Neutralizing antibodies to HIV-1 induced by immunization

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Most neutralizing antibodies act at the earliest steps of viral infection and block interaction of the virus with cellular receptors to prevent entry into host cells. The inability to induce neutralizing antibodies to HIV has been a major obstacle to HIV vaccine research since the early days of the epidemic. However, in the past three years, the definition of a neutralizing antibody against HIV has been revolutionized by the isolation of extremely broad and potent neutralizing antibodies from HIV-infected individuals. Considerable hurdles remain for inducing neutralizing antibodies to a protective level after immunization. Meanwhile, novel technologies to bypass the induction of antibodies are being explored to provide prophylactic antibody-based interventions. This review addresses the challenge of inducing HIV neutralizing antibodies upon immunization and considers notable recent advances in the field. A greater understanding of the successes and failures for inducing a neutralizing response upon immunization is required to accelerate the development of an effective HIV vaccine.

Some protective antibodies can act later in the replication cycle, for example, antibodies involved in ADCC (antibody-dependent cytotoxicity) and ADCVI (antibody-dependent cell-mediated virus inhibition), in addition to or in the absence of neutralizing properties (Forthal and Moog, 2009).

HIV presents special hurdles to generating broad and potent neutralizing antibodies. It was already apparent from the first reports of neutralizing antibodies against HIV-1 (Robert-Guroff et al., 1985; Weiss et al., 1985) that the neutralizing response in infected patients was weak compared with non-neutralizing HIV antibodies. For instance, although anti-envelope glycoprotein (Env) antibody titers were equivalent to those in patients infected with HTLV-1 (human T-lymphotropic virus type 1; measured by binding or by immunofluorescence) neutralizing titers were 100-fold lower (Weiss et al., 1985). Moreover, difficulties in eliciting neutralizing antibodies by vaccination as opposed to infection quickly became apparent with the observation that the neutralizing responses

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elicited by gp120 immunization were more type specific than those produced in natural infection (Weiss et al., 1986).

There are several reasons why HIV is a challenging target for neutralizing antibodies. First, the sheer genetic diversity of concurrent HIV subtypes (clades), circulating recombinant forms, and strains is greater than for any other virus, except possibly hepatitis C virus, and this is reflected in the antigenic diversity of Env which is the target of neutralizing antibodies (Burton et al., 2012; Ndung’u and Weiss, 2012). Second, the neutralizing epitopes are, for the most part, hidden beneath a glycan shield which makes them inaccessible to antibodies, although some epitopes include carbohydrate moieties (Sattentau, 2011). Third, although all strains of HIV bind to the CD4 cellular attachment receptor, the CD4 binding site resides in a pocket to which antibody access is restricted (Kwong et al., 2012). Nevertheless, during the last three years a new generation of mAbs has been identified which offers broad and potent neutralization of diverse HIV strains. Previously, there was concern that a gain in the breadth of neutralization might be accompanied by loss of potency, but we now know that this is not the case. These discoveries have led to increased optimism that vaccines which induce cross-clade neutralizing antibodies will be achieved. The challenge now is to translate the new knowledge of neutralizing epitopes into immunogens that will elicit potent and lasting immunity to HIV infection.

Recently, our understanding of what constitutes a broadly neutralizing antibody against HIV has been revolutionized by the isolation of extremely broad and potent neutralizing mAb from HIV-infected individuals (Walker et al., 2009, 2011; Corti et al., 2010; Wu et al., 2010; Scheid et al., 2011). These mAbs were identified by dissecting the broad neutralization activity seen in specific patient serum samples and by characterizing mAbs from B cells (Beirnaert et al., 2000; Dhillon et al., 2007; Binley et al., 2008; Scheid et al., 2009; Simek et al., 2009; Walker et al., 2009). The application of single B cell cloning techniques (Tiller et al., 2008) enabled the leap forward in neutralizing antibody identification via the use of soluble antigens (Scheid et al., 2009) or baits and recently cell-based antigens (Klein et al., 2012a), alongside the use of direct screening of B cell supernatants for neutralization activity as reviewed extensively in Moir et al. (2011). Although the knowledge garnered from the new generation of HIV neutralizing antibodies can directly inform future immunogen design, as reviewed elsewhere (Pejchal and Wilson, 2010; Walker and Burton, 2010; McMichael and Haynes, 2012), this review aims to provide further insight by considering the efforts made to elicit broadly neutralizing antibodies by vaccination in contrast to their generation during natural HIV infection.

