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Viva la Revolución: Rethinking Influenza A Virus Antigenic Drift

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Abstract

Rapid antigenic evolution of the influenza A virus hemagglutinin has precluded developing vaccines that provide durable protection. The yearly costs of influenza (circa \$10¹¹ in the USA alone) easily justify investments in better understanding the interaction of influenza with antibodies and other inducible elements of the immune system that potentially limit or circumvent antigenic variation. Here, I summarize exciting new findings that offer the possibility of a quantum improvement in vaccine efficacy, focusing on studies clearly documenting robust neutralizing antibody responses to the conserved stem region of the hemagglutinin.

Introduction

Influenza A virus (IAV) expresses two complementary glycoproteins whose functions bookend the infectious cycle. The hemagglutinin (HA) initiates infection by attaching virus to host cell surface sialic acids and catalyzing the fusion of viral and cellular membranes. The neuraminidase (NA) ends the infectious cycle by releasing nascent virions from the infected cell surface. Antibodies (Abs) to HA block viral entry and play a critical role in immunity following infection or vaccination. Due to the rapid appearance of HA-escape mutants in human populations, vaccines must be constantly reformulated, sometimes even yearly, leading to poor population coverage and high disease incidence with considerable mortality, morbidity, and enormous costs. The cost of influenza in the USA (estimated to be nearly \$10¹¹ per annum) [1] is roughly 3-times the *total* NIH budget, putting the investment in this one area of biomedical research in perspective to the costs of ignorance.

The enormous savings promised by improved influenza vaccines provide clear incentive for deeper understanding of immunity to influenza. The increase in funding of influenza research in the past decade, triggered by fears of highly lethal avian IAVs becoming highly transmissible and accelerated by the introduction of swine origin IAV in 2009, has generated a quantum leap in understanding human immunity to IAV. “Facts” about influenza immunity are crumbling before a tsunami of new information. It now seems possible that future generations will be genuinely puzzled over why it was so difficult to develop an effective influenza vaccine strategy.

Here, I will review recent progress in several areas of influenza immunity. Although I focus on recent remarkable progress in potentially thwarting antigenic variation through induction of neutralizing monoclonal antibodies (mAbs) that recognize conserved HA domains, to start I will highlight some surprising findings in other areas with implications for rethinking immunity to IAV and broadening vaccine coverage.

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Cross-reactive Immunity to Internal Proteins

Adaptive innate cellular immunity

In the initial encounter between host and pathogen, the innate immune system plays a critical holding action for five or more days until T and B cells can reach sufficient numbers to participate in the immune response. Natural killer (NK) cells potentially play an important role during this phase, though their role in human influenza is unclear. Classically, NK cells, like other elements of the innate immune system, recognize large classes of antigens, unlike T or B cells, which demonstrate exquisite specificity and limited degeneracy for cognate antigens. NK cells divide upon activation, but this does not lead to generation of a memory pool that exhibits more robust responses upon challenge; again believed to be the exclusive province of B and T lymphocytes.

Extending prior work that demonstrate NK cell memory to haptenylated antigens in mice [2], Paust *et al.* [3]** showed that immunization of mice with virus like particles containing IAV M1 protein produced by insect cells induce a protective NK response to influenza infection that demonstrates the hallmarks of adaptive immunity with regard to protein specificity and memory.

This remarkable finding raises a number of questions regarding the molecular basis for NK antigen specificity, the duration of immunity, and not the least, the relevance to human immunity to IAV. Regardless of practical applications, it provides a shining example of the importance of disregarding dogma and following the data to discovery and enlightenment.

Nucleoprotein Based Humoral Protection

Many moons ago (371, to be precise), while investigating the basis for cross-subtype recognition by CD8+ T cells, I helped to show that nucleoprotein is expressed on the surface of infected cells [4,5] (sadly, a red herring for T cell recognition), and can serve as a target of antibody-targeted cellular cytotoxicity (ADCC) [6]. Though the pathway that traffics NP to the surface remains an intriguing mystery (that likely functions for many viral and host “internal” proteins), the potential importance of surface of NP as a target for protective Abs was resurrected by Randall and colleagues [7–9], who demonstrated a clear protective effect in mice following immunization with NP. The mechanism likely involves Fc-based association with NK cells or other innate immune ADCC effector cells.

