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Broadly neutralizing antibodies against influenza virus and prospects for universal therapies

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Abstract

Vaccines are the gold standard for the control and prevention of infectious diseases, but a number of important human diseases remain challenging targets for vaccine development. An influenza vaccine that confers broad spectrum, long-term protection remains elusive. Several broadly neutralizing antibodies have been identified that protect against multiple subtypes of influenza A viruses, and crystal structures of several neutralizing antibodies in complex with the major influenza surface antigen, hemagglutinin, have revealed at least 3 highly conserved epitopes. Our understanding of the molecular details of these antibody-antigen interactions have suggested new strategies for the rational design of improved influenza vaccines, and has inspired the development of new antivirals for the treatment of influenza infections.

Evasion of host antibody responses

Because antibodies play a central role in the recognition and elimination of invading microbes, many pathogens have developed strategies to evade the humoral immune response. Many viral pathogens, such as influenza and HIV, have evolved low-fidelity polymerases that result in high mutation rates [1]. While this process leads to a large number of deleterious mutations that inactivate or attenuate individual virus particles, the large diversity of the resulting virus quasispecies allows these viruses to rapidly adapt to a changing environment and escape immune recognition by the host. Thus, the main challenge in developing vaccines that elicit a more broadly neutralizing antibody response is to counter the unrelenting variation generated by the virus by harnessing the equally diverse repertoire of the immune system.

Despite the hypervariability in the amino-acid sequence of these viral surface antigens to escape recognition by neutralizing antibodies, functional constraints can severely restrict the variability in key locations. For example, attachment is typically mediated by a specific interaction between the viral surface protein and a receptor on the target cell. Any mutations affecting these surfaces are more likely to interfere with receptor engagement and reduce the fitness of the virus, making this region less prone to mutation. Similarly, regions of the viral antigen that are important for membrane penetration or fusion are expected to be less tolerant of mutations. As a result, regions of the viral surface protein that carry out functions

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essential for infection and replication are weak points in the virus' defenses and present sites of vulnerability for recognition by more cross-reactive and broadly neutralizing antibodies. The influenza virus hemagglutinin (HA) is required for attachment to sialic acid receptors and, after endocytosis, for fusion of the viral and cellular membranes (Fig. 1). Thus, the receptor binding site on the HA1 subunit and the fusion machinery of the HA2 subunit are prime targets for antibody intervention.

Broadly neutralizing antibodies

Broadly neutralizing antibodies against a number of highly variable viruses have been reported, including hepatitis C [2–4], dengue [5], RSV [6], and influenza [7–15], but they have been explored most fruitfully and most abundantly so far in the case of HIV. Neutralizing antibodies against HIV target the Env protein (gp120/gp41), which is functionally and perhaps even distantly related evolutionarily to influenza HA [16]. A number of broadly neutralizing antibodies targeting Env have been isolated, targeting four distinct epitopes: 1) the CD4 receptor binding site, 2) the membrane-proximal external region (MPER), 3) conserved glycan structures, and 4) the V1/V2/V3 loops and associated glycans. Some parallels can be drawn between these conserved elements of the HIV spike and the influenza hemagglutinin, and similar classes of antibodies are now being generated against flu. In particular, antibodies against the stem and the sialic binding pocket of influenza HA have been identified (epitopes that are functionally homologous to the HIV env MPER and CD4 binding site, respectively) and will be discussed in detail below. However, no clear examples of antibodies targeting glycans on HA have yet been reported, suggesting this may be a fertile area for future discovery efforts, although the density of glycans on the HA does not approach that of HIV-1 Env.

