REVIEW

Broadly Neutralizing Antibodies Present New Prospects to Counter Highly Antigenically Diverse Viruses

Dennis R. Burton,[1,2]* Pascal Poignard,[1,4] Robyn L. Stanfield,[3] Ian A. Wilson[3]*

Certain human pathogens avoid elimination by our immune system by rapidly mutating the surface protein sites targeted by antibody responses, and consequently they tend to be problematic for vaccine development. The behavior described is prominent for a subset of viruses—the highly antigenically diverse viruses—which include HIV, influenza, and hepatitis C viruses. However, these viruses do harbor highly conserved exposed sites, usually associated with function, which can be targeted by broadly neutralizing antibodies. Until recently, not many such antibodies were known, but advances in the field have enabled increasing numbers to be identified. Molecular characterizations of the antibodies and, most importantly, of the sites of vulnerability that they recognize give hope for the discovery of new vaccines and drugs.

Mutation and recombination in genetic material have allowed the evolution of the living world and of our species. But these mechanisms also cause us great problems, notably in the many faces of cancer and from the onslaught of certain highly antigenically diverse pathogens that can change sequence rapidly enough to avoid timely elimination by our immune system. Pathogens that can rapidly change their antigenic profiles include RNA viruses such as HIV, influenza virus, and hepatitis C virus (HCV); bacteria such as *Streptococcus pneumoniae*; and protozoa such as *Plasmodium falciparum*, which causes human malaria, and *Trypanosoma brucei*, which causes sleeping sickness. Preventing infection by these pathogens through design and development of vaccines has proven challenging. Antigenic diversity has also caused notable difficulties in developing certain classes of drugs against these pathogens, in particular those targeting surface-associated glycoproteins. For drugs that are successfully introduced, the ability of many pathogens to rapidly generate variants can lead to drug resistance. Recent breakthroughs in the generation and characterization of broadly neutralizing antibodies against the highly antigenically diverse viruses HIV, influenza virus, and HCV have helped identify potential targets for vaccines and drugs and presented new opportunities to counter these viruses. These types of approaches may also help in designing strategies to counter other highly antigenically diverse pathogens.

Sequence variability of pathogens is not confined to surface antigens and is not only the result of immune pressure; replication in the host, transmission, and survival of a pathogen may also depend on genetic diversification. It is instructive to consider highly antigenically diverse pathogens in the general context of pathogen variability. A number of pathogens have evolved rapid genetic diversification, including various bacteria and protozoa, but the champions of diversity are viruses. RNA viruses in particular have high mutation rates, largely because of the involvement of virus-encoded error-prone RNA and DNA polymerases in their replication cycle, leading to mutation rates as high as 1.5×10^{-3} mutations per nucleotide per genomic replication (*1*). Certain DNA viruses, especially single-strand DNA viruses, can also have high mutation and substitution rates, sometimes as high or higher than RNA viruses (*1*). For many viruses, sequence variability is crucial to escape host immune cellular and humoral responses, leading to great antigenic diversity in the proteins targeted by the adaptive immune system. However, other variable viruses have evolved modes of replication and transmission that allow them to survive without the need to escape the pressure of their host's adaptive immune responses, for example, by transmitting from one host to the next before an effective immune response is mounted. As a consequence, these viruses do not display a high level of antigenic diversity despite an inherent capacity to do so. Therefore, high sequence diversity per se is not necessarily an obstacle to vaccine development, and effective vaccines have been developed against relatively highly diverse viruses such as measles virus, hepatitis B virus, polio virus, and rabies virus. Conversely, vaccines have been difficult to develop against pathogens with low diversity, such as herpes simplex virus. However, high antigenic diversity, in which there is a very high level of variability in the viral protein sites principally targeted by the immune system (immunodominant or immunoprominent epitopes), does consistently impair vaccine development. Classical vaccine approaches will tend to afford protection against a very limited fraction of circulating virus populations. Because the most important contributors to protection by the majority of antiviral vaccines are neutralizing antibodies targeting the surface envelope (Env) proteins, the highly dynamic antigenic diversity of these proteins is the major obstacle to the development of practical vaccines for viruses such as HIV, influenza virus, and HCV.