### First generation HIV neutralizing mAbs

During the course of chronic HIV infection, neutralizing antibodies are elicited to a variable degree in different individuals (Willey and Aas-Chapman, 2008; Gray et al., 2011; Bonsignori et al., 2012; Lynch et al., 2012) to Env, the key characteristics of which are summarized in Table 1. For many years, only a limited number of neutralizing mAbs from infected patients were described, but between the available clones the key regions targeted during the neutralization were largely identified and have been thoroughly reviewed elsewhere (Sheppard and Sattentau, 2005; Fig. 1). To summarize, the mAb with the best anti-HIV potency and breadth before 2009 was b12, which was shown to target the CD4-binding site of Env and neutralize up to 40% of strains tested (Burton et al., 1994). Once Env has bound CD4, a conformational change occurs that presents induced epitopes (CD4i epitopes) which can be targeted by neutralizing antibodies (Moulder et al., 2002). The neutralization activity of identified CD4i mAbs, e.g., 17b, is enhanced in the presence of subinhibitory concentrations of sCD4. Moreover, potency and breadth are boosted when this mAb is assayed as a FAB rather than full-length IgG, whereas the reverse is true for the CD4-binding site mAb b12 (Labrijn et al., 2003). These results suggest that antibody access to CD4i epitopes is restricted by steric factors, and thus, although CD4i binding activity is induced in the sera of immunized humans, the sera do not have broad neutralization activity (Vaine et al., 2010).

In addition, an anti-Env glycan-specific neutralizing mAb, 2G12, was identified (Trkola et al., 1996), which has an intriguing domain-swapped architecture (Calarese et al., 2003). Initially N-linked glycans in conserved regions (C) 1, C2, C3, C4 and variable regions (V) 4 were identified with 2G12 binding. More recently, modifications in V1/V2 and V3 have been linked to 2G12 sensitivity (Chaillon et al., 2011). The V3 loop has also been identified as the target of neutralizing antibodies (Durda et al., 1988), notably the mAbs 447-52D (Stanfield et al., 2004; Rosen et al., 2005) and B4E8.
(Bell et al., 2008). Gp41 was also identified as a target for neutralizing antibodies isolated from HIV-infected humans; the two most studied mAbs are 2F5 (Barbato et al., 2003) and 4E10 (Stiegler et al., 2001), which both bind within the membrane-proximal region (MPER) and also have a lipid binding ability (Alam et al., 2009). 4E10 exhibits a great breadth of neutralization across subtypes (Mehandru et al., 2004); however, neutralization is only observed at weak to moderate potency (Binley et al., 2004).

Although much was learnt from the first generation of neutralizing mAbs against HIV, there was residual doubt as to whether antibodies with greater breadth and neutralization potency could ever be elicited, even in natural infection in humans, and thus there was speculation as to whether a humoral response induced by vaccination could ever be truly protective. Furthermore, although protection from infection was seen when these mAbs were passively transferred to NHP (Mascola et al., 1999, 2000; Shibata et al., 1999; Parren et al., 2001; Veazey et al., 2003; Hessell et al., 2009; Watkins et al., 2011), viral escape mutants emerged in animal models (Andrus et al., 1998; Poignard et al., 1999) and in patients who had interrupted antiretroviral treatment concurrently with passive transfer of mAbs (Trkola et al., 2005). With the failure of the initial human vaccination trials, based on gp120 protein antigens postulated to induce neutralizing antibodies (Flynn et al., 2005; Pitisuttithum et al., 2006; Gilbert et al., 2010), the prospects for a neutralizing antiserum-inducing HIV vaccine appeared bleak and there was a shift within the HIV prevention field toward vaccines designed to induce mainly T cell responses, as previously reviewed (Barouch, 2010; McElrath and Haynes, 2010; Pantaleo et al., 2010; Picker et al., 2012).

Second generation HIV neutralizing mAbs
In 2009, two landmark papers described mAbs isolated from the sera of HIV-infected donors (Scheid et al., 2009; Walker et al., 2009). The use of single B cell cloning, coupled with an antigen-binding-based selection method, revealed that underlying the broad serum neutralization activity of elite neutralizer patients are multiple antibody lineages targeting a range of epitopes on gp120 (Scheid et al., 2009). Concurrently, the adaptation of single B cell cloning to a high-throughput screen of >30,000 B cell clone supernatants for the ability to neutralize HIV led to the isolation of two mAbs, PG9 and PG16 (Walker et al., 2009). These mAbs neutralize between 70 and 80% of >150 strains of HIV tested (Walker et al., 2009) by binding preferentially to trimeric Env via the V1/2 loops of the gp120 subunit (Walker et al., 2009; McLellan et al., 2011). Shortly afterward, a set of mAbs was isolated from

