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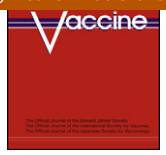

Review

Monoclonal antibodies for prophylactic and therapeutic use against viral infections

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ABSTRACT

Neutralizing antibodies play an essential part in antiviral immunity and are instrumental in preventing or modulating viral diseases. Polyclonal antibody preparations are increasingly being replaced by highly potent monoclonal antibodies (mAbs). Cocktails of mAbs and bispecific constructs can be used to simultaneously target multiple viral epitopes and to overcome issues of neutralization escape. Advances in antibody engineering have led to a large array of novel mAb formats, while deeper insight into the biology of several viruses and increasing knowledge of their neutralizing epitopes has extended the list of potential targets. In addition, progress in developing inexpensive production platforms will make antiviral mAbs more widely available and affordable.

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1. Passive immunization with polyclonal sera

Passive immunization is based on the administration of serum from convalescent/vaccinated human donors or animals to attempt to prevent or control infection [1,2]. Whilst vaccines require time to induce immunity and depend on the host's ability to mount an immune response, passive immunization can provide immediate protection and is theoretically independent of the recipient's immune status. Following the development of anti-diphtheria serum by von Behring and Kitasato in the early 1890s [3], immune sera from convalescent humans were used to prevent or treat a range of viral diseases including measles, the 1918 pandemic flu, varicella-zoster virus, Bolivian hemorrhagic fever, Argentine hemorrhagic fever as well as Ebola and Lassa hemorrhagic fevers [4]. Moreover, some of the earliest attempts to cure veterinary diseases involved passive immunization with serum from recovered animals as was described in seminal attempts to 'cure' rinderpest in the 1890s [5]. Today, several pooled antiviral immunoglobulin products are still available on the US market including

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hyperimmune immunoglobulin preparations against rabies virus, cytomegalovirus, hepatitis B and C viruses, vaccinia virus, varicella-zoster virus, respiratory syncytial virus (RSV) and West Nile virus.

A common disadvantage of polyclonal preparations is that many of their constituent virus-specific antibodies are non-neutralizing [4]. Moreover, polyclonal sera have to be screened and treated due to risks related with the use of blood products. Problems associated with the use of polyclonal sera might also include batch-to-batch variation and difficulties in obtaining immune donors [1,6]. An alternative to polyclonal antibody preparations is offered through the development of monoclonal antibodies (mAbs).

2. Development of monoclonal antibodies

In 1975, Köhler and Milstein developed hybridomas at the Medical Research Council Laboratory of Molecular Biology in Cambridge, UK [7]. Since then, technologies for generating and engineering mAbs have greatly improved and the industrialization of mAb production has resulted in a large number of antiviral mAbs being developed for preclinical and clinical studies. Fully human mAbs (Fig. 1A) with minimized immunogenicity can now be generated using methods such as phage display [8] and purified envelope glycoproteins in either monomeric or oligomeric forms and viral particles are two types of antigen that are commonly used as bait for panning antibody libraries [4]. These antibody libraries are either naïve for the viral antigen [9,10], or can be obtained from convalescent or immunized patients or animals.

The first antiviral mAb approved by the US Food and Drug Administration (FDA) was palivizumab (Synagis/MedImmune), a humanized IgG1 antibody that confers RSV prophylaxis in high risk infants [11,12]. Prior to palivizumab, prophylaxis of RSV disease depended on a polyclonal serum preparation called RespiGam (or RSV-IGIV). This polyclonal preparation showed relatively low specific activity, and dosing required the application of relatively large volumes of antibody in low weight infants [13,14]. The greater potency of palivizumab reduced the volume required to deliver a therapeutic dose to an infant and has improved RSV treatment by avoiding the side effects of pooled serum [13,14].

3. Antiviral immunity

Specific antibody titers have been identified as correlates of protection against various viral infections. Antibodies operate through various mechanisms, mediated by either their variable or constant regions. Highly selective binding to specific epitopes on the target antigen is a functionally crucial property that is mediated by the antibody variable domains [15]. The antibody constant domains include the Fc region and perform other important functions including antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) [15]. ADCC and ADCP are mediated by Fcγ receptors while CDC is mediated by complement cascade proteins such as C1q and C5 [16]. Another function of the Fc region is extension of antibody half-life (21 days for human IgG) through interaction with the neonatal Fc receptor (FcRn) [17].