While this remains to be determined, note that there has been tremendous recent progress in understanding how oligosaccharides on Abs govern their interaction with Fc receptors and enable the immune system to distinguish between Abs secreted by distinct B cell subtypes, enabling “time stamping” of B cell responses [10]. This fine control on Ab function means that functional assays must be used in place of simple binding assays (*e.g.* ELISA) to accurately assess the anti-viral activity of Abs.

What’s in the Vaccine Anyway?—Vaccinologists might consider the preceding sections to be highly esoteric and of little practical import. Aside from the obvious possibility of devising vaccines to exploit cross-reactive immunity, there is a highly pragmatic, if completely ignored consideration. Standard seasonal influenza vaccines, ostensibly consisting of purified HA and NA, can contain considerable amounts of contaminating M1 and NP [11]. It is clearly of interest to correlate the protection afforded by vaccination with the immunogenicity of these highly conserved proteins in terms of Ab, T cell and NK/ADCC responses.

Rethinking Drift

The Big Question: Why Flu?

HA has 4 to 5 major antigenic sites recognized by neutralized antibodies [12,13], and mAbs against each site select escape variants present at a frequency of $\sim 10^{-5}$ in virus populations [14]. The effects of mutations are site-limited [15], meaning that multiple mutations, which simultaneously occur at low frequency (10^{-5n} , where n = number of sites mutated), are required to change multiple sites. This raises the question of how IAV escapes polyclonal Ab responses. Do some individuals only respond to one (or two) sites, enabling sequential selection?

This begs the larger question, why don't many of the other highly mutable RNA viruses that cause acute human infections demonstrate significant drift? Parainfluenza viruses, for example, have a similar number of antigenic sites recognized by neutralizing Abs on their attachment protein [16], and demonstrate a similar frequency of escape mutants [17], yet exhibit minor variation over decades of circulation [18,19]. Is this due to the greater structural plasticity of IAV HA that enables it to accept substitutions that alter antigenicity without complex multiple compensatory substitutions to enable proper folding/assembly [20]? Is it due to unique features of IAV replication the immune response it induces, global population size, number/type of animal reservoirs, or geographical/immunological/age-related metapopulations?

Surprising Insights from Modeling Drift in Mice

To better understand IAV drift, Hensley *et al.* [21]** turned back the clock to a drift model published in the 50's based on vaccination of outbred mice with formaldehyde-inactivated PR8 (a prototype H1N1 virus) dosed to induce a partially protective Ab response [22]. Essentially repeating the passage protocol verbatim, Hensley *et al.* found that mice rapidly selected PR8 escape mutants with up to a 20-fold decrease in virus neutralization (VN) titer. For each of three independent isolates, escape was based on a single amino acid substitution in the HA. Two of the substitutions occurred in the Sa and Sb sites (as defined by Caton *et al.* [12]). The Sb mutation reduced the interaction of HA with serum Abs by $\sim 50\%$, confirming the immunodominance of the Sb site in mice [23] (and raising the issue of the mechanisms underlying immunodominance in Ab responses, of which little is known). The mutation in the Sa site had little effect on serum Ab HA antigenicity, and the third mutation occurred in a non-antigenic region. The common feature of the substitutions is that all increased HA avidity for sialic acid receptors (avidity is the net affinity that results from multivalent interactions). Co-infecting vaccinated mice with *wt* virus and a mutant with an avidity-enhancing substitution in the receptor-binding pocket confirmed rapid immune selection of the receptor mutant.

This points to an oft-neglected feature of antibody mediated neutralization: it represents a ternary competition between receptor and antibody for virus. In the face of a partially neutralizing antibody response, virus can escape by increasing receptor avidity. Partial neutralization must be a common occurrence in nature due to many factors, including variability in human immune responses and particularly for IAV, infection with drifted variants that demonstrate fractional escape from the Ab response to (un-drifted) parent. Selection of mutants with increased viral receptor avidity ("absorptive mutants") was initially proposed by Fazekas de St Groth [24], and subsequently confirmed using a mixture of mAbs to select escape mutants *in vitro* under partially neutralizing conditions [25].

