y-shaped structures consisting of two main parts the upper arms of the Y containing two identical variable region antigen binding sites and the lower region called the constant region responsible for initiation of effector functions leading to removal and destruction of the pathogen or cells harboring the pathogen. The antigen binding sites on an antibody can by themselves bind to and neutralize bacterial toxins and viruses thereby preventing them from binding to their target cells or receptors causing toxic effects or spread of the infection.

Antibodies consist of two pairs of heavy and light chains which as described above are held together in a Y shaped arrangement. Each of the heavy and light chains in these pairs are separated in constant and variable chains. The upper arms of

Table 13.4 IgG subclasses and characteristics

Subclass	IgG1	IgG2	IgG3	IgG4
Serum half-life (days)	21	21	7	21
FcRn binding	++	++	1	++
FcγRI binding	++++	++	1	++
FcγRIIA binding	+++	+	++	+
FcγRIIB binding	++	+	++	+
FcγRIIC binding	++	+	++	+
FcγRIIIA binding	+++	+/_	+++	+
FcγRIIIB binding	++	–	++	–
C1q (complment activation)	++	+	+++	–

Four different heavy chain versions exist for IgG, creating IgG1, 2, 3, and 4 isotypes each with different characteristics in their serum half-lives and ability to trigger different effector functions. The combined advantage in triggering of effector mechanisms, long half-life (21 days), their ability to be transported over the placenta and their stability in the production process, antibodies of the IgG class and of the IgG1 isotype are preferred as basis for new antibody based therapies. The effector functions are triggered by the Fc part of an antibody when the variable pars of the antibody is bound directly to an infectious agent or to proteins from an infectious agent expressed on an infected cell. This binding, leaves the Fc part free to interact with Fc receptors on phagocytic cells and neutrophils, eosinophils and natural killer cells capable of inducing phagocytosis or lysis of infectious agents or infected cells

FcRn is the neonatal FcR expressed on monocytes, macrophages, DCs, hepatocytes, epithelial cells, endothelium of small blood vessesl, intestinal endothelium and placenta. Binding to and re-cycling in FcRn expressing cells is the main factor providing the long half-life for the IgG antibodies, and also ensures transfer of maternal antibodies to the fetus during pregnancy during the last trimester

FcγRI (CD64) is the high affinity receptor for IgG expressed on monocytes, macrophages, neutrophils, eosinophils, DCs, mast cells, platelets, microglia. C

FcγRIIA (CD32A) is the low/medium affinity receptor for IgG expressed on monocytes, macrophages, neutrophils, eosinophils, NK cells, platelets, microglial cells, Langerhans cells

FcγRIIB (CD32B1) is the low affinity inhibitory receptor for IgG expressed on B cells and mast cells

FcγRIIC (CD32B2) is the low affinity receptor for IgG expressed on macrophages, neutrophils, eosinophils

FcγRIIIA is the low/medium affinity receptor for IgG expressed on NK cells, monocytes, macrophages, subset of T cells(?). FcγRIIIB is the low affinity receptor for IgG expressed on eosinophils, neutrophils, monocytes, macrophages, mast cells, follicular DCs

C1q is the first component of complement

the Y each contain a variable and a constant section of the light and the heavy chain, where the upper variable parts of the heavy and light chains contain the antigen binding site and the constant parts are connected via disulfide bonds. The lower part of the Y the called the constant part or Fc part, consist of 2–3 constant segments (immunoglobulin domains) from each of the two heavy chains interacting and also linked via disulfide bonds. The sections of the heavy chains connecting the constant part of the upper arms to the constant parts of the constant region contains special hinge regions providing flexibility to the different sections of the antibody to bind to antigens and effector cells. The constant regions of the heavy chain also contains attached oligosaccharide moieties which also provides functional specialization to the antibodies [6].

The variable domains of the heavy and light chains forms the antigen binding sites and contain special hyper-variable segments also named CDRs (complementarity- determining regions) allowing the B cells via genetic recombination to generate antibodies to all the different antigens (specific of amino acid sequences or 3-dimentional structural motifs (polysaccharides, DNA and RNA) found on pathogens (Table 13.4).