Early work on bnAbs against influenza

In contrast to the relatively large number of broadly neutralizing antibodies known for HIV, until recently only one such cross-protective antibody against influenza had been identified. Antibody C179 was isolated many years ago from a mouse that had been immunized with H2N2 virus, but was later found to cross-neutralize H1, H2, H5, H6, and H9 subtypes [7,17,18]. All of these subtypes are members of the group 1 hemagglutinins, one of the two major subdivisions of the influenza A viruses. In contrast, none of the group 2 hemagglutinins are bound or neutralized by C179, suggesting that the epitope is only fully conserved in some group 1 viruses. Two lines of evidence suggested that C179 binds the HA stem. First, unlike essentially all other neutralizing antibodies isolated previously, which bind epitopes near the top of the HA “head” and interfere with receptor binding, C179 has no effect on virus attachment [7]. Instead, it was found to act by blocking membrane fusion, consistent with binding distant from the receptor binding site and possibly binding to the membrane fusion subunit, HA2. Second, two virus escape mutations were identified in the stem, in a region that was otherwise well conserved among neutralized strains and divergent in non-neutralized subtypes [7]. Taken together, these data suggested that C179 binds the HA stem of several group 1 subtypes, but the molecular details of the C179-HA interaction remained elusive, and it was unclear whether similar antibodies were present in the human immune repertoire.

Human antibodies with broad activity specific for group 1 viruses

The next major advance in the field came ~15 years later, with the discovery of a novel class of human antibodies encoded by the V_H1-69 gene [9–12]. CR6261 [9,10] and F10 [11] are the best studied among these first reported antibodies from this family. These antibodies were all isolated by phage display and are all remarkably similar to one another at the sequence level and had a relatively small number of mutations from the germline antibody

sequence. Further, these antibodies had very similar patterns of reactivity and neutralization when tested against a large number of influenza A viruses and hemagglutinin (Fig. 2A). Like C179, CR6261 and F10 exhibit broad activity against group 1 hemagglutinins, including the H1, H2, H5, and H9 subtypes. Importantly, CR6261 and F10 are protective in mouse models, suggesting that these antibodies may have therapeutic potential and that immunogens that elicit similar antibodies may result in broader immunity than current vaccines. Unlike all structurally characterized antibodies studied previously, which bind the HA1 “head”, crystal structures of CR6261 bound to H1 and H5 HA, and F10 bound to H5 HA revealed that both of these antibodies recognize the same, conserved epitope in the stem (Fig. 2B). Remarkably, these antibodies bind HA using only their heavy chains, whereas antibodies against protein antigens typically employ both heavy and light chains for binding. The epitope lies close to the virus membrane, and consists of an α -helix from HA2 and adjacent loops derived from HA1. A comparison of the antibody contact residues on the HA in the pre- and post-fusion states suggests that the epitope is highly conserved due to functional constraints on the protein sequence. While in the pre-fusion state of the HA, the epitope is solvent exposed and accessible to the antibody, many epitope residues make intramolecular interactions in the post-fusion state that likely are critical for efficient membrane fusion. Consistent with the location of the epitope in the membrane fusion subunit, far from the receptor binding site, the V_H1-69 antibodies have little effect on virus attachment. However, the binding of CR6261 and F10 inhibit key conformational changes in the hemagglutinin that drive the fusion of the viral and endosomal membranes. As a result, release of the viral RNA from the endosome is blocked and presumably the virus particle is ultimately degraded in the lysosome.

Human antibodies with broad activity specific for group 2 viruses

In contrast to the now relatively large number of V_H1-69 antibodies that have been identified against group 1 HAs, few antibodies thus far been reported with broad activity against group 2 HAs. As group 2 viruses are no more antigenically diverse than the group 1 subtypes, it is unclear whether the dearth of antibodies specific to group 2 HAs represents a unique obstacle to the neutralization of this lineage, limitations in the technologies used to screen large numbers of antibodies, or simply an under-explored subset of the anti-influenza repertoire. However, one human antibody with broad activity against group 2, called CR8020, was recently reported [13]. Unlike most of the V_H1-69 antibodies discussed previously, which were identified using phage display, CR8020 was isolated using cell-based methods, by screening immortalized antibody secreting cells. CR8020 was found to bind HAs from all six group 2 subtypes and neutralize H3, H7, and H10 viruses in vitro (Fig. 3A). In addition, CR8020 protects mice from lethal challenge with H3 and H7 viruses, the two subtypes in group 2 of greatest concern for human health. A crystal structure of CR8020 bound to H3 HA revealed that, like the V_H1-69 antibodies specific for group 1 viruses, CR8020 also binds an epitope on the HA stem (Fig. 3B). However, the CR8020 epitope is largely distinct from the site recognized by CR6261/F10, and is even lower down on the HA stem (Fig. 3C). Thus, the structure of the CR8020 complex has defined a second site that may be targeted by antiviral therapies. Many of the residues on HA that are contacted by CR8020 map to key elements of the membrane fusion machinery, such as the fusion peptide, suggesting that variation in the epitope may be functionally constrained. CR8020 blocks membrane fusion, perhaps by preventing the efficient release of the fusion peptide from its location in the pre-fusion HA. Interestingly, CR8020 also blocks the proteolytic activation the HA0 precursor to HA1 and HA2 and may, therefore, prevent the maturation of nascent virions. Further work is warranted to explore how blocking maturation may contribute to CR8020's activity and whether this strategy is employed by other antibodies.

