Preventive and therapeutic applications of neutralizing antibodies to Human Immunodeficiency Virus Type 1 (HIV-1)

Ther Adv Vaccines
(2013) 1(2) 67–80
DOI: 10.1177/2051013613494534

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Abstract: The development of a preventive vaccine to neutralize the highly variable and antigenically diverse human immunodeficiency virus type 1 (HIV-1) has been an indomitable goal. The recent discovery of a number of cross-neutralizing and potent monoclonal antibodies from elite neutralizers has provided important insights in this field. Neutralizing antibodies (NAbs) are useful in identifying neutralizing epitopes of vaccine utility and for understanding the mechanism of potent and broad cross-neutralization thus providing a modality of preventive and therapeutic value. In this article we review the current understanding on the potential use of broadly neutralizing antibodies (bNAbs) in their full-length IgG structure, engineered domain antibody or bispecific versions towards preventive and therapeutic applications. The potential implications of NAbs are discussed in the light of the recent developments as key components in vaccination against HIV-1. The development of a vaccine immunogen which elicits bNAbs and confers protective immunity remains a real challenge.

Keywords: HIV-1, neutralizing antibodies, vaccines

Introduction

Acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus type 1 (HIV-1) is a major public health problem and warrants the urgent need for the development of a preventive vaccine. Evidence gathered from recent discoveries backed by basic scientific research and clinical trials indeed provides optimism about the possibility of developing a vaccine against HIV-1. Antibodies that block the virus entry are referred as neutralizing antibodies (NAbs) and these are thought to be a crucial correlate of protection in some of the successful viral vaccines and can also be effective against HIV-1 [Haynes and Montefiori, 2006; Mascola, 2003; Montefiori *et al.*, 2007a; Plotkin, 2008]. However, for complete protection, both arms of the adaptive immune response, antibody response and T-cell response, seem to be necessary [Walker and Burton, 2008]. Both of these responses work in concert: the antibodies control the cell free virus entry into the target cells whereas the T-cell response controls the viral reservoirs and is required to check viral replication [McMichael and Hanke, 2002; Pantaleo and Koup, 2004]. Virus transmission after breaching the mucosal barrier into the submucosal environment leads to the infection of surrounding $CD4^+$ T cells and thereafter disseminates into the systemic infection. The persistent viral replication leads to the destruction of $CD4^+$ T cells culminating in AIDS [Letvin, 2006; McMichael, 2006]. Envelope protein (gp160) encoded by HIV *env* is the outermost protein expressed on HIV. It acts as a molecular machine that binds the virus to the target cell receptors, thus mediating the cell membrane fusion and virus entry [Wyatt *et al.* 1998]. Env is a crucial component of viral entry and represents an attractive target for vaccine-induced antibodies that has potential to bind with Env and block the entry of virus into the target cell [Burton *et al.* 2004; Haynes and Montefiori, 2006; Montefiori *et al.* 2007b]. In recent years substantial progress has been made on antibody discovery and highly potent and broad NAbs have been isolated from chronically infected HIV-positive patients with broadly neutralizing serum activity, also referred to as elite neutralizers. These antibodies upon passive immunization of animals conferred protection in nonhuman primates and

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humanized mice [Burke and Barnett, 2007; Klein *et al.* 2012b; Mascola, 2003, Moldt *et al.*, 2012]. The *in vivo* expression of broadly neutralizing antibodies (bNAbs) by vector-mediated gene transfer also showed high efficacy in humanized mice [Balazs *et al.* 2012]. However, attempts to elicit such antibodies by immunization have not been very successful [Burton *et al.* 2004; Haynes and Montefiori, 2006]. The initial recombinant protein vaccine based on gp120 protein induced only immunogen-specific antibodies which could neutralize lab-adapted virus strains but not the primary isolates and thus showed no clinical relevance [Flynn *et al.* 2005; Gilbert *et al.* 2005; Graham and Mascola, 2005]. However, the recent RV144 HIV-1 vaccine trial of the canarypox vector (ALVAC-HIV) plus the gp120 AIDSVAX B/E vaccine demonstrated moderate efficacy and promise that the antibodies induced by vaccination can provide protective immunity against HIV-1 [Baden and Dolin, 2012; Rerks-Ngarm *et al.* 2009]. Intriguingly, the antibodies in RV144 trial were mostly non-neutralizing; however, it is the binding of IgG antibodies to the V1V2 region of the gp120 Env that likely was the correlate of protection in this trial [Haynes *et al.* 2012a]. Although this regimen failed to produce NAbs, the results of this trial may provide a valuable guide as to the immunogen improvement efforts and antibodies required for protection against HIV-1 infection. Efforts are being made to build improved immunogens based on the newer detailed structural insights in Env protein that exhibit a better antibody response [Kovacs *et al.* 2012; Phogat and Wyatt, 2007]. The improved knowledge of the Env structure and neutralization epitopes will help improve the rational immunogen design in order to elicit potent bNAbs [Dormitzer *et al.* 2008; Kwong and Wilson, 2009; Montefiori *et al.* 2007b; Phogat and Wyatt, 2007; Stamatatos *et al.* 2009]. The present paper reviews the current understanding about the progress in the discovery of broad and potent NAbs to HIV-1 as well as their potential in HIV-1 therapeutics and prophylactics.