Antibodies can interfere with virus entry into a cell by various mechanisms [4]. One mechanism is inhibition of virus attachment to cell surface receptors. This can be achieved through antibody binding to viral spikes, thereby interfering with their ability to bind to cellular receptors [18]. The same effect is achieved by antibodies targeting receptors or co-receptors, thereby making the binding sites for viruses unavailable [19]. Another mechanism is post-binding/pre-fusion neutralization and interference with required conformational changes at the cell membrane or endosomal membrane by antibodies that target non-receptor binding regions [20]. Additional mechanisms of virus neutralization include antibody-mediated crosslinking of virions [21,22], resulting in their immobilization and agglutination, or inhibition of the release of progeny virus, observed e.g. for antibodies against influenza virus [23].

In general, virus neutralization is considered to occur when a sufficient number of epitopes on the viral surface are occupied by antibody. This 'occupancy' model, sometimes referred to as the 'multi-hit model', proposes that obtaining a sufficient antibody density on a virion is the most critical factor for neutralization, leading to inhibition of attachment to cellular receptors or interference with endosomal or plasma membrane fusion processes [24,25]. An alternative model of neutralization is the 'critical binding site' model which is compatible with both a single-or multi-hit theory of neutralization [4]. According to this model, neutralization depends on targeting essential binding sites and is less dependent on obtaining high antibody densities on the viral surface [4,26].

In addition to their ability to directly interfere with virus entry into a cell, antibodies can counteract viral infection by means of their Fc effector functions [27,28]. The extent to which effector functions contribute to protection appears to be specific for different viruses. For HIV-1, it has been demonstrated that a neutralizing mAb engineered not to activate complement is as protective as the wildtype antibody [29]. However, when both complement and the FcRn (neonatal Fc receptor) were abolished, the same antibody showed reduced *in vivo* protective capacity [29]. While these observations point to an important role of ADCC in HIV neutralization, Fc effector functions do not seem to be required for neutralization of several other viruses, e.g. the antibody Fc region and its associated effector functions are not necessary for neutralization of rabies virus [1]. Equine sera for rabies post-exposure-prophylaxis (PEP) in humans routinely consists of $F[ab']_2$ fragments which are prepared by pepsin digestion and are devoid of the Fc region [30].

In some cases, antibodies may also act as immunomodulators and certain antiviral mAbs have been shown to have a 'vaccine-like effect' [31,32]: mice infected with a murine retrovirus and subjected to a short immunotherapy with a neutralizing mAb of the IgG2a isotype remained healthy and mounted a long-lasting protective antiviral immunity with strong humoral and cellular immune responses. The endogenous antiviral antibodies generated in mAb-treated mice allowed containment of viral propagation and enhancement of memory cellular responses after disappearance of the injected mAb. The administration of the mAb permitted the development of a long-lasting endogenous antiviral immunity, pointing to an important role for infected-cell/antibody immune complexes for long-term protection mediated by short passive immunotherapy [31,32].

4. Viral escape mutants

For an effective immunoprophylaxis, the antigenic variability of circulating viral strains and the potential for emergence of viral escape mutants need to be considered. These considerations are of special importance in the case of influenza A viruses where both antigenic drift and antigenic shift occur naturally and in the case of HIV where formation of different quasispecies with many different virus variants drives immune evasion. RNA viruses possess RNA polymerases devoid of proofreading and repair capabilities which may result in the emergence of resistant mutants under selective pressure, such as mAb administration. Escape mutants can be generated *in vitro* under selective pressure of antibodies [33,34], as observed e.g. with mAbs against chikungunya virus. Intriguingly, high-throughput sequencing also detected the mutated residues associated with the chikungunya viral escapes in sequences derived from virus treated with a non-specific antibody although

