Is It Possible to Develop a “Universal” Influenza Virus Vaccine?

Outflanking Antibody Immunodominance on the Road to Universal Influenza Vaccination

Davide Angeletti and Jonathan W. Yewdell

Laboratory of Viral Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Correspondence: jyewdell@niaid.nih.gov

Influenza remains a major human pathogen despite seasonal vaccination. At long last, there is energy and resources to develop influenza vaccines that provide more predictable and durable protection. Vaccines based on inducing antibodies to the conserved stem of the viral hemagglutinin (HA) have emerged as leading candidates for broadening population immunity and ultimately limiting antigenic drift. Here, we discuss the knowns and unknowns of HA-specific B-cell and antibody responses. In particular, we focus on how immunodominance sculpts antibody responses and drives antigenic drift. We propose a number of strategies to overcome immunodominance and improve the breadth and efficacy of antibody responses.

GREAT DEBATES

What are the most interesting topics likely to come up over dinner or drinks with your colleagues? Or, more importantly, what are the topics that *don't* come up because they are a little too controversial? In ***Immune Memory and Vaccines: Great Debates***, Editors Rafi Ahmed and Shane Crotty have put together a collection of articles on such questions, written by thought leaders in these fields, with the freedom to talk about the issues as they see fit. This short, innovative format aims to bring a fresh perspective by encouraging authors to be opinionated, focus on what is most interesting and current, and avoid restating introductory material covered in many other reviews.

The Editors posed 13 interesting questions critical for our understanding of vaccines and immune memory to a broad group of experts in the field. In each case, several different perspectives are provided. Note that while each author knew that there were additional scientists addressing the same question, they did not know who these authors were, which ensured the independence of the opinions and perspectives expressed in each article. Our hope is that readers enjoy these articles and that they trigger many more conversations on these important topics.

Editors: Shane Crotty and Rafi Ahmed

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THE PROBLEM

Seasonal influenza imposes a significant socioeconomic burden on humanity. Vaccination, the best hope for reducing the impact of influenza, is, even under optimal circumstances, effective in only 60% of individuals. The difficulty, of course, stems from the protean ability of influenza A virus (IAV) to rapidly escape existing immunity.

IAV evolved an error-prone polymerase that drives the rapid antigenic evolution of the two virion surface glycoproteins, neuraminidase (NA) and hemagglutinin (HA). Since the most potent antibodies (Abs) at neutralizing viral infectivity (neutralizing Abs [NAbs]) are directed to the head of the HA, amino acid substitutions in this region enable IAV to evade antibody (Ab)-based immunity.

Despite the obvious problem with antigenic drift, apparent for 70 years, the goal of current vaccines remains inducing NAbs to the HA head. Compounding the problem, vaccine quality is benchmarked by induction of ferret Abs-inhibiting IAV-mediated agglutination of avian erythrocytes. This is an imperfect proxy of neutralization, since blocking binding to erythrocytes versus the airway cells that support viral replication in vivo is not identical. Further, it ignores other mechanisms of virus neutralization, which include preventing fusion of viral and cellular membranes as well as innate immune cell-mediated eradication of virus-infected cells (DiLillo et al. 2014, 2016).

THE SOLUTION?

After decades of complacency, there is now great energy, interest, and, most importantly, substantial government resources directed at improving IAV vaccination. Of a number of feasible strategies, the most promising, in our opinion, is inducing Abs specific for the conserved stem of the HA that can neutralize strains within, and even between HA subtypes.

More than 20 years ago, Okuno et al. (1993) identified a neutralizing mouse monoclonal Ab (mAb) that binds to the stem of nearly all H1 and H2 HAs tested. Unfortunately, at the

time of publication, the world was inappropriately complacent regarding influenza. This important finding was essentially ignored until human monoclonal Abs were isolated with similar properties after 15 years had ticked away. Since 2008, dozens of HA stem-specific mAbs have been isolated that neutralize across and between HA subtypes (Krammer and Palese 2015).