![Figure 1. Neutralizing mAbs targeting HIV Env. The diagram was adapted from Burton and Weiss (2010).](image-url)
multiple patients. Although not as broadly neutralizing as PG9 and PG16, novel epitopes were identified and, in the case of HJ16, showed atypical patterns of virus sensitivity (Corti et al., 2010). Subsequently, a CD4-binding site mAb was isolated, again by a large-scale screening process, but this time using a resurfaced gp120 core protein as bait (Wu et al., 2010). This mAb, VRC01, along with its somatic variant VRC02, neutralizes up to 90% of strains tested (Wu et al., 2010; Zhou et al., 2010; Li et al., 2011). Further improvements to previously used isolation methods (Moir et al., 2011) resulted in the identification of PG9/16-related mAbs designated PGT121-145, which range in breadth from 16 to 80% but exhibit remarkable potency (Walker et al., 2011) and interact with the glycans of HIV, with potency potentially arising as a result of Env cross-linking by these mAbs (Pejchal et al., 2011). Substantial advances were also made in terms of the potency of broadly neutralizing CD4-binding site antibodies isolated (Scheid et al., 2011) and differences in mechanism of action of different mAbs targeting the virus at this crucial site (Falkowska et al., 2012). Structural studies on one new anti-CD4 binding site mAb, NIH45-46, revealed that it did not have a large aromatic residue to contact the hydrophobic pocket between the CD4 binding loop and the bridging sheet on Env, as does CD4 itself (Diskin et al., 2011). By mutating a phenylalanine into this position on NIH45-46, an extremely potent CD4bs mAb was generated which could provide protection against ~75% of circulating strains at levels as low as 0.1 µg/ml (Diskin et al., 2011). Furthermore, the study of CD4bs mAbs from multiple individuals has suggested a common pathway for their development in humans from particular heavy chain germline genes (West et al., 2012). In addition, recent work has shown the potential of combining these broad and potent neutralizing antibodies (Doria-Rose et al., 2012; Klein et al., 2012a,b). These mAbs do not impair another one's function (Doria-Rose et al., 2012), and, given the distinctive neutralization patterns observed, their combined use could provide enhanced breadth and potency. If used either in a multi-antibody therapy/prophylactic or in the ideal case of a vaccine capable of inducing a range of these broad and potent antibodies that between them target multiple epitopes, this could result in serum activity reminiscent of the multispecificity neutralization activity seen in some elite neutralizer patients (Scheid et al., 2009). In addition, combining antibodies that target both gp41 and gp120 subunits has recently been rendered more appealing by the isolation of an anti-MPER 10E8 which effectively neutralizes 98% of viruses tested with a median IC50 of 0.35 µg/ml (Huang et al., 2012).

Now, after 25 years of HIV research, truly cross-reactive antibodies isolated, again by a large-scale screening process, but this time using a resurfaced gp120 core protein as bait (Wu et al., 2010). This mAb, VRC01, along with its somatic variant VRC02, neutralizes up to 90% of strains tested (Wu et al., 2010; Zhou et al., 2010; Li et al., 2011). Further improvements to previously used isolation methods (Moir et al., 2011) resulted in the identification of PG9/16-related mAbs designated PGT121-145, which range in breadth from 16 to 80% but exhibit remarkable potency (Walker et al., 2011) and interact with the glycans of HIV, with potency potentially arising as a result of Env cross-linking by these mAbs (Pejchal et al., 2011). Substantial advances were also made in terms of the potency of broadly neutralizing CD4-binding site antibodies isolated (Scheid et al., 2011) and differences in mechanism of action of different mAbs targeting the virus at this crucial site (Falkowska et al., 2012). Structural studies on one new anti-CD4 binding site mAb, NIH45-46, revealed that it did not have a large aromatic residue to contact the hydrophobic pocket between the CD4 binding loop and the bridging sheet on Env, as does CD4 itself (Diskin et al., 2011). By mutating a phenylalanine into this position on NIH45-46, an extremely potent CD4bs mAb was generated which could provide protection against ~75% of circulating strains at levels as low as 0.1 µg/ml (Diskin et al., 2011). Furthermore, the study of CD4bs mAbs from multiple individuals has suggested a common pathway for their development in humans from particular heavy chain germline genes (West et al., 2012). In addition, recent work has shown the potential of combining these broad and potent neutralizing antibodies (Doria-Rose et al., 2012; Klein et al., 2012a,b). These mAbs do not impair another one’s function (Doria-Rose et al., 2012), and, given the distinctive neutralization patterns observed, their combined use could provide enhanced breadth and potency. If used either in a multi-antibody therapy/prophylactic or in the ideal case of a vaccine capable of inducing a range of these broad and potent antibodies that between them target multiple epitopes, this could result in serum activity reminiscent of the multispecificity neutralization activity seen in some elite neutralizer patients (Scheid et al., 2009). In addition, combining antibodies that target both gp41 and gp120 subunits has recently been rendered more appealing by the isolation of an anti-MPER 10E8 which effectively neutralizes 98% of viruses tested with a median IC50 of 0.35 µg/ml (Huang et al., 2012).

