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# Broadly Neutralizing Antiviral Antibodies

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## Abstract

A fascinating aspect of viral evolution relates to the ability of viruses to escape the adaptive immune response. The widely held view has been that the great variability of viral glycoproteins would be an absolute obstacle to the development of antibody-based therapies or vaccines that could confer broad and long-lasting protection. In the past five years, new approaches have been developed to interrogate human memory B cells and plasma cells with high efficiency and to isolate several broadly neutralizing antiviral antibodies against highly variable pathogens such as HIV-1 and influenza virus. These antibodies not only provide new tools for prophylaxis and therapy for viral diseases but also identify conserved epitopes that may be used to design new vaccines capable of conferring broader protection.

## INTRODUCTION

The role of antibodies in host protection against viral infections has been formally demonstrated through passive immunization of immunodeficient animals and is best exemplified by the protection conferred to neonates by maternal antibodies (1). Antibodies neutralize viral infection by targeting viral glycoproteins of enveloped viruses or the protein shell of nonenveloped viruses. These proteins have two major functions in the viral life cycle: They bind to cellular receptors and mediate the fusion of the viral and cellular membranes (in the case of enveloped viruses) or the penetration into the cytosol (in the case of nonenveloped viruses). A distinctive property of viral glycoproteins is their capacity to vary the antigenic surface while maintaining their function, a property that endows the virus with the capacity to escape the antibody response.

For a long time, the conventional view was that the high variability of viral glycoproteins and the capacity of viruses to escape the antibody response would be an absolute obstacle to the development of therapies and vaccines that confer broad and long-lasting protection. This view was challenged by the recent isolation of antibodies, mostly from infected individuals, that can neutralize highly variable viruses by binding to conserved regions. The importance of isolating these antibodies and of identifying their corresponding targets is twofold. First, the antibodies can be used for passive immunization as prophylaxis or therapy for viral infection. Second, structural information about

the antibodies can be used to improve vaccination strategies by focusing the response against the conserved regions.

In this review, we first discuss the mechanisms of viral neutralization and escape. We then discuss recent advances in the fields of HIV-1, influenza virus, and other viruses as well as the impact of these findings on serotherapy and vaccine design.

## MECHANISMS OF PROTECTION BY ANTIBODIES: NEUTRALIZATION AND BEYOND

Neutralization of infectivity is thought to be the main mechanism of protection by antibodies and can be studied *in vitro* using infectious virus and susceptible cells (2, 3). Infection can be inhibited either by (*a*) blocking binding to cellular receptors or (*b*) interfering with the fusion machinery in enveloped viruses or with penetration in naked viruses. It is debated whether neutralization requires antibody binding to critical sites (critical binding site model) or is simply mediated by the occupancy of the viral surface (occupancy model) (3, 4). In the case of nonenveloped viruses, antibodies may also neutralize infectivity by promoting cytosolic degradation of incoming virions through a TRIM21-proteasome pathway (5). A special case of neutralization is mediated by antibodies that target the cellular receptor of the virus, as occurs for anti-CD4 or anti-CCR5 antibodies that broadly neutralize HIV-1 infection (6, 7), and by antibodies that target host molecules on viral particles, as happens with ICAM-1 or HLA molecules on HIV-1 particles (8, 9).

In addition to neutralization, other mechanisms contribute to *in vivo* protection. In the case of enveloped viruses that bud from the plasma membrane, antibodies can recruit effector cells or complement, leading to the killing of infected cells or the lysis of viral particles. In addition, the same antibodies can inhibit virus assembly, budding, and maturation. These mechanisms can be engaged by neutralizing as well as nonneutralizing antibodies that bind to viral

## RECOGNITION OF SIMILARITY

Similarity is not an absolute property of objects but rather relies on a subjective judgment. Antibodies can discriminate between two highly homologous structures or bind to molecules that share very little homology, depending on the fine details of the epitope recognized. Recognition of similarity is therefore a property of unique antibodies and thus, like beauty, is in the eye of the beholder.

antigens, such as the extracellular domain of the influenza M2 protein (M2e), that are expressed on the surface of infected cells but not on virions (10, 11). Given the complexity of the mechanism and of the viral life cycle, it is perhaps not surprising that for a given antibody there is no precise correlation between in vitro neutralization and in vivo protection, as discussed below.

Finally, it is worth considering that there is no evidence that neutralizing antibodies are preferentially selected in the immune response as compared with nonneutralizing antibodies. Indeed, B cells are selected for their capacity to capture and present viral antigens irrespective of their nature and topology. Consequently, only a small fraction of virus-specific memory B cells make neutralizing antibodies, whereas the majority make antibodies to denatured or internal proteins (12). Although some nonneutralizing antibodies might be able to restrict viral infection in vivo, the largest fraction of the response appears not to have protective value. The aim of an efficient vaccine would therefore be to enhance the fraction of memory B cells making neutralizing antibodies while minimizing the junk component of the response.

### Antigenic Drifting of Viral Glycoproteins

Genetic diversification is an intrinsic property of each virus and is required for the virus to adapt to the host and evade the immune response. The extent of diversification varies for different viruses depending on the fidelity of the viral RNA and DNA polymerases, which leads to different mutation rates (13). The mechanism of mutation is often biased toward transitions rather than transversions and therefore reduces the number of amino acid substitutions at any given codon. In addition, the diversification process is limited by the requirement for the virus to maintain fitness by preserving the receptor-binding site (RBS), the fusion machinery, and the overall global structure. Consequently, some regions in viral glycoproteins are more variable because they can accept mutations without loss of function,

whereas other regions are more conserved because mutations may compromise their function. As a rule of thumb, the most variable regions are also the most immunogenic and define the viral serotypes.

For many years, the classification of viruses was based on their susceptibility to specific neutralizing antisera, which defined distinct viral serotypes. This functional classification of viruses reflects their genetic distance but has survived due to its usefulness; it has recently been adapted to a cartographic representation to describe the annual drift of influenza A viruses (14). Influenza virus has 17 subtypes, and within each subtype there is a continuous antigenic drift. The genetic distance between the more distant influenza virus subtypes is similar to that between the F proteins of two different viruses, such as human respiratory syncytial virus and human metapneumovirus (**Figure 1**).

The race between viruses and the antibody response can be easily documented within the same individual in the case of HIV-1 or within the human population in the case of influenza virus. However, in several instances, such as dengue or rhinoviruses, different viral serotypes or genotypes coexist and segregate in distinct geographical areas.

To understand the basis for broadly neutralizing antibodies, it is worth considering the characteristics of the conserved and constrained sites in viral glycoproteins. The RBS of viral glycoproteins, depending on the virus tropism, has to accommodate different types of ligands, such as proteins, glycolipids, or sugar moieties, that can differ considerably in their size. In turn, the antibody-binding site has a large surface of approximately  $700 \text{ \AA}^2$  which for most antibodies is made by the heavy- and light-chain variable complementarity determining regions (CDRs). In the case of small RBSs, the antibody may also interact with polymorphic residues that surround the RBS, thus limiting the breadth of the antibody, as observed for rhinoviruses (15). In contrast to the RBS, the fusion machinery may be poorly accessible due either to the tight packing of

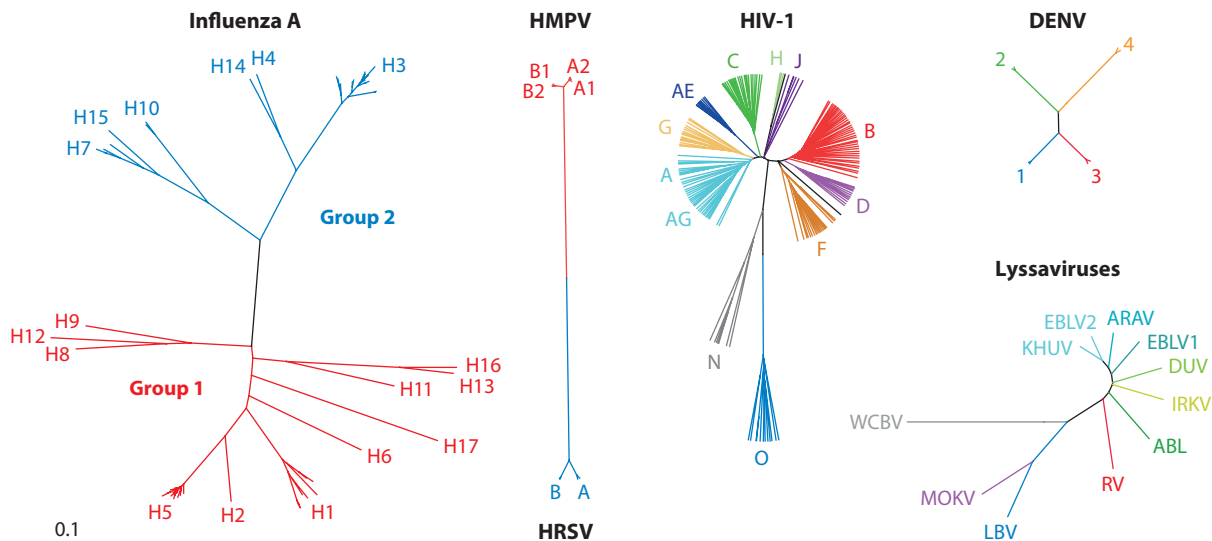


**Figure 1**

Protein distance of viral glycoproteins. Dendrograms, made by the neighbor-joining method, show the protein distance of viral glycoprotein amino acid sequences representative of the variability of each virus family. The amino acid distance scale is indicated with a value of 10% distance. Abbreviations: DENV, dengue virus; HMPV, human metapneumovirus; HRSV, human respiratory syncytial virus.

viral glycoproteins or to the transient exposure during the fusion process for those viruses that fuse at the cell membrane.

## BROADLY NEUTRALIZING ANTIBODIES AGAINST HIV-1

### First- and Second-Generation Antibodies

The target of HIV-neutralizing antibodies is the trimeric Env complex, which is present at low density on the viral membrane. The gp120 component of the Env protein engages CD4 and the coreceptors CCR5 or CXCR4 on macrophages and CD4<sup>+</sup> T cells and undergoes a conformational change that triggers the fusogenic activity of gp41 (16). HIV-1 is characterized by extraordinary genetic diversity, reflected by the presence of several clades. *Env* is the most variable gene, with up to 35% sequence diversity among clades, 20% within clades, and 10% in a single infected individual (17, 18). The virus has evolved to keep the most vulnerable conserved regions of the Env trimer

hidden and employs various mechanisms to escape from neutralizing antibodies, such as conformational masking of vulnerable epitopes; amino acid substitutions, insertions, or deletions (especially in the variable loops); and changes in the glycan shield, without significant loss of viral replicative capacity (19–21). In addition to their genetic diversity, HIV-1 strains are characterized by differing sensitivities to antibody-mediated neutralization; these differences led to their clustering into three subgroups, with tier 1 the most sensitive and tier 3 the most resistant (22). The structural basis for the differential sensitivity to neutralizing antibodies is poorly understood. In this context it is important to consider that a crystal structure of the entire HIV-1 Env trimer has not yet been determined at atomic-level resolution.

The isolation of antibodies that broadly neutralize HIV-1 has been a long-sought goal pursued through a major international effort. For several years, only a few such antibodies, all derived from clade B–infected individuals, were available (23): b12, specific for the

CD4-binding site (CD4bs) (24, 25); 2G12, specific for a complex gp120-glycan (26, 27); and 2F5, Z13, m66, and 4E10, specific for the membrane-proximal external region (MPER) of gp41 (28–31). These first-generation antibodies were limited either in their breadth or in their potency. For instance, b12 neutralized on average only one-third of the HIV-1 isolates, whereas 4E10 neutralized up to 98%, but only at high concentrations (32). Other neutralizing antibodies such as 447-52D and 17b targeted the V3 loop (33–36) and the CD4-induced site (37), but they showed an overall limited breadth of reactivity, a finding that may be related to the poor accessibility of their epitopes in HIV-1 primary isolates.