Predictably, increased viral receptor avidity incurred fitness costs *in vivo*. Re-passage of the absorptive mutants in naïve mice selected HA-single substitution mutants with diminished receptor avidity. Despite the lack of Ab pressure, some of the substitutions occurred in

antigenic regions. Indeed, analysis of previously described mAb escape mutants [12] unexpectedly revealed that approximately half the mutants demonstrate alterations in avidity. There was a clear positive relationship between net charge change of substitutions and avidity, retroactively consistent with a prior study on H3 escape mutants [26] (interestingly, epidemic H3 (but not H1 viruses) demonstrate a clear increase in net positive charge during their first 20 years evolution in humans [27]). Similarly, single amino acid substitutions in the major antigenic sites of the H5 HA were shown to modulate receptor avidity [28,29] and immune escape in VN and HI assays [29]. Selection of adsorptive mutants is likely to be relevant for other pathogens subject to antibody competition for attachment, and should be relatively easy to detect in highly variable viruses like norovirus, which demonstrate rapid antigenic drift in humans [30].

These findings demonstrate that single substitutions in the HA globular domain can simultaneously modulate antigenicity and receptor binding, confounding retrospective analysis of genetic variation in HA. The situation is complicated further by the occurrence of compensatory (or “epistatic” changes within HA to maximize viral fitness following selection [31]*, and even between HA and NA [32]*. Thus, for example, substitutions in defined antigenic sites could be selected not for Ab escape but to modulate receptor avidity or to improve HA folding/function in response to selection for an epistatic residue. Alternatively, amino acid substitutions in the receptor site that modulate avidity may represent substitutions that modulate avidity in the opposite direction from their epistatic partners. Moreover, substitutions selected to modulate receptor avidity will inevitably modify receptor specificity for various sialic acid terminated glycans [21] and *vice versa*.

From trees to forest: even in the simplest species (viruses), evolution is complicated, and oversimplified analysis leads to all sorts of errors, including those with practical ramifications in interpreting sequences for choosing vaccine strains.

The Holy Grail: Broadly Neutralizing Abs

Nearly 20 years ago Okuno *et al.* [33] reported a mAb, C179, that neutralizes strains across H1 and H2 subtypes (but not H3), likely based on its interaction with a conformational epitope in stem region comprised of residues from HA1 and HA2 chains. C179 derives from a mouse immunized twice with infectious H2N2 by intraperitoneal injection (i.e. nothing unusual). It fails to block viral attachment (as measured by hemagglutination inhibition (HI) assay, which is a convenient, highly useful, but imperfect measure of blocking viral attachment [34]), yet exhibits weak VN activity *in vitro*, likely based on blocking HA-mediated fusion, and is capable of reducing mortality of H1, H2 and H5 subtype viruses in mice [35,36]. Unlike typical attachment-inhibiting neutralizing mAbs, C179 does not prevent infection, but limits spread *in vivo*. Although amino acids constituting the epitope are highly conserved in H1, H2 and H5 viruses, neutralization escape can be conferred by a single substitution in HA2 [33].

These studies, which clearly established the possibility of inducing broadly cross-protective antibodies, remained unexploited for 15 years. A critical breakthrough was applying methods to generate human mAbs to IAV [37,38].

Throsby *et al.* [39]** identified cross-reactive, stem reactive mAbs (StRAbs) with VN activity in a combinatorial expression library generated from memory B cells from patients recently immunized with seasonal influenza vaccine. Library Abs were converted to standard IgG1 antibodies, which were used for further characterization. The cross-reactive epitopes recognized were similar to the C179 epitope, and the mAbs did not block virus attachment as measured by HI. Epitopes were conserved across H1 and H9 clade viruses (the 16 HA subtypes fall into 4 clades: the H1 clade contains H1, H2, and H5 of its seven

subtypes, the H9 clade has 3 subtypes, and the more distant H3 and H7 clades contain 6 subtypes). The most active mAb, CR6261, prophylactically protected mice from lethal H1 and H5 pneumonia, and remarkably, provided partial protection when given 5 days after infection when mice were in respiratory distress.