Now, after 25 years of HIV research, truly cross-reactive human antibodies have been identified which target multiple sites on Env. This demonstrates that improved neutralizing antibody elicitation on vaccination should be possible if the correct immunogens, adjuvants, and immunization schedules can be determined; yet, this is in itself a formidable challenge. Detailed descriptions of these second generation mAb and their mechanisms of action have been reviewed in detail elsewhere (Hessell and Haigwood, 2012) alongside how this information can be used to improve vaccine design (Burton and Weiss, 2010; Walker and Burton, 2010). When used in combination, these second generation mAbs are predicted to provide neutralization coverage approaching 100% breadth (Doria-Rose et al., 2012). In addition, the high potency demonstrated by some mAbs means the titers required for protection are postulated to be within an achievable range for vaccine-induced antibodies (Diskin et al., 2011). Thus, what constitutes an HIV neutralizing antibody has been redefined and the benchmark to aim for is now significantly higher. Despite the extensive study of these mAbs, it is not a straightforward endeavor to elicit them by immunization. They remain rare antibodies in the repertoire of a few patients. A range of attempts has been made with some limited success, as outlined below. However, there remains much uncertainty regarding how such antibodies mature during natural infection let alone after immunization. Thus, there remains a black box in HIV vaccinology which needs to be decoded: how can we elicit broadly neutralizing responses, preferably involving multiple antibody lineages as the predominant response in anti-HIV sera? To begin to unravel this code, it is helpful to reflect on the progress in experimental HIV immunizations to date.

**HIV neutralizing responses after animal immunization: successes and failures**

Initial immunization studies demonstrated that although high anti-Env titers are inducible, the neutralization phenotype of both sera and isolated mAbs is limited (Robey et al., 1986; Berman et al., 1988; Ho et al., 1988; Nara et al., 1988; Earl et al., 1989; Haigwood et al., 1990). Given the largely unsuccessful immunizations with monomeric gp120 (Barnett et al., 1990; Porin et al., 1995; Mascola et al., 1996; Barnet et al., 1997; Belshe et al., 1998; Connor et al., 1998), attempts have been made to present the immune system with a more accurate representation of the neutralizing epitopes displayed on functional HIV Env spike by using trimeric Env. Although these recombinant immunogens have proved more effective than their monomeric counterparts at inducing neutralizing antisera in side by side comparisons (Kim et al., 2005; McBurney et al., 2007; Beddows et al., 2007; Forsell et al., 2009), particularly in terms of boosting neutralizing titers (VanCott et al., 1997; Barnet et al., 2001; Yang et al., 2002; Bower et al., 2004; Hammonds et al., 2005; Zhang et al., 2007), trimeric Env immunization did not result in the great jump hoped for in the level of neutralization breadth seen in postimmune sera. However, questions have been raised as to the quality of trimer preparations, and recent data suggests homogeneous and stable trimers are advantageous over their monomeric counterparts (Kovacs et al., 2012). Furthermore, it has been demonstrated that sequential immunization of macaques with Env from distinct subtypes results in greater antibody maturation than simultaneous exposure (Malherbe et al., 2011). In addition, some animal immunizations, including trimeric Env immunizations, have led to the production of cross-subtype neutralizing sera or mAbs, as detailed in
Table 2 (cross-reactivity is defined as activity against three or more subtypes of HIV). Indeed, neutralization breadth equivalent to that of the new generation of neutralizing mAbs from infected patients has been observed in one study (McCoy et al., 2012). However, this breadth is exhibited by a purified mAb fragment rather than the postimmune sera, which is in fact relatively weakly neutralizing (although cross-reactive). Second, this mAb fragment is derived from a heavy chain–only antibody and so the limits on recognition of recessed epitopes may be less restrictive.

Attempts to focus the immune response on epitopes recognized by neutralizing antibodies and also to increase the breadth of the response have been made by modifying the Env protein immunogen. For example, alterations to the variable loops of Env, including in a study describing a subtype C gp140 immunogen containing a deletion in V2, resulted in rabbit sera that neutralized another subtype C virus and a few heterologous primary isolates (Lian et al., 2005). However, use of the same subunit immunogen in rhesus macaques produced only moderate levels of autologous neutralizing mAb (Lian et al., 2005). Similarly, cynomolgus macaques immunized with a different V2-deleted Env produced sera that neutralized only the homologous HIV strain and a closely related SHIV, although three of the four animals were protected against intrarectal challenge with the same SHIV strain (Ferrantelli et al., 2011). More drastic Env modifications, such as mutation of the CD4-binding site or disruption of the bridging sheet, result in less potent macaque sera neutralization.

Table 2. Animal immunizations yielding sera or mAbs that neutralize three or more subtypes