Human antibodies against both group 1 and 2 viruses

HAs from human influenza viruses that have caused pandemics come from group 1 (H1 and H2) and group 2 (H3). Further, zoonotic subtypes with the potential to trigger future pandemics are also found in both groups (H5 and H9 from group 1, H7 from group 2). A universal therapy for influenza will need to be effective against both groups (as well as influenza B), and consequently, there is increasing interest in antibodies that can span these two phylogenetic lineages.

Perhaps the first antibody that was reported to neutralize both group 1 and group 2 viruses was S139/1 [8]. S139/1 is a murine monoclonal antibody that was isolated as a hybridoma from a mouse immunized against an H3 virus. Further study revealed that S139/1 has activity against both group 1 (H1, H2, H5, H9, and H13) and group 2 (H3) viruses (Fig. 4A). Most remarkably, S139/1 interferes with virus attachment, suggesting that it binds the HA1 head. Based upon escape mutants selected by growth of virus in the presence of S139/1, its epitope is likely close to the receptor binding site (Fig. 4C). This location is in stark contrast with essentially all other broadly neutralizing antibodies to influenza described to date, which bind to the stem, and is surprising since the head is significantly more variable than the stem. While the molecular mechanism by which S139/1 accomplishes group 1/group 2 cross-reactivity remains elusive, it seems that some regions of the HA1 head, such as the receptor binding site, may be sufficiently well conserved to allow such broad activity. It is worth mentioning that several other antibodies have subsequently been reported that also target the head, although the modest breadth of these clones seems to be restricted to single subtype [19–21]. However, the structure of one such antibody, CH65, which cross-neutralizes a large subset of H1 viruses, revealed that its epitope is centered on the receptor binding site, but contacts hypervariable residues that limit its cross-reactivity to one subtype. Taken together, these head antibodies confirm the notion that functional constraints imposed by the essential interaction with sialic acid receptors restrict variation in the receptor binding site and allow broad antibody recognition, providing that the antibodies can avoid in large part the surrounding hypervariable regions

In contrast to the approach to cross-group neutralization taken by S139/1 and CH65 against the HA1 head, one other antibody has been reported that achieves exceptionally broad activity by targeting an epitope in the stem. FI6 was isolated by high-throughput screening of immortalized antibody secreting cells, and was found to bind both group 1 and group 2 viruses, including 11/16 HA subtypes [15](Fig. 4B). FI6 neutralizes H1 and H3 viruses in vitro, protects mice from challenge with H1, H3, and H5 viruses, and inhibits the replication of virus-like particles pseudotyped with H7 HA. While further work will be needed to define its full breadth of neutralization, FI6 may be able to neutralize the majority of influenza A virus subtypes. Crystal structures of the antibody bound to H1 and H3 HAs revealed that FI6 binds to an epitope on the stem that is very similar to that of CR6261 and F10 (Fig. 4, C and D). However, FI6 recognizes the epitope in an entirely different way, using both light and heavy chain, particularly HCDR3. Thus, FI6 sheds light on some previously puzzling aspects of neutralization by CR6261. In particular, it was noted previously that the CR6261 epitope is highly conserved across all 16 influenza A subtypes, yet CR6261/F10 and most other V_H1-69 antibodies against the stem are group 1 specific. While possible explanations for the lack of reactivity with group 2 HAs were proposed, such as interference from a nearby glycan, the cross-reactive binding of FI6 clearly demonstrates that this surface on HA is indeed well conserved across groups. FI6 appears to avoid the group 2 glycan by gently nudging the sugar moiety out of the way so as to access only the highly conserved protein residues. In light of these findings, it will be interesting to explore this epitope further in order to better define the structural requirements for antibodies to achieve cross-group neutralization.