In the past four years, a renewed effort has led to the isolation of several second-generation human monoclonal antibodies characterized by greater breadth and potency. The success of this effort was based on the combination of three main strategies: (*a*) the selection of chronically infected individuals with potent and cross-clade-reactive serum antibodies; (*b*) the use of novel selection and screening approaches; and (*c*) the development of efficient methods to isolate human monoclonal antibodies.

Using a strict protocol termed protocol G (38) to identify the best out of 1,800 donors and a pseudovirus-based neutralization assay to select monoclonal antibodies from in vitro-activated memory B cells, Burton and coworkers (39) isolated from a clade A-infected African donor two antibodies, PG9 and PG16, that bind to a quaternary conformational epitope and neutralize 70–80% of the isolates tested. An antibody specific for a quaternary epitope, 2909, was already described but showed a very limited breadth of reactivity (40, 41). Both PG9 and PG16 antibodies bind to a proteoglycan epitope through an extended CDR3 region of the heavy chain (HCDR3), characterized by a hammer-like conformation that penetrates N156 and N160 glycans to reach strand C of the V1/V2 region (42, 43). Using a similar approach, Haynes and coworkers (44) isolated four antibodies (CH01 to CH04) similar to PG9 and PG16 in terms of specificity but with less

breadth. Another class of glycan-dependent antibodies was identified using the same selection approach by Pognard and coworkers (45), who isolated, from four selected donors, four sets of clonally related antibodies (PGT121-123, PGT125-131, PGT135-137, and PGT141-145) comparable to PG9 in terms of breadth but with higher potency. The PGT141-145 antibodies showed a specificity similar to that of PG9/PG16, whereas the PGT121-137 antibodies recognized conserved glycan clusters located on the outer domain of gp120 in proximity to the V3 or V4 loops. The crystal structure of PGT128 complexed with a glycosylated gp120 outer domain revealed that the antibody penetrates the glycan shield between N301 and N332 glycans to reach a short  $\beta$  strand at the base of the V3 loop (46). Whereas PG9 and PGT128 interact with high-mannose N-glycans, PGT121 and the clonally related antibody 10-1074 (47) bind to complex-type N-glycans in the vicinity of the V3 loop. These findings indicate different modalities of glycan recognition ranging from interaction with high-mannose glycans alone (2G12), to the interaction with high-mannose or complex-type glycans and amino acid residues of the Env surface in the V1/V2, V3, or V4 regions (PG and PGT series).

Using a similar microneutralization approach for the screening of antibodies produced by in vitro-activated memory B cells, Connors and coworkers (48) recently isolated an antibody termed 10E8 that binds to conserved residues within the MPER and partially overlaps with the 4E10 epitope without, however, interacting with the lipid bilayer. Interestingly, 10E8 carries a high load of somatic mutations (*i.e.*, 22% in VH) and neutralizes up to 98% of viruses at concentrations 5- to 10-fold lower as compared with 4E10 but lacks the polyreactivity and autoreactivity characteristic of the first-generation MPER antibodies (6, 7, 49).

Using an EBV-immortalization method and an ELISA-based screening, Corti *et al.* (50) isolated from memory B cells of non-clade B donors a panel of antibodies with significant breadth. Among them, HJ16, which targets

the CD4bs core region, is particularly effective against tier 2 viruses (51). Another antibody, HK20, binds to the heptad repeat-1 (HR-1) region of gp41 and neutralizes more than 90% of isolates when tested as an scFv, but is much less effective as an intact IgG. This finding is consistent with the limited accessibility in time and space of HR-1 during viral entry from the cell surface (52). A third antibody, HGN194, targets a conserved epitope on the tip of the V3 loop that, in spite of its conservation, is variably accessible on different isolates.

In contrast with previous studies that selected neutralizing antibodies regardless of their site specificity, Mascola and colleagues (53) focused on the CD4bs as a target of broadly neutralizing antibodies and identified two patients in whom the broadly neutralizing activity of the serum was absorbed by wild-type gp120 but not by a gp120 engineered not to bind CD4. Using antigenically resurfaced gp120 molecules (termed RSC3) with preserved or mutated CD4bs, they were able to stain the rare CD4bs-specific memory B cells; from these cells they retrieved, by single-cell RT-PCR, the VRC01 antibody that neutralizes with high potency more than 90% of the isolates tested (54). VRC01 is highly mutated and mimics the CD4 binding to gp120 (55). The same approach was subsequently applied to two other patients selected within the International AIDS Vaccine Initiative and Center for HIV-AIDS Vaccine Immunology consortia, leading to the isolation of additional CD4bs-specific broadly neutralizing antibodies: PGV04 and the CH30–34 series (56).

Using a cell sorting strategy designed to isolate all gp120-binding antibodies, Nussenzweig and coworkers (57) isolated from six patients 502 antibodies that target different sites on the Env protein. The breadth of the individual antibodies was limited and did not reflect the breadth observed in the serum. Interestingly, upon a second attempt using a different set of primers that did not anneal in the V region, and were therefore less affected by the high level of somatic mutations characteristic of these

antibodies, they isolated 576 antibodies from the same as well as other patients (including the VRC01 patient described above) (58). Most of these antibodies are clonally related, broadly neutralize HIV-1 by targeting the CD4bs, and carry a very high load of somatic mutations, as well as insertions and deletions in both CDRs and framework regions. The three most potent antibodies, 3BNC117, 3BNC60, and NIH45-46 (which is clonally related to VRC01), show greater potency as compared with VRC01. Interestingly, the crystal structure of 3BNC60 revealed a remarkable structural convergence with the structure of the nonclonally related VRC01 (58). The presence and the repertoire of several of these antibodies were also characterized in the patient's serum using mass spectrometry. Using a structure-based design, Bjorkman and colleagues (59) further improved the breadth and potency of NIH45-46 by introducing a glycine-to-tryptophan substitution in the HCDR2 (G54W) that mimics the interaction of a critical phenylalanine of CD4 with gp120.

Most of the antibodies directed to the CD4bs (VRC01, 3BNC60, and NIH45-46) induce conformational changes in the HIV-1 gp120 monomer similar to those induced by the CD4 receptor, as shown by the enhanced binding of anti-CD4i antibodies (60). This property is not shared by another broadly neutralizing antibody, PGV04, that targets the CD4bs without inducing a conformational change of the gp120 (56, 60). Overall these results indicate that antibodies can bind to the CD4bs in many different ways that may or may not mimic CD4 binding. However, only the rare antibodies that avoid clashes with glycans and variable residues that surround the CD4bs are broadly neutralizing (61) (**Figure 2**). In this respect, heavy chain-only antibodies, such as those made by llamas, offer a considerable advantage due to the small size of the combining site. This was recently demonstrated by the isolation of the J3 antibody from a VHH phage library generated from immunized llamas; J3 binds to the CD4bs and neutralizes 96% of viruses tested with high potency (62).

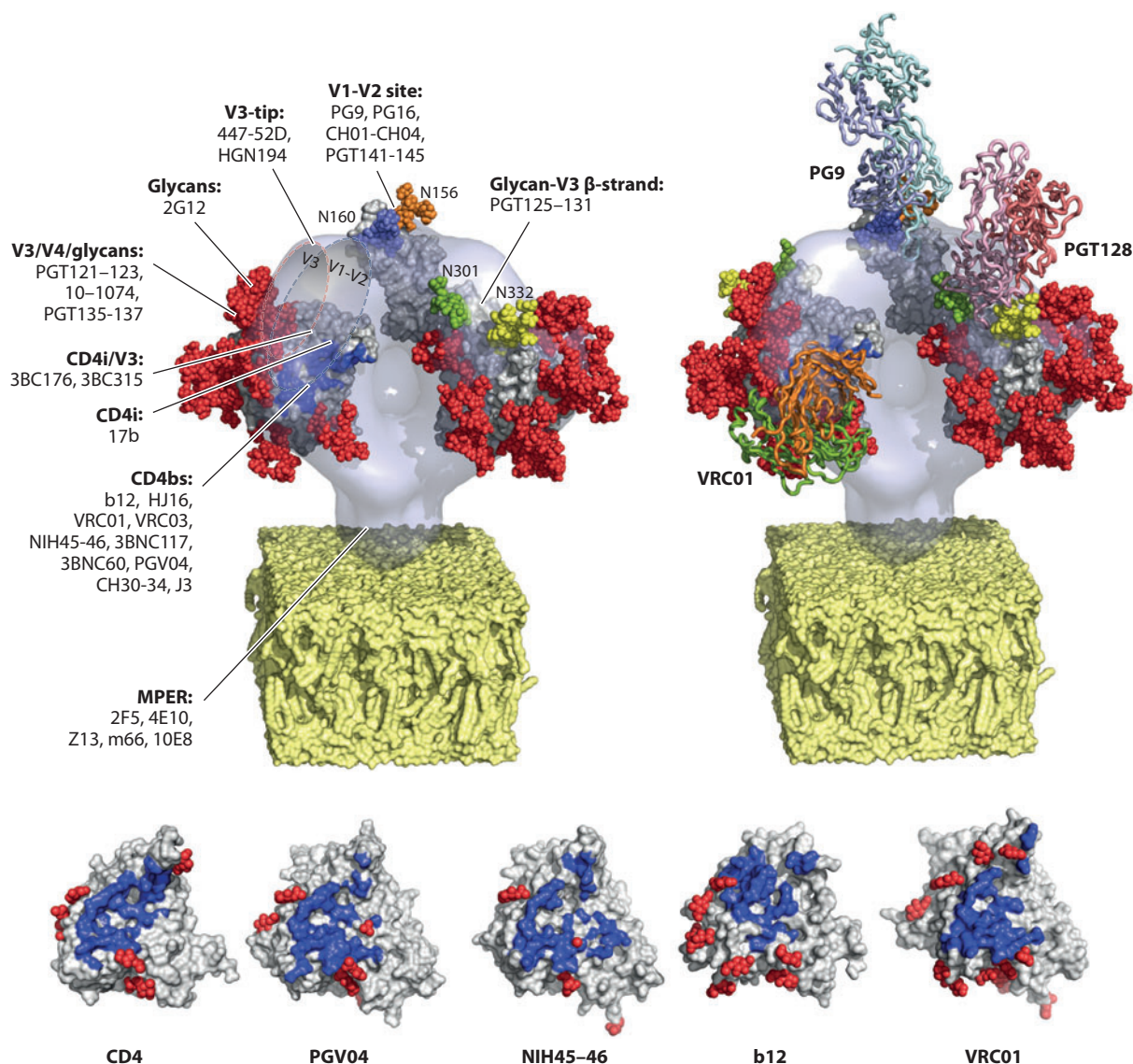


**Figure 2**

Model of the HIV-1 Env trimeric glycoprotein bound to broadly neutralizing antibodies. The tridimensional model of the Env trimer (Electron Microscopy Data Bank code EM-5019) with two of the three gp120 subunits highlighted in gray was positioned on a modeled lipid bilayer (64). The left panel shows the major sites targeted by broadly neutralizing antibodies. The approximate positioning of the V1/V2 and V3 loops is shown, and the CD4 footprint on the gp120 monomer is highlighted in blue. The right panel shows the Fabs of broadly neutralizing antibodies VRC01 (3ngb), PG9 (3us2), and PGT128 (3tyg) bound to gp120. Carbohydrates (oligomannose, *red spheres*) were modeled on the unliganded YU2 gp120 core (3tgq) using GlyProt, with the exception of the glycans bound by PGT128 and PG9 (depicted with different colors), which were taken from the structures. The location of PG9 above the trimeric gp120 is approximate; VRC01 and PGT128 Fabs were docked by superposition with the unliganded YU2 gp120 model. The bottom panel shows in blue the footprints of CD4 (1gc1) and the CD4bs-specific antibodies PGV04 (3se9), NIH45-46 (3u7y), b12 (3dnl), and VRC01 (3ngb). Figures were made with PyMOL.

To isolate antibodies that preferentially target the intact Env trimers, Nussenzweig and coworkers (63) recently developed an alternative sorting strategy based on capturing single B cells that recognize the HIV-1 Env protein expressed on the surface of transfected cells. Using this approach, they identified antibodies, such as 3BC176 and 3BC315, that neutralized 60–70% of the isolates tested by targeting a novel neutralizing epitope in the vicinity of the V3 loop and the CD4i site (63), which is not displayed on soluble Env proteins.