13.4.2 Treatment of Special Populations

Safety and flexibility of mAb based therapeutics are also valuable when treating special populations like children, elderly or pregnant women. This, due to the lower tolerability and higher

risks associated with potential side effects in these populations. In children and juveniles additionally, potential detrimental effects to developing organs or organ systems can occur. In elderly and children with undeveloped or less well functioning immune system potential risks in relation to treatment with immunomodulatory mAb treatments the potential higher risk from opportunistic infections. For mAb treatments targeting juveniles, a special part of the nonclinical and clinical development is to do juvenile toxicity studies and careful PK assessments and introduction of treatment in a stepwise fashion ensuring safety in adolescents/teenagers (age 13–18) prior to going into to school children (age 6–12) and then again before going into pre-school children and toddlers (age 2–6). When treating pregnant women, there are both potential pros and cons to consider. Antibodies are normally transferred to the fetus via the FcRn expressed in maternal and fetal parts of the placenta. In humans, transfer starts slowly in the second trimester and increases through the third trimester, to the extend where concentration of maternal antibodies in the fetus at birth can be even higher than in the maternal circulation. This makes the fetus very sensitive to the potential pharmacological and adverse effects of a mAb-based treatment. When choosing IgG subtype, IgG2 has the lowest placental transfer and could be considered in antibodies targeting pregnant women. Also, if Fc related effector functions are wanted or to be avoided it is important to consider that IgG2 has very low affinity for Fc-γIII and thus does not support antibody effector functions like ADCC or CDC. The placental transfer through FcRn, can however also be used to support treatment. This e.g. when treating congenital infections with cytomegalovirus where transfer of the infection during pregnancy causes developmental and hearing defects in the fetus. Here mAb treatment could potentially prevent the transfer of the virus and viral infection to the fetus by killing the virus early in the mother in general but especially in the placental tissues.

13.4.3 Specificity: Good or Bad?

The high specificity of mAbs contributes to their safety but also means mAb treatments are restricted to one pathogen, based on binding to specific toxins, or surface molecules expressed on their surface. This in contrast to antibiotics which target more general functions in e.g. bacteria like cell wall synthesis, membrane function, inhibition of protein or nucleic acid synthesis or metabolic processes allowing them to function in a more broad way in bacteria or vira sharing special features in these general functions. However their vast overuse has resulted in development of resistance and life-threatening untreatable infections like MRSA, creating a demand for new treatment modalities. For this, antibody based therapeutics which alone or in combination with antibiotics could delay or circumvent the resistance would be creating extremely valuable future treatment perspectives. Several features of mAbs, are however working against them when comparing with antimicrobial therapies. First, and most importantly, the very high cost of production. This in the absence of resistance makes them unsuitable for first line or prophylactic treatment. In addition, since antibodies are proteins, they need to be treated carefully and be kept refrigerated, and they are administered by intravenous or subcutaneous injection. In contrast, antibiotics usually come in the form of tablets that can be taken orally and can be kept in a bag or in a closet at room temperature. Antibiotics normally target general mechanisms, e.g., cell wall formation in bacteria, and can act against, e.g., a broad spectrum of bacteria; whereas antibodies are very specific to a single virus, bacteria, or bacterial subtype, and a clear diagnosis should be made before treatment with a monoclonal antibody. This, both from the perspective of a patient in need of treatment and from an economical perspective contributes to the overall cost of monoclonal antibody therapeutics. This highlights the need for improving and developing diagnostics

in parallel to developing antibody therapies but also that these diagnostics must be providing quick answers to avoid delays in treatment and must be easy to handle under challenging weather conditions or less advanced medical facilities and by less trained staff in developing countries. The specificity also has a general economical dimension since it results in a smaller market compared to broad-spectrum antibiotics, making antibody-based therapies less attractive for pharmaceutical companies to develop. The specificity can however, in other ways, be considered a further safety benefit. Antibodies work only on a specific infectious agent, and although mutations in that agent can render the antibody ineffective, this does not affect other similar agents and thus does not cause the spread of resistance. The specificity also ensures that the gastrointestinal effects often observed with antibiotics due to broad effects on bacterial flora in the gut are avoided.

