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Influenza vaccine immunology

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Summary: Studying the spread of influenza in human populations and protection by influenza vaccines provides important insights into immunity against influenza. The 2009 H1N1 pandemic has taught the most recent lessons. Neutralizing and receptor-blocking antibodies against hemagglutinin are the primary means of protection from the spread of pandemic and seasonal strains. Anti-neuraminidase antibodies seem to play a secondary role. More broadly cross-reactive forms of immunity may lessen disease severity but are insufficient to prevent epidemic spread. Priming by prior exposure to related influenza strains through infection or immunization permits rapid, potent antibody responses to immunization. Priming is of greater importance to the design of immunization strategies than the immunologically fascinating phenomenon of dominant recall responses to previously encountered strains (original antigenic sin). Comparisons between non-adjuvanted inactivated vaccines and live attenuated vaccines demonstrate that both can protect, with some advantage of live attenuated vaccines in children and some advantage of inactivated vaccines in those with multiple prior exposures to influenza antigens. The addition of oil-in-water emulsion adjuvants to inactivated vaccines provides enhanced functional antibody titers, greater breadth of antibody cross-reactivity, and antigen dose sparing. The MF59 adjuvant broadens the distribution of B-cell epitopes recognized on HA and NA following immunization.

Keywords: *influenza, vaccine, immunology, pandemic, immunization, memory*

Introduction

Influenza is one of a group of viruses, including rhinoviruses, noroviruses, lentiviruses, and hepaciviruses, for which a multitude of antigenically distinct strains cause human disease. Observation of the natural history of successive antigenic variants spreading through human populations, including the occasional pandemic (three to four per century), frames our understanding of immunity to influenza. Understanding the immunological basis for successful influenza immunization has been facilitated by the ready adaptation of many strains to growth in culture and the availability of animal models. Moreover, influenza virus is unique among variable viruses causing human disease because it can be prevented by current vaccines. The study and practical experience of human immunization against influenza over more than five decades (1) has provided unparalleled insights into vaccine immunology and the relationship between findings at the laboratory bench or in animal models and outcomes in humans. In recent years, human

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Petitioner Merck, Ex. 1179, p. 167
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00009

infections caused by avian H5N1 strains, the 2009 H1N1 influenza pandemic, and the vaccine responses to these events provide some of the largest natural and designed 'experiments' ever performed in human immunology. The H5N1 zoonoses and 2009 H1N1 pandemic have been first major influenza events to have occurred at a time when virologists and immunologists have had the tools to follow the molecular evolution of the responsible strains in real time and to analyze antigen-specific B and T cells at the single cell level. In addition, for the first time, vaccinologists have had the safe novel adjuvants needed to make more potent vaccines. This review describes how these events allow us to draw some important conclusions about influenza vaccine immunology. Ongoing studies will reveal much more information in the near future.

Neutralizing antibodies prevent infection, other immune effectors mitigate disease

The 2009 H1N1 influenza pandemic caught the experts by surprise, because it propagated in a population with extensive experience of seasonal H1N1 viruses. Very early in the pandemic it was shown that despite having been exposed to or vaccinated against seasonal H1N1 viruses, the vast majority of the population, especially people born after 1950, lacked antibodies able to neutralize viral infectivity or inhibit red blood cell aggregation caused by the pandemic strain's hemagglutinin (HA) (2, 3) (*Fig. 1*). The observation that within a few months the virus spread globally in a population that had plenty of B cell and T cell immunity to the H1N1 viruses that had circulated recently but (except for the elderly) did not have significant titers of neutralizing or HI antibodies to the 2009 H1N1 strain confirmed that robust protective human immunity against influenza is primarily provided by antibodies targeting the virus' variable epitopes, those found on portions of its surface glycoproteins. Ferret studies confirmed the strain specificity of protection against the 2009 pandemic strain. Hyperimmunizing influenza-naive ferrets with an adjuvanted seasonal vaccine failed to induce neutralizing or HI antibodies against the pandemic 2009 H1N1 strain or protect from infection (4).