Overall, it seems that strategies based on binding to soluble recombinant Env proteins select antibodies with different specificities, but the broadly neutralizing antibodies have a clear preference for the CD4bs, which is well preserved on the recombinant Env molecules. In contrast, screening strategies based on neutralization or binding to native Env proteins on cells appear useful for the identification of novel epitopes including both glycan and protein determinants that are not well displayed on soluble recombinant proteins. The discovery of broadly neutralizing antibodies continues at a rapid pace. However, in spite of their considerable potency and breadth, none of the second-generation antibodies appears able to neutralize all HIV-1 isolates tested.

### **The Protective Role of Broadly HIV-1-Neutralizing Antibodies**

Formal proof for the *in vivo* efficacy of broadly neutralizing antibodies can be obtained by passive transfer into naive animals. For HIV-1, the only suitable animal model is nonhuman primates challenged with SIV–HIV-1 chimeric virus (SHIV). In spite of the complexity of these studies, several data demonstrate the efficacy of neutralizing antibodies in preventing infection. First-generation neutralizing antibodies protect nonhuman primates against vaginal or intravenous challenge with SHIV (65–70). Further evidence for the prophylactic efficacy of antibodies came from studies showing an association between maternal neutralizing antibodies

and lower rates of HIV-1 transmission to the child (71).

On the basis of several *in vivo* experiments, Burton and coworkers (72) proposed that *in vivo* protection requires a serum concentration of the administered neutralizing antibody at least 100-fold higher than the concentration needed for 90% viral neutralization *in vitro* (IC<sub>90</sub>). This so-called 100 × rule applies to antibodies such as b12, 2F5, and 4E10. Surprisingly, however, other antibodies, such as the antiglycan 2G12 and the anti-V3 crown HGN194, are effective *in vivo* at 100-fold lower concentration (73, 74). The lack of correlation between *in vitro* neutralization and *in vivo* efficacy is reminiscent of previous observations in a mouse model of influenza infection (75) and highlights the importance of determining the antibody efficacy using *in vivo* models and challenge protocols that are more physiological in terms of virus inoculum and route of infection. The discrepancy may be explained, at least in part, by the capacity of some antibodies to efficiently recruit effector functions *in vivo*. To address these issues, Burton and coworkers (76) compared in a mucosal SHIV macaque infection model the b12 antibody with b12 variants lacking either complement binding (b12-KA) or complement and Fc receptor binding (b12-LALA) and found that *in vivo* protection was remarkably decreased for the LALA variant but not for the KA variant. These results indicate that, at least for this antibody, engagement of Fc receptors plays an important role *in vivo*. In a more recent study (77), this group showed that a nonfucosylated variant of b12 (that binds with increased affinity to the human and macaque FcγRIIIa, mainly expressed on NK cells) does not confer enhanced protection as compared with the original fucosylated antibody, suggesting that the protective role of antibody-dependent cell-mediated cytotoxicity (ADCC) is not mediated by NK cells.

An emerging issue is whether antibodies that lack *in vitro* neutralizing activity can nonetheless restrain HIV-1 replication *in vivo*. The nonneutralizing anti-gp41 antibody F240 reduces the number of infected animals as well

as the viremia in those that became infected, whereas another nonneutralizing antibody specific for gp120, b6, does not show any effect (78). The authors suggest that F240 antibody may reduce the inoculum by targeting the incoming virus for degradation by phagocytes. Indeed, F240 is more effective than b12 in capturing SHIV virions from solution, most likely by binding to functionally inactive gp41 stumps that remain exposed on infectious virions.

To circumvent the need for repeated injection of antibodies, a vectored immunoprophylaxis approach has been proposed. Clark and coworkers (79) used in macaques an adeno-associated virus (AAV) as a gene transfer vector expressing SIV-neutralizing immunoadhesins consisting of small, artificially fused antibody fragments. SIV-specific antibodies are synthesized in myofibers and passively distributed to the circulation, delivering long-lasting neutralizing activity in serum that can protect macaques against intravenous challenge with SIV (79). However, this approach was hampered by the production of antibodies directed against the immunoadhesin proteins. More recently, Baltimore and coworkers (80) showed that a single intramuscular injection of an AAV vector encoding the full-length VRC01 antibody led in humanized mice to sustained levels of serum antibody that was able to fully protect from high-dose challenge with HIV-1.

Studies aimed at evaluating the therapeutic efficacy of antibodies have been performed in HIV-1-infected patients and, more recently, in humanized mice. A cocktail of first-generation neutralizing antibodies (2G12, 2F5, and 4E10) was tested in a therapeutic setting in patients undergoing interruption of antiretroviral treatment and was found to initially reduce viral loads in some individuals. However, this reduction was only transient, due to the emergence of 2G12 escape mutants that were still susceptible to 2F5 and 4E10 and therefore did not appear to exert a significant antiviral effect in vivo (81, 82). Although it represents a proof of concept for the use of passive serotherapy, this finding

illustrates the limitation of using a single, low-potency antibody.

In a recent study (83), the therapeutic efficacy of second-generation HIV-1-neutralizing antibodies was tested in HIV-1-infected humanized mice. Five antibodies selected on the basis of their specificity for different epitopes on the Env protein (NIH45-46<sup>G54W</sup>, PG16, PGT128, 10-1074, and 3BC176) were tested therapeutically alone or as mixtures of three (tri-mix) or five (penta-mix) antibodies. Whereas single antibodies and the tri-mix selected escape mutants, the penta-mix effectively suppressed viral loads below the level of detection for the entire course of treatment and failed to select escape mutants. This mouse study suggests that a cocktail of potent and broadly neutralizing antibodies represents an alternative or possibly synergistic therapeutic modality to small-molecule antiretroviral therapy.

In the context of an antibody-based therapy, it should be noted, however, that the individuals from whom broadly neutralizing antibodies were isolated do not appear to benefit from such antibodies in controlling virus replication. The prevalence of broadly neutralizing antibodies is similar in long-term progressors and nonprogressors, and in both groups serum-neutralizing activity against autologous HIV-1 variants fades over time due to viral escape (84). Of note, HIV-1 isolates from contemporary seroconverters have become more resistant to antibody neutralization by broadly neutralizing antibodies, such as PG9, PG16, and VRC01 (85), thus suggesting an evolution toward more neutralization-resistant strains.

In conclusion, serotherapy can be effective prophylactically, given the very small number of viruses responsible for transmission of infection. However, its long-term therapeutic effectiveness in individuals who harbor high titers of viral quasi species awaits clinical trials in view of the possibility that escape mutants exist even in the presence of several antibodies targeting different sites. Another problem that needs to be addressed relates to the potential immunogenicity of these highly mutated antibodies.

## Maturation Pathways for Broadly HIV-1-Neutralizing Antibodies

The large number of antibodies available outlines some general mechanisms for their generation during the immune response to HIV-1. It is well appreciated that the neutralizing antibody response develops at late points after infection (generally 2–4 years after infection) (86, 87), and all broadly neutralizing antibodies have been isolated from patients in the chronic phase of the disease, suggesting that they result from a continual selection process by continually mutating viruses. The reconstruction of this process is made difficult by the lack of information on the original infecting virus. The broadly neutralizing antibodies isolated so far have very high numbers of somatic mutations in CDRs and frameworks and are also frequently characterized by extensive nucleotide insertions or deletions. Antibodies typically accumulate 5–20% changes in heavy- and light-chain amino acid sequences during the affinity maturation process, but in the case of HIV-1-neutralizing antibodies, the level of somatic mutation is markedly increased, ranging from 15% to 44% amino acid substitutions in the variable region (**Table 1** and **Figure 3**). Notably, a very high load of somatic mutations is a characteristic of CD4bs-specific antibodies, whereas extraordinarily long HCDR3s (up to 33 amino acids long) represent a hallmark of V1/V2-specific antibodies. The antibodies to MPER and V3-glycans have a moderately long HCDR3 and a relatively high load of amino acid substitutions, often involving insertion and deletions. Importantly, in most cases examined, the germlined predecessors of broadly neutralizing antibodies bound poorly or not at all to HIV-1 Env (55, 88). It is possible that the primary response was elicited by a particular HIV-1 strain that was not available for testing and that the breadth was subsequently engineered by somatic mutations in response to continually evolving viruses.

Mascola and coworkers (56) developed an approach to trace pathways of affinity maturation for VRC01-like antibodies in

several donors using deep sequencing and structural bioinformatics. This analysis revealed a common pathway for development of antibodies to the CD4bs starting from VH1-2. Common maturation intermediates with 20 to 30 nucleotide changes were found in different individuals; these intermediates gave rise to mature, broadly neutralizing VRC01-like antibodies characterized by 70 to 90 changes from the VH1-2 precursor.

An interesting hypothesis was proposed by Nussenzweig and coworkers (89) on the basis of their observation that the germlined version of several Env-specific antibodies, while having no or low-affinity binding to Env protein, retained a substantial degree of polyreactivity. They suggested that the recognition of unknown low-affinity sites on the HIV-1 virion may mediate bivalent heterologation, reaching an avidity threshold sufficient for B cell priming (89). This concept is consistent with previous observations on the polyreactivity of the MPER-specific antibodies 4E10 and 2F5 (49). It should be noted, however, that not all broadly neutralizing antibodies are polyreactive (90) (**Table 1**).

Beyond the high level of somatic mutations, an additional level of complexity is added by posttranslational modifications. Tyrosine sulphation was found on several HIV-1 antibodies (91), a modification that was suggested to mimic the tyrosine sulphation of the N terminus of CCR5, which is known to be important for the interaction with gp120 (92). Tyrosine sulphation has also been described in the protruding anionic HCDR3s of PG9, PG16, and PGT145, although in these cases mimicry can be excluded because the antibodies bind to the V1/V2 region (42, 45).

In conclusion, it appears that the generation of broadly HIV-1-neutralizing antibodies is the result of an extremely elaborated and long process of selection. This maturation pathway explains the rarity of such antibodies but represents a formidable challenge for the development of an HIV-1 vaccine capable of eliciting broadly neutralizing antibodies.

## BROADLY NEUTRALIZING ANTIBODIES AGAINST INFLUENZA VIRUS

The target of influenza virus–neutralizing antibodies is the hemagglutinin (HA) molecule. The globular HA head domain mediates binding to cell surface sialic acid, and the HA stem mediates the subsequent fusion between the viral and cellular membranes that is triggered by the low pH of the endosomal compartment (93). Seventeen subtypes of HA that share between 40% and 60% amino acid sequence cluster in two phylogenetic groups: group 1 (comprising H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, and H17) and group 2 (comprising H3, H4, H7, H10, H14, and H15) (94, 95). The globular head is subject to continual genetic evolution, with amino acid substitutions in antibody-combining sites, known as antigenic drift (96, 97), whereas the structure of the stem region, which is primarily formed by the HA2 domain, is more conserved (98). The globular head is the major target of neutralizing antibodies that inhibit virus binding to target cells and are classically detected by the HA inhibition (HAI) assay. Distinct antigenic sites have been mapped mainly on the globular head using sequence information from naturally occurring and antibody-selected antigenic variants (99, 100). A common feature of these antigenic sites is that they are located primarily in protruding loops, and mutations in these regions are tolerated without affecting the overall framework and functionality of the HA.

The first broadly neutralizing antibody to influenza A, named C179, was isolated by Okuno and coworkers (101, 102) in the early 1990s from H2N2-immunized mice. C179 showed considerable breadth, able to neutralize in vitro and in vivo multiple viruses belonging to the H1, H2, H5, H6, and H9 subtypes within group 1. The epitope recognized by this antibody was mapped through the isolation of escape mutants bearing a single mutation in the HA stem region. The existence of stem-specific broadly neutralizing antibodies was underes-

timated, and it was only after 15 years that Goudsmit and coworkers (103) reported on human antibodies with similar characteristics. The authors isolated from a phage display library of IgM memory B cells several antibodies that neutralized influenza A viruses belonging to multiple group 1 subtypes. The most effective of these antibodies, CR6261, neutralizes several group 1 viruses (H1, H5, H6, H8, and H9, but not H2, subtypes). In a subsequent study, Marasco and coworkers (104) isolated from random phage libraries several human antibodies, including F10, that recognize viruses of all group 1 subtypes. Surprisingly, both CR6261 and F10 use the VH1-69 gene, and structural studies revealed that they bind with virtually identical modalities to a conserved epitope in helix A of the HA stem (104, 105) (**Figure 4**). Remarkably, for both antibodies the most prominent HA contact residues are in the HCDR1 and HCDR2 and are germline-encoded, with a major contribution by phenylalanine 54, whereas the light chain does not contribute to antigen binding.