Neutralizing epitopes on the HIV-1 envelope

Although antibodies are elicited against most of the viral proteins, those that bind to Env protein and prevent viral entry are referred to as NAbs [Mascola and Montefiori, 2010; Pantophlet and Burton, 2006; Zolla-Pazner, 2004]. The unique subunit architecture of HIV-1 Env trimer that induces NAbs is particularly challenging to achieve [Mao *et al.* 2012]. The antibodies in the early infection are generally strain specific but in some patients bNAbs develop in the chronic stage of infection. Around 20% of HIV patients with chronic infection develop NAbs with potential to neutralize diverse HIV-1 strains, and 2–4% of such subjects have even greater serum neutralizing activity that neutralize most HIV-1 strains from different clades [Simek *et al.* 2009]. Antigenically Env protein is highly variable and virus can quickly escape from the selective pressure from existing NAbs. Nevertheless, sera from certain chronically infected patients exhibit broader neutralizing activity which attributes to single, few or multiple specificities [Scheid *et al.* 2009; Walker *et al.* 2009; Walker *et al.* 2010; Wu *et al.* 2010]. The first broadly neutralizing human monoclonal antibody (mAb) b12 was isolated from a clade B infected patient and binds to gp120 at its CD4 binding site (CD4bs) [Burton *et al.* 1994]. b12 was found to neutralize more than 50% of clade B viral isolates and about 30% of non–clade B viruses [Binley *et al.* 2004; Kulkarni *et al.* 2009]. Recently, novel forms of broad and potent CD4bs antibodies have been isolated from elite neutralizer using reverse vaccinology approaches [Falkowska *et al.* 2012; Wu *et al.* 2011a]. These human mAbs were isolated by exploiting the ability of a resurfaced stabilized gp120 core protein mimicking the CD4 binding site to capture only broadly NAbs based on affinity and deep recognition (weakly binding non-NAbs were kept at bay). Using this modified gp120 core as a fluorescent-labeled probe to capture antigen-specific memory B cells, CD4bs-specific VRC antibodies were also isolated. VRC01 and VRC02 are the broadest, neutralizing 91% of primary Env pseudoviruses while VRC03 was found to neutralize 57% of the circulating viruses of the panel (Table 1) [Wu *et al.* 2010]. NIH45-46 is a more potent clonal variant of VRC01 and has a four-residue insertion in the heavy chain complementarity determining region 3 (CDR3) which enhances gp120 binding [Scheid *et al.* 2011]. 3BNC117 [Scheid *et al.* 2011] and VRC-PG04 [Wu *et al.* 2011b] are also CD4bs antibodies with neutralization breadth and potency similar to VRC01. Although all of these antibodies bind to CD4bs there are differences in the way they interact with this site. Structural analyses of the CD4bs interaction with mAbs b12 (prototype CD4bs NAb), F105 (non-neutralizing), and VRC01 (very broadly neutralizing) revealed the unusual binding into a narrow site [Schief *et al.* 2009; Zhou *et al.* 2010]. VRC01