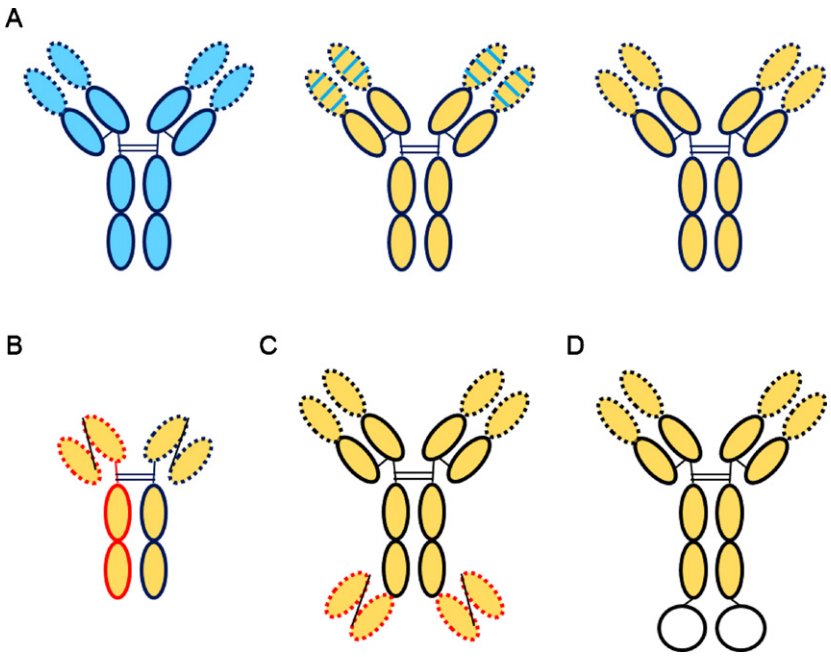

Fig. 1. Antiviral mAb formats. A: Murine (left panel), humanized (middle) and fully human mAbs (right). The humanized mAb (e.g. palivizumab) contains both murine (blue) and human (yellow) sequences. B: Scheme of bispecific immunoadhesins. Immunoadhesins were generated using the Knob-into-hole technology which involves the introduction of certain 'knob' and 'hole' mutations in the CH3 domain of the Fc region to fuse two scFv-Fc molecules with different specificities. The mutated Fc regions favor HC heterodimerization over homodimerization, thereby minimizing the pairing of identical halves. C: Scheme of Morrison-type bispecific mAbs. Full-size mAbs and scFvs were fused to each other and issues of antibody stability were addressed by design optimization, including disulfide stabilization of scFvs and various linker designs. D: Scheme of multimeric mAb-fusion molecule. This transgenic plant-derived molecule combines the functional activities of the anti-HIV mAb b12 and the small microbicidal protein cyanovirin.

their proportion was extremely low (0.05–0.20% of the total nucleotides at each position), suggesting that minor pre-existing viral quasi-species were amplified under selective pressure [34]. In addition to isolating viral escape mutants *in vitro*, they can also be isolated *in vivo*, e.g. influenza H5N1 escape mutants have been isolated from the lungs of mice receiving anti-H5N1 mAbs [35]. Moreover, resistant RSV variants could be isolated from patients receiving palivizumab [36]: nucleotide sequence analysis of RSV isolates collected directly from infants who received palivizumab and still developed acute lower tract respiratory infection revealed specific mutations in the RSV fusion protein, allowing the virus to escape neutralization [36]. A second generation, affinity-matured variant of palivizumab, termed motavizumab, has recently been developed and investigated in a large comparative phase 3 clinical study of the two preparations. Similar to palivizumab treatment, resistant RSV variants containing certain sequence changes in the RSV envelope protein were generated either *in vitro* or collected from RSV breakthrough patients receiving motavizumab [36].

The emergence of viral escapes can be accompanied by alterations in viral fitness which can affect virus growth both *in vitro* and in the infected host. Mutations may render the viral escape mutant resistant to a specific mAb, but alterations in growth and infectivity may render the virus attenuated so that it can be cleared by the host's immune system [37].

5. Cocktails of mAbs

Broad coverage of different strains as well as prevention of viral escape mutants are important considerations in the development of passive immunotherapies. As such, various combinations of mAbs have been developed and assessed (Table 1) [38–40]. The mAbs are selected for inclusion in a cocktail based on specificity and functionality, such that they complement each other with regards to breadth and specificities and do not compete for antigen binding [41–43].

Cocktails of mAbs might be required if the target epitope of a single mAb is not conserved on all strains of a virus, especially in the case of human infections that emerge from heterogeneous pools circulating in various animal reservoirs. For example, the genus *Lyssavirus* comprises numerous different closely related virus strains which circulate in a range of different hosts of the orders *Carnivora* (dogs, wildlife) and *Chiroptera* (bats) [44,45]. Following a severe exposure to a rabid animal, the prompt administration of rabies PEP including the administration of human or equine rabies immunoglobulins (HRIG and ERIG, respectively) can prevent development of rabies and death in previously unvaccinated victims [46,47]. Crucell/Sanofi are developing CL184, a cocktail of two potent mAbs to replace HRIG and ERIG in PEP [48,49]. This cocktail was designed by applying two main criteria [50,51]. First, the mAbs should cover a wide range of viral variants, targeting distinct, non-overlapping epitopes and preferably should not compete for antigen binding. Secondly, in vitro-generated mAb-resistant escape mutants selected using one antibody should be neutralized by the other nonselecting mAb in the cocktail and vice versa [50,51]. Crucell's mAb cocktail is undergoing clinical trials [52] and exemplifies how issues of viral heterogeneity and

Table 1
Examples for antiviral mAb cocktails under investigation.