To address whether antistem antibodies are produced in the course of the immune response, Corti et al. (106) analyzed the immune response to seasonal influenza vaccination (H1N1 and H3N2) and found that some, but not all, individuals produced significant levels of serum antibodies able to neutralize H5 pseudoviruses. Using H5-pseudotyped neutralization as a screening assay, they isolated from EBV-immortalized memory B cells a panel of 19 antibodies that bound to the HA stem and showed considerable breadth of neutralization against several group 1 subtypes in vitro and in vivo. Most of these antibodies use VH1-69, but some use other VH genes such as VH3-53, VH3-23, and VH3-30, indicating that the antistem response is not restricted to VH1-69. In a subsequent study, Wilson and coworkers (107) analyzed the repertoire of plasma cells produced in response to the 2009 pandemic swine-origin influenza virus (SOIV). Unlike antibodies elicited by seasonal influenza vaccinations, most neutralizing antibodies induced by SOIV are broadly cross-reactive against

**Table 1** Characteristics of first- and second-generation human broadly neutralizing antibodies against HIV-1

| Antibody      | Specificity                  | Breadth <sup>a</sup> | VH   | SHM <sup>b</sup> | HCDR3 <sup>c</sup> | Insertion/<br>deletion | Poly-<br>reactivity <sup>d</sup> | Year | Isolation<br>Method     | Screening<br>Strategy | Reference |
|---------------|------------------------------|----------------------|------|------------------|--------------------|------------------------|----------------------------------|------|-------------------------|-----------------------|-----------|
| 2F5           | gp41 MPER                    | ~60–70%              | 2–5* | 15.2             | 24                 |                        | ✓                                | 1994 | EBV-electrofusion       | gp160 and p24 binding | 28        |
| 4E10          | gp41 MPER                    | ~96–98%              | 1–69 | 15.6             | 20                 |                        | ✓                                | 1994 | EBV-electrofusion       | gp160 and p24 binding | 28        |
| 10E8          | gp41 MPER                    | ~98%                 | 3–15 | 22.1             | 22                 |                        | ×                                | 2012 | Stimulated B cells      | Microneutralization   | 48        |
| 2G12          | gp120 glycans                | ~25–30%              | 3–21 | 33.6             | 16                 |                        | ×                                | 1994 | EBV-electrofusion       | gp160 and p24 binding | 28        |
| PGT128        | Glycans and V3<br>β-strand   | ~70–75%              | 4–39 | 27.9             | 21                 | +6 aa in<br>HCDR2      | ×                                | 2011 | Stimulated B cells      | Microneutralization   | 45        |
| PGT127        | Glycans and V3<br>β-strand   | ~50%                 | 4–39 | 23.2             | 21                 | +6 aa in<br>HCDR2      | ×                                | 2011 | Stimulated B cells      | Microneutralization   | 45        |
| PGT121        | Complex type V3<br>N-glycans | ~65–70%              | 4–59 | 21.2             | 26                 |                        | NA                               | 2011 | Stimulated B cells      | Microneutralization   | 45, 47    |
| 10-1074       | Complex type V3<br>N-glycans | ~55–60%              | 4–59 | 24.4             | 26                 |                        | NA                               | 2012 | Cell<br>sorting/RT-PCR  | gp140 binding         | 47        |
| PGT135        | Glycans and V4               | ~30–35%              | 4–39 | 26.8             | 20                 | +6 aa in<br>HCDR1      | NA                               | 2011 | Stimulated B cells      | Microneutralization   | 45        |
| PG9/PG16      | Glycans and V1/V2            | ~75–80%              | 3–33 | 15.4–16.8        | 30                 |                        | ×                                | 2009 | Stimulated B cells      | Microneutralization   | 39        |
| CH01–<br>CH04 | Glycans and V1/V2            | ~50%                 | 3–20 | 23.3–19.5        | 26                 |                        | CH03<br>✓                        | 2011 | EBV-<br>immortalization | Microneutralization   | 44        |
| PGT145        | Glycans and V1/V2            | ~75–80%              | 1–8  | 22.8             | 33                 |                        | ×                                | 2011 | Stimulated B cells      | Microneutralization   | 45        |

(Continued)

|           |             |         |      |           |    |                |    |      |                     |                                                       |    |
|-----------|-------------|---------|------|-----------|----|----------------|----|------|---------------------|-------------------------------------------------------|----|
| bl2       | gp120 CD4bs | ~30–35% | 1–3  | 17.3      | 20 |                | ✓  | 1991 | Phage library       | gp120 binding                                         | 24 |
| HJ16      | gp120 CD4bs | ~30–35% | 3–30 | 36.7      | 21 | –1 aa in HCDR2 | ×  | 2010 | EBV-immortalization | gp140 binding                                         | 50 |
| VRC01     | gp120 CD4bs | ~90–95% | 1–2  | 38.8      | 14 |                | ×  | 2010 | Cell sorting/RT-PCR | B cell staining RSC3 <sup>+</sup> /ΔRSC3 <sup>–</sup> | 54 |
| VRC03     | gp120 CD4bs | ~50%    | 1–2  | 34.9      | 16 | +7 aa in HFR3  | ×  | 2010 | Cell sorting/RT-PCR | B cell staining RSC3 <sup>+</sup> /ΔRSC3 <sup>–</sup> | 54 |
| 3BNC117   | gp120 CD4bs | ~85–90% | 1–2  | 36.9      | 12 | +4 aa in HFR3  | ✓  | 2011 | Cell sorting/RT-PCR | B cell staining with 2CC-gp120                        | 58 |
| 3BNC60    | gp120 CD4bs | NA      | 1–2  | 36.9      | 12 | +4 aa in HFR3  | ×  | 2011 | Cell sorting/RT-PCR | B cell staining with 2CC-gp120                        | 58 |
| NIH45-46  | gp120 CD4bs | ~90%    | 1–2  | 44        | 18 |                | ✓  | 2011 | Cell sorting/RT-PCR | B cell staining with 2CC-gp120                        | 58 |
| CH30–CH34 | gp120 CD4bs | ~80%    | 1–2  | 31.9–31.9 | 15 | +9 aa in HCDR1 | ×  | 2011 | Cell sorting/RT-PCR | B cell staining RSC3 <sup>+</sup> /ΔRSC3 <sup>–</sup> | 56 |
| PGY04     | gp120 CD4bs | ~85–90% | 1–2  | 38.2      | 16 |                | NA | 2011 | Cell sorting/RT-PCR | B cell staining RSC3 <sup>+</sup> /ΔRSC3 <sup>–</sup> | 56 |
| 3BC176    | CD4/V3      | ~60–70% | 1–2  | 29.4      | 19 |                | NA | 2012 | Cell sorting/RT-PCR | Cell-based single B cell capturing                    | 63 |

<sup>a</sup>The indicated breadth refers to the approximate percentage of viruses neutralized with IC50 values below 50 µg/ml by each monoclonal antibody.

<sup>b</sup>Percentage of amino acidic somatic hypermutations (SHMs) calculated on the full amino acid sequence by comparing the full amino acid sequences of original (mutated) and germlined antibodies, as assessed using the international ImMunoGeneTics information system (IMGT) database. Insertions or deletions are not considered in the calculation.

<sup>c</sup>HCDR3 amino acid length according to IMGT.

d ✓ = yes; × = no.

<sup>e</sup>Sequence not available in the Genbank; information shown was retrieved from Haynes et al. (90).

Abbreviations: aa, amino acids; EBV, Epstein-Barr virus; MPER, membrane-proximal external region; NA, not available.

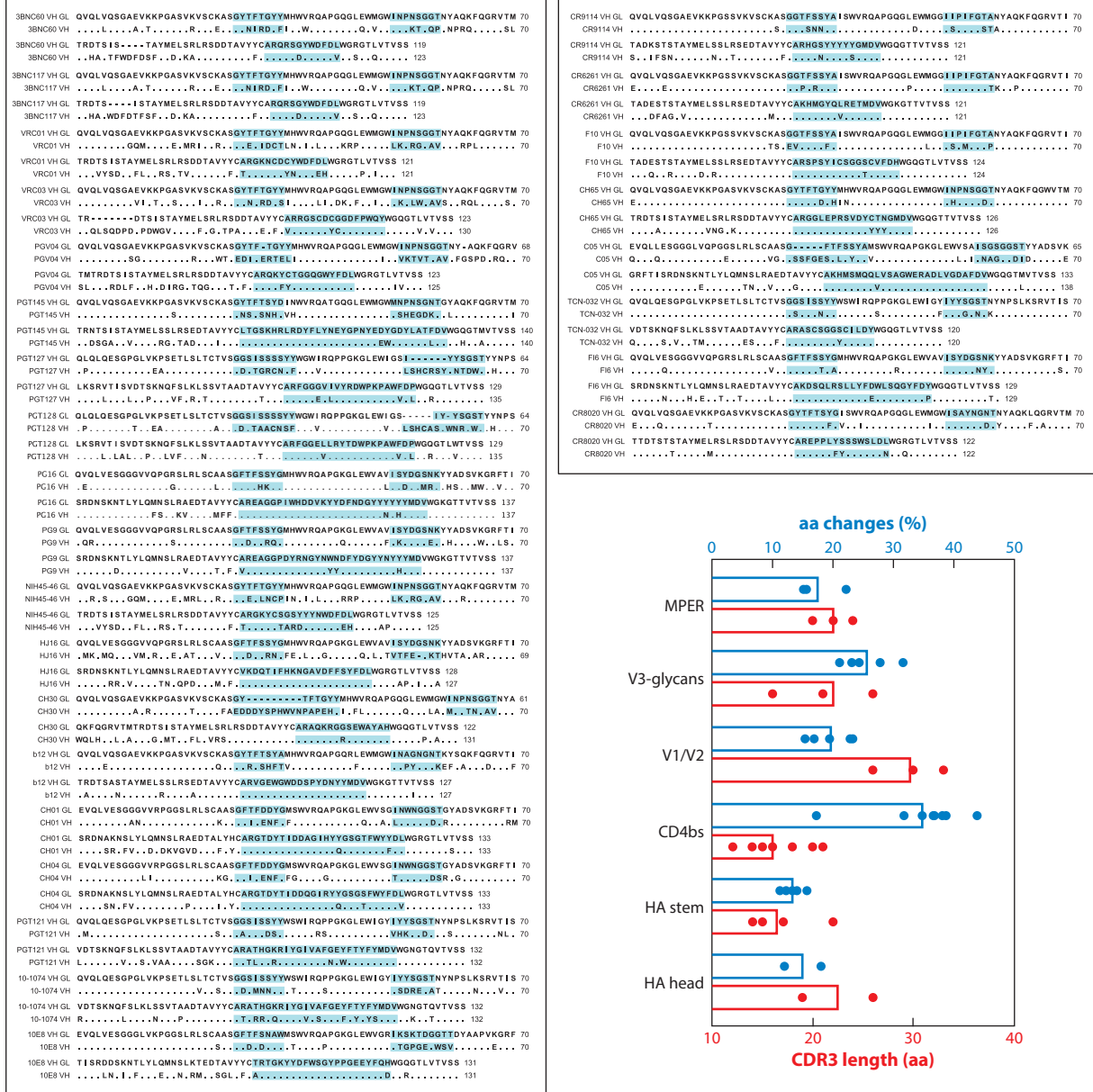


**Figure 3**

Molecular features of HIV-1 and influenza A broadly neutralizing antibodies. Variable heavy chains of HIV-1 (*left panel*) and influenza A (*right panel*) antibodies are aligned to the corresponding nonmutated ancestor germline sequences. Dots indicate identical residues, dashes indicate amino acid insertions or deletions. HCDRs are highlighted in blue. The bar graph shows the percentage of amino acid (aa) substitutions and the HCDR3 lengths for antibodies of different epitope specificity.