Sui *et al.* [40]** isolated multiple StRAbs from a non-immune human phage library, and demonstrated therapeutic and prophylactic efficacy in mice challenged with highly lethal H5 viruses with high potential for tissue dissemination. Structural analysis by x-ray crystallography of one of their mAbs bound to H5 HA [40] or the CR6262 mAb bound to H1 or H5 HAs [41] ** directly confirmed the interaction of this class of mAbs with the stem region just under the globular head. In conjunction with the structure, functional evidence [41] clearly points to a mechanism of viral neutralization based on preventing conformational alterations in the region required to trigger viral fusion activity.

Given the snug packing of virion HA visualized by electron microscopy [42,43], it is puzzling how StRAbs overcome steric interference between neighboring spikes to access their epitope. Binding almost certainly must occur prior to internalization, for two reasons.

First, the volume of endosomes is so minute (4×10^{-15} ml, the volume of a sphere with a radius of 0.1 μ), a StRAb would have to be present extracellularly at 1 μ M (150 μ g/ml) to achieve just one molecule per endosome. Second, although acid triggering of HA would expose the epitope, this nearly by definition would be too late to block viral fusion, and would also destroy many of the epitopes recognized by StRAbs [41].

Access to HA stems might be increased at the edge of “NA islands” present in the otherwise uniform HA sea, a possibility that can be addressed by high resolution electron microscopy of virus-Ab complexes. Inasmuch as human isolates are thought to typically be filamentous (a property rapidly lost upon growth in eggs or cultured cells) [44,45], it will be important to compare Ab interaction with filamentous vs. spherical/elliptical virions, since anti-stem Ab access may be influenced by viral morphology. Consistent with this idea, Corti *et al.* [46]** reported that StRAbs neutralize HA-containing pseudovirions up to 1000-fold more efficiently than IAV itself, in contrast to globular-binding mAbs, which neutralized with similar efficiency.

Understanding the mechanism of StRAb-mediated neutralization is obviously critical to understanding their function *in vivo*. *In vivo* neutralization can be considerably more complicated than *in vitro* neutralization, due to the interaction of Abs with serum proteins [47], the potential for prolonged interaction of Abs with virions resulting in binding to dynamically exposed, otherwise buried epitopes [48], and virion pleomorphism. Further, despite what appears to be a clear correlation between protection and *in vitro* neutralization, protection can actually result from alternate mechanisms acting in parallel, such as ADCC-based recognition of HA expressed on infected cells.

Water, water, everywhere: the paradox of drift

Given historic difficulties to demonstrate robust heterosubtypic immunity in animals [49,50], or detect cross-subtype neutralizing Abs, and the recurrent nature of human influenza, it was widely believed that StRAbs are unusual and difficult to elicit. Surprise, surprise.

A number of groups independently and essentially simultaneously reported that StRAbs are common components of Ab responses. Wei *et al.* [51]** found that priming mice, ferrets, or monkeys with H1 HA-encoding plasmids and boosting with the homologous HA either as a standard vaccine or expressed by a recombinant adenovirus generated a robust StRAb

response that provided protection against virus challenge when tested in mouse and ferrets. Corti *et al.* [46] found that vaccination with standard seasonal H1 vaccine induced or increased H5 neutralizing Abs in all 24 patients tested. Importantly, there was a 30-fold range in the post-vaccine levels of H5-neutralizing Ab titers among individuals that poorly correlated with standard neutralizing Ab titers. Limiting dilution analysis of B cells from 3 patients showed that vaccination increased the number of cross-subtype specific B cells from non-detectable to 0.03% to 1.6% of all B cells. Nineteen of the twenty H1-H5 subtype cross-reactive mAbs generated from immortalized B cells obtained from vaccinated patients recognize epitopes present in the stem region. A representative StRAb protected mice against lethal infection with H1, H5 and H6 viruses but not an H7 virus.