A number of these NAbs (bNAbs) have been characterized in fine structural detail bound to HA (Lee and Wilson 2015). They neutralize virus in vitro through several proposed mechanisms, including preventing:

1. Conformational alterations required for HA-mediated fusion of viral and cellular membranes, thus blocking viral cell membrane fusion in the endosome.
2. Proteolytic cleavage of HA to generate the fusion active form (though it is not clear that Abs could gain sufficient access to the Golgi complex, site of HA cleavage during biogenesis) (Ekiert et al. 2009).
3. Release from infected cells (Brandenburg et al. 2013).

Despite this, the impressive in vivo activity of antistem bNAbs appears to exclusively be based on mediating Ab-dependent cellular cytotoxicity (ADCC) of infected cells (DiLillo et al. 2014). Perplexingly, even bNAbs specific for the head are reported to depend on ADCC for in vivo activity (DiLillo et al. 2016), even if they block viral attachment. This intriguing finding demands an explanation, as it points to a major gap in our understanding of Ab-based neutralization.

THE STEM PARADOX

Original antigenic sin (OAS) describes the phenomenon whereby secondary immunization with a drifted IAV elicits antibodies that bind the priming virus more avidly than the boosting virus (Fazekas de St. Groth and Webster 1966). Given the conserved nature of stem epitopes, repeated exposure to a drifted series of viruses, as happens with humans in the natural course of

events, should preferentially boost responses to stem epitopes.

Flaunting OAS, stem-binding Abs are present in human sera at 0.1 $\mu\text{g}/\text{mL}$ in the general population, and increase only 1.5- to 2-fold following immunization with a partially heterologous IAV vaccine (swine origin IAV) (Sui et al. 2011). By comparison, the sera of children, who have only limited exposure to influenza, contain 100–500 $\mu\text{g}/\text{mL}$ of anti-HA Ab, presumably nearly all head-specific (Fig. 1) (Fazekas de St. Groth and Webster 1966).

To make these numbers real, animal studies reveal that, for therapeutic or protective effect, antistem mAbs must be present at $\sim 2 \mu\text{g}/\text{mL}$ (Throsby et al. 2008; Corti et al. 2011) or 20-fold higher than is achieved naturally, but surprisingly similar to standard neutralizing mAbs specific for the head (Mozdzanowska et al. 2003).

Perhaps the stem is intrinsically poorly immunogenic. But in a number of recent studies, stem antigens cleverly designed to achieve the native structure in the absence of the head appear to be highly immunogenic (Lu et al. 2014; Malajosyula et al. 2014; Impagliazzo et al. 2015; Yassine et al. 2015). This strongly implies that stem antibodies are victims of immunodominance (ID), the marked tendency of the immune sys-

tem to respond to complex antigens in a reproducibly hierarchical manner, with higher-ranking antigens sometimes suppressing responses to lower-ranking antigens (immunodomination). Indeed, adding glycosylation sites to the head to block antibody binding increased stem antibody responses up to 8-fold at the expense of head-specific antibodies in multiply immunized mice (Eggink et al. 2014), providing a clear case of head immunodomination over stem.

AB ID: A CENTRAL FEATURE OF VIRAL IMMUNITY

The term “immunodominant” first appeared in a classic 1966 review on Ab responses to *Salmonella* to describe the predominant Ab response to a bacterial oligosaccharide (Lüderitz et al. 1966). ID studies were eventually extended to IAV and other viruses by determining the frequency of hybridomas specific for defined antigenic sites (Staudt and Gerhard 1983). While the field was primed for a great leap forward with monoclonal Ab analysis, interest petered out as immunologists became obsessed with T-cell responses.

Consequently, antiviral T-cell ID has been fairly well characterized, particularly CD8^+ T-cell responses. The peptide-based nature of T-cell receptor recognition makes it far easier to quantitate responses than B cells, whose antibody receptors typically recognize discontinuous epitopes on viral proteins that require native structures, making it difficult to measure Ab responses at the level of individual antigenic sites. Remarkably, even at the level of individual proteins, essentially nothing is known about the prevalence of B cells, and very little is known about even the magnitude of Ab responses.