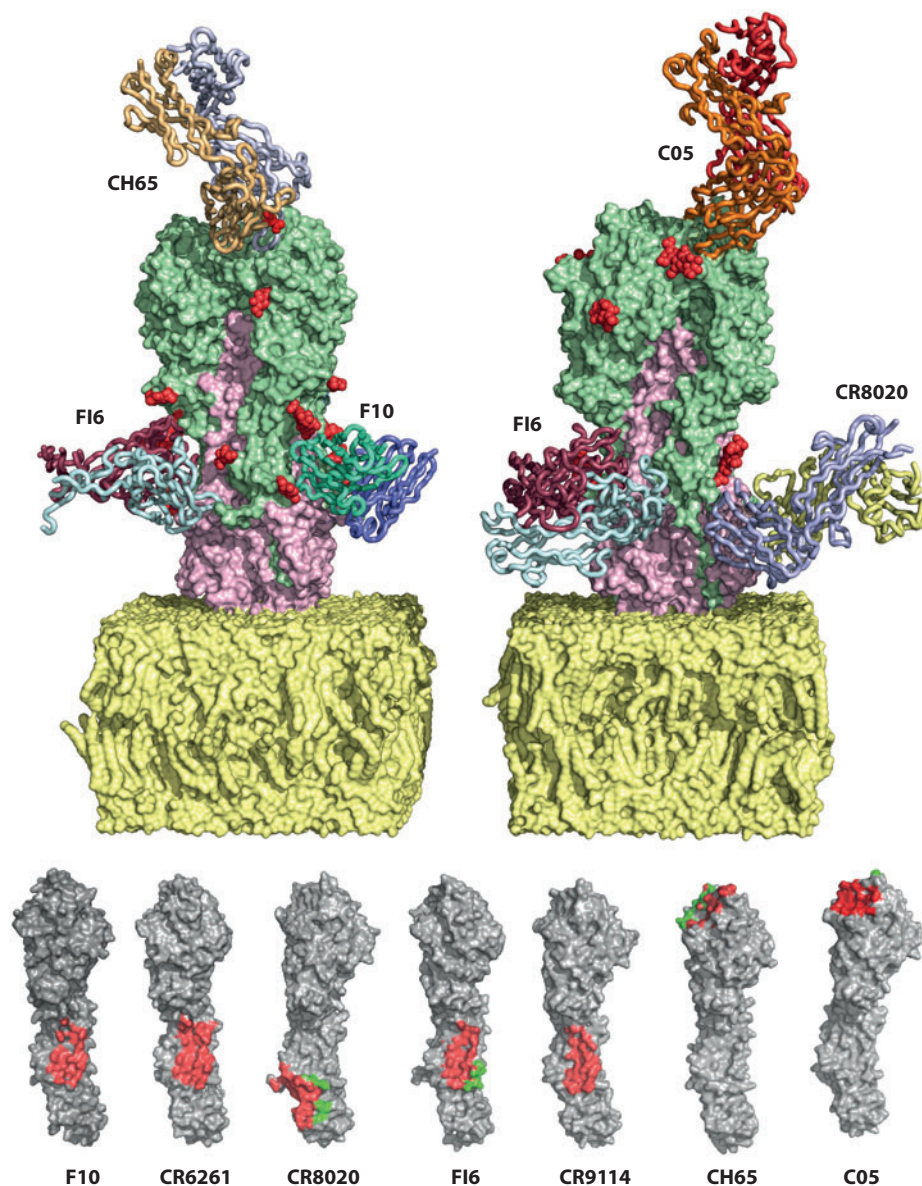


**Figure 4**

Model of the H1 and H3 hemagglutinin (HA) trimeric glycoproteins bound to broadly neutralizing antibodies. HA trimers were positioned on a modeled lipid bilayer. In the left upper panel, the Fabs or scFvs of antibodies FI6 (3ztn), F10 (3fku), and CH65 (3sm5) were modeled on the trimeric HA from H1N1 A/California/04/09 (3ztn). In the right upper panel, the Fabs of antibodies FI6 (3ztj), CR8020 (3sdy), and C05 (4fqr) were modeled on the trimeric HA from H3N2 A/Hong Kong/68 (3ztj). The bottom panel shows the footprints of antibodies F10 (3fku), CR6261 (3gbn), CR8020 (3sdy), FI6 (3ztn), CR9114 (4fqy), CH65 (3sm5), and C05 (4fqr) on their cognate HA ligand. The heavy- and light-chain contact residues are depicted in red and green, respectively. HA monomer molecules are shown with the same orientation. Figure was made with PyMOL.

epitopes in either the HA stem or globular head domain of multiple influenza H1N1 strains.

As discussed above, broadly neutralizing antibodies that target the HA stem of group 1 viruses are produced in many individuals, although this response remains subdominant upon conventional vaccination and becomes dominant only after heterologous boost (106, 107). In contrast, broadly neutralizing antibodies against group 2 viruses are much more rare. A possible explanation is the presence in group 2 viruses of a conserved N38 glycan in HA1 that may interfere with the accessibility of the conserved antigenic site on helix A (104). However, Goudsmit and coworkers (108) were able to isolate from human memory B cells an antibody, CR8020, that binds HAs belonging to several group 2 subtypes including H3 and H7. The crystal structure of CR8020 in complex with H3 HA revealed that this antibody binds to an epitope in the HA stem that is distinct and closer to the viral membrane as compared with the epitope recognized by the group 1-specific antibodies (108). In group 1 HAs, this site is hindered by the highly conserved N21 glycosylation site in HA1, which is adjacent to CR8020 HCDR1, explaining why CR8020 does not bind to group 1 HAs.

The first group 1 and group 2 influenza A-neutralizing antibody, named FI6, was isolated by Corti et al. (109) using a novel plasma cell culture method. More than  $10^5$  plasma cells were maintained alive in single-cell cultures, and the supernatants were screened for the presence of antibodies that bound to both H5 and H7 HAs. FI6 was isolated multiple times from plasma cells collected from the same donor after seasonal vaccination and after infection by the 2009 pandemic SOIV. FI6 uses VH3-30, recognizes HAs of all subtypes, and neutralizes all the group 1 and group 2 influenza A viruses tested (109). Besides inhibiting the conformational changes in HA that are required for membrane fusion (93), FI6 prevents fusion and also inhibits HA0 processing, which is required for virus infectivity—at least for those viruses in which cleavage occurs extracellularly. The crystal structure of FI6 in complex with

group 1 (H1) and group 2 (H3) HAs indicated that the antibody binds to a quaternary epitope using both heavy and light chains, with a major contribution from a long and flexible HCDR3 that contacts helix A and from the light-chain CDR1 that contacts the fusion peptide region of the neighboring HA molecule. Interestingly, FI6 binding to H3 HA is enabled by the reorientation of the N38 glycan away from the HA surface and by the flexibility of HCDR3 that can accommodate the different conformations of the tryptophan 21 loop (W21 loop), which are distinctive between group 1 and group 2 HAs (109).

Recently, Dreyfus et al. (110) isolated from a phage library an antibody, CR9114, that binds to both influenza A and influenza B HAs. CR9114 uses VH1-69 and binds to an epitope in the HA stem nearly identical to that bound by CR6261, using the same HCDR1 and HCDR2 loops with no light-chain contacts. The ability of CR9114 to achieve this remarkable breadth of reactivity has been attributed to several factors, such as the ability to accommodate different conformations of the W21 loop and to displace the N38 group 2-specific glycan [also observed for FI6 (109)] and the capacity to accommodate threonine or asparagine HA2 residues at position 49 and to displace the HA1 N332 glycan characteristic of influenza B (110). However, it should be noted that when the antibody affinity was accurately measured by biolayer interferometry, CR9114 was found to bind with high affinity (1–10 nM) to most group 1 HAs (with the exception of H2 HA), with lower affinity (10–100 nM) to group 2 HAs, and with very low affinity (100 nM) to influenza B HA. This low affinity for influenza B viruses is consistent with the failure to neutralize influenza B viruses in vitro, although, surprisingly, the antibody showed prophylactic activity in mice challenged with influenza B strains.

Antibodies capable of neutralizing viruses belonging to different subtypes can also target globular head sites. An example is provided by the FE17 antibody isolated from memory B cells of a vaccinated donor (106). FE17 binds to the Ca<sup>2+</sup> site in the globular head (as determined

by the isolation of escape mutants) and neutralizes some, but not all, H1 and H5 viruses. Another example is the S139/1 antibody isolated from immunized mice, which binds to the Sb site and cross-reacts with some strains of group 1 and group 2 subtypes (111). Although shared by multiple subtypes, these sites are not conserved, as shown by the fact that escape mutants were easily isolated for both antibodies.

In the context of the globular head, the sialic acid-binding pocket represents a potential target for broadly neutralizing antibodies due to its conservation, but its accessibility is constrained by neighboring residues, which are highly variable. One example of an antibody that binds to the sialic acid-binding site is the CH65 antibody, which inserts the tip of the HCDR3 into the receptor-binding pocket, mimicking in many respects the interaction with sialic acid (112). However, CH65 neutralizes only a fraction of H1N1 viruses (30 out of the 36 tested); the resistant strains, including the H1N1 SOIV, show an insertion near the rim of the sialic acid-binding pocket. Recently, another antibody that targets the sialic acid-binding site has been described (113). This antibody, named C05, interacts with a small HA footprint primarily through the extended HCDR3 and has a broader reactivity than CH65, being able to neutralize, in addition to several H1N1 viruses, some H2N2 and H3N2 viruses.

Besides breadth of reactivity, there are two major differences between anti-globular head and antistem HA antibodies. Antibodies to the globular head are generally more potent, able to neutralize both infectious viruses and pseudoviruses at low concentration (5–500 ng/ml). In contrast, stem-reactive antibodies neutralize pseudoviruses at comparable or even lower concentrations but are much less effective against infectious viruses, which are neutralized at antibody concentrations 100- to 1,000-fold higher (104, 106). The different neutralizing potency of antistem antibodies is consistent with the notion that accessibility to the target epitope is limited by the tight packing of HA molecules in intact viruses as compared with pseudoviruses (114). It should be noted, however, that these

antibodies can efficiently recognize the less tightly packed HA on the membranes of infected cells.

The second important difference between anti-globular head and antistem antibodies relates to the mechanisms of viral escape. The globular head of HA is characterized by several loops, as well as glycosylation sites, that can tolerate substitutions without compromising the functionality of the protein. Indeed, selection of escape mutants is a well-established method to map anti-globular head antibodies (100), and the current broadly neutralizing antibodies that bind to the globular head are not exceptions. As previously discussed, whereas the sialic acid-binding pocket has to be conserved, the residues at the rim of this pocket can vary substantially among different HAs, thus limiting the breadth of antipocket antibodies as exemplified by C65 and C05 (112, 113). In contrast, the core epitope of antistem antibodies is made of helical regions that are intrinsically less permissive for mutations due to the need to maintain inter-chain packing and to undergo conformational changes during the fusion process. Indeed, attempts to isolate escape variants from antistem antibodies were either unsuccessful (104, 109) or showed only a partial reduction of binding (103).

## The Protective Role of Broadly Influenza-Neutralizing Antibodies

The discovery that broadly neutralizing antibodies are produced in response to influenza virus infection or vaccination is still recent, and we do not yet know whether this response confers protection against antigenically new viruses. The HAI assay, which is used universally to measure the immune response to influenza virus, does not detect stem-specific antibodies; therefore, information on their prevalence and titers is currently scanty. Stem-specific neutralizing antibodies can be efficiently detected using pseudovirus-based neutralization assays (115) or binding assays based on heterologous or chimeric HA molecules (116). A site-specific binding assay,

which can be generally applied to any antigen, is based on the capacity of the serum to inhibit binding of an antibody of known specificity—for instance, an antistem or anti-RBS antibody. This blocking-of-binding (BOB) assay has been used to determine human serum titers of antistem or anti-globular head antibodies targeting the heterologous H5 HA (106). The antistem antibodies were largely cross-reactive on H5 HA, but antibodies to the H5 globular head were not detected. In the field of influenza, the BOB assay can be easily adapted to dissect the specificity of polyclonal antibodies against different sites of heterologous HA molecules.