| Immunogen                                                                 | Species          | Neutralization                                                                 | Reference                      |
|---------------------------------------------------------------------------|------------------|--------------------------------------------------------------------------------|--------------------------------|
| Oligomeric electrophilic gp120                                            | Mice             | mAb which neutralizes 11 strains from subtypes A, B, and C, including tier 2 and 3 isolates, with intermediate potency. | Nishiyama et al., 2009         |
| Multi-subtype (ABC) gp160 DNA immunization + GM-CSF                       | Mice             | Sera from 6 animals neutralized 1 strain from subtype A and C and 3 strains from subtype B. | Rollman et al., 2004           |
| Synthetic peptide derived from C2 residues 218-239 from CRF01_AE Env    | Mice             | mAb neutralizes 10 strains out of 14 tested, including subtypes A, C, D, and AE but not B | Sreepian et al., 2009          |
| Formaldehyde-stabilized, heat-inactivated virion subtype B cytoplasmic tail Env mutant (more Env on surface) | Rabbits          | Sera from 3 animals neutralized 4 strains from subtypes A, B, C, D, and AE but less potently than murine sera | Poon et al., 2005              |
| VEE virus replicon expressing: gp140/gp160, gp160 lacking cytoplasmic tail | Rabbits          | Sera from 3 animals neutralized subtype B (SF162) and sera from 2 of these animals also neutralized subtype B (R2) | Dong et al., 2003              |
| Gp140 with novel carbopol-971p and MF59 adjuvant combination              | Rabbits          | Sera from 6 animals in the combined adjuvant group neutralized 6 strains from subtypes A, B, C, D, and AE | Lai et al., 2012               |
| 2FS used to select immunogen from combinatorial libraries of recombinant human rhinoviruses displaying ELDKWA | Guinea pigs      | Sera from 1 animal neutralized 9 strains from subtypes A, B, C, D, and AE with moderate/weak potency | Arnold et al., 2009            |
| Multiclade (A, B, C) gp140ΔCFI DNA (mutations in the cleavage site, fusion peptide, and interhelical regions) followed by replication defective adenovirus expressing gp140ΔCFI | Guinea pigs      | Sera from 4 animals neutralized 13 strains from subtypes A, B, C, and D with weak potency | Chakrabarti et al., 2005       |
| Gp140 (subtype A or C), verified as homogenous and stable                 | Guinea pigs      | Sera from animals immunized with either gp140 neutralized 1 subtype A, 2 subtype B, and 4 subtype C strains with statistically higher titers for sera resulting from subtype C gp140 rather than gp120 immunization | Kovacs et al., 2012            |
| Gp120 protein from subtype B                                             | Llamas           | VHH mAb, neutralizes up to 40% of 59 strains tested from subtypes A, B, C, BC, AG, and AE with variable potency. | Forsman et al., 2008           |
| Gp140 proteins from subtypes A and B/C                                   | Llamas           | VHH mAb, neutralizes 70–96% of strains, with 30–100 strains tested from subtypes A, B, C, BC, AG, AE, AC, ACD, D, and G | McCoy et al., 2012; Strokappe et al., 2012 |
| Gp140                                                                     | Rhesus macaques  | Sera from 6 animals potently neutralized 5 strains from tier 1 (subtypes B, C, and A) and weakly neutralized 2 tier 2 strains. Protection: modest, nonsterilizing impact on acquisition of heterologous SHIV | Sundling et al., 2010          |
of sensitive viruses compared with sera raised against wild-type trimers (Douagi et al., 2010). Given the extensive glycan shield used by HIV to evade neutralization, modifying the glycosylation of Env immunogens has also been investigated. Several studies have shown increased titers of anti-Env activity in sera but not a concurrent increase in neutralizing activity (Joyce et al., 2008; Ma et al., 2011; Ahmed et al., 2012). However, an increase in neutralization titers was seen in cynomolgus macaques boosted with DNA and Env after a prime comprised a poxvirus expressing an Env mutant lacking an N-linked glycosylation site in the V2 loop, which has been previously characterized as more sensitive to the first generation mAbs b12 and 447-52D (Li et al., 2008). The animals that received the mutant rather than wild-type Env also had lowered viral loads and increased survival upon homologous challenge (Li et al., 2008).

Env immunization has focused primarily but not exclusively on gp120/140, as many immunodominant but non-neutralizing antibodies interact with gp41 (Wilson et al., 1990). However, recently gp41 immunogens have been investigated with a degree of success. An MPER mimotope linked to lipid carriers induced subtype B (SF162.LS) MPER–specific neutralizing sera responses in mice (Zhou et al., 2012). Furthermore, immunization of rabbits with a mimetic of the coiled-coil of gp41 presented by phage display led to the isolation of a single-chain Fv antibody that neutralizes primary HIV of subtypes B and C with modest potency (Nelson et al., 2008). Grafting MPER onto the more highly exposed V1/V2 loop induced neutralizing sera in mice but, unfortunately, not specific for MPER or the V1/V2 loop (Law et al., 2007). Also disappointingly, an NHP study with a gp41 proteoliposome induced MPER binding, but not neutralizing sera (Dennison et al., 2011). Mucosal vaccination with gp41 peptide immunogens has, however, produced sera that can neutralize a limited number of strains (Devito et al., 2004) and block viral transcytosis in vitro (Jain and Rosenthal, 2011). The broadest neutralization induced thus far to gp41 involved immunization of guinea pigs with human rhinoviruses displaying modified versions of the 2F5 epitope which were preselected for binding to this mAb. The postimmune serum from one animal neutralized nine HIV-1 strains from subtypes A, B, C, D, and AE with moderate to weak potency (Table 2; Arnold et al., 2009).