Unlike B- and T-cell responses, measuring Ab responses in body fluids is confounded by the problem of Ab affinity for the immunogen/antigen. The magnitude of responses measured in simple binding assays and more complex functional assays is governed by the product of Ab concentration and avidity for antigen. Few studies have measured these values in antiviral responses, and even fewer have used this information to explore the rela-

Figure 1. Cartoon representing the hemagglutinin (HA) trimer. The head (highlighted in orange) and the stem (highlighted in blue) domains of one monomer are shown with the amount of specific binding Abs present in human sera (Fazekas de St. Groth and Webster 1966; Sui et al. 2011). Stem-Abs are present at levels too low to confer in vivo protection (PDB: 1RVZ).

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tionship between Ab avidity and antiviral functional activity.

Ab responses are further complicated by the presence of many thousands of species that simultaneously compete for binding and exert allosteric affects after binding that can positively and negatively impact Ab binding to other sites on the same antigen. On top of this, antibodies interact with serum proteins (e.g., C1Q) that modulate their function and also innate immune cells that exert complex antiviral activities by secreting cytokines and cytotoxic molecules when they encounter virus-infected cells.

Rational vaccine design requires better understanding of how B-cell responses are influenced by the specificity of the naïve B-cell repertoire and the physical nature of viral immunogens, and in non-naïve individuals, the balance between naïve and memory B cells (and T cells), and critically, the presence of pre-existing Abs. This entails defining the ID hierarchy of Ab responses at the level of proteins, antigenic sites, and epitopes, and then delineating the underlying cellular and molecular mechanisms during naïve and memory responses.

Building a foundation of ID basics, Altman et al. (2015) found that approximately 2/3 of the serum responses of mice following immunization with inactivated IAV focuses on the HA head, with the remaining Abs recognizing NA, and to a lesser extent nucleoprotein (NP). Notably, there is a poor correlation of ID with virion protein abundance (copies per virions: M1,

2200, NP 530, HA 294, NA 23, other viral proteins each <10 [Hutchinson et al. 2014]). M1 did not induce a measurable Ab response, and NP ranked below NA, which, on a molar basis, might well be the most immunogenic protein (Fig. 2).

Most remarkably, the mouse ID Ab response hierarchy to IAV was recapitulated in lampreys, a nonjawed vertebrate that evolved a completely different type of immune cell Ab receptor, termed a variable lymphocyte receptor. As with mice, guinea pigs, and chickens, lamprey Abs recognized the five principal antigenic sites on the head, and binding for Abs from all species was abrogated by the same mutations that enable escape from mouse antibodies. Further, in all species tested, primary antistem responses were not detected.

These findings suggest that the rules governing B-cell ID are largely conserved between organisms, even those with a completely different basis of recognizing immunogens. This points to physical features of complex antigens as the governing principal in their immunogenicity. Pragmatically, this is very good news as it supports the use of a diverse range of animal models in designing human vaccines for IAV and other viruses.

DOES ID DRIVE ANTIGENIC DRIFT?

The sine qua non of HA is variability. With few known exceptions (Das et al. 2011), only a sin-

Figure 2. Pattern of immunodominance (ID) to influenza A virus (IAV) virion. Cartoon of a virion depicting hemagglutinin (HA) (blue), neuraminidase (NA) (red), M2 (violet), and ribonucleoproteins (green) (courtesy of the Centers for Disease Control website). Indicated is the relative abundance of surface and internal proteins (Hutchinson et al. 2014). The relative ID of serum Abs in response to infection (Altman et al. 2015) and immunization is not dependent on the number of proteins presented on the virion. n.d., not determined.

gle amino acid substitution in an epitope is needed to reduce the binding of any given Ab by several orders of magnitude, rendering it functionally non-neutralizing at physiological Ab concentrations. Whether this is a general feature of Ab–Ag interactions, or evolutionary honing of HA’s antigenic knife is an interesting and important question.

Mutants that escape individual monoclonal antibodies are generated at high frequency and present in virus populations at frequencies of $\sim 10^{-5}$ (Yewdell et al. 1979; Valcarcel and Ortin 1989). Human upper respiratory infections have been estimated to generate 10^{12} virions (Perelson et al. 2012), and more severe infection involving the lung would conservatively generate 100-fold more virions. In each person, then, escape mutant populations should exceed 10^9 single escape virions for each mAb, and 10^2 “double mutants” (i.e., mutants that escape neutralization from two mAbs with nonoverlapping epitopes) (Das et al. 2011).