It is particularly important to establish whether preexisting antibodies and memory B cells specific for the stem region can confer protection against a new pandemic virus. This could be tested in the context of the recent SOIV infection by asking whether preexisting antistem cross-reactive antibodies correlate with a favorable clinical outcome. This type of observational study could validate the role of antistem antibodies in protection, but we are not aware of any such study. Along the same line of thought and following the observation that new pandemic viruses can displace previous circulating strains (117), it has been suggested that the SOIV infection, by boosting group 1 stem-specific immunity at the population level, may lead to the extinction of H1N1 viruses, as may have happened in 1957 when the circulating H1N1 disappeared following the emergence of the group 1 H2N2 virus (118). It has also been speculated that boosting the N2 immunity might have caused the disappearance of H2N2 virus following the emergence of the H3N2 virus in 1968. Indeed, studies suggest that antineuraminidase antibodies, although devoid of virus-neutralizing activity, can confer homologous as well as heterologous protection in vivo (119–122). These observations are consistent with a role of cross-reactive antibodies in the modulation of viral fitness at the population level.

Although in vitro neutralization is an excellent correlate of protection, it is also clear that antibodies that are poorly neutralizing or even

nonneutralizing can exert significant protection in vivo (75, 123). Recruitment of C1q can contribute to antibody-mediated neutralization by enhancing the coating of viral particles (124), and activation of the complement cascade can lead to the killing of infected cells (125). Interestingly, some stem-specific antibodies are particularly effective in inducing lysis of infected cells, possibly due to the recruitment of the complement cascade in proximity with the cell membrane (126). Antibodies can also engage Fc receptors on different types of effector cells, which can result in killing of infected cells and enhanced antigen presentation (127).

The role of complement and Fc receptors in antibody-mediated protection is illustrated by antibodies specific for M2e, which is highly conserved and abundantly expressed on the membrane of influenza virus-infected cells but virtually absent on virions. Anti-M2 antibodies, such as TCN-032 and D005, lack in vitro neutralizing activity but show significant therapeutic efficacy in vivo (10, 11). The protective effect of vaccine-induced anti-M2 antibodies is dependent on Fc receptors expressed by alveolar macrophages, thus identifying not only the mechanism but also the types of effector cells involved in this animal model (128). The role of Fc-dependent mechanisms was tested in mice using FI6 variants lacking either complement binding (FI6-KA) or complement and Fc receptor binding (FI6-LALA). When tested prophylactically, FI6-LALA showed a substantial loss of activity, whereas FI6-KA showed only a modest reduction, indicating that ADCC plays a significant role in mediating in vivo protection (109). These findings are consistent with the observation that the epitope recognized by FI6, as well as by other antistem HA antibodies, is expressed at high density and is readily accessible on the surface of influenza-infected cells. Interestingly, when tested for their ability to induce ADCC by human CD16<sup>+</sup> NK cells, antistem antibodies were significantly more effective than anti-globular head antibodies (D. Corti & B. Guarino, unpublished data).

The contribution of Fc-dependent effector mechanisms to in vivo protection has practical

implications given the possibility to modulate the effector functions of antibodies by modifying the glycan composition or engineering the Fc portion (129). It is worth considering that the impact of Fc-dependent mechanisms in protection could be much higher than estimated using the conventional animal models, in view of the mismatch between human antibodies and mouse Fc receptors as well as the different distribution of Fc receptors on human versus mouse cells (130). The availability of a transgenic mouse model that fully recapitulates the cellular distribution of human Fc receptors (131) will be instrumental for dissecting the role of Fc receptors, antibody isotypes, and cell types in antibody-mediated protection against influenza virus.

Passive transfer of convalescent sera has shown efficacy in humans against the pandemic 1918 and 2009 H1N1 viruses as well as the H5N1 avian virus (132–134). The newly isolated broadly neutralizing antibodies offer considerable advantages over blood-derived products in terms of safety, availability, and efficacy. Most importantly, stem-specific neutralizing antibodies fail to select escape mutants, a characteristic that is compatible with their development as a universal anti-influenza therapeutic. These antibodies could be used for the treatment of severely infected patients and the prophylaxis of individuals with concomitant disease or poor antibody response to vaccines, as occurs in elderly, newborn, and immune-compromised individuals. Furthermore, the stockpiling of broadly neutralizing antibodies may be an effective measure not only for the management of animal-to-human transmission of highly pathogenic viruses but also for the early management of a new pandemic virus before a vaccine becomes available.

### Maturation Pathways to Broadly Influenza-Neutralizing Antibodies

In the context of identifying the pathways that lead to the selection of broadly neutralizing antibodies, the group 1-specific neutralizing antibodies that use VH1-69, such as CR6261 and

F10, represent a very interesting case. The crystal structures of CR6261 and F10 are very similar and indicate that the interaction with the HA stem is mediated primarily by germline residues of the VH1-69 gene, with minimal contributions from the HCDR3 and no contribution from the light chain (104, 105). Because VH1-69 is used by 2–6% of B cells (135), there is in principle an extraordinarily high number of naive B cell precursors capable of responding to the stem of group 1 HAs. To address the mechanisms that lead to the generation of such antibodies, Nabel and coworkers (136) removed the somatic mutations from three VH1-69 group 1-specific neutralizing antibodies (CR6261, 1009-3B05, and FE53) and found that the germlined antibodies lost binding to influenza HA as determined in standard assays. Surprisingly, however, cells transfected with membrane versions of the germlined antibodies were capable of binding liposomes displaying HA molecules, suggesting that the germlined antibodies, when expressed in the membrane-bound form, have enough affinity to capture multivalent antigen and therefore to start a T cell-dependent antibody response. The authors suggest that a large fraction of human naive B cells are able to respond directly to influenza HA and subsequently increase the binding affinity through somatic mutations in the CDR1 and framework 3 of the heavy chain. This model, however, does not explain why the VH1-69 response is subdominant and is observed only in some individuals (106). It is still possible that somatic mutations induced in response to antigens other than HA may be required to achieve enough affinity to drive the antistem antibody response. This alternative model could be addressed using transgenic B cells carrying a rearranged germline VH1-69 gene.

Another approach to understanding the mechanisms that lead to broadly neutralizing antibodies consists in the analysis of sister clones and the reconstruction of their genealogical tree. An interesting example is provided by the FI6 antibody. In contrast to the VH1-69 antibodies, the germlined configuration of FI6

**Table 2** Characteristics of human broadly neutralizing antibodies against influenza virus

| Antibody | Specificity                   | VH     | SHM <sup>a</sup> | HCDR3 <sup>b</sup> | Insertion /deletion | Poly-reactivity <sup>c</sup> | Year | Isolation method                     | Screening strategy               | Reference |
|----------|-------------------------------|--------|------------------|--------------------|---------------------|------------------------------|------|--------------------------------------|----------------------------------|-----------|
| C179     | Group 1 HA stem               | Murine | NA               | NA                 |                     | ×                            | 1993 | Hybridoma fusion                     | Staining of infected cells       | 101       |
| CR6261   | Group 1 HA stem               | 1-69   | 12.4             | 14                 |                     | ×                            | 2008 | Phage library                        | Binding to H5 HA                 | 103       |
| F10      | Group 1 HA stem               | 1-69   | 11.3             | 17                 |                     | ×                            | 2009 | Phage library                        | Binding to H5 HA                 | 104       |
| F16      | Group 1 and 2 HA stem         | 3-30   | 13.2             | 22                 |                     | ×                            | 2011 | Single-plasma cell culture           | Binding to multiple HAs          | 109       |
| CR8020   | Group 2 HA stem               | 1-18   | 13.9             | 15                 |                     | ×                            | 2011 | Lentiviral B cell immortalization    | Sorting with H3 HA               | 108       |
| CR9114   | Group 1, 2, and B HA stem     | 1-69   | 15.7             | 14                 |                     | NA                           | 2012 | Phage library                        | Binding to influenza A and B HAs | 110       |
| CH65     | Several H1N1 strains          | 1-2    | 11.9             | 19                 |                     | NA                           | 2011 | Single-cell RT-PCR from plasma cells | Secondary screening for binding  | 112       |
| C05      | Several group 1 and 2 strains | 3-23   | 18               | 26                 | +5 aa in HCDR1      | NA                           | 2012 | Phage library                        | Binding to H1 and H3 HAs         | 113       |
| TCN-032  | H1N1 and H5N1                 | 4-59   | 13.3             | 14                 |                     | NA                           | 2010 | B cell proliferation                 | Staining of M2-transfected cells | 10        |
| D005     | H1N1 and H5N1                 | NA     | NA               | NA                 |                     | NA                           | 2009 | Single-cell sorting and RT-PCR       | Staining of B cells with M2-VLPs | 11        |

<sup>a</sup>Percentage of amino acidic somatic hypermutations (SHMs) calculated on the full amino acid sequence by comparing the full amino acid sequences of original (mutated) and germlined antibodies, as assessed using the international ImMunoGeneTics information system (IMGT) database.

<sup>b</sup>HCDR3 amino acid length according to IMGT.

<sup>c</sup> × = no.

Abbreviations: HA, hemagglutinin; NA, not available.

binds with high affinity to group 1 HAs but not to group 2 HAs. The genealogical tree of FI6 showed that somatic mutations required for binding to group 2 HAs were acquired in a stepwise fashion (109). This finding demonstrates how an increased breadth of reactivity can be acquired through somatic mutations following serial stimulations by divergent viruses, in this case most likely an H1N1 followed by an H3N2 virus. Another example is provided by a recently isolated antibody (MPE8) capable of neutralizing both respiratory syncytial virus (RSV) and metapneumovirus (MPV), which in the germline configuration is RSV-specific and acquires MPV reactivity through a few critical mutations in light-chain CDRs (D. Corti, manuscript in preparation). The possibility of using high-throughput cellular screens to interrogate memory B cell and plasma cell repertoires offers a straightforward approach for studying the mechanisms that increase the affinity and breadth of antiviral antibodies in the course of the immune response. In the case of influenza, this approach is facilitated by the structural information on the antigen and by the lower number of mutations required as compared with HIV-1-neutralizing antibodies (Tables 1 and 2; Figure 3).

## **BROADLY NEUTRALIZING ANTIBODIES AGAINST DENGUE VIRUS, WEST NILE ENCEPHALITIS VIRUS, AND HEPATITIS C VIRUS**

Dengue virus (DENV), West Nile encephalitis virus (WNV), and hepatitis C virus (HCV) belong to the Flaviviridae family of RNA enveloped viruses along with yellow fever virus (YFV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV). The envelope (E) proteins, which form the surface of the viral particle, are the main target of neutralizing antibodies. Structural data on the E proteins are available for DENV, TBEV, JEV, and WNV (137–140), but not for HCV. Whereas effective vaccines are available for YFV and TBEV, there are no vaccines for

DENV, WNV, or HCV; therefore, the isolation and characterization of broadly neutralizing antibodies against these viruses represent an important opportunity for serotherapy and for understanding host-pathogen interactions.

A remarkable aspect of DENV biology is that antibodies can not only neutralize but also enhance infectivity. The phenomenon of antibody-dependent enhancement (ADE) of infection has been illustrated in newborns, when maternal DENV-specific antibodies wane, and in DENV-immune donors infected by a DENV virus of a different serotype (141, 142). In both cases, the enhanced infection results in a severe hemorrhagic fever that may be caused by an exuberant T cell response and cytokine storm (143). Studies on the ADE mechanism in WNV revealed that the concentrations of antibody that promote maximal enhancement of infection correspond to those that neutralize half of the infectious virions (144). This reflects the fact that neutralization and ADE are two coexisting phenomena that ultimately relate to the number of antibodies bound to the virion, consistent with the occupancy model of viral neutralization.