Table 1. Characteristics of broad and potent neutralizing antibodies to HIV-1

Antibody Name	Site of Contact	Study	Antibody Type	Glycan dependence	Quaternary Structure dependence	Neutralization Breadth (%)	Infecting Subtype	Therapeutic Potential
IgG1b12	CD4bs	Burton *et al.* (1991) Proc. Natl. Acad. Sci. USA; 88: 10134-10137	Whole Antibody	No	No	35 (n=190)	B	Effective against SHIV vaginal challenge and protects macaques.
VRC01,02,03	CD4bs	Wu *et al.* (2010) Science 329: 856-861	Whole Antibody	No	No	91, 91, 57 (n=190)	B	Effective against SHIV mucosal challenge and protects macaques.
NIH45-46	CD4bs	Scheid *et al.* (2011) Science, 333: 1633-1637	Whole Antibody	No	No	96 (n=118)	B	Not tested
VRC-PG04	CD4bs	Wu *et al.* (2011) Science; 333:1593-602	Whole Antibody	No	No	76 (n=178)	A-D recombinant	Not tested
3BNC117	CD4bs	Scheid *et al.* (2011) Science, 333: 1633-1637	Whole Antibody	No	No	96 (n=118)	B	Not tested
VHH J3	CoRbs	McCoy, L. and Weiss, R. (2013). J Exp Med 210: 209-223.	Domain Antibody	NA	NA	96 (n=100)	-	Not tested
m36	CoRbs	Chen (2008) Proc Natl Acad Sci USA 2008; 105: 17121-17126	Domain Antibody	No	No	91 (n=11)	-	Not tested
2G12	Glycan	Trkola *et al.* (1996) J. Virol. 70: 1100-1108)	Whole Antibody	Yes	No	32 (n=162)	B	Reduces viral load and increases CD4 T cell count in combination with 2F5, 4E10
PGT121-123	Glycan	Walker *et al.* (2011) Nature; 477:466-470)	Whole Antibody	Yes	No	65-70 (n=162)	A	Not tested
PGT125-128,130-131	Glycan	Walker *et al.* (2011) Nature; 477:466-470)	Whole Antibody	Yes	No	40-72 (n=162)	CRF02_AG	Not tested
PGT135	Glycan	Walker *et al.* (2011) Nature; 477:466-470)	Whole Antibody	Yes	No	33 (n=162)	C	Not tested
PGT141-145	Glycan	Walker *et al.* (2011) Nature; 477:466-470)	Whole Antibody		Yes	38-78 (n=162)	A or D	Not tested
PG9, PG16	Quaternary structure including V1V2,V3	Walker *et al.* (2009) Science 326: 285-289	Whole Antibody	Yes	Yes	79 and 73 (n=190)	A	Not tested
2F5	MPER	Purtscher *et al.* (1994) AIDS Res Human Retroviruses 10: 1651-1658	Whole Antibody	No	No	57 (n=177)	B	Tested in combination with 2G12. Help reduce viral load and increase CD4+ T cell count in HIV infected individuals.
4E10	MPER	Stiegler *et al.* (2001) AIDS Res. Hum. Retroviruses 17:1757-1765	Whole Antibody	No	No	98 (n=180)	B	Moderately suppresses viral load in combination with 2F5
10E8	MPER	Huang *et al* (2012) Nature, 491: 406-412)	Whole Antibody	No	No	98 (n=180)	B	Not tested