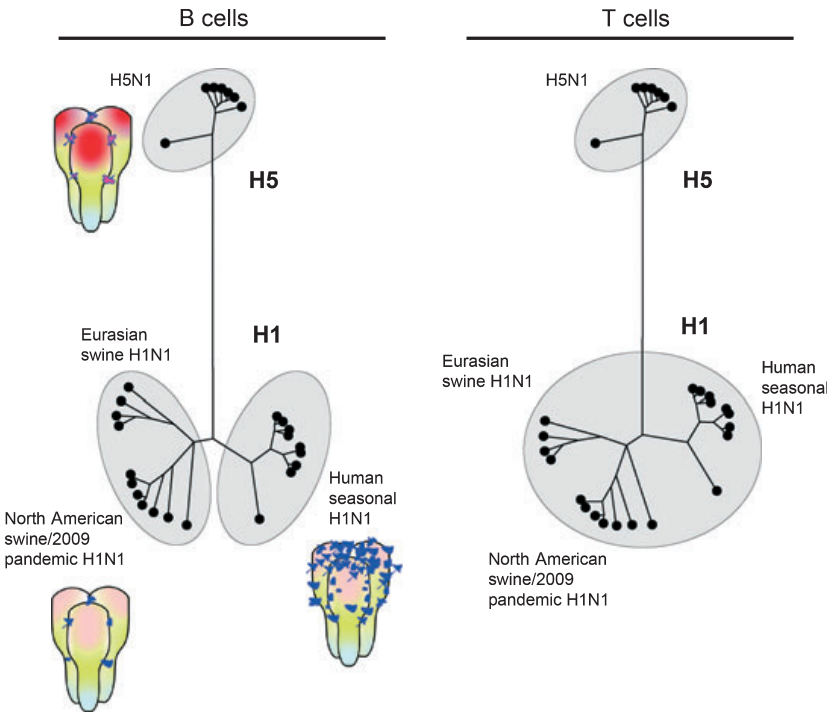


***Fig. 1.* How the immune system sees H1N1 and H5N1 HAs**. The dendrograms show the relatedness of H5N1 and H1N1 influenza strains isolated from humans and swine. The gray ovals illustrate the range of cross-reactive HI or neutralizing activity between strains anticipated after infection or immunization with an adjuvanted vaccine on the basis of B-cell responses (dendrogram on the left) and the range of cross-priming activity between strains on the basis of T cell responses (dendrogram on the right). Note that, although there is significant cross-priming between swine origin and seasonal H1N1 strains based on shared T-cell epitopes, there is no significant cross-neutralization. The 2009 H1N1 pandemic strain falls in North American swine cluster. The cartoon diagrams depict the HAs characteristic of each cluster. The difference in coloring between the H5 and H1 HA heads indicate their divergence in primary amino acid sequence. The swine-origin and human seasonal H1 HA heads are more similar to each other on the basis of sequence than they are on the basis of antigenic topography because of the much heavier decoration of the human seasonal H1 HA heads with glycans (depicted in blue), masking many epitopes (5, 6, 93).

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Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00009

The mystery of the absence of pre-existing protective antibodies titers in people born after the 1950s was recently explained by a series of elegant studies (5–7). These studies demonstrated that the 1918 and 2009 H1N1 strains share immunodominant neutralization epitopes in the Sa site on the HA head. After H1N1 strains crossed into humans in 1918 and began to circulate, several glycosylation sites were added to the HA head, masking the shared protective epitopes (*Fig. 1*). Other HA epitopes on the H1N1 strains circulating in humans drifted extensively, while epitopes on the strains circulating in pigs drifted much less. Humans exposed only to subsequent masked and drifted seasonal H1N1 strains did not develop significant titers of antibodies capable of protecting against the 2009 H1N1 strain. The 1976 H1N1 swine flu strain, like the 1918 and 2009 swine-origin strains, also had a 'bald' HA head. A history of immunization against the 1976 strain correlates with higher pre-existing functional antibody titers against the 2009 strain (8). Therefore, exposure of the elderly to infection or immunization with bald H1N1 strains explains the presence of pre-existing neutralizing antibodies against the 2009 H1N1 strain among the elderly.