Future outlook

Over the last few years, our understanding of the antibody response against influenza HA expanded in ways that may have been unimaginable even 5 years ago. In 1996, we knew that cross-reactive murine antibodies like C179 could exist [7], yet little progress had been made in the 13 subsequent years towards understanding how this antibody worked and its implications for human health. Until very recently, it was unclear whether humans could even generate such a broadly neutralizing antibody against flu. But in a very short period of time (2008–2011), literally hundreds of broadly neutralizing antibodies have been isolated, targeting at least 3 distinct sites on HA: the CR6261/F10/FI6 (V_H1-69) epitope (stem region [10,11,15]), the CR8020 epitope (stem region, second site [13]), and the HA1 head [8].

A large amount of data has been generated on influenza over a period of just a few years, and the challenge in the future will be to apply this new knowledge towards the development of improved therapies and vaccines. In the near term, the antibodies themselves are attractive lead molecules for further clinical development, and many of the antibodies discussed here are in development as monoclonal antibody therapies. Taking this one step further, one could imagine trying to take what we have learned from these antibodies and apply it in a new way. For example, these antibodies have effectively identified weak spots in the virus defenses, which can now be exploited by non-immunoglobulin therapies. Binding proteins, such as HB36 and HB80 [22], which mimic to some extent the interaction with the same epitopes as the broadly neutralizing antibodies, may be a viable alternative to antibody therapies and have several distinct advantages. Such binders can be produced more cheaply than antibodies due to high yields from bacterial expression, may be easier to administer (as small proteins and peptides are more orally bioavailable and can even be delivered via an inhaler), and because they are structurally unique, they may be able to achieve results that antibodies cannot. For example, unlike antibodies, which use loops to recognize antigens, HB36 and HB80 interact with HA predominantly using a single α -helix, and the best HB80 variants are even more cross-reactive than the antibody that inspired its design, CR6261. More challenging still, but perhaps with greater reward and wider applicability, would be the design or identification of small molecules that target these antibody binding sites and similarly inhibit virus entry and replication. Unlike protein therapies, such as monoclonal antibodies or small proteins like HB36/HB80, small molecules are the mainstay of the pharmaceutical industry, as they are more convenient (frequently formulated as a pill instead of an injectable like most biologics) and can be used more widely.

However, the ultimate goal, perhaps still on the horizon, is a universal vaccine for flu. The antibodies that we have described here represent optimal solutions to the neutralization of influenza A viruses. If we could re-elicite such antibodies with a vaccine, and also find antibodies that cross-react with influenza B, it may be possible to confer life-long immunity (or at least, longer-lasting and broader protection). The identification and molecular understanding of how broadly neutralizing antibodies function is just a first step towards this goal, but many obstacles remain to be overcome. The challenge will be to develop strategies to selectively stimulate the B cell clones that produce antibodies like CR6261, CR8020, FI6, and S139/1. This has proved to be quite challenging for other viruses such as HIV, where a number of high quality antibodies have been identified, but there has been little success in re-eliciting a similarly broad response by vaccination. However, there are several reasons to think that influenza may be a more tractable target, at least in the near future. Most notably, the influenza envelope protein, hemagglutinin, is much more stable and homogeneous than HIV gp120/gp41. Moreover, a number of high-resolution crystal structures of the hemagglutinin ectodomain have been determined, including the first decades ago [23], but the structure of a native HIV trimer is a highly sought after but a yet elusive goal.