Given the acknowledged role of lipid reactivity in some mAb derived from HIV-positive individuals, and the restrictions placed on the angle of approach of antibodies to the Env spike on a viral particle, strategies for presenting Env in a membrane-bound context have been investigated. A range of viral vectors have been exploited to express Env, including Semliki Forest virus (Forsell et al., 2005), alphavirus replication particles (Dong et al., 2003; Mörner et al., 2009; Barnett et al., 2010; Table 2), adenoviruses (Lubeck et al., 1997; Zolla-Pazner et al., 1998a; Chakrabarti et al., 2002; Mascola et al., 2005), measles (Lorin et al., 2004), varicella (Traina-Dorge et al., 2010), vesicular stomatitis virus (Schell et al., 2009), and multiple poxviruses (Verrier et al., 2000; Radaelli et al., 2007). A parallel aim of many of these studies was the effective induction of T cell immunity which has been reviewed elsewhere (McElrath and Haynes, 2010). Only two of these studies (Dong et al., 2003; Chakrabarti et al., 2005) resulted in cross-reactive postimmune sera able to neutralize at least three subtypes (Table 2). However, a range of these immunizations resulted in protection from infection (Lubeck et al., 1997; Zolla-Pazner et al., 1998b) or control of viral load in infected animals (Schell et al., 2009; Traina-Dorge et al., 2010). To address concerns about the integrity of recombinant Env immunogens, particularly regarding posttranslation glycosylation, DNA encoding Env has been included in immunization strategies given the preferential uptake of DNA by antigen presenting cells. However, DNA immunization without subsequent viral vector or protein boosts has only produced sera that neutralize three or more subtypes when administered with granulocyte-macrophage colony-stimulating factor (Rollman et al., 2004; Table 2). Overall, the use of viral vectors combined with either Env or DNA has shown some success but, again, has not resulted in a great leap forward in terms of neutralization titers or breadth.

Recent studies have highlighted the importance of including protein components, particularly Env, in immunization protocols to achieve protection against heterologous challenge strains in NHPs (Watkins et al., 2011; Barouch et al., 2012). Although greater understanding of how Env subunit immunogens elicit antibodies has been gained, attempts thus far to focus the immune system on particular epitopes have not resulted in highly specific mAbs. Given that such mAbs have now been identified in multiple infected humans, both mAb structure and mode of action studies should provide clearer insight into how to modify Env to focus the immune response to induce broadly neutralizing sera. Investigations into more selectively focused Env immunizations include several approaches: mutation of immunodominant epitopes; masking of immunodominant epitopes by cross-linking, for example, with aldehydes (Poon et al., 2005; Table 2); and selective alteration of carbohydrate moieties. In addition, when considering the handful of studies (Table 2) that have given rise to cross-reactive responses or mAb (defined as neutralization of three or more HIV subtypes) it is noteworthy that almost half of these studies evaluate individual mAbs rather than serum neutralization, in contrast to most HIV immunization studies. In at least one case, a mAb fragment that neutralizes 96% of strains tested was isolated from weakly neutralizing serum (McCoy et al., 2012). Thus, it may be that if the large-scale techniques applied to searching for neutralizing mAb from infected humans were applied to postimmune sera, it could be clarified whether rare broadly neutralizing antibodies were in fact elicited but at levels too low to dominate the sera neutralization activity. In turn, this knowledge could be used to optimize immunization protocols and improve the stimulation of the appropriate B cell precursors.
Postimmunization protection from HIV and neutralizing responses

Huge efforts have been made to elucidate how humoral responses are induced by Env proteins and to characterize the anti-HIV activity of the resulting sera. However, it is important to ask not only what neutralization activity has been induced upon immunization but also whether the animals are protected from viral challenge. Indeed, protection from both heterologous and homologous challenge in NHP has been observed despite suboptimal serum neutralizing activity and there is residual doubt regarding the role of vaccine-induced neutralizing antibodies in such studies. However, correlation between neutralizing antisera and protection has been noted after Env immunization (Barnett et al., 2008), adenovirus priming followed by Env or alphavirus replicon particles (Bogers et al., 2008), and alphavirus replicon particles with a trimeric Env boost (Barnett et al., 2010). Furthermore, this association between neutralizing antisera and protection is not at the expense of effective cellular immunity. In fact, a separate study of rhesus macaques immunized with alphavirus replicon particles, followed by an Env boost with or without vectors expressing SIV gag/pol, showed not only that there is no interference between cellular and humoral immunity but that protection to heterologous challenge was higher for serum neutralization titers and improved cellular immune responses with a few subjects completely protected from infection (Quinnan et al., 2005). Furthermore, peak viral loads after repeated low-dose challenge of rhesus macaques immunized solely with recombinant protein showed a significant inverse correlation with both cross-reactive neutralizing antibodies levels and cellular immunity (Lakhashe et al., 2011). Recently, viral vector combination regimens expressing SIV gag, pol, and env have resulted in 80% or more reduction in infection after repeated challenge with a heterologous, neutralization-resistant SIV, and the inclusion of env was shown to be essential to this protection, highlighting a potential role for neutralizing antibodies in the success of this immunization protocol (Barouch et al., 2012). Thus, the challenge is now to identify how this protection is being mediated and to improve immunogens and immunization regimens to promote the protective component of the response. Strategies to improve antibody elicitation have been reviewed elsewhere (Dimitrov et al., 2011; Moir et al., 2011; Verkoczy et al., 2011; Klasse et al., 2012).