approaches the conformationally invariant site following the initial CD4 attachment, escaping the hindrance by conformational masking which most CD4bs antibodies experience, thus diminishing their neutralization potency. Partial receptor mimicry and extensive affinity maturation thus facilitate effective neutralization of HIV-1 by natural human antibodies such as VRC01 [Zhou *et al.* 2010]. Two prototype membrane-proximal external region (MPER) gp41 NAbs are 2F5 and 4E10 [Cardoso *et al.* 2005; Ofek *et al.* 2004; Stiegler *et al.* 2001]. The gp41 subunit is far more conserved than gp120 but kinetic and steric constraints potentially protect its vulnerable regions from NAb attack. MAbs 2F5, 4E10 and Z13 were isolated from an HIV-positive patient and show considerable potency, with 4E10 being the most broadly neutralizing [D'Souza *et al.* 1997]. These MAbs bind to the intermediate conformation of gp41 during the fusion process [Frey *et al.* 2008]. The native gp41 seems to be inaccessible to antibodies and reveals epitopes during the fusion process. Therefore, either limited antibodies are produced against these epitopes, as their exposure is too short to be recognized by B-cell receptors to elicit antibodies against them, or the antibody has a little window of opportunity to bind to short-lived epitopes that are exposed only during fusion [Alam *et al.* 2011; Ringe and Bhattacharya, 2012; Shen *et al.* 2010]. 2F5 binds to a core target at ELDKWA in the heptad repeat-2 region of gp41, whereas 4E10 binds to the NWFDIT sequence in the MPER [Cardoso *et al.* 2005; Ofek *et al.* 2004]. These antibodies bind to both lipid–MPER peptide complexes and to HIV virions [Alam *et al.* 2009] in a two-step conformation change model wherein they bind first to virion lipids, then surf the viral membrane while awaiting transient exposure of their neutralizing epitopes during fusion with a target cell. These antibodies are autoreactive because of their reactivity with lipids [Alam *et al.* 2009]. Recently, a very potent and broadly cross-neutralizing gp41 MPER-specific antibody, named 10E8, has been reported by Huang and colleagues [Huang *et al.* 2012]. 10E8 neutralizes 98% of tested viruses (Table 1). Moreover, unlike 2F5 or 4E10, 10E8 does not bind phospholipids, is not autoreactive and binds to the cell-surface envelope, suggesting that the highly conserved epitope in the gp41 ectodomain is directly accessible to 10E8 and can potentially increase the window of opportunity to access the epitope. The study also showed that 8% of the sera from healthy HIV-1-positive individuals contained 10E8-like specificities suggesting that the corresponding epitope is immunogenic in nature and 10E8-like antibodies are not deleted from the B-cell repertoire because of autoreactivity. The structure of 10E8 in complex with the complete MPER and the mutagenesis studies revealed a binding site comprising a narrow stretch of highly conserved gp41-hydrophobic residues and a critical arginine or lysine just before the transmembrane region [Huang *et al.* 2012]. The highly conserved MPER is a target for potent, non-self-reactive NAbs. The frequent generation of 10E8-like antibodies in HIV-1-infected individuals suggests that this specificity can be induced by vaccination in a larger fraction of HIV-negative or HIV-positive individuals than other gp41 specific antibodies. Another immunodominant region in Env is the V3 loop on gp120 and antibodies are often made against the epitopes at the tip of this loop. V3 specific antibodies have generally a narrow reactivity and can neutralize only tier 1 or T-cell line adapted strains [Davis *et al.* 2009]. Many V3-specific antibodies with some broad reactivity from chronically infected HIV-1 subjects have been isolated that recognize a strain-specific quaternary epitope involving gp120 V2 and V3 loops in the context of Env trimer [Zolla-Pazner and Cardozo, 2010]. The new technical advances in single memory B-cell stimulation and high-throughput microneutralization assay facilitated the antibody screening to higher levels. The isolation of potent and broadly neutralizing mAbs PG9 and PG16 (closely related somatic mutants) from a patient was carried out by Walker and colleagues using this methodology [Walker *et al.* 2009]. PG9 and PG16 recognize a novel epitope that is composed of V1V2 and V3 loops on the Env trimer and exhibit remarkable neutralization breadth and potency [McLellan *et al.* 2011; Walker *et al.* 2011]. More recently, by using the same technology, many additional monoclonal antibodies, referred to as PGT antibodies, have been isolated from four elite neutralizers which target a glycan-dependent epitope and exhibit about 10 times more potency than VRC01, PG9 and PG16 and 100 times more than old-generation prototypic antibodies. Among these PGT antibodies, PGT141-145 targets glycan-dependent quaternary epitopes on gp120 whereas PGT 125-128 and PGT130-131 interact specifically with the Man8/9 glycans on gp120. These antibodies are very potent, with some neutralizing >70% of HIV-1 env pseudotyped virus panel of 162 envelopes (Table 1) [Pancera *et al.* 2010; Walker *et al.* 2009, 2011]. The crystal structure of PGT128, the most potent and broad PGT, complexed with

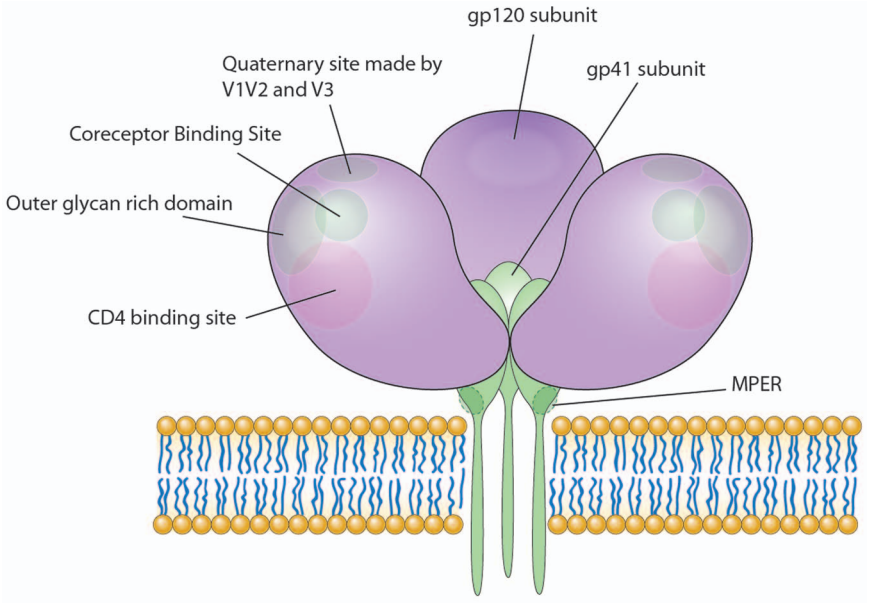

Figure 1. Potential sites of antibody response on Env. The Envelope trimer is composed of three gp120 surface protein subunits (purple) and three transmembrane gp41 subunits (green) as shown. gp120 has multiple potential clusters (indicated by circles) against which neutralizing antibodies are elicited in HIV-infected individuals (many have been isolated from broadly neutralizing sera). The gp41 protein has so far shown one site in the ectodomain region (indicated by dotted circles) against which broadly neutralizing antibodies are isolated. The envelope trimer is shown in an open conformation (post-CD4 engagement) to reveal the coreceptor binding site cluster which is exposed after gp120 is engaged with CD4. MPER, membrane-proximal external region. Illustration courtesy of Alessandro Baliani. Copyright © 2013.