Disease Impacts

HIV establishes a chronic infection that, over a period of years and if left untreated, leads to AIDS. About 25 million people have died of AIDS, and about 35 million are currently infected (*2*). The epicenter of the plague is sub-Saharan Africa, with an adult infection rate reaching 5%. The high relative cost of treatment has begun to consume a large proportion of development aid to this region with diverse negative consequences. Influenza virus infection produces acute respiratory and systemic symptoms and leads to between one-quarter and one-half million deaths on average per year and societal costs of billions of dollars annually in health care and lost productivity (*3*). At 10- to 50-year intervals, the virus triggers recurring deadly pandemics; the great influenza pandemic of 1918 killed about 50 million people worldwide (*4*), and the other pandemics of the past century in 1957, 1968, and 2009 caused millions of deaths. HCV chronically infects 120 to 170 million people globally (*5*) and is a major cause of chronic liver disease and liver cancer in the developing world. The societal costs of HCV in the United States are expected to approach $85 billion by 2024 (*6*).

Antibody Discovery

HIV, influenza, and HCV show great sequence heterogeneity in their Env proteins, which are the essential targets of an effective antibody response. This heterogeneity is seen between different isolates or strains, particularly in their surface-exposed amino acid residues. Nevertheless, at least two regions on these viral Env proteins are expected to be both conserved and accessible, even if only transiently in some instances, to permit virus infection. All enveloped viruses are required to bind to one or more receptors on their target host cell and have a mechanism for entry into that cell. Therefore, the Env proteins contain a receptor binding site(s) and the viral fusion machinery, both of which are likely to be exposed, at least transiently, during cell attachment and viral entry. However, these conserved accessible regions may be relatively small; for example,

[1]Department of Immunology and Microbial Science and International AIDS Vaccine Initiative (IAVI) Neutralizing Antibody Center, The Scripps Research Institute, La Jolla, CA 92037, USA. [2]Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard University, Cambridge, MA 02129, USA. [3]Department of Molecular Biology, Skaggs Institute for Chemical Biology, and IAVI Neutralizing Antibody Center, The Scripps Research Institute, La Jolla, CA 92037, USA. [4]IAVI, New York, NY 10038, USA.

*To whom correspondence should be addressed. E-mail: burton@scripps.edu (D.R.B.); wilson@scripps.edu (I.A.W.)