Target	No. of mAbs included	Reference
Rabies virus	2 or 3 mAbs	[6,38,40]
HIV	2, 3 or 4 mAbs	[39,54–56]
SARS-CoV	2 or 3 mAbs	[9,53]
Hepatitis B virus	3 mAbs	[41]
Ebola virus	2 or 3 mAbs	[42,43]
Influenza virus	2 mAbs	[35]

emergence of resistant virus variants can be overcome with a combination of two mAbs.

In addition to these anti-rabies mAbs, combinations of mAbs have been developed against several other viral diseases including influenza. In this instance two mAbs that target the influenza A H5N1 hemagglutinin molecule were developed [35]. These mAbs were shown to target different epitopes and demonstrated reciprocal coverage of escape mutants. In combination, the two mAbs showed broad coverage of different clades and no escape variants were detected after therapy [35]. Similarly, a combination of two non-competing human mAbs against SARS-coronavirus (SARS-CoV) has been developed [53]. This combination potentially controls immune escape, extends the breadth of protection and may allow for a lower total antibody dose to be administered for passive immune prophylaxis of SARS-CoV infection. Synergism of neutralizing mAbs has not only been reported for SARS-CoV, but has also been observed e.g. for combinations of two, three, or four mAbs directed against different epitopes on the HIV-1 envelope glycoprotein, leading to a 2–10-fold increase of neutralization titers [54–56].

6. Multivalent and multispecific mAbs

When considering the biological requirements for an antiviral mAb, antibody valency is an important factor, as observed for varicella-zoster virus, HIV and rabies virus [21,57,58]. Bivalent antibody binding can mediate the cross-linking of virions, resulting in their immobilization or agglutination. It has been shown that certain epitopes, e.g. on Herpes simplex virus (HSV), can be efficiently targeted only with bivalent antibody formats (full-size mAb, F[ab']$_2$) while the use of monovalent antibody formats (scFv, F[ab]) severely diminished neutralization [22]. HSV neutralization by F[ab] fragments could be restored by cross-linkage of F[ab]s, using IgGs reacting with murine F[ab] fragments. These observations demonstrated that neutralization by this mAb is dependent on cross-linkage of glycoprotein B (gB) trimers and that immobilization of gB trimers inhibits activation of the fusogenic signal. Consequently only bivalent mAb derivatives exhibited adequate *in vitro* neutralizing activity [22].

In nature, multivalency is achieved through dimerisation or multimerisation of immunoglobulin sub-units, resulting in polymeric or secretory IgA or IgM antibodies. Whilst IgM antibodies are generally considered to have low binding affinity and to be important in primary immune responses, secretory IgA (SIgA) is the predominant protective antibody in all mucosal secretions. Its somewhat complicated assembly requirements, which naturally requires a plasma cell to produce dimeric IgA and an epithelial cells that contributes the secretory component, has resulted in slow progress in the development of these mAbs. Although expression is possible in mammalian cell expression systems [59], this approach is difficult to scale-up. However, recombinant secretory antibody production has also been described in plant systems [60] and offers hope for SIgA based prophylaxis of mucosal infections for the future.

Generally, bivalent antibody binding contributes to neutralization of viruses that express high densities of surface spikes, such as RSV and influenza virus [61,62]. In contrast, HIV has only a limited number of surface spikes and it has been proposed that this low density of gp160 trimers renders mAbs less efficient for viral neutralization by interfering with their bivalent binding to the virus [63,64]. Mature HIV particles express 10–15 randomly distributed viral spikes, which would be spaced too far apart for a bivalent antibody to bridge [58,63,64]. However, multivalent binding could theoretically still be achieved by altered antibody geometry, e.g. a dimeric form of mAb 2G12 demonstrated substantially increased neutralization potency [65–67]. Other examples for multivalent mAbs with increased potency (compared to original IgGs) include polymeric IgA and IgM versions of the anti-HIV mAbs 2F5 and 2G12 [68].