With this vast number of escape mutants ripe for selection, ID can obviously influence drift if it limits selection pressure to a subset of antigenic sites. This problem would be exacerbated by OAS-mediated generation of lower avidity Abs, which would be easier to evade and therefore increase the repertoire of escape mutants. Early studies using mAb escape mutants (Natali et al. 1981) or monoclonal Fab fragments in competition assays (Wang et al. 1986) demonstrated great heterogeneity among humans in the magnitude of human serum responses to different antigenic sites. More recently, Linderman et al. (2014) and Huang et al. (2015) reported that, respectively, for two of 195, and two of 41 individuals, functional serum responses were focused on individual antigenic sites, as revealed by diminished activity in functional assays comparing wild-type virus to mutants with single amino acid substitutions. These individuals should immediately select escape mutants.

With the widespread application of deep sequencing technology, it is now possible to match a patient’s Ab response with the repertoire of variants selected. Although a comprehensive study awaits, Dinis et al. (2016) showed

the potential of this approach by sequencing upper airway swaps from infected patients, finding that mutants encoding changes in many amino acids in defined antigenic sites were present at high frequencies (from 1% to 25%). Because infection is likely based on less than 10 particles transmitted among patients (Varble et al. 2014), the variety of mutations detected by Dinis et al. and the low frequencies of most of the mutations can only mean that they arose during virus replication in these individuals.

These data imply that drift should occur rapidly. Each patient with a dominant response should immediately select escape mutants for transmission. From this perspective, the most remarkable feature of drift is not its speed but rather the opposite. H1 and H3 HAs accumulate only slightly more than one substitution per year between 1978 and 2008 in their antigenic regions (Bhatt et al. 2011). Importantly, N1 and N2 NAs accumulated amino acid substitutions in surface residues at essentially the same rate, pointing to the overlooked importance of the anti-NA Ab response. These rates reflect the accumulation of substitutions on the main evolutionary IAV tree, excluding the branches, which are pruned in less than 2 years on average (Bhatt et al. 2011) and not at the rate of evolution of the branches. The rapid extinction of most escape variant lineages points to their poor fitness in human populations, perhaps because of their inability to maintain amino acid substitutions as their fitness landscapes change (Das et al. 2013).

This pruning would appear to be a fortunate feature of antigenic drift; if the various branches persisted and expanded, efficacy would be even lower with currently used vaccines. On the other hand, faced with such antigenic diversity, humanity may have made greater efforts much sooner in searching for conserved targets for vaccines or in developing antiviral drugs targeted at the polymerase or other conserved targets.

STRATEGIES FOR OUTFLANKING ID

To reduce IAV’s burden on humanity, we need to outflank ID and modify the typical Ab response to vaccines and virus infections. In addition to providing better protection to individuals at the

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herd level, this should also retard antigenic drift. The goal is to reach a tipping point where the immune response of a sufficient fraction of humanity no longer permits drifted viruses to prevail at the population level. There are a number of strategies of interest and potential impact.

1. Develop vaccines that induce a balanced response against all of the globular antigenic sites to limit generation and transmission of escape mutants with changes in a subset of antigen sites.

- This will require a great deal more knowledge about ID. It is well worth the effort, however. In addition to its applications to IAV, this would likely have practical applications in many other circumstances. As a step in the right direction, Khurana found that adding the MF59 adjuvant to standard vaccines expands the Ab repertoire for head antigenic sites following human vaccination, while increasing Ab avidity as well (Khurana et al. 2011).

2. Develop vaccines that limit OAS.

- OAS compromises protection by reducing the average avidity of responding Abs to the challenge virus, which likely contributes to drift by enabling easier virus escape. Jacob and colleagues (Kim et al. 2012) have shown in the mouse model that a number of adjuvants (including a squalene adjuvant similar to MF59) prevent both future OAS to challenge virus when given with priming vaccination, and OAS induced by secondary challenge after exposure to immunogen when given without priming vaccination. While in theory OAS could boost Ab responses to conserved stem epitopes, in practice, stem responses seem to still be dominated by head-specific responses, despite their lower avidity.