Recently, Screaton and coworkers (145) isolated human monoclonal antibodies from memory B cells and found that antibodies to the structural precursor–membrane protein (prM) form a major component of the human response to DENV. These antibodies are highly cross-reactive among the four DENV serotypes and, even at high concentration, do not neutralize infection but potently promote ADE (145). Beltramello et al. (146) characterized a large panel of DENV-specific antibodies against different domains of the E protein as well as against prM. Antibodies against domain III (DIII), the most variable domain, were potently neutralizing but were serotype specific, able to neutralize one, two, and only exceptionally three of the four DENV serotypes. In striking contrast, antibodies to domains I and II (DI/II) were generally less potent but were cross-reactive with the four serotypes. These findings are consistent with the notion that the potent DIII-neutralizing antibodies would

confer lifelong protection from homologous challenge. In contrast, upon heterologous infection, in the absence of cross-reactive DIII antibodies, the DI/II and prM cross-reactive antibodies would be present in the serum at a concentration insufficient to neutralize the incoming virus but sufficient to enhance infection. Hence, it appears that in the case of DENV the broad reactivity of weakly neutralizing DI/II and prM antibodies may play a pathogenic role.

Previous studies have addressed the therapeutic role of a potent and broadly reactive WNV-neutralizing humanized antibody named E16, which showed impressive therapeutic efficacy in mice even when administered five days after infection (147). This antibody binds to a conserved epitope in the lateral ridge of DIII and inhibits infection after viral attachment by interfering with conformational changes required for viral entry (140).

In the context of a possible serotherapy for DENV infection, three neutralizing monoclonal antibodies (DV87, DV82, and DV22) that cover all four DENV serotypes by binding to two nonoverlapping epitopes on E protein were produced in the LALA form and shown to be therapeutically effective in a lethal mouse model of antibody-enhanced infection (72, 146). Similar findings were obtained by Harris and coworkers (148) using the mouse antibody E60 engineered with a mutation (N297Q) that disrupts Fc receptor binding.

In contrast to WNV and DENV, in which the structural bases of the antibody-dependent neutralization are available, little is known about the HCV E1 and E2 glycoprotein structures. E2 binds to the cellular CD81 receptor (149), and infection also requires claudin-1 (150), occludin (151), and scavenger receptor class B type I (SR-B1) (152). Recent progress in the establishment of surrogate, pseudotype-based and infectious virus-based neutralization assays (153) has been instrumental to isolating and characterizing several HCV-neutralizing antibodies. These antibodies against HCV were isolated from immunized human Ig-transgenic mice or normal mice [HCV1 and 95-2 (80,

154); H77.39 (155)] or from HCV chronically infected patients [#4 (156); 1:7 and A8 (157); AR3A and AR3B (158); AR4A (159); HC-84 series (160)] and potentially neutralize viruses or pseudoviruses belonging to multiple, or even all, genotypes. These antibodies target different sites on the E2 glycoprotein by recognizing linear or conformational epitopes, and most neutralize by targeting the CD81-binding site (CD81bs) or a common binding site between CD81 and SR-B1, such as in the case of H77.39 antibody. An interesting exception is antibody AR4A, which was suggested to interact with the E2 MPER, a region supposed to be involved in membrane fusion (161). The only E1-specific broadly neutralizing antibodies described to date are IGH505 and IGH526, which neutralize HCV infection by targeting a 15 amino acid region near the C terminus of E1 (162). Some of the broadly neutralizing antibodies (AR3A, AR3B, and AR4A) show prophylactic efficacy, although at very high doses (100–200 mg/kg), when tested in a human liver chimeric mouse model (158) or in a genetically humanized bioluminescent mouse model (159, 163).

The development of an HCV vaccine is hampered not only by the lack of structural information but also by other aspects such as (*a*) the rapid evolution of viral quasi species that rapidly generates antibody escape variants (164); (*b*) the capacity of nonneutralizing antibodies to interfere with neutralizing antibodies (165); and (*c*) the findings that some antibodies may enhance HCV infection in non-HCV-permissive cell lines (166). Finally, there is no evidence that neutralizing antibodies are produced in individuals who spontaneously clear HCV infection.

## NEUTRALIZING ANTIBODIES AND VACCINE DESIGN

The current approach to vaccine design relies on the identification of antigens either by empirical testing using inactivated or attenuated pathogens or by genomic analysis followed by high-throughput expression and testing of individual proteins for their capacity to induce

a protective antibody response. The latter approach, defined as reverse vaccinology, was pioneered by Rappuoli and colleagues (167) with the development of a multivalent vaccine for meningococcus B. An alternative approach is based on the identification of antigens and epitopes targeted by neutralizing antibodies generated in the course of the immune response to the pathogen. This approach, which was initially proposed as a strategy to make an HIV-1 vaccine, has been dubbed reverse (168), structure-assisted (169), or analytic vaccinology (170). In this context, potent and broadly neutralizing monoclonal antibodies offer a shortcut solution to the challenge of pathogen complexity and variability (**Figure 5**).

An example of how the study of the human antibody response can lead to antigen discovery is provided by the isolation of unusually potent neutralizing antibodies against human cytomegalovirus (HCMV) (171), a pathogen responsible for severe infection in immunosuppressed patients and in the fetus. These antibodies, which neutralize infection in the picomolar range, recognize several conformational epitopes on a pentameric complex of gH/gL/UL128/UL130/UL131. This pentamer was known to be required for HCMV infection of endothelial, epithelial, and myeloid cells, but not to be a target of neutralizing antibodies (172–174). In contrast to other HCMV genes, those encoding for the pentamer are relatively conserved among HCMV isolates (175), as shown by the fact that the antibodies cross-neutralize different field isolates (171). Another viral glycoprotein, gB, was tested as a candidate vaccine mainly because of its abundance on HCMV virions, but it failed to provide significant efficacy in clinical trials (176). This negative result might be explained by the finding that the vast majority of gB-specific antibodies lack virus-neutralizing activity (177). Using a BOB assay with antipentamer antibodies, it was shown that in pregnant women with primary HCMV infection, the appearance of antipentamer, but not anti-gB, antibodies correlates with a lower risk of viral transmission to the fetus (178).

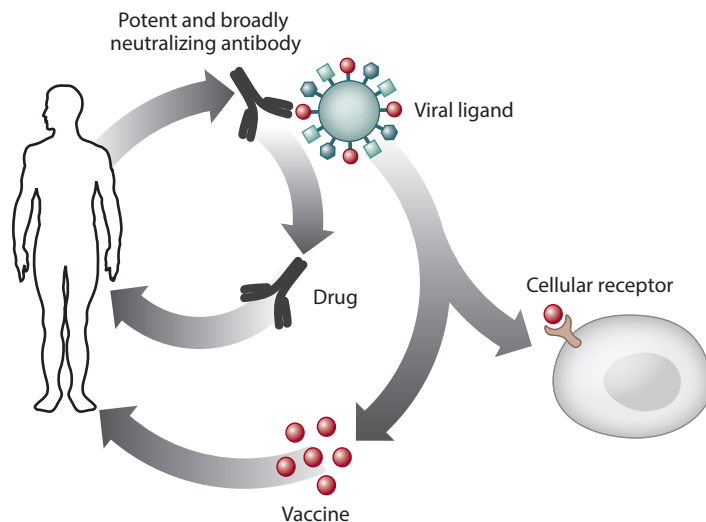


**Figure 5**

Neutralizing monoclonal antibodies as candidates drugs for serotherapy, as tools for vaccine design, and for the dissection of host-pathogen direction.

Although it might be relatively straightforward to formulate a conserved protein as a vaccine, it is highly challenging to design a vaccine around the conserved epitope defined by broadly neutralizing antibodies, which represents only a small portion of the antigenic protein. Current efforts focus primarily on HIV-1 Env and influenza A HA and will be illustrated in more detail in the following two sections. In both cases the largest fraction of the antibodies produced are directed against the most variable regions of the proteins, while broadly neutralizing antibodies represent only a minor proportion of the response. The challenge is how to reverse this trend by increasing the immunogenicity of conserved regions and decreasing that of the most variable regions. Several aspects described throughout this review represent significant obstacles in translating epitope discovery into antigen design. There are two series of factors: The first relates to the structure and topology of the viral antigens, such as (*a*) epitope masking or shielding by glycans or protein loops; (*b*) transient exposure in the entry process or via other mechanisms such as viral “breathing;” (*c*) small size of the conserved epitopes, which constrains interaction with the

large surface of the antibody combining site, as in the case of the sialic acid-binding site; (d) limited accessibility of the epitope, as in the case of tightly packed envelope proteins; and (e) epitope mutability leading to viral escape. The second series of factors that constrain the response to conserved sites relates to the requirement for the generation of antibodies to these sites, such as (a) the need for extensive somatic mutations and focused evolution; (b) the use of specific germline allelic variants and HCDR3s of particular length and structure; and (c) the molecular mimicry of host molecules, as in the case of glycan epitopes.

In addition to their use for epitope discovery and vaccine design, antibodies may also serve as adjuvants. Several studies have shown that, when administered at the right time and the right concentration, antibodies can enhance the antibody response (179). Interestingly, administration of b12 to newborn macaques, at levels insufficient to block mucosal SHIV infection *in vivo*, led to enhanced neutralizing response and significantly reduced plasma viremia, indicating that antibodies can act as natural adjuvants (180).

## HIV Vaccine Research

Initial attempts to construct an HIV vaccine based on inactivated virus (181) or recombinant gp120 (182, 183) failed to show efficacy in non-human primates; approaches based on attenuated SIV showed efficacy in animal models (184) but were not translated to human trials due to safety issues raised in the neonatal macaque model (185). The failure of a T cell-based vaccine in 2007 (186) and the demonstration that neutralizing antibodies can prevent infection in nonhuman primates (187) revamped the idea that an effective HIV-1 vaccine must also elicit anti-Env protective antibodies (188, 189).

In the past decade, several attempts were made using different immunogens delivered as protein (administered with alum, MF59, or AS02A adjuvants), naked DNA, or viral vectors. These studies led to variable results with either no protection or only a low degree of protection

(190). A note of optimism came from the RV144 phase 3 trial, which enrolled heterosexual individuals at low risk of HIV-1 infection in Thailand. The vaccine was based on a canarypox vector encoding gag, pol, and nef and a membrane-anchored gp120 followed by a protein boost with a bivalent gp120 in alum. At the end of the trial, the number of infections was 74/8197 in the placebo group versus 51/8198 in the vaccine group, consistent with a modest yet significant efficacy of 31% (191). These findings suggested for the first time that prevention of HIV-1 infection is an achievable goal. However, the correlates of protection were not entirely clear. Haynes et al. (192) reported that noninfected individuals had on average higher levels of IgG antibodies directed to the V1/V2 region and lower levels of anti-Env IgA antibodies following vaccination as compared with those who became infected. However, Montefiori et al. (193) found that the weak neutralizing antibody response in the RV144 trial was largely V3 specific and mostly effective against neutralization-sensitive viruses, and it was lower than that elicited by another vaccine (VAX003) that did not show clinical efficacy. An important limitation of all early trials, including RV144, is that peak antibody levels waned rapidly with half-lives of a few weeks, in contrast with most current vaccines, in which the serological memory can last from several years to a lifetime (194). Indeed, in the RV144 trial the efficacy in the first year was approximately 60%, thus suggesting that the limited efficacy may be due primarily to the failure to maintain sufficiently high antibody levels. More studies are therefore required to understand the B cell biology and the mechanisms that drive long-lived serum antibodies.

A major problem encountered in the development of an antibody-based HIV vaccine is the presence of neutralization-resistant tier 2 and tier 3 viruses, which account for the majority of clinical isolates and which are hardly neutralized by vaccine-elicited antibodies. The structural basis of neutralization resistance is not understood but is well illustrated by the case of the V3 loop, a very immunogenic functional region characterized by a certain degree

of conservation. Anti-V3 loop antibodies show considerable breadth, able to bind V3 loops of tier 1 and tier 2 viruses. However, these antibodies neutralize the corresponding tier 1, but not tier 2, viruses (50) and often neutralize virus infectivity only partially (195). The level of neutralization observed was associated with the degree of V3 loop accessibility, which is supposed to be very limited on tier 2 and tier 3 viruses. The concept of an inaccessible V3 loop on neutralization-resistant viruses is also supported by the finding that soluble CD4 broadens neutralization by V3 antibodies (196). In this respect, the production of CD4bs-specific antibodies able to mimic the CD4-induced conformational change of gp120 might unmask V3 epitopes on tier 2 or tier 3 viruses, thereby making these viruses vulnerable to the abundantly produced V3-specific antibodies.