a fully glycosylated gp120 outer domain, reveals that the antibody penetrates the glycan shield and recognizes two conserved glycans as well as a short β-strand segment of the gp120 V3 loop, accounting for its high binding affinity and broad specificity [Pejchal *et al.* 2011]. The 2G12, prototypic carbohydrate specific mAb, is unusual in its structure and binding specificity. Unlike PG9 or PGT mAbs, it is a canonical glycan-binding bNAb with a unique domain-swap structure. Its Fab region is composed of a heavy chain and a light chain which are shared by the other arm of the 2G12 antibody [Calarese *et al.* 2003; Scanlan *et al.* 2002]. The glycans on gp120 are the result of host cell posttranslational modifying glycosidases and therefore resemble host carbohydrates, possibly reducing the immunogenicity of gp120 [Scanlan *et al.* 2002, 2003]. The glycans recognized by 2G12 comprise a unique conformational epitope of oligomannose glycans in the outer domain of gp120 that is poorly immunogenic [Astronomo *et al.* 2008]. However, the newer glycan-binding antibodies recognize these domains far more strongly and neutralize many primary isolates [Walker *et al.* 2011]. This suggests that the immune response in HIV infection takes a complex pathway to evolve and recognize such complex and difficult epitopes [Mouquet *et al.* 2012]. Thus, the HIV-1 envelope has at least five conserved regions, each with overlapping epitopes that can be targets for bNAbs (Figure 1).

HIV-1 NAbs and antibody-based inhibitors

NAbs are very powerful agents against most of the animal viruses and in vaccine-induced immune responses [Casadevall, 2002; Klasse and Sattentau, 2002]. However, the treatment of HIV-1 with NAbs has not been successful because of the rapid mutability of the virus [Manrique *et al.* 2007; Wei *et al.* 2003]. Increasing the potency and breadth might overcome this daunting problem. Substantial efforts have been made in this direction and several antibody-based inhibitors have been made with desirable features. In addition to informing strategies to design vaccine immunogens and elicit similar antibody responses, therapeutic application of bNAbs in patients failing antiretroviral therapy (ART) or those who developed resistance represents a promising approach towards the eradication of virus. Thus, bNAbs can be used individually or in

combination with ART for effective elimination of virus [Chen *et al.* 2013; Corti and Lanzavecchia, 2013]. In this review, we discuss how the antibody modifications are made to achieve the desirable characteristics and how monoclonal antibodies, in their native form or as engineered versions, can be used to confer protection in HIV infection.

