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

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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].

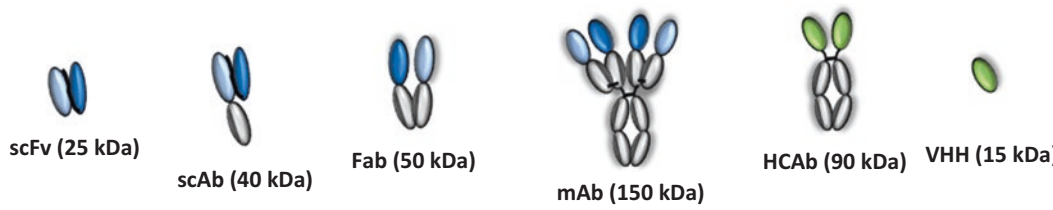

Fig. 6.1 Various antibody formats described in this chapter and their molecular mass

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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