The disproportionately high mortality and hospitalization rates because of influenza among young people in the 2009 pandemic (9) contrasts with relatively low influenza mortality and hospitalization rates among the elderly and emphasizes the importance of the variable HA head B-cell epitopes for mediating clinical protection against influenza. The ability of the traditional HI assay and more sensitive microneutralization assays to detect functional antibodies against these epitopes explains the continuing utility of these assays, despite many advances in our ability to probe antigen-specific immune functions (10, 11).

Human challenge studies with seasonal H1N1 and H3N2 strains and clinical outcomes following immunization of schoolchildren with a vaccine that matched the NA but not the HA of circulating strains indicate that NA-inhibiting antibodies may also contribute to protection (12, 13). Observations during the pandemic of 1968, when H3N2 influenza replaced H2N2 strains, provide a perspective on the role of anti-NA immunity in protection. The antigenic similarity of NA of the seasonal and new pandemic strains did not prevent a pandemic when the HA subtype shifted; however, individuals with higher pre-existing titers of NA-inhibiting antibodies had a lower frequency of H3N2 infection and less severe disease when infection with the pandemic strain did occur (14). Thus, anti-NA immunity appears to play a secondary but still significant role in protection as compared to anti-HA immunity, leading to consideration of including anti-NA antibody assays in vaccine evaluations (15). The poor physical stability of NA limits its utility in inactivated influenza vaccines (16).

Fortunately, the severity of illness from the 2009 pandemic strain among young people, although significant, did not approach the levels seen in the 1918 H1N1 pandemic or following zoonotic transmissions of avian H5N1 strains (17). It was also fortunate (and surprising) that a single dose of vaccine was sufficient to induce protective antibody levels in adults (18), and a single dose of adjuvanted vaccine induced protective antibody levels in young children (19). Based on the experience of immunization against H5 and H9 avian influenza strains (20, 21), it had been anticipated that two doses of an adjuvanted vaccine might be necessary for robust antibody responses. The mortality and vaccine response observations from the 2009 pandemic suggest that the pre-existing immunity, not detected in HI or neutralization assays, to T- or B-cell epitopes shared between the pandemic and seasonal H1N1 viruses, although not sufficient to prevent the spread of infection, did mitigate the severity of disease and primed for a rapid and effective response to immunization (*Fig. 1*).

Ferret experiments confirm the role of seasonal priming for pandemic immunization. Although a dose of seasonal vaccine did not protect ferrets from infection with the 2009 pandemic strain, it did prime for a robust functional antibody response and increased protection from infection following a single dose of pandemic vaccine (4). The priming effect of immunization with seasonal vaccine was more pronounced if the seasonal vaccine was adjuvanted with MF59, which promotes potent $CD4^+$ T-cell help (22). This disease mitigating and priming immunity, not measured in neutralization or HI assays, is likely to be mediated by T cells that either eliminate infected cells or help naive B cells to mount a more rapid neutralizing antibody response. In addition, memory B cells bearing antibodies that cross-react with the pandemic strain, but with affinities too low to detect by neutralization or HI assays, might have quickly undergone affinity maturation and expansion following exposure to the pandemic surface glycoproteins, lessening disease severity and enhancing pandemic vaccine responses.

Differences between mice and men

The influenza virion contains a number of structural antigens, such as HA, the neuraminidase (NA), a capsid protein (M1), an ion channel (M2), a nucleoprotein (NP), and, in small quantities, the components of the polymerase (PA, PB1, and PB2). Many studies in mice report protection from lethal infection mediated by conserved antigens such as M2 and NP

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Petitioner Merck, Ex. 1179, p. 169
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00009

(23–26). Such experiments have been interpreted as pointing the way to a universal influenza vaccine. It is our experience that protecting mice against influenza virus is very easy. Although mice have been very helpful in uncovering basic immunological mechanisms, mouse models of human disease have limitations (27), and animal studies are, at best, imperfect predictors of vaccine efficacy in humans. Although sufficiently rigorous immunization with M2 and NP can protect ferrets from low doses of a highly pathogenic influenza strain (26), protection by these conserved antigens is not seen following more rigorous ferret challenge (28).