Neutralization of HIV-1 by native forms of mAbs

As discussed earlier, few crucial neutralizing epitopes have been characterized against which bNAbs have been isolated. One of the best characterized broadly cross-reactive and potent human mAbs targeting the CD4bs of gp120 is b12. This was selected almost 20 years ago by phage display from an antibody library made from the bone marrow of an HIV-1-infected donor [Burton *et al.* 1994; Roben *et al.* 1994]. Intravenous transfusion of b12 was found to partially protect the macaques from vaginally challenged R5 virus SHIV162P4 and later it was found that vaginal administration of b12 can partially protect macaques from vaginal simian/human immunodeficiency virus (SHIV) transmission [Parren *et al.* 2001; Veazey *et al.* 2003]. These observations suggest that mAbs can be a potential modality to prevent the sexual transmission of HIV-1 to humans. The synergistic effect of multiple mAbs together can improve the protective effect and may compromise the virus to the level from which viral rebound is difficult [Klein *et al.* 2012a]. However, the mechanism by which NAbs confer protection *in vivo* is not very clear. In addition to a direct neutralization, antibody Fc receptor-mediated effector functions are also important for protection against HIV-1. Removal of Fc-receptor binding of b12 resulted in a loss of its protective function suggesting that the antibody has both cell-free and cell-associated virus neutralizing abilities [Hessell *et al.* 2007]. Moldt and colleagues [Moldt *et al.* 2012] demonstrated that an increase of the b12 interaction with FcgammaRIIIa enhanced the antiviral activity *in vitro* but could not exert the same effect *in vivo*. Thus, the exact antiviral mode of action of NAbs *in vivo* remains unclear. The carbohydrate-specific antibody 2G12 has been tested intravenously in the animal models for its efficacy against vaginally challenged SHIV. Results showed a substantial protection of macaques or reduced viral load and delay in $CD4^+$ T-cell loss [Chen and Dimitrov, 2012; Mascola *et al.* 1999, 2000]. Hessell and colleagues [Hessell *et al.* 2009] have also shown that low intravenous administration of 2G12 can protect the animals from vaginal challenge of SHIV, in contrast to the observation that high-dose b12 was required to confer protective immunity. In these studies the challenge virus used was highly sensitive to the NAb tested. *In vitro* studies of 2F5 and 4E10 demonstrated that the neutralizing activity of these mAbs is enhanced when tested in combination [Mascola *et al.* 1997] and therefore they were evaluated for their antiviral activity *in vivo* mainly in combination in animal models and in HIV-infected persons. Alone, neither 2F5 nor 2G12 completely protected macaques from intravenous challenge but treated animals showed a less profound drop in $CD4^+$ T cells suggesting that these antibodies compromise virus replication *in vivo* [Mascola *et al.* 1999]. These initial studies in animal models documented some success in protecting against infection or delaying the viral rebound/$CD4^+$ T-cell loss upon the vaginal challenge of SHIV. Later the 2F5/2G12 combination was also evaluated in a phase I clinical trial in seven HIV-1-infected patients [Armbruster *et al.* 2002] which confirmed the safety of these mAbs. In addition, transient reductions in viral loads were observed in five of the seven subjects while improvement in $CD4^+$ T-cell counts and CD4+/CD8+ ratios were observed in all individuals. However, the HIV virus can develop resistance to 2G12 making this approach ineffective. For this reason, a combination of three mAbs 4E10, 2F5 and 2G12 [Armbruster *et al.* 2004] was also evaluated in seven individuals. The study confirmed the safety of this combination and some protection. In a phase II clinical trial [Trkola *et al.* 2005] the capacity of these antibodies to suppress or delay the viral rebound caused by the ART was evaluated. A substantial delay in viral rebound was observed in four of six acutely infected individuals and only in two of eight chronically infected patients. This suggests that the mAbs can prevent disease progression when HIV infection is at early stage and viral diversity is limited [Bar *et al.* 2012]. In chronic infection viral diversity and viral reservoirs may be too high to be controlled by mAbs. Resistance to 2G12 but not to 2F5 or 4E10 was observed in 12 of 14 individuals, suggesting that the glycosylation pattern can be readily changed without compromising the gp120 function to resist the binding to 2G12 whereas MPER is crucial in the fusion process and the acquisition of resistance to the corresponding antibodies may require a higher fitness cost [Manrique *et al.* 2007]. Subsequent studies

indicated that the antiviral effect of these three mAbs is the result of direct *in vivo* neutralization in addition to antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis mechanisms [Huber *et al.* 2008].