13.4.4 Antibody Engineering

Another strategy for increasing likelihood of clinical efficacy and success is to after choice of antibody format and/or subtype to use antibody engineering, to increase the ability of the treatment to reach the intended target, and avoid unwanted side effects resulting from the killing of non-target cells.

Optimizing effector functions:

This more precisely refers to, genetic manipulation of the Fc domain (mainly in the CH2 domain) or changes to the glucosylation pattern of the N-linked oligosaccharide moieties attached at antibody N297 in the Fc part of the heavy chain [39]. For generating antibodies with enhanced effector functions, different mutations have been identified that have increased affinity to the FcγIIIa receptor and a significant enhanced cellular cytotoxicity (S239D/A330L/I332E, also known as 3M [37, 38], F243L [41], and G236A). These antibodies, either directly or indirectly enhance binding of Fc receptors and thus significantly enhance cellular cytotoxicity. Enhanced effector function can also be achieved by modulating the oligosaccharide moieties [40]. Removal of fucose from the A297 linked oligosaccharide moieties, which creates so-called afucosylated Fc domains, has been shown to greatly increase the potency for inducing antibody-dependent cellular cytotoxicity [42]. This can be achieved by manufacturing the antibodies in cell lines lacking the enzyme fucosyl transferase, which renders them unable to add fucose to the Fc oligosaccharide moieties [42].

Similarly, ways to reduce or ablate the ability of antibodies to trigger effector functions have been described and are being used broadly in cases where the aim is to block specific membrane-bound receptors/targets and where killing of the cell harboring the target is not desired. Again, mutations in the Fc part, e.g., the mutations L234A and L235A, also called the LALA mutation, greatly reduce but do not completely remove effector functions by removing amino acids important for the C1q factor of complement [41–43]. Modulation of the glycosylation pattern, in this case creating completely aglycosylated antibodies, has also been shown to remove the ability to properly bind Fc receptors on effector cells and trigger effector functions. One alternative approach used especially when developing immuno-modulatory agonistic antibodies is the use of antibodies of the IgG4 isotype, which does not trigger effector functions. Finally, mutations in the Fc part that increase the affinity to the FcRn receptor have also been used to create antibodies with an increased half-life. Introduction of three mutations in the Fc domain (M252Y, S254T, and T2556E, also called the YTE) has been shown to provide a half-life extension of three to fourfold [45]. From a convenience point of view, a long half-life is obviously attractive, but it can be a down-side in the case of severe adverse effects due to the long duration of action, demonstrated the potential for developing

such antibodies. However, they are not widely used in cancer therapy.

13.5 Considerations Specific to Developing Antibody-Based Therapies for Infectious Diseases

As mentioned above, the past 15–20 years saw a revolution in development and approvals of monoclonal antibody-based therapies for inflammatory and neoplastic diseases. As can be seen in Table 13.1, more than 60 antibody-based therapies are now approved for therapeutic use. This revolution, however, did not include treatments for infectious diseases, with still only three therapeutic antibodies for infectious diseases being approved. This is, in these times of increasing multidrug resistance, inability to treat immunocompromised patients, risk of bioterrorism and new emerging diseases, surprising since there is a real need for alternatives to the current armament.