Robust universal protection by conserved antigens such as M2 or NP is not consistent with the epidemiological and experimental evidence of human protective immunity. If conserved antigens, such as M2 and NP, mediated robust protection, influenza type A would be a one-time childhood illness, like mumps, measles, or chicken pox. Instead, drifted influenza viruses, differing only slightly in the sequences of their surface glycoproteins, have spread globally every year, re-infecting individuals who have experienced previous influenza infections or received past years' influenza vaccines, including vaccines (such as live attenuated or whole virus inactivated vaccines) that express or contain M2 and NP. Human experimental results reflect this epidemiological pattern. Immunization of adults with inactivated whole virus vaccines provides some protection from experimental challenge with drifted, attenuated influenza strains, but the degree of protection correlates with the antigenic match between the HA of the vaccine virus and the challenge virus (29, 30).

Most experimental investigations of vaccine efficacy in animal models test potential immunogens in influenza-naive hosts. In contrast, almost all human vaccinees, starting from late infancy or early childhood, have been exposed to influenza viruses. An initial infection primes the immune system against influenza for an individual's entire life. As a consequence, most human immunization against influenza occurs in the context of pre-existing immunity, which alters the response to vaccination. Only during infant or early childhood immunization do we vaccinate some truly influenza-naive hosts. Recipients of vaccines against zoonotic influenza strains, such as those of H5, H7, or H9 subtypes, are largely naive, because these strains are only distantly related to human seasonal influenza strains and only sporadically cause human infections. The lack of prior exposure to influenza in most animal immunization experiments complicates extrapolation of their results to human immunization.

Even the difference in exposure history does not fully explain the difference between results of vaccine studies in animals and humans. Ferrets, unlike mice, develop an acute respiratory illness after intranasal inoculation of modest doses of human influenza strains, making influenza infection of ferrets a more accurate animal model for human influenza than infection of mice (31). Yet, ferret results do not always predict vaccine protection, even of young children with limited previous influenza exposure. Infection of ferrets with a H1N1 or a H3N2 virus provides partial, but significant, cross-protection from challenge with the hetero-subtypic strain (32). In contrast, previous infection of children less than 3 years of age with H1N1 or H3N2 influenza provides no protection against experimental challenge with attenuated hetero-subtypic strains (33). Similarly, observation of the 1957 pandemic, when H2N2 viruses spread through a population in which H1N1 influenza was endemic, showed no evidence of cross-protection of children, although there was evidence of partial hetero-subtypic protection of adults with extensive past influenza exposure (34). Thus, however, promising novel approaches to universal influenza immunization may appear in preclinical studies, such results always must be weighed against the evidence from observing human natural infection and immunization.

The human response to different types of influenza vaccines

Two major types of influenza vaccines are commercially available, live attenuated cold-adapted influenza strains (LAIV) and inactivated vaccines. The latter may contain either the entire whole inactivated virus (whole virus vaccines), virus disrupted by detergents or solvents (split vaccines), or purified HA and NA (subunit vaccines). Recombinant vaccines composed of insect cell-expressed virus-like particles (VLPs), insect cell-expressed trimeric subunits, or *E. coli*-expressed fusion proteins between the HA head domain and flagellin from *Salmonella typhimurium* type 2 are also in clinical development (35–37). Inactivated vaccines are licensed for those greater than 6 months of age; seasonal LAIV is licensed only for those from 2 to 49 years of age. In children younger than 2 years, LAIV is not licensed because of concern over wheezing and excess hospitalizations (38); licensure for those 50 and older has not been obtained because, in a *post hoc* analysis of a critical study, efficacy was not demonstrated for subjects aged 50–64 (39).