3. Develop vaccines that limit B-cell differentiation.

- This highly counterintuitive strategy is based on the Keating et al. (2013) remarkable report that rapamycin, a mechanistic target of rapamycin (mTOR) inhibitor,

limits B-cell differentiation and Ab class switching, generating mostly IgM antibodies with high bNAb activity. It implies that either class switching itself from IgM to IgG (by decreasing Ab valence perhaps) or somatic mutation reduces the cross-reactivity of germline Abs. It is of obvious interest to determine the targets of such Abs. Whereas there would be great reluctance to use general pharmacologic inhibitors in mass vaccinations of healthy individuals, there are probably other treatments that would achieve the same effect. At the same time, it is worth noting that mTOR inhibitors have been shown to increase vaccine effectiveness in the elderly (Mannick et al. 2014).

4. Immunize with chimeric head-stem HA vaccines to selectively boost antistemic responses.

- This approach is based on selectively activating stem-specific memory B cells, since the chimeric head is derived from avian IAVs with the stem derived from circulating H1 or H3 viruses. It has the advantage of using standard vaccine-production technology already licensed for use in humans (Krammer and Palese 2015). A potential disadvantage is the induction of primary antibodies for the head of an irrelevant pathogen. This flips to an advantage, however, should the avian virus ever become widely transmissible in humans. If routine reimmunization is required, it would be necessary to use different heads to prevent head ID, but there are 15 other zoonotic HA subtypes to choose from, as well as H2 HA, which can be used in individuals born after 1968.

5. Immunize with stem-only constructs.

- This is the simplest approach and, in our opinion, the most likely to work as it completely bypasses the problem of head immunodominance.

BE CAREFUL WHAT YOU WISH FOR?

One potential problem applies to all stem-based strategies. There seems to be a poor correlation

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with the immediate B-cell response to vaccination and the long-term persistence of antistem serum Abs. Wrammert et al. (2011) and Huang et al. (2015) reported a far greater frequency of stem-specific Ab-secreting plasmablasts (50% and 8%, respectively) following vaccination than would be expected from the low titers of Abs present in plasma. This suggests that either stem-specific B cells are disfavored from becoming long-lived plasma cells, or that antistem Abs are cleared from circulation, perhaps in both cases because of self-cross reactivity. Stem-specific antibodies may have a marked tendency toward “polyreactivity” (i.e., binding to charged surfaces such as assay plates, cellular lipids, and nucleic acids) (Andrews et al. 2015). Whether because of structural constraints or natural selection for immune evasion, the HA stem may be sufficiently related to self-antigens to cross-react serologically, perhaps even to the point of autoimmunity. This would be curtains for stem-based vaccines.

Another potential problem is IAV escape from antistem immunity. Antistem Abs readily select for escape mutants in vitro (Chai et al. 2016). While it is generally assumed that stem escape mutants will exhibit compromised fitness in humans (based on the high conservation in the stem among IAV isolates), this must be established empirically initially in animal studies, but ultimately in human populations if antistem vaccination is ever used on a wide scale.

LONG-TERM PROSPECTS

It is highly likely that effective drugs targeting various viral proteins (the polymerase is a prime target) can be developed for IAV. While these will be a boon for patient care, particularly very ill patients, they will not solve the problem of seasonal influenza unless they are taken by a high percentage of individuals prior to infection, a highly unlikely proposition.

The burden of IAV can only be effectively addressed by vaccination. Whereas T-cell-based vaccines are possible, they have a greater potential for exacerbating immunopathology, and also suffer from allowing considerable viral rep-

lication, which may not effectively limit transmission.

We have focused on Ab responses to HA, but NA is also an important Ab target, and we should not write off M2 and NP as targets for ADCC. Above all, we need to demonstrate patience and not rashly write off discouraging results in clinical trials. The development of cancer therapies provides a shining example of the need to exploit multiple approaches and revisit failed approaches as advances in knowledge and technology improve the odds.

IAV has plagued humanity for thousands of years. It is about time we got serious about minimizing its impact.

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Davide Angeletti and Jonathan W. Yewdell

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