Antibody modifications to enhance antiviral activity

A whole antibody is a large molecule which generally binds to a larger portion of an antigenic protein to enhance its avidity. The problem associated with these large molecules is that they may not access the epitopes when these are sterically occluded. This is especially common for many epitopes of the HIV-1 envelope proteins resulting in resistance against the antibodies induced by HIV infection [Burton *et al.* 2005; Mascola and Montefiori, 2010]. For such changing epitopes small fragments of antibody could be more effective and more able to control virus replication. The binding site for such entities is smaller and therefore steric occlusion can be avoided [Labrijn *et al.* 2003]. Labrijn and colleagues [Labrijn *et al.* 2003] showed that the neutralization potency of CD4-induced (CD4i) epitope binding antibody is inversely correlated with the size of the antibody fragments scFv (~25 kDa). It appears that smaller antibody formats can access the binding site more easily than the whole antibody as the access for the latter is sterically restricted due to the small space between the viral envelope and cell membranes after or before the engagement of Env trimer with the CD4 receptor [Labrijn *et al.* 2003]. Few CD4i NAbs have been isolated from HIV patients are represented by 17b (IgG), X5 [Chen and Dimitrov, 2009; Moulard *et al.* 2002], m9 and m16 [Zhang *et al.* 2004]. These CD4i antibodies have strong neutralizing activities and therapeutic relevance as their target site is mostly conserved on the Env structure. A newer class of antibodies are called domain antibodies (dAbs) which are represented by engineered antibody fragments that are fairly potent and smaller in size compared with Fab or scFv [Chen *et al.* 2008a, 2008b; Holt *et al.* 2003; Vanlandschoot *et al.* 2011]. These small antibody fragments, ranging from 11 to 15 kDa in size, lack either the VL or VH domain and are highly directed against conserved domains of the epitope. This is a particularly important characteristic as most of the immunogenic conserved epitopes of the Env are guarded by conformational masking by variable loops or the glycan shield and escape from immune recognition [Kwong *et al.* 1998; Pantophlet and Burton, 2006; Wyatt *et al.* 1998]. Epitopes which are beyond the reach of a full-size antibody or a Fab/scFv because of steric constraints are easily accessed by dAbs [Chen and Dimitrov, 2009; Chen *et al.* 2008a; Gong *et al.* 2012]. The coreceptor binding site (CD4i) is the most sterically occluded immunogenic structure in gp120 as its formation is induced only following gp120 binding to CD4. It was therefore hypothesized that small antibody fragments (derived from whole antibody domains) targeting such epitopes could neutralize the virus with breadth and potency [Chen *et al.* 2010]. The highly potent dAb m36 reported by Chen and colleagues is directed at a conserved structure of the coreceptor binding site (CD4i) [Chen *et al.* 2008a]. This antibody is broadly cross-neutralizing, potent in its neutralizing activity and showed higher potency than scFv antibody m9 [Chen and Dimitrov, 2009]. Such dAbs can be particularly effective against kinetic signatures such as CD4i and MPER epitopes. Some dAbs have also been isolated recently from recombinant gp120-based immunization of llamas and exhibited potent neutralizing activity against HIV-1 [Forsman *et al.* 2008]. Some variable domains derived from heavy-chain antibodies (VHH) such as A12, D7 and C8 were able to neutralize HIV-1 clade B and C isolates [Forsman *et al.* 2008; Strokappe *et al.* 2012]. Using the more stringent criteria of selection, VHH was able to neutralize 42% of the strains tested *in vitro* with IC_{50} in the range of <0.2–2533 nM although they have in general a higher specificity towards the immunizing strain or clade. These dAbs block the binding of CD4 to gp120 and can compete with the binding of CD4bs mAbs to gp120 [Forsman *et al.* 2008]. The crystal structure of a llama heavy-chain antibody fragment VHH D7, revealed the presence of two canonical CDR1 and CDR2 but a longer and highly mobile CDR3 which is probably required for recognizing and conferring more binding energy for the interaction with gp120 CD4bs and virus neutralization [Hinz *et al.* 2010; Koh *et al.* 2010; McCoy and Weiss, 2013]. Using a family-specific approach, Koh and colleagues have recently isolated the largest possible diversity of related VHH antibodies that compete with soluble CD4 for binding to the HIV-1 envelope glycoprotein [Koh *et al.* 2010; Strokappe *et al.* 2012]. The dAb list was then expanded by Matz and colleagues with the immunization of llamas using trimeric gp140, free or bound to a CD4 mimic, in order to isolate CD4bs and CoRbs dAbs [Matz *et al.* 2013]. The single-domain antibodies

(sdAbs) isolated in this study potently neutralized subtype B viruses but also showed neutralizing activity against viruses carrying envelopes from A, C, G, CRF01_AE and CRF02_AG, subtypes including tier 3 viruses. A new modified screening process able to distinguish between neutralizing and non-NAbs, allowed the isolation of the extremely broad and potent VHH J3 by screening of a phagemid VHH library generated from a llama immunized with two recombinant HIV gp140 proteins. This VHH dAb neutralized 96% of the large panel of HIV-1 strains and represents a potential therapeutic candidate [McCoy and Weiss, 2013]. This work demonstrated that broad and potent smaller format antibodies can be obtained upon immunization and considered for several applications such as anti-HIV-1 microbicide and for the rational HIV immunogen design of HIV to define vulnerable epitopes on the Env protein. However, few limitations are associated with these antibodies. They have smaller half-life and low retention *in vivo*. In addition, the antiviral action of the antibody fragment lies in competitive binding and neutralization. However, other IgG functions such as the Fc-mediated ADCC or phagocytic effects are absent which may reduce the antiviral effect of these antibodies. Finally, the development of resistance to a single mode of antiviral action can be less difficult than that observed in antibodies with multiple antiviral mechanisms.