Wrammert *et al.* [52]** isolated plasmablasts (surface Ab+ B cells actively secreting antibody) from the blood of nine patients infected with swine origin H1N1 virus, cloned 86 Ig heavy and light chains gene pairs from individual cells, and expressed the mAbs in transfected cells. Of the 51 virus-binding mAbs isolated, 15 were HA specific, and 11 demonstrated VN activity. Six of the 11 mAbs proved to be H1-H5 HA cross-reactive StRAbs. Further, three other mAbs specific for the globular domain, bound to H1 HAs separated by 70 years of evolution in humans.

Extending these findings to H3 viruses, Wang *et al.* [53]** generated standard mAbs from mice by sequential immunization with extensively drifted H3N2 viruses and identified three mAbs that neutralize and protect mice across the drifted viruses. Mapping one of the Abs revealed the epitope to reside in the stem region, but restricted to a continuous epitope on the long α -helix of the HA2 chain.

Hasta la vista, influenza?

Taken together, these findings demonstrate that HA-specific Abs exist in both animal models and humans that are capable of providing broad protection within subtypes and between closely related subtypes. At the very least, such Abs represent promising therapy for patients with life-threatening influenza. At the very most, the existence of such antibodies offers the possibility of developing broadly cross-reactive vaccines.

Before raising expectations too high, however, these findings raise an obvious and central question: if neutralizing StRAbs Abs exist in humans, and are boosted by traditional vaccines, why does influenza remain prevalent and re-infect individuals who are repeatedly vaccinated/infected [54]? There are two general possibilities (and everything in between).

1. Neutralizing StRAbs are present in many individuals, but at insufficient concentration at the relevant sites of infection to provide protection.
2. Neutralizing StRAbs are present in some individuals, are boosted by vaccines and provide protection, but the protection afforded is not obvious when the effectiveness of vaccines is measured on entire populations.

Clearly, there is a tremendous need to step up the sophistication of influenza vaccination studies and correlate clinical outcome with more precise measures of immune responses: in this case neutralizing StRAb titers in serum and nasal washes. There are many possibilities. StRAbs may not provide sterilizing immunity, but greatly reduce morbidity and mortality. StRAb titers may wane with age, and be difficult to boost. StRAbs might be absent in children.

The technology for measuring neutralizing StRAbs in vaccinees developed by the recent ground breaking studies should not be prohibitively expensive. In any event, the yearly costs

of influenza easily justifies whatever the R&D costs are of improving IAV vaccines. The time is ripe for taking a quantum leap in influenza vaccine effectiveness.

Given an effective vaccine targeted to stem epitopes, the virus will likely evolve resistance. Aside from simple amino acid substitutions that can abrogate antigenicity, HA can shield the epitopes by adding N-linked glycosylation sites. Oligosaccharide shielding likely accounts for the poor binding of many of the H1-H2-H5 binding StRAbs with H3 [55]**. H1 and H2 viruses, seem however, to pay a steep fitness price for adding oligosaccharides, [56], likely limiting this escape mechanism. Further, the geometry of stem mediated-viral entry may preclude complete oligosaccharide cloaking of all potential neutralizing epitopes. In this case, new epitopes that arise could be rapidly targeted by rationally designed vaccines.

Viruses don't always win the battle with vaccines, indeed, they frequently lose. Given sufficient effort, enthusiasm, and open mindedness, humanity might relegate IAV to the list of the subdued within a decade or so.

Highlights

- Breakthroughs in basic understanding in influenza immunity suggest novel vaccine strategies for inducing broader immunity that circumvents antigenic drift
- Internal proteins may provide effective targets for vaccines based on NK cell recognition or antibody dependent cellular cytotoxicity
- A key feature of immune escape may be modulation of viral receptor avidity which triggers epistatic alterations that secondarily modulate antigenicity
- The human immune system is fully capable of generating neutralizing antibodies that interact with epitopes conserved within and even between hemagglutinin subtypes

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