Comparative analysis of the efficacy of LAIV and trivalent inactivated vaccines (TIV) has yielded differing results, depending on the population and end point studied. A meta-analysis of comparative studies in all age groups revealed no significant overall differences in reactogenicity or efficacy between the vaccine types and no significant difference in the

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Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00009

ability to protect against drifted strains (40). However, a meta-analysis of studies in children showed greater efficacy of LAIV (41). The durability of protection induced by LAIV in children appears to be greater (42). In US military populations, LAIV is more efficacious than trivalent inactivated vaccine (TIV) among recruits, who have had limited previous influenza immunization, but TIV is more efficacious among non-recruits, who have relatively high rates of annual immunization (43, 44). LAIV protects despite its limited ability to elicit serum HI titers (40), the correlate of protection enshrined in regulations for TIV. Overall, the data suggest an advantage of LAIV in those with limited prior immunity to influenza and an advantage of TIV among those with greater prior exposure. The comparative efficacy of LAIV and MF59-adjuvanted TIV (discussed below) is unknown.

Among inactivated vaccines, whole virus preparations can be more immunogenic but are often less well tolerated than more highly purified preparations (45–47). In already primed subjects, the immunogenicity of the different forms of inactivated vaccine are generally equivalent; differences are more apparent during immunization of naive hosts, such as younger subjects and those receiving prepandemic immunization against potential zoonotic strains (45, 48). The robust primary responses to whole virus inactivated vaccines may be because of the presence of viral RNA, known to be an agonist of Toll-like receptor 7 (TLR7) (49).

The addition of adjuvants, such as the oil-in-water emulsions MF59, licensed in 1997, and AS03, licensed in 2009, has provided a more controlled technique to increase the immunogenicity of seasonal and pandemic inactivated influenza vaccines (50, 51). MF59 enhances immunity through a TLR-independent pathway. It targets muscle cells at the injection site, upregulating the expression of a broad array of genes to induce a strong immunocompetent environment (52). MF59 recruits mononuclear cells into the muscle, induces differentiation of monocytes towards dendritic cells, and promotes antigen uptake into dendritic cells (53). Thus far, the oil-in-water emulsion adjuvants have an acceptable overall safety profile but are associated with a mild increase in reactogenicity (50, 51). In addition to increasing antibody titers, the oil-in-water emulsions also expand the cross-reactivity of the antibody response and reduce the amount of antigen needed to elicit protective responses (20, 54–58). The effect of adjuvants in immunization of the elderly, with or without underling chronic health conditions, indicates that they are also useful to enhance the responses of the experienced but senescent immune system (59, 60). In addition, based on the ability of oil-in-water emulsions to enhance antibody responses elicited by the immunization of adults with H5N1 vaccines and young children with seasonal or H1N1 pandemic vaccines, adjuvants appear to be particularly important when immunizing in the absence of pre-existing immune memory (19, 58, 61). In particular, recent trials of an MF59-adjuvanted seasonal vaccine in 6- to 36-month-old children have shown robust enhancement of immunogenicity, suggesting that an adjuvanted vaccine may be the best solution at an early age (58).

Broadly neutralizing antibodies recognizing HA

Although most HA-directed neutralizing monoclonal antibodies (mAbs) recognize the variable HA head and are subtype- or even strain-specific (62), in 1993 a mAb that neutralizes across several subtypes by recognizing an epitope in the HA stem and blocking virus-cell fusion was obtained from a mouse that had been hyperimmunized with an H2N2 influenza strain (63). More recently, similar antibodies have been found in the repertoire of human memory B cells. Their binding has been analyzed by X-ray crystallography, and the antibodies have been shown to block the low-pH-induced conformational change that mediates membrane fusion (64–66). Because HI only detects antibodies that block receptor binding, the standard assay used as a correlate of vaccine protection systematically overlooks such broadly neutralizing antibodies, which could be detected by microneutralization. Broadly neutralizing anti-stem antibodies can be elicited by immunization, but they seem to make up a generally small proportion of the elicited repertoire, but a proportion that varies between individuals, suggesting that the stem epitope is poorly immunogenic (67). There are many hypotheses to explain the sub-dominance of the broadly conserved neutralizing stem epitope, such as an inherently poorly immunogenic structure, inaccessibility on the virion, similarity to human host determinants, or an overwhelming immunodominance of the globular HA head (68).