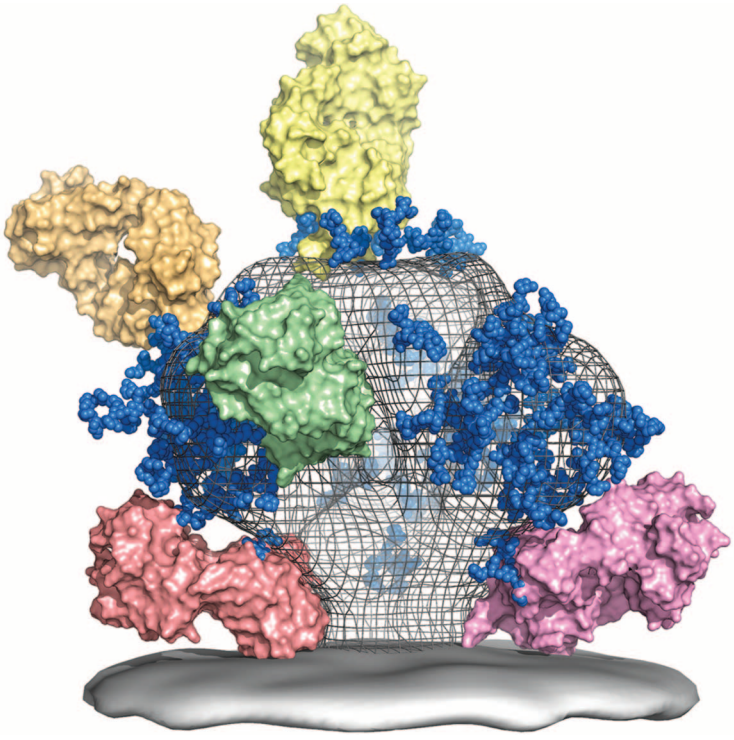

Fig. 1. A model of the HIV-1 Env spike with select bnmAbs bound. The spike model is derived from cryoelectron tomography from (*73*), Electron Microscopy (EM) Data Bank codes EM-5019 and EM-5021. The Fabs are color-coded in the figures to represent different sites of interaction on the Env proteins. The pinkish red Fabs bind to the Env stem that houses the fusion machinery, the green Fabs bind at or around the receptor binding site (CD4 receptor for HIV-1 and sialylated glycans for influenza, as shown in Fig. 2), and the yellow Fabs bind and penetrate the glycan shield for HIV-1. Anti-HIV Fabs shown are PG9 [bright yellow, on top, Protein Data Bank (PDB) code 3u4e] (*34*), PGT128 (orange-yellow, 3tyg) (*33*), VRC01 (green, 3ngb) (*32*), 4E10 (salmon at bottom left, 2fx7) (*74*), and 2F5 (pink at bottom right, 2f5b) (*75*). Carbohydrates (Man_9, blue spheres) were modeled onto an unliganded YU2 gp120 core (3tgq) (*76*) with GlyProt (*77*) from glycosciences.de, except for the glycans binding to PGT128 and PG9, which were taken directly from their structures in complex with outer domain (*33*) and scaffold (*34*), respectively. The locations of PG9, 4E10, and 2F5 are approximate; however, the VRC01 and PGT128 were docked by superposition of gp120 core and outer domain, respectively, with the unliganded YU2 gp120 model (that is invisible under the EM mesh). Figure was made with Pymol (*78*).

the hemagglutinin (HA) protein that mediates influenza viral entry is only required to recognize a relatively small glycan moiety, containing a terminal sialic acid, as its receptor on target cells. The small size of the receptor severely limits the conserved footprint that can be specifically recognized by an antibody in order to acquire cross-reactivity against different strains and subtypes. For HIV-1, that footprint is larger because the receptor is a protein, CD4, but CD4 has a molecular width of only a single immunoglobulin (Ig) domain. An antibody antigen-binding fragment (Fab) has twice that width, with two Ig domains. Suggestions that antibody footprint-sized conserved regions may be exposed at the Env surface came originally from observations of serum cross-neutralizing activity against diverse influenza and HIV isolates (*7*, *8*) from individuals infected with the corresponding viruses. Serum cross-neutralizing activity could arise, in principle, from a combination of a large number of antibodies directed to variable regions. However, the isolation of individual monoclonal antibodies (mAbs) that were able to neutralize in vitro multiple, diverse isolates [broadly neutralizing monoclonal antibodies, bnmAbs (*9*)] confirmed the presence of conserved antibody footprint-sized accessible regions on Env antigens on the viral surface. BnmAbs to HIV were first isolated from infected individuals in 1992 by electrofusion (*10*) and by phage display (*11*) but only shown convincingly to be broadly neutralizing to primary, as distinct from laboratory-adapted, virus isolates in 1994 (*12*, *13*). A mouse bnmAb to influenza virus was isolated and characterized in 1993 (*14*), and a mouse bnmAb to HCV was first generated in 2001 (*15*) and shown to be broadly functional in 2005 (*16*). Until the late 2000s, only a small number of bnmAbs against these viruses had been isolated and characterized, but in the past 3 years an explosion in the rate of generation and characterization of such Abs has occurred. These exciting breakthroughs have unearthed many novel Abs with unexpected epitopes, as well as novel modes of antibody-antigen recognition, and have suggested a plethora of new vaccine and drug targets.

For many years, only a handful of bnmAbs against HIV, all human, were known (*17*, *18*). Of these Abs, two that were directed to the Env glycoprotein gp120 (b12, 2G12) and two to the transmembrane Env glycoprotein gp41 (2F5, 4E10) were the most notable and were intensely studied. Importantly, these bnmAbs were all shown to protect against free virus challenge in relatively robust macaque models of HIV infection (*19*–*22*). Crystal structures of these mAbs in complex with Env antigens were determined, and a variety of novel antibody features were identified in their hypervariable regions (*23*, *24*). These features included long heavy-chain variable complementarity–determining regions 3 (HCDR3s), which had scarcely been reported at that time, antibody domain exchange to recognize a glycan cluster on gp120 (2G12), and very hydrophobic HCDR3s (2F5, 4E10), which appeared to be associated with recognition of epitopes very close to the virus membrane (i.e., membrane proximal external region) for the two anti-gp41 mAbs.