Human HIV vaccine studies have in many ways mirrored the studies undertaken in animals. Initial human gp120 immunization resulted in serum neutralization against the strain from which the vaccine was derived (Win and Nunberg, 1994; Graham et al., 1996; Mascola et al., 1996; Pincus et al., 1997; Bartlett et al., 1998; Gorse et al., 1998). Subsequently, the first phase III efficacy trial (VAX003/004) involving vaccination with two subtype B gp120 subunits was unable to provide protection from acquisition but did result in serum neutralization of related CXCR4-tropic subtype B strains but not of primary CCR5-tropic isolates (Flynn et al., 2005; Pitpisuttithum et al., 2006). The VAX004 trial was also not protective against acquisition but resulted in neutralizing activity against virus expressing the subtype B gp120 immunogen administered to vaccinees. Thus, this weak level of subtype-specific neutralization, which is reminiscent of that shown to protect from homologous challenge in many animal studies, was not protective in humans in a phase III trial (Gilbert et al., 2010).

In 2009, a degree of efficacy was observed in a phase III HIV vaccine trial, with 30% of vaccinees protected relative to placebo for the first time (Rerks-Ngarm et al., 2009). The RV144 trial involved priming with the poxvirus vector ALVAC followed by AIDSVAX B/E gp120 boost, and recently two correlates of risk have been identified. Both focus on the humoral response to vaccination, with an increased risk correlated with high titers of anti-immunogen IgA and decreased risk correlated with high titers of anti-Env IgG that target the V1/V2 loop (Haynes et al., 2012). Interestingly, a previous canarypox-based phase II trial was deemed inadequately effective based on poor cytotoxic T lymphocyte responses despite the elicitation of MN neutralizing sera in 57–94% of subjects (Russell et al., 2007). However, it should be noted that the humoral correlates of risk identified in the analysis of the RV144 trial do not include neutralizing serum responses (Rerks-Ngarm et al., 2009; Haynes et al., 2012; Montefiori et al., 2012).

There is some similarity between the outcomes of many animal immunization experiments and human vaccination trials in that the majority of studies have resulted in serum neutralization which is limited in breadth to highly sensitive viruses. The contrast is that the animal studies often show protection from acquisition in challenge studies, mostly with homologous virus, whereas for human vaccination trials, protection has generally not been evident, presumably as a result of the highly diverse nature of real world exposure to HIV. As outlined in the introduction, until recently the field did not have a clear idea of what levels of breadth and potency could be achieved by human neutralizing antibodies and, as such, the relatively narrow breadth of serum neutralization usually seen upon immunization was not considered suboptimal as it is now judged to be. The knowledge of the caliber of neutralizing antibody that humans can produce during infection provides a high benchmark against which to evaluate immunization studies and the techniques used to isolate these second generation neutralizing mAb can help to assess how close we come to the mark.

It is noteworthy that mAb-mediated protection via passive transfer in NHP is not thought to act solely via the mechanism of neutralization (Hessell et al., 2009; Watkins et al., 2011; Moldt et al., 2012), and there is increasing evidence in support of the importance of antibody effector functions and also trans-cytosis blocking antibodies (Bomsel et al., 1998; Burke and Barnett, 2007; Tudor et al., 2009). Overall, despite the progress in understanding how antibodies can effectively neutralize HIV it is still the major challenge of the HIV vaccine field to induce a high titer sera response comprising such antibodies. This challenge is even greater when
the predominant mode of HIV transmission is considered, as to induce high concentrations of neutralizing antibody at the mucosal level is even more difficult than on a systemic level (Wright et al., 2004). Thus, it is likely that improving immunization protocols to the level where they can elicit such antibodies will require considerable effort so it is worthwhile to consider how the identified neutralizing mAbs can be used as preventative reagents in the meantime.

Prospects and considerations for the therapeutic use of second generation bnAbs

Given the protection seen in passive immunization studies with the first generation of neutralizing mAbs, which are substantially less potent, it is hypothesized that the second generation of neutralizing mAbs could be used via passive transfer at much lower doses (Diskin et al., 2011), and in combination (Doria-Rose et al., 2012; Klein et al., 2012b), to provide enhanced protection from infection. When could such dosing be useful to prevent transmission in a clinical setting? It seems unlikely that such an intensive intervention would be suitable for prophylactic daily use. Whether passive transfer could be used as postexposure prophylaxis is unclear; data from infusion of polyclonal neutralizing antibodies into NHP 6 h after challenge did provide sterilizing immunity but at 24 h did not (Nishimura et al., 2003). Studies are needed with the second generation mAb, most critically in combination, to assess the time limits and potential advantages of such an approach alongside whether viral escape is feasible in vivo, which in turn will have implications for the combinations of epitopes to be included future vaccination studies.