Bispecific antibodies

Dealing with efficacy issues at the clinical level and viral resistance to bNAbs has prompted the need to enhance the potency and breadth of the NAbs. Few studies in recent years have described the generation of antibody conjugation products with two different specificities on the Env protein. The soluble form of CD4 is a virus-neutralizing protein and has been used to conjugate with the antibodies that target the epitopes that are induced or exposed upon CD4 binding to Env [Chamow *et al.* 1992; Chen *et al.* 2013; Traunecker *et al.* 1992; West *et al.* 2010]. External domains of CD4 receptor D1D2 conjugated with the antibody specific to CD4i epitope (CD4-CD4i antibody) showed enhanced neutralization potency and breadth [Chen and Dimitrov, 2009; West *et al.* 2010]. The bivalent reagent that fuses CD4 to the heavy chain of the CD4i antibody E51 showed similar or higher neutralizing potency than that of the well-known bNAbs [West *et al.* 2010]. Similarly, immunoadhesin–antibody hybrids in which scFv is conjugated with the Fc domain has been explored and their neutralization potencies compared to those of the parent IgG. Immunoadhesins made from PG9, PG16 and VRC01 showed reduced potency likely because of the reduced affinity of the cognate epitope. However, the attachment of the VRC01 scFv to PG16 IgG yielded a bispecific reagent whose neutralization activity combined activities from both parent antibodies and also fewer strains escaped neutralization [West *et al.* 2012]. Such approach of combining antigen-binding sites together in a single antibody enhances the avidity of the antibody that likely translates into increased potency [Cavacini *et al.* 1994; Kausmally *et al.* 2004]. In addition to the valency, improved flexibility and size of antigen binding sites can have an impact on the neutralization potency and breadth. The efficiency of b12 to neutralize a panel of clade B envelopes was seen to increase with valency and flexibility between antigen binding sites. Engineered b12 displayed the ability to bind bivalently and cross-link envelope spikes on the virion surface. This was not observed with similarly engineered 4E10 antigen-binding sites probably because the 4E10 epitope is difficult to access in a native trimer and such design may be of least relevance [Klein *et al.* 2009]. Recent studies have established the fact that most bNAbs are polyreactive and hypermutated [Mouquet *et al.* 2010]. The bNAbs are generated in the chronic stage of HIV infection and are affinity matured by virtue of somatic hypermutations in mostly but not limited to the antigen-combining sites [Klein *et al.* 2013; Mouquet and Nussenzweig, 2012; Mouquet *et al.* 2010; Scheid *et al.* 2011; Wu *et al.* 2011b]. The affinity maturation and polyreactivity is explained to corroborate the binding to high-affinity anti-Env-combining site and a second low-affinity site on another molecular structure on HIV-1 [Mouquet *et al.* 2010]. Antibody engineering methods aimed at enhancing the apparent affinity and neutralization potency were reviewed recently by Mouquet and Nussenzweig [Mouquet and Nussenzweig, 2012]. Bispecific anti-HIV-1 antibodies (BiAbs) that can bind bivalently by virtue of one scFv arm that binds to gp120 and a second arm to the gp41 subunit of gp160 showed enhanced neutralization [Mouquet *et al.* 2012]. Thus, antibodies engineered to contain different combining sites could be potential neutralizers and therapeutic candidates against HIV-1. The actual *in vivo* therapeutic or prophylactic value could also be measured in efficacy trials in the future.

Summary

A major goal towards finding an effective intervention to HIV/AIDS is to develop an effective vaccine which can check the new acquisitions and the therapy for already infected individuals. Antiretroviral therapy based on small-molecule drugs against reverse transcriptase has paid rich dividends and increased the life expectancy of infected people; however, several limitations need to be considered. The circulating virus can develop resistance to therapy and rebounds after cessation of therapy. In addition, side effects associated with antiviral therapy translate into an increasing economic burden. While an HIV vaccine has been sought after for some time, the development of a suitable immunogen has been hampered by the extreme genetic diversity, mutability of envelope protein, and immunological constraints such as affinity maturation. In the recent years several potent bNAbs have been isolated from HIV-positive patients which showed a great potential to neutralize primary viruses and their combination has yielded promising results as a therapeutic intervention [Burton *et al.*, 2012b; Klein *et al.* 2012b]. In addition, many new dAbs have also been isolated which showed enhanced antiviral activity. While designing appropriate immunogens towards eliciting NAb response is of high priority [Burton *et al.*, 2012a; Haynes *et al.* 2012b], passive immunity mediated by the combination of antibodies can be an effective prophylactic control against HIV in high-risk people and at the same time represents an alternative to combination therapy in infected individuals. The alteration of properties in engineered antibodies such as improved accessibility of recessed epitopes and enhanced delivery to lymphoid and mucosal tissues can improve their efficacy. The inclusion of such antibodies in the mainstream repertoire of full-size antibodies may enhance the overall antiviral effect by combining effector and neutralization functions. No vaccine has provided complete protection against HIV but substantial progress and understanding has been made as to how potent antibodies neutralize the virus and what are the vulnerable epitopes on the HIV-1 envelope. However, it is less clear how these bNAbs are eventually developed in HIV-positive patients due to the extremely complex antibody repertoire activation and maturation pathways. A deeper understanding of crucial specificities and the isolation of more broad and potent antibodies can coalesce to pave the way to the design of an effective therapy.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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