There are however despite extensive research and development and promising treatments in clinical testing, challenges preventing a real "take-off" of mAb based treatments. First the price and narrow indication of mAb therapeutics compared to e.g. antibiotics. With respect to price antibiotics can be purchased from $10–$3500 (https://www.goodrx.com/) whereas treatments with therapeutic antibodies are in the range of $30–$50,000. Here both development and manufacturing costs play in but prices for mAbs are also highly regulated by the market. A recent case highlights the effect of the market and show that anti infectious agents can be very costly as well. This case is Sovaldi (Sofosbuvir) which is an important new treatment for Hepatitis C infection. The manufacturer Gilead is charging $1,000 per pill making it up to $84,000 for a simple cause of Hepatitis C treatment. This price is preventing treatment of numerous patients who could benefit from treatment. For early and broader treatments in pandemics the high prices raises extra challenges for health providers worldwide. In that context, vaccine development programs should be intensified in aftermath of pandemics like the recent one caused by Ebola ensuring that proper vaccines are available to limit extend and spread of a new one. WHO based library of screened high avidity antibodies or IVIGs could be tested/developed for such diseases in an expedited fashion.

Apart from price there is also as previously mentioned due to the specificity of the mAbs, the need for diagnostics. Although diagnostics are becoming more available, this is an extra hurdle and cost in the development and price and can require equipment and facilities not present in rural and less developed country sites. Furthermore mAb based products are more sensitive to shifting conditions and usually require storage at <25°C or at 2°C–8°C and in the dark. Treatment with mAbs usually require IV or SC injections, often in a hospital setting by qualified staff whereas antibiotics can be carried in a handbag and swallowed with a glass of water.

13.5.1 Strategy to Avoid Escape Mutants

Although antibody treatments do not induce resistance in non-target bacteria or viruses, both bacteria and viruses have the ability to escape the host immune system and specific antibody treatments via mutations that change their surface proteins or structure, creating so-called escape mutants that are no longer neutralized by the specific antibody. The best way to circumvent this is by using a combination of antibodies directed at different viral targets. The use of cocktails of two or more antibodies was shown to provide synergism or additive effects in neutralizing, e.g., hepatitis B virus (HBV) and respiratory syncytical virus (RSV) infections [44]. Combinations of antibodies have also been used in HIV, targeting GP41 and GP120 viral proteins [45], and rabies [46] and are part of the strategy in most pharmaceutical companies that are developing antiviral antibodies this even more extensively in developing mAb treatments for cancer where especially combination with so-called immune check-point inhibitors like PD1/PDL1. To assess potential for escape

mutations, part of the early preclinical development of such antibody combinations consists of serial passage of virally infected cells (>20 generations) to ensure continued efficacy and the absence of escape mutants. This is then combined with testing against known patient isolates, when available. Cocktails of antibodies would also be an interesting approach to target groups of infectious agents often seen in parallel in, e.g., burn wounds and with a potential wider application such treatment cocktails might be more attractive to pharmaceutical companies although it poses additional challenges in the manufacturing and control of the compounds [48].

13.5.2 Challenges in Obtaining Relevant Safety and PD Data

As described above, most of the targets in treatment of infectious diseases are foreign (viral, bacterial, fungal) and there are often no relevant animal species where the antibodies can be tested. Also the diseases themselves can be very specific and thus disease models or relevant surrogate models for safety or efficacy testing either don't exist or are of questionable relevance. As a result the safety data that can be generated are very limited and usually come from just short single species (mainly rat) hazard identification studies. These are then furthermore done in healthy animals and not in animals in a more relevant diseased state. No relevant studies can be performed to assess potential chronic or reproductive toxicity effects. This lack of safety data poses extra challenges when treating e.g. children, elderly or pregnant women or women of child-baring potential. In such cases assessment of tissue cross-reactivity on placenta and on fetal human tissues might be the only additional information to inform of potential off-target toxicity pregnant women of their fetus. Another example of the limitations in obtainable data was seen in the case of raxibacumab (ABThrax®), indicated for treatment of infections with bacillus anthrax. Here clinical PK information and tolerability could be assessed in human healthy volunteers, but since it is unethical to conduct studies with experimentally induced infections with b anthrax in humans, this antibody was approved based on animal efficacy data only.