It cannot be overlooked that there are resource limits which need to be considered when discussing the use of purified mAb to combat HIV, and which may restrict the use of the applications above to a very small proportion of the global population at risk of infection. Efforts are under way, however, to overcome the limits imposed by cost and time intensive production of mAbs so that they could be used prophylactically on a larger scale. The first option is that of mAb-based microbicide. The protection seen with an antiretroviral-based microbicide gel has reinvigorated the HIV microbicide field (Abdool Karim et al., 2010), and although gels containing mAbs have been considered, the cost implications are similar to those for passive transfer of mAbs, with additional problems caused by the need for cold storage of such a microbicide (Gorlani et al., 2012). An alternative prospect is that of a persistent biological microbicide, namely Lactobacilli, genetically modified to express anti-HIV molecules. Vaginal colonization of Macaca mulatta with Lactobacilli jensenii expressing cyanovirin has already been shown to prevent mucosal SHIV transmission (Lagenaur et al., 2010) and attempts are underway to express mAb fragments in this system. Although L. jensenii is a naturally occurring human commensal strain, thorough evaluation of the effects of colonization will be required alongside monitoring of the level of mAb expression. Another attractive approach to using mAbs prophylactically is a gene therapy–based approach. Such an approach was first described 10 years ago (Lewis et al., 2002; Johnson et al., 2009). Last year, this approach made a major advance with the description of a highly effective vector-mediated immunoprophylaxis in SCID mice reconstituted with human PBLs and challenged with a high dose of HIV (Balazs et al., 2012). A range of mAbs were shown to be effective in this system and secreted at 20–250 mg/ml in sera (Balazs et al., 2012), with protection from infection with serum concentrations for b12 and VRC01 of 34 and 8.3 mg/ml, respectively. How well vector-mediated immunoprophylaxis protects from mucosal transmission remains to be established. It will be particularly interesting to see whether combinations of mAbs result in a higher transmission barrier. Other obstacles to this approach are those associated with gene therapy in general. However, it should be noted that the adeno-associated virus vector used is nonintegrating but persistent and thus advantageous over other gene therapy vectors.

Conclusions

Single B cell cloning and systematic high-throughput methods involving the development of novel selection methods have resulted in the identification of the second generation neutralizing mAbs from HIV infected patients, despite such mAbs being relatively rare within the broadly neutralizing B cell repertoires of selected individuals. There is now a need for a similarly systematic approach to identify immunogens that may be capable of eliciting neutralizing antibodies, given that many novel immunogens to date have produced somewhat similar clade-specific weak serum neutralizing responses. In fact, a huge variety of immunization protocols have produced what could almost be termed a consensus humoral immune response comprising homologous neutralization activity in the sera as per initial immunization studies (Weiss et al., 1986), with some exceptions showing an increasing breadth in heterologous activity. A more detailed analysis of the components of the postimmune sera could enable advanced evaluation of the degree to which the immunization was effective, which could in turn inform immunogen and immunization study design. Despite the association observed between neutralizing antisera and a level of protection in some vaccination regimens, neutralization activity in immunized animals and humans is markedly inferior to those in rare HIV-infected individuals who are termed elite neutralizers. Passive immunization studies have shown that if certain levels of neutralizing mAbs are present, complete protection can be achieved (Mascola et al., 1999, 2000; Shibata et al., 1999; Parren et al., 2001; Veazey et al., 2003; Hessell et al., 2009; Watkins et al., 2011). Furthermore, it has been shown that combinations of recently described neutralizing antibodies together could block infection by the vast majority of strains (Doria-Rose et al., 2012; Klein et al., 2012a) and can control infection in humanized mice (Klein et al., 2012b). Therefore, there is a need for a bilateral approach to (1) identify immunogens able to induce neutralization activity (even at low titers) by investigating mAb as well as serum neutralization, and (2) understand how to modulate the immune system to promote the
affinity maturation of these rare clones to predominate over the abundant nonneutralizing antibodies. The desired outcome of such a strategy would be that the required level of neutralizing antibodies are present at the mucosa during the short window between exposure and infection to provide protection from HIV. Although the epidemic continues, we need to find ways to use all the available resources, in this case, the new generation of neutralizing mAb to prevent spread and lower disease burden. Approaches that bypass the difficult elicitation stage to provide broadly neutralizing mAb at the point of need, such as gene therapy and biological microbicides, show much promise. However, many more years of development may be required before these options can be rolled out to the populations most in need of protection.

In conclusion, even though we now have an expanding array of broadly neutralizing antibodies, which block a large proportion of HIV strains by binding to various epitopes on Env, there remains an immense challenge to convert our understanding of how these mAbs bind antigen and block infection into immunogens that will elicit a major humoral response to these targets. Despite the potential applications of HIV neutralizing antibodies detailed above, the challenge of effective immunogen design remains a priority in HIV research as part of the overall goal of developing a prophylactic HIV vaccine. It is to be hoped that the same level of rigor used to identify the new generation of broad and potent neutralizing mAbs will be applied to the challenge of immunogen design, leading to successful vaccine candidates.

Submitted: 13 August 2012
 Accepted: 11 December 2012

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