Many immunogens have been designed based, to varying degrees, on molecular understanding of the interaction of these bnmAbs with HIV Env antigens. No immunogen tested to date has "re-elicited" antibodies with broadly neutralizing character. Several explanations have been proposed, including the limitations imposed by the availability of such a small panel of bnmAbs, that make the drawing of general conclusions for immunogen design hazardous. Therefore, considerable effort by a number of laboratories went into the discovery of more bnmAbs. Two factors appear to have been most crucial in the success of that effort: first, the screening of large cohorts of infected donors to identify individuals with broadly neutralizing sera by using reproducible high-throughput neutralization assays (*25*) and, second, the application of novel single B cell technologies to samples from these donors to facilitate the isolation of bnmAbs from a background of many other non-neutralizing antibodies against Env (anti-Env). The application of direct neutralization screening to a large number of B cells (about 30,000) from a donor with broad and potent serum-neutralizing activity led to the isolation of a pair of bnmAbs to a novel epitope in 2009 (*26*). The new mAbs were about an order of magnitude more potent in standard neutralization assays than the previously identified bnmAbs while generally maintaining or improving breadth. This development was quickly followed by the sorting of B cells using an engineered gp120 molecule to identify a potent bnmAb directed to the CD4 binding site of gp120 of similarly enhanced potency and even greater breadth (*27*). The application of direct neutralization screening to further donors with broadly neutralizing sera led to the

identification of yet more bnmAbs that targeted novel mixed glycan/protein epitopes on gp120 with even greater potency (*28*). Larger panels of highly potent anti-CD4bs bnmAbs have also been isolated by single B cell sorting using Env baits and specially designed polymerase chain reaction (PCR) primers to account for a high degree of antibody mutation (*29*). Engineering of one such mAb has generated remarkable potency and breadth (*30*). Deep sequencing of antibody repertoires from donors from whom bnmAbs have been isolated has revealed many additional potent related mAbs (*31*). BnmAb targets on HIV Env are represented in Fig. 1.

The structures of many of the newer HIV bnmAbs in complex with Env antigens (*31*–*34*), together with analysis of the bnmAb sequences, provide opportunities and potential lessons for vaccine and drug discovery. Several new targets are available for immunogen and drug design, and the understanding of existing targets has also been greatly enhanced. For example, recognition of a favored vaccine target, the CD4 binding site, has now been shown to not necessarily require a long HCDR3 but appears to be strongly dependent on certain gene segments of the variable region of immunoglobulin heavy chain (V_H) (*29*, *32*). High-affinity glycan recognition on Env can also be achieved by conventional antibodies in the absence of 2G12-like domain exchange (*33*). Other recurring themes of HIV bnmAbs have been a relatively high degree of somatic hypermutation, a prevalence of insertions and deletions in the antibody variable regions, and an inability of germline versions of the bnmAbs to bind Env (*26*–*29*, *35*–*38*). It is still unclear whether a high degree of hypermutation reflects a general property of HIV Abs that has arisen because of the chronic antigen stimulation from natural infection or is a requirement for the evolution of such Abs toward broad cross-reactivity. The affinity threshold required for the Env-germline antibody to trigger a response is also unclear. Nevertheless, a number of immunogens are currently being designed to bind to germline, as well as mature, antibodies to activate naive B cells (*37*).

Besides informing vaccine design, the discovery of conserved exposed regions, particularly associated with the glycan shield of gp120 (Fig. 1), provides a number of new potential viral entry inhibitor targets.