13.5.3 Combine with and Supplement/Rescue Antibiotics

Another consideration for the future is to focus more on developing treatments that are adjunct to existing ones, e.g., antibiotic treatment instead of a stand-alone treatment. This might provide extra efficacy in patients with failing immune response and potentially also compensate for and reduce the development of resistance, ensuring availability of future efficacious treatment options.

On the cost side, research into the use of the smaller and cheaper antibody-based fragments could contribute to making antibody-based treatments more attractive and more efficient, and cheaper ways of manufacturing antibodies would be vital contributions to this.

13.6 Summary and Conclusions

There is no doubt that antibody-based treatments are very attractive treatment modalities. The flexibility to design specific format, size, half-life and effector functions makes them ideal to hit a specific target at the desired site of action. This even more when combined with their good safety and the available screening tools like phage display enabling selection for specific targets on infectious agents or host immune cells which might not be feasible using more traditional immunization strategies.

The good safety profile of mAb and mAb derived therapies is definitively a great attribute, and adverse effects normally related to exaggeration of the intended pharmacology. However, as mentioned in the sections above there are potential safety issues like cytokine release syndrome, inflammatory reactions local and systemic from killing of infected cells and ADE where the first treatment might have solved one problem, curing the acute infection, but on the other hand created

a bigger problem in case of subsequent infection with the same pathogen but of different serotype. This highlight the statement important to all involved in developing medicines and especially biologics and mAbs – "KNOW YOUR TARGET". This, both with respect to biology and physiological context, but also very much expression and expression in humans relative to the animal species used for nonclinical safety testing during development. More in-depth knowledge of target biology and expression might also help in selection of and development of better models for pharmacology and safety studies and increasing the translatability of the animal data to the human setting and understanding the limitations of the models applied. For the development of anti-infectious disease treatments the species specificity of the infectious agents and the foreign nature of the target often makes it even more difficult to test the actual human therapeutic in a relevant species or in a relevant animal infection model. This limits the data that can be obtained to provide useful dose-response information and informing on potential efficacious doses in humans or information on special safety issues or reactions in diseased animals which might have provided vital information of issues to monitor in diseased humans.

At this time only three mAb based treatments are approved for infectious diseases and for many years and until recently only one mAb, palivizumab (Synagis®) was approved. How do we now get from 1 to 3, to 1 to 3 to MANY?

There is definitively a huge need and demand for alternatives to or supplements to antibiotics to fight the growing problem of resistance to antibiotics and resulting life-threatening inability to treat bacterial and viral infections. A large part of the medical need for anti-infective treatments is however in less developed countries where health care systems and patients are struggling with the prices for the currently available treatments. In that context the current business models and prices which could be taken for new mAb based treatments would not make development and marketing of those attractive to pharmaceutical companies putting a brake on development and approval of more of these. Therefore new approaches and initiatives are needed.

The main driving force and passion for people and companies involved in pharmaceutical development is to get medicine to patients in need, to save patients' lives, to help patients and improve their quality of life. Pharmaceutical companies are however businesses where shareholders and or investors require growth and some return on their investments. It is therefore necessary to find a balance where companies can both "do the right thing" from a patient and ethical point of view and still deliver acceptable profit.

One initiative called "Access to Medicines" supported by the Access to medicines Foundation, is by many pharmaceutical companies seen as a way to develop their business in emerging markets. Every second year an Access to Medicines Index is published where pharmaceutical companies are ranked based on the initiatives and progress within areas like – General access, Market Influence, R&D, Pricing Manufacturing and Distribution, Patents and licensing activities, Capacity building and Donations. Apart from supporting the development strategy in emerging markets efforts to improve you ranking in this index also improves the public image of the individual companies. Through such initiatives hopefully collaborations with local governments, collaborations between companies in consortia or projects run in collaboration with organisations/institutions like WHO, FDA, EMA, EFPIA can be established and facilitate business models and research and development in antibody based therapies which definitively holds great potential to provide new and safe treatments for infectious diseases and help in dealing with the increasing heath problem from resistance to the currently available antibiotic treatments.

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