A consistent, yet unexplained, observation is that gp120-based vaccines fail to elicit broadly neutralizing antibodies, even though gp120 can absorb the broadly neutralizing activity present in immune sera (53). Indeed, with the exception of PG9-like and 3BC176-like antibodies, all broadly neutralizing antibodies found so far bind well to gp120 proteins. Why do antigens that express the right epitopes fail to elicit broadly neutralizing antibodies? A possible explanation is that the epitopes targeted by the broadly neutralizing antibodies are only a minute fraction of all epitopes that antibodies can recognize on the surface of the trimeric spike. For example, many antibodies bind to the CD4bs, but only the few broadly neutralizing ones can interact with conserved residues without being affected by surrounding glycans or variable residues. It is therefore possible that in a naive individual only very few B cells, if any, express the right VH/VL combinations that are compatible with the development of broadly neutralizing antibodies through multiple rounds of mutations and selection. This view is supported by the finding of the convergent evolution of the anti CD4bs broadly neutralizing antibodies VRC01, PGV04, and CH30-34 (56). The reconstruction of the molecular events that lead to the generation of broadly

neutralizing antibodies starting from their germline versions will provide information that may be relevant to developing vaccination strategies that accelerate this selection, for instance through successive immunizations with divergent Env molecules.

Another important aspect for vaccine design is the stability of the neutralizing epitopes in the recombinant proteins, an aspect that can be easily probed using broadly neutralizing antibodies. In relation to the differential immunogenicity and antigenicity of Env, it would be important to rule out the trivial possibility that the epitopes targeted by broadly neutralizing antibodies are lost when antigen is formulated with adjuvants, such as alum, that have protein-denaturing activity. A recent study by Sattentau and coworkers (197) showed that the nondenaturing adjuvant carbopol (a polyanionic carbomer gel) is superior to alum and equivalent to Freund's complete adjuvant in eliciting antibodies against HA and gp120 in mice; more recently, Heeney and coworkers (198) showed that a combination of carbopol and the licensed squalene-based MF59 adjuvant optimally preserved antigenicity while increasing immunogenicity of a variety of HIV-1-neutralizing epitopes.

The overall limited success of conventional vaccine approaches forms the basis of current efforts to focus the response to the newly identified conserved epitopes using novel types of immunogens that belong to the following categories: (*a*) mimics of the trimeric Env such as virus-like particles (VLPs), DNA, and stabilized trimeric Env; (*b*) antigens in which immunodominant regions have been removed; (*c*) mixtures of antigens to cover multiple clades matching the epitopes recognized by broadly neutralizing antibodies; and (*d*) epitope grafting on scaffold proteins. The latter approach was pioneered by Kwong and coworkers (199), who grafted the continuous helical 2F5 epitope into an acceptor scaffold. The best 2F5-epitope scaffolds that have been generated possessed nanomolar affinity for 2F5 antibody and induced 2F5-like antibodies in mice (199); however, these antibodies lacked neutralizing

activity (200). More recently, Schief and coworkers (201) grafted the b12 discontinuous epitope into an unrelated scaffold protein by combining computational design with multiple rounds of experimental selection. The chimeric protein bound the b12 antibody with high affinity, but its capacity to elicit b12-like antibodies has not yet been assessed. This approach may become successful if more epitopes are tested, in particular those recognized by second-generation broadly neutralizing antibodies. Concerns remain that epitope-specific cells would be too rare in the naive repertoire and that the path of somatic mutations required would be too complex for an effective response to be generated. For this reason, it makes sense to use the epitope-grafting approach when the molecular pathway to generate neutralizing antibodies from naive B cells is relatively short.

It is still debated whether antibodies elicited against the more conserved gp41 Env protein can have any role in protection. In this respect, a gp41-based virosomal vaccine administered intranasally or intramuscularly protected macaques from vaginal challenge with SHIV in the absence of neutralizing IgG antibodies in serum (202). The presence in vaginal secretions of gp41-specific IgA antibodies with HIV-1 transcytosis-blocking properties was suggested as the possible mechanism that prevented mucosal infection. The recent discovery of a potent antibody to the MPER, 10E8 (48), characterized by very high breadth and potency and lack of autoreactivity, revamps the idea that this region may be a primary target for HIV-1 vaccine design.

## Influenza Vaccine Research

The current influenza vaccines are made by the combination of three or four strains (H1N1, H3N2, and one or two influenza B strains) in the form of inactivated viruses (whole-virus or split vaccines), virosomes, or live-attenuated influenza viruses (FluMist®) (203, 204). These vaccines are reformulated annually to include the best predictions of which strains will circulate in the following

year. In spite of better methods to identify the new antigenic variants on the basis of HAI data and antigenic cartography (14), the overall rate of success is not impressive. Furthermore, current influenza vaccines may be less effective than previously thought, especially in elderly individuals (205). Although the use of new adjuvants, such as MF59, can increase the magnitude and potentially the breadth of the response to influenza vaccine (206), it is still foreseen that a new vaccine must be formulated every year to match the circulating strains.

The holy grail of influenza vaccine research is a universal vaccine that protects against all strains, subtypes, and groups of influenza A viruses, including seasonal and pandemic strains, and thereby renders the annual vaccine redundant. In this context, the identification of the HA stem as the main target of broadly neutralizing antibodies provides a new and powerful rationale for the generation of a universal vaccine based on “headless” HA molecules that lack the globular head region while retaining the stem region in the prefusion conformation.

In an early attempt to produce a HA stem-based vaccine, Palese and coworkers (207) treated influenza virus with acid and a reducing agent to remove the globular head. They found that rabbit sera elicited by these headless subviral particles cross-reacted against multiple subtypes but lacked neutralizing activity. In a subsequent study, Sagawa et al. (208) immunized mice with transfected cells expressing a headless H2 HA protein that was recognized by the stem-specific neutralizing antibody C179 and reported partial protection from heterologous challenge with H1N1. Unfortunately, there was no follow-up on this study, possibly due to the complexity of using transfected cells as vaccine. More recently, Palese and coworkers (209) immunized mice with VLPs carrying headless H1 or H3 HAs and showed production of cross-reactive binding antibodies and partial protection from homologous H1N1 challenge. These authors also described HA2 stabilized immunogen that was produced in bacteria and conferred protection in mice against homologous and heterologous H3N2 virus challenge

(210). They also reported that immunization with a peptide spanning the helix B of H3 HA, recognized by the H3-neutralizing antibody 12D1 (211), confers modest antibody-mediated protection against heterologous H1N1 and H3N2 viruses (212). The latter results are intriguing given that helix B is not exposed on the surface of the trimeric HA and may become available only during the fusion process. Finally, Wilson and coworkers (213) immunized mice with icosahedral VLPs expressing helix A as a minimal component of the HA stem. These VLPs, which were not recognized by CR6261 or F10 antibodies, elicited antibodies that recognize multiple group 1 subtype HAs but failed to neutralize infectious virus.

In conclusion, despite several attempts, the engineering of a headless HA vaccine has proven particularly difficult, and there is no direct evidence that the modest degree of protection achieved in some of the above studies was due to the elicitation of neutralizing antibodies specific for the HA stem. The availability of a large panel of broadly neutralizing antibodies now offers the possibility of better characterizing the immunogens to ensure that they maintain the correct conformation. In addition, in future studies it will be important not only to use *in vivo* protection of vaccinated animals as a readout but also to analyze the effect of passively transferred antibodies and to document the production of broadly neutralizing antibodies through the isolation of monoclonal antibodies from immunized animals.

Another candidate for a universal influenza vaccine is M2e, which is a highly conserved nonglycosylated stretch of 23 amino acids expressed at high levels on infected cells but virtually absent on virions. Antibodies to M2e are produced at low levels only upon infection and are not elicited by conventional vaccines. Several studies in mice showed that vaccination with different forms of M2e, including synthetic peptides, fusion proteins, and M2-displaying VLPs, conferred different levels of protection against lethal influenza A challenge (214–217). As discussed above, M2-specific antibodies are not neutralizing and therefore cannot prevent

infection but rather limit viral spread by facilitating the elimination of infected cells.

An alternative approach to focus the antibody response to the HA stem region relies on a heterologous prime-boost strategy. Three recent studies demonstrate that infection or vaccination with the new pandemic H1N1 virus, either of which is equivalent to a heterologous boost, can selectively recall memory B cells that make broadly neutralizing antibodies against the conserved stem region, including some that react with both group 1 and group 2 HAs (107, 109, 218). This phenomenon was known since 1966 as the “original antigenic sin” (219) and was initially viewed as a mechanism that prevents an effective primary response to new viruses. However, in view of the recent findings, the original antigenic sin can now be considered as a natural mechanism of immunofocusing. Thus, a possible strategy to focus the immune response to the HA stem is to sequentially immunize with chimeric HAs encompassing a variety of globular head and stem combinations from different subtypes (116). Seasonal vaccines may not achieve this goal because the globular heads of drifting viruses are relatively conserved and therefore continuously boost the response against this immune-dominant region.

In conclusion, the new antibodies and the new information on the mechanisms of defense have given new impetus to the search for a universal influenza vaccine based on the relatively conserved stem region. An important proof of concept toward the development of a stem-based vaccine is the demonstration that antistem neutralizing antibodies are effective in humans in a prophylactic setting. Given the limitations of animal models with regards to the effector mechanisms and the biology of the virus, it is crucial that these studies are performed in humans. We must also consider that a universal influenza vaccine must elicit neutralizing antibodies against group 1 and 2 and possibly against influenza B viruses. Whereas the HA stem of group 1 viruses is clearly immunogenic, as demonstrated by the isolation of plenty of neutralizing antibodies,

this evidence is still lacking for the stem of group 2 influenza A and influenza B viruses, perhaps because of the presence of conserved glycans that prevent access to helix A.

## FUTURE PERSPECTIVES

The recent progress in the discovery of potent and/or broadly neutralizing antibodies has been promoted by the development of new technologies for the efficient isolation of antibodies from the human immune response, combined with sophisticated screening strategies based on functional assays or recognition of cross-reactive epitopes. These findings highlight the importance of leveraging the extraordinary potency and diversity of the human immune system, which is stimulated by multiple variant viruses in the context of acute and chronic infections. It is foreseen that the effort of isolating broadly neutralizing antibodies will be extended to other viruses as well as to bacteria and parasites. Although currently there is only one licensed anti-infective monoclonal antibody (the anti-RSV palivizumab), in the future antibodies might be successfully used to prevent and treat viral diseases such as those caused by rabies virus, RSV, MPV, rhinovirus, and norovirus.

Although the prophylactic use of antibodies is relatively straightforward in view of the small

load of infectious virus, their therapeutic use in acutely or chronically infected individuals remains to be determined and will be influenced by the viral load and the selection of escape mutants. It will be fundamental to understand the mechanisms of protection in appropriate animal models and, whenever possible, in the human system in order to appropriately tune the antibody effector functions and dosage. A potential synergy between antibodies and antimicrobial drugs (antivirals or antibiotics) may be exploited to restrain pathogen replication while boosting the immune response through the enhancement of microbial antigen presentation.

From the vaccine perspective, the isolation of broadly neutralizing antiviral antibodies offers a new benchmark to analyze the antibody response and find correlates of protection using the new serological assays developed. There is no doubt that the structural information gained through the new potent and broadly neutralizing antibodies will impact vaccine design. However, major issues remain, such as how to focus the antibody response to the most conserved regions of the target viral glycoproteins. The analysis of the antibody response in vaccinated individuals as well as the use of the most appropriate animal models, such as human immunoglobulin transgenic mice, will be instrumental to solve this problem.

## DISCLOSURE STATEMENT

A.L. is the scientific founder and shareholder of and D.C. is employed at Humabs Biomed, a company that develops human antibodies for treatment of infectious diseases.

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## Errata

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