For influenza virus, the first bnmAb was isolated in 1993 by immunization of a mouse with H2N2 virus (HA subtype 2, neuraminidase subtype 2) and shown to neutralize group 1 viruses bearing HAs of subtypes 1, 2, 5, 6, and 9 (*14*). The antibody was proposed to bind to a conserved region in the stem of HA, in contrast to typical strain-specific antibodies that bind to hypervariable regions on the head of HA (Fig. 2). In 2008 and 2009, a number of novel human bnmAbs that cross-neutralized group 1 subtypes were isolated from phage libraries by different laboratories. The antibodies had remarkably similar sequences to one another, close to the corresponding human germline sequence of the V_H1-69 family with relatively few somatic mutations; showed similar patterns of group I subtype neutralization (representing 10 of the 16 known flu subtypes); and were protective in mouse models (*39*–*42*). Structural studies showed this group of V_H1-69 bnmAbs recognized a conserved region of the stem of HA by using the heavy chain alone (*42*, *43*) (Fig. 2). More recently, high-throughput screening of immortalized antibody-secreting cells generated a bnmAb that recognizes this conserved region in a different way, using both heavy and light chains, and is able to neutralize both group 1 and group 2 viruses (*44*). In addition, a different cell-based method has been used to isolate a bnmAb that binds to a second site on the stem of HA, again quite close to the virus membrane; neutralizes group 2 viruses; and protects mice against virus challenge (*43*) (Fig. 2). Furthermore, bnmAbs have recently been described that target the head region of HA (*39*, *45*–*48*) around the receptor binding site. One of these bnmAbs neutralizes selected isolates from both group 1 and 2 viruses (*49*), but most neutralize only a single subtype. The structure of a mAb that broadly neutralizes viruses from the H1 subtype does so by targeting an epitope that overlaps the HA receptor binding site (*48*) (Fig. 2).

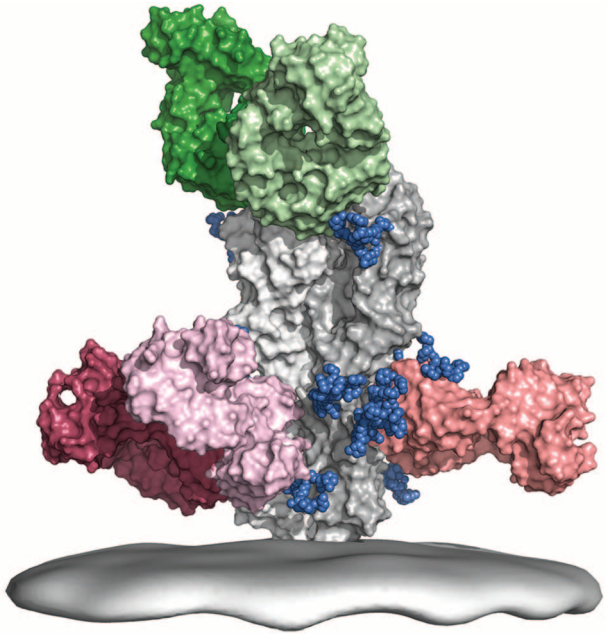

Fig. 2. A model of the influenza virus spike with select bnmAbs bound. Anti–influenza virus Fabs are 2D1 (top, light green, 3lzf) (*79*), CH65 (top, dark green, 3sm5) (*48*), FI6 (bottom right, link pink, 3ztj) (*44*), CR6261 (bottom center, salmon, 3 gbm) (*41*), and CR8020, (bottom left, red, 3sdy) (*43*). The HA trimer spike is from the 1918 strain (gray, 3r2x) (*56*), with Man_9 carbohydrates (blue spheres) modeled on with GlyProt (*77*) to give approximate dimensions for the attached glycans. EM-5021 membrane density was used to approximate the membrane surface.

The holy grail of influenza virus vaccine research is a universal vaccine that protects against all strains and subtypes of the virus, including seasonal and pandemic strains, and thereby renders the annual vaccine redundant. If the vaccine is long-acting as, for example, the yellow fever or smallpox vaccines, it could be given at a relatively young age and protect into old age. There is a great need for such a vaccine because current seasonal influenza vaccines tend to be less effective in elderly individuals as the capacity of the immune system wanes (*50*). Also, the vaccine needs to be reformulated annually to include the best predictions of which strains will circulate in any given year, and the match is not often optimal. Indeed, the efficacy and effectiveness of current influenza vaccines may be notably lower than previously thought (*50*). Furthermore, the vaccine takes some months to develop and manufacture, which can be problematic in the face of a potential pandemic or epidemic. As a new approach to the standard live or inactivated influenza vaccines, the identification of the stem of HA as a vaccine target has already led to design of "headless" HA immunogens, which have thus far produced modest improvements in neutralization breadth (*51*, *52*), and the engineering of scaffolds incorporating crucial structural elements recognized by the stem bnmAbs. A number of different immunization strategies are also being intensively investigated, resulting in some success in improving the breadth of neutralizing anti-influenza virus responses (*53*, *54*). It is also worth noting that the pandemic H1N1 vaccine does induce broadly cross-reactive antibodies to the HA stem region (*55*). Lastly, as with HIV, the influenza bnmAbs identify promising drug targets, and, indeed, a small protein has been computationally designed to bind to the HA stem region defined by bnmAbs and experimentally shown to neutralize influenza virus by inhibiting critical HA conformational changes required for fusion (*56*) by a mechanism similar to that used by the stem antibodies. Presumably, a small-molecule drug could also be targeted to this region and used to specifically inhibit influenza virus infection.