Furthermore, many of the broadly neutralizing antibodies to HIV-1 are highly mutated compared, for example, to the V_H1-69 stem binding antibodies that are remarkably close to the germline [24,25]. Thus, many of the roadblocks in HIV are entirely absent in the case of influenza. Consequently, efforts in rational vaccine design can focus on immunofocusing, re-engineering the HA to eliminate or mask immunodominant regions, or novel immunizations strategies that favor the production of a broadly neutralizing response. Considerable progress has already been made in this regard. In an order to try to focus the immune response against the more conserved stem region instead of the head, several groups have created “headless” HA immunogens by deleting most of HA1 [26–29]. While these efforts have been met with modest success, perhaps the most effective approach thus far has been simply changing the way vaccination is performed. By immunizing with a series of antigenically distinct HAs [14] or by using a two-stage prime-boost strategy [30], a much more broadly neutralizing response can be achieved. Indeed, this success is consistent with recent observations that following vaccination or infection with the 2009 H1N1 virus (which was antigenically divergent from previously circulating H1N1 strains), the frequency of stem antibodies similar to CR6261 is enhanced significantly [31]. Thus, while there are clearly many questions remaining, the future of antibody-guided rational vaccine design remains promising and we may be closer to a universal influenza vaccine than anyone would have guessed.

Highlights

Broadly neutralizing antibodies to influenza virus
 Three major protective epitopes on influenza hemagglutinin
 Design of novel immunogens
 Therapeutic antibodies and small proteins

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Figure 1. Structure of the major influenza viruses surface glycoprotein, hemagglutinin
HA is a trimer consisting of three identical copies of the HA protein (one protomer is colored, the other two are in gray). Each protomer contains two subunits, HA1 (red) and HA2 (blue). HA1 is the receptor binding domain and contains the sialic acid binding pocket. A human $\alpha(2,6)$ sialoglycan receptor is depicted (yellow spheres). HA2 contains the membrane fusion machinery. Broadly neutralizing antibodies can block receptor engagement and virus attachment or they can block membrane fusion.

Figure 2. V_H1-69 mAbs, such as CR6261 and F10, have broad activity against group 1 influenza (A) Phylogenetic tree of the 16 influenza A virus subtypes, classified into two major groups (1 and 2). Subtypes bound or neutralized by CR6261 or F10 are highlighted in red, and include most group 1, but no group 2 subtypes. (B) CR6261 and F10 recognize essentially identical epitopes on the HA stem. HA is depicted as a molecular surface, with the HA1 and HA2 subunits from one protomer highlighted in pink and cyan, respectively. CR6261 is shown as red and yellow ribbons (heavy chain and light chain, respectively).

Figure 3. CR8020 has broad activity against group 2 influenza A

(A) Phylogenetic tree of the 16 influenza A virus subtypes, classified into two major groups (1 and 2). Subtypes bound or neutralized by CR8020 are highlighted in red, and include all group 2, but no group 1 subtypes. (B) CR8020 recognizes an epitope on the HA stem proximal to the viral membrane. HA is depicted as a molecular surface, with the HA1 and HA2 subunits from one protomer highlighted in pink and cyan, respectively. CR8020 is shown as red and yellow ribbons (heavy chain and light chain, respectively). (C) The CR8020 epitope (green surface) is largely distinct from the CR6261/F10 epitope (lilac surface), but overlaps slightly with two residues shared between the two sites (orange surface).

Figure 4. Cross-group neutralization by FI6 and S139/1

Phylogenetic trees with subtypes bound or neutralized by FI6 (**A**) or S139/1 (**B**) highlighted in red. Note that both antibodies neutralize subtypes from both major virus groups (1 and 2). (**C**) Like CR6261 and CR8020, FI6 also recognizes an epitope on the HA stem. HA is depicted as a molecular surface, with the HA1 and HA2 subunits from one protomer highlighted in pink and cyan, respectively. FI6 is shown as red and yellow ribbons (heavy chain and light chain, respectively). While the precise location of its epitope is unknown, escape mutations suggest that S139/1 recognizes the HA1 “head”. (**D**) The FI6 and CR6261/

F10 epitopes overlap extensively (orange surface), with only a small number of HA residues being recognized by FI6 only (green surface) or CR6261 only (lilac surface).