An alternative to homotypic bivalent binding is heterotypic bivalent (=bispecific) binding, e.g. by designing scFv-Fc molecules ('immunoadhesins') that can bind bivalently by virtue of one scFv arm targeting gp120 and a second arm targeting the gp41 subunit of gp160 (Fig. 1B) [69]. The special geometry of the immunoadhesins was shown to overcome the lack of bivalent binding to HIV surface spikes [69]. Another study investigated several novel tetravalent, bispecific antibody derivatives for simultaneous targeting of two different epitopes on the HIV coreceptor CCR5 [70]. These molecules were based on Morrison-type bispecific antibodies which are whole IgGs connected to scFvs via flexible linkers (Fig. 1C). The bispecific mAbs maintained their binding activity toward both individual epitopes, were able to simultaneously block two docking sites of CCR5-tropic HIV strains, and showed 18–57-fold increased antiviral activities compared to the parent monospecific antibodies. Interestingly, one prototypic tetravalent CCR5 antibody had antiviral activity against virus strains resistant to the single parental antibodies. In summary, the increased valency and bispecificity translated into enhanced antiviral potency and increased threshold for antiviral resistance [70].

Multispecific antibodies have also been generated by fusing small molecules and antibodies, e.g. by constructing a single multimeric recombinant protein that combines the functional activities of the anti-HIV mAb b12 and the small microbicidal protein Cyanovirin (Fig. 1D) [71]. Importantly, these two molecules do not compete with each other for antigen binding as b12 recognizes a conformational amino acid epitope on HIV gp120 whereas Cyanovirin binds a glycan epitope [71]. Strategies similar to the bispecific b12-Cyanovirin construct have also been applied to other molecules, e.g. bifunctional HIV fusion inhibitor (BFFI) molecules were generated by linking either an anti-CCR5 or anti-CD4 antibody to a small fusion inhibitor [72–74] and multimeric molecules targeting murine cytomegalovirus-infected cells were constructed by linking cytomegalovirus-specific antibodies to a cellular toxin (deglycosylated ricin A chain) [75].

7. Antibody engineering

The antibody variable domains can be engineered into small fragments (Table 2), including scFvs and F[ab] molecules [76–78] which do not require production in costly eukaryotic expression systems. Several of these small antibody fragments have been investigated regarding their antiviral activities [79,80], including camelid VHH domains. The serum of camels, dromedaries and llamas contains a unique type of antibodies devoid of antibody light chains [21]. These camelid heavy-chain antibodies have attracted interest because they can recognize antigens via a single VHH domain that can be expressed with inexpensive bacterial or yeast

Table 2
Small antibody fragments, developed for rabies post-exposure-prophylaxis in humans.

Antibody format	Derivation	Reference
scFv	Ribosome display	[76]
dsFv	Human mAb 57	[77]
scFv-Fc	scFv library	[78]
Fab	Fab library	[79]
Fab on nanoparticles	Fab library	[80]
Nanobody	Camelid antibody library	[21]

scFv, single chain variable fragment; dsFv, disulfide-stabilized single chain variable fragment; scFv-Fc, single chain variable fragments fused to antibody Fc region; Fab, antigen-binding fragment.

expression systems [21]. VHH have been developed against several infectious diseases, e.g. as potent HIV-1 entry inhibitors [81]. In addition to the heavy chain immunoglobulins of the *Camelidae* family, single domain antibodies have been discovered in cartilaginous fish (sharks and possibly rays) [82]. Shark antibodies, also called Ig new antigen receptors (IgNAR), have been developed against hepatitis B virus [82] and Zaire Ebolavirus [83].

Engineering efforts have also been aimed at modifying the antibody variable or constant domains, e.g. the identification of palivizumab (Synagis; MedImmune/Abbott) has been followed by the development of the second-generation version motavizumab (MEDI-524; MedImmune) which has affinity matured complementary-determining regions (CDRs) [84]. Moreover, mAb MEDI-557 (MedImmune), a third-generation version of motavizumab currently investigated in clinical trials, contains engineered Fc domains for a longer half-life [85].

The optimization of Fc effector functions has been a focus of antibody engineering and two main approaches, site-specific mutagenesis and deglycosylation, have been applied to engineer antiviral antibodies with greatly enhanced binding to FcRIIIa and/or FcRIIa. For example, a panel of eleven variants of the anti-HIV mAb b12 with a broad range of affinities for FcRIIa and FcRIIIa has been investigated [86]. All variants with increased affinity for either of the main activating receptors (FcRIIa and FcRIIIa) also demonstrated an increase in viral inhibition compared to the original b12 antibody.