For HCV, the first bnmAb was isolated in 2001 (*15*) and shown to be broadly neutralizing

in 2005 (*16*). The mouse bnmAb and a human bnmAb identified later (*57*) define a continuous epitope on the E2 Env glycoprotein of the virus and inhibit interaction of E2 with the receptor CD81. In the past few years, a range of human and murine bnmAbs, mostly directed to discontinuous epitopes on E2, have been identified (*58*–*63*), with one report of a bnmAb directed to the E1 glycoprotein (*64*). Of note, a single bnmAb was shown to provide protection against HCV quasi-species challenge in an animal model (*60*). Unfortunately, no structure is yet available for E2 or the E1 E2 complex, limiting the value of the bnmAbs for immunogen and drug design. However, the crystal structure of a human bnmAb complexed with a peptide corresponding to a continuous epitope on E2 (*57*) has recently been determined (*65*), providing a template for immunogen design.

For the highly antigenically diverse viruses described above, an effective vaccine would seek to induce antibodies that recognize conserved regions and neutralize as broadly as possible. Challenges will always remain because of the diverse sequences and constant antigenic variation in these viruses, which will lead to differences in neutralization sensitivity across the spectrum of circulating isolates and could lower overall efficacy of vaccine-induced antibody responses or, as in the case of dengue virus, enhance disease (*66*).

Lastly, we have focused here on harnessing the information derived from bnmAbs to help define vaccine and drug targets on highly antigenically diverse viruses. These bnmAbs may also have direct application as prophylactic and/or therapeutic passively administered reagents. For HIV in a prophylactic setting, one might consider systemic passive administration of bnmAbs for high-risk individuals or topical application in a microbicide. In a therapeutic setting, combining passively administered antibody with anti-retroviral drugs could be considered. One advantage of systemic antibody is long half-life, but a serious disadvantage is high cost. This problem could be ameliorated by the expression of bnmAbs from vectors such as adeno-associated viruses (*67*, *68*). For influenza virus, therapy with bnmAbs could be considered, especially given indications that antibody administration relatively late in disease course may be beneficial (*69*). For HCV, a clear application of bnmAbs is to prevent re-infection of the grafted liver that typically occurs after a liver transplant (*70*).

Summary

Highly antigenically diverse pathogens are a major health concern and are particularly difficult to counter through vaccination. The generation of a whole new armamentarium of broadly neutralizing antibodies to several highly antigenically diverse viruses in the past 3 to 4 years has revealed new and unexpected vulnerabilities in these previously impregnable viruses. The stage is now well and truly set for rational vaccine design based on exploiting these vulnerabilities. Immunogen design based on computational approaches is advancing rapidly (*71*, *72*). Small-animal models expressing human antibody repertoires suitable for immunogen evaluation are being evaluated and developed. Technologies for the detailed evaluation of antibody responses, including deep sequencing and single B cell approaches, will facilitate iterative improvements of immunogens. Immunization strategies based on a better understanding of the antibody requirements for broad neutralization and of the roles of innate immunity and T cell help in eliciting the appropriate antibody responses will also likely be crucial. The challenges are manifest, but the advances made against HIV-1, influenza viruses, and HCV may have ramifications and implications that go well beyond these highly diverse viruses to the many other microbial pathogens that threaten the health and well-being of mankind.

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Dennis R. Burton, Pascal Poignard, Robyn L. Stanfield and Ian A. Wilson

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