Role of cell-mediated immunity in influenza infection

Because of the evidence for the dominant role of antibodies in protecting humans from influenza and because regulatory agencies require manufacturers seeking licensure to demonstrate that new influenza vaccines either elicit HI titers > 1:40 or protect from disease (a more challenging but relevant endpoint for clinical trials), the vast majority of clinical studies on influenza vaccination focus on antibody responses. Cellular immunity is much less frequently investigated. Nevertheless, achieving high and sustained titers of high affinity antibodies through immunization requires coordinated innate and adaptive responses, including $CD4^+$ T-cell-mediated help for B-cell

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Petitioner Merck, Ex. 1179, p. 171
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00009

activation and affinity maturation through somatic hypermutation. Antibody generation is the final consequence of cellular interactions that occur earlier after immunization. Understanding the impact of distinct vaccination strategies on the various arms of the immune system can guide optimal vaccine design.

The longevity of memory B-cell responses after influenza infection or immunization

Influenza infection induces life-long HA-specific B-cell memory, either through long-lived antibody-secreting cells (ASCs) or re-circulating resting memory B cells (MBCs). The presence of detectable MBCs and high titers of antibodies specific for HA of the 1918 H1N1 pandemic virus among individuals who were born in or before 1915 but who had not yet been exposed to the 2009 pandemic virus (69) suggests the persistence of memory for almost a century (although boosting by swine flu immunization in 1976 is possible). Similarly, neutralizing antibodies against drifted variants of the 1918 H1N1 virus that circulated until the 1950s persisted for at least 50 years in sera from subjects older than 60 years of age (2, 3). Immunization with LAIV or TIV also generates MBCs and ASCs (70, 71). A MF59-adjuvanted subunit vaccine (but not the non-adjuvanted subunit) induces pools of MBCs recognizing H5N1, against which there was no pre-existing memory (22). There is no consensus on the duration of B cell memory after vaccination. However, we do know that 6–8 years after priming with two doses of a H5N3 (clade 0) MF59-adjuvanted subunit vaccine, subjects have rapid, high titer antibody responses to boosting with a H5N1 (clade 1) MF59-adjuvanted subunit vaccine (61). The rapidity and magnitude of the booster response suggests that the initial immunization produced memory lasting at least 8 years.

Is B-cell memory against influenza virtuous or sinful?

Many observational studies in humans document the important role of priming to potentiate robust subsequent responses to immunization. Following initial exposure to influenza antigens, cross-reactive T-helper cells potentiate B-cell activation on re-exposure, and cross-reactive MBCs affinity mature and differentiate into plasma cells that produce protective neutralizing antibodies. Thus, pre-existing MBCs allow a more rapid response to subsequent vaccination or infection with a close variant of a previously experienced influenza strain. Pre-pandemic vaccination strategies against H5N1 are built on the 'virtue' of memory, which has been verified by observing sequential immunizations with drifted H5 variants (61, 72–75).

Why, then, could prior exposure to closely related influenza antigens be considered 'sinful'? The hypothesis of original antigenic sin (OAS) suggests that cross-reacting MBCs can actually reduce the effectiveness of subsequent vaccination against drifted strains. According to the hypothesis, a virus that causes an initial infection permanently imprints the immune system to preferentially recognize its own antigenic features, and all subsequent responses to variants of the original virus will be dominated by, if not restricted to, antibodies that cross-react with the old strain (76). This theory emerged early after the discovery of the influenza virus and before modern understanding of antibody diversity. It was based on the observation that sera from individuals recovering from secondary influenza infections can display higher reactivity against a virus that caused an earlier influenza episode than against the virus responsible for the more recent infection (77). Contemporary observations of responses to influenza immunization provide evidence that pre-existing immunity can sometimes interfere with the response to immunization. For example, subjects who lack detectable HI