In certain viral disease applications, specific modifications that reduce or eliminate specific Fc effector functions may be desirable, e.g. altering the Fc region has been explored as a way to reduce or eliminate antibody-dependent enhancement (ADE) of infection [4]. ADE is a well-recognized phenomenon observed in various infections including numerous flavivirus infections, e.g. West Nile virus and dengue virus. Both active immunization and passive transfer of antibody have been shown to mediate this phenomenon, resulting from increased uptake of virus in the presence of neutralizing antibody [87,88]. Virus-specific antibodies enhance viral entry into, and in some cases, replication in monocytes/macrophages and granulocytic cells through interaction with Fcγ and/or complement receptors.

ADE is the proposed mechanism responsible for dengue hemorrhagic fever and dengue shock syndrome, two clinical conditions that are frequently seen in patients infected with a second heterotropic infection and infants with maternally transferred anti-dengue antibodies. For dengue virus, four serotypes exist and the generation of antibodies following exposure to one serotype may affect the response to repeat exposure with the same or an alternative serotype [89,90]. Experimental passive transfer of a high dose of serotype-specific antibodies enable elimination of viremia, but lower doses of such antibodies or cross-reactive polyclonal or monoclonal antibodies may all cause enhanced disease *in vivo* [88–90]. In contrast, genetically engineered mAb variants (e.g. E60-N297Q) that cannot bind Fcγ receptors exhibited prophylactic and therapeutic efficacy against ADE-induced lethal challenge [90].

8. Recent developments

The recent identification of human mAbs that broadly neutralize different HIV strains may allow the reverse engineering of potent vaccines. The human serologic response to HIV-1 infection targets both internal and viral surface proteins, but only antibodies targeting the HIV envelope spike gp160 achieve viral neutralization [91]. The conformational flexibility is considered to be the main obstacle to the development of an HIV-1 vaccine, besides the sequence variability and the glycan shield [92]. However, the observation that mAbs targeting certain epitopes can be protective suggests that a vaccine that elicits such antibodies could have a similar effect. These broadly neutralizing mAbs are directed either against gp120 or gp41 [92]. Efforts are focused on designing epitope mimics, in order to direct humoral responses toward these neutralizing epitopes after vaccination. Similar strategies might also be applied to develop more potent vaccines against influenza virus, following the recent identification of broadly neutralizing human mAbs with V_H1-69 germline heavy chains [93,94]. These mAbs were shown to broadly neutralize many influenza A group 1 viruses and crystal structures of a mAb in complex with H1 and H5 hemagglutinins (HAs) revealed a highly conserved epitope in the HA stalk [93]. Subsequent studies have identified human mAbs that show broad neutralizing activity against group 2 viruses and that target conserved epitope in the HA stalk distinct from the epitope recognized by the V_H1-69 group 1 antibodies [95]. The mAbs targeting groups 1 and 2 viruses are potentially complementary and may hence open up the prospect of developing a universal influenza vaccine, as opposed to current vaccines which are restricted to the circulating seasonal strains [93–95].

Applications for antiviral mAbs may also include infections of the central nervous system (CNS) and several mAbs have shown promise in clearing established neurological diseases, including West Nile virus and Hendra virus infections [10,96]. However, the use of antibodies for neurological infections may frequently be limited due to the presence of the blood–brain-barrier (BBB), especially in infections like rabies during which the BBB remains largely intact [97]. Patients with clinical rabies do not respond to PEP and so advances in delivering therapeutic mAbs specifically to the CNS [98] should be further explored.

9. Outlook

Polyclonal antibodies are increasingly being replaced by mAbs, e.g. hepatitis B immunoglobulin (HBIG), varicella-zoster immunoglobulin (VZIG) and rabies immunoglobulin (RIG) [1]. RIG is part of the WHO Essential Medicines List for both adults and children, and the use of mAbs could help to overcome the current insufficient supply of antiserum across the developing world, thereby contributing to meet the vision of the United Nations Millennium Declaration. Importantly though, the costs of mAb production and the choice of expression system need to be carefully considered to make any candidate preparations widely available and affordable. The relatively high expenses and the usually short-lived protection of mAbs (due to their limited half-life) may impede their widespread application for diseases for which small molecule drugs and vaccines are available. The costs of 5 monthly doses of palivizumab for RSV prevention are up to 6000 British pounds per patient [99], indicating that the high expenses for mAb development, production and storage can be prohibitive. Access to antiviral mAbs may be restricted, especially in low-income countries, so efforts are being made to develop inexpensive production platforms that are amenable for transfer to the developing world. In particular, the use of transgenic plants has raised hopes that several mAb preparations may become more widely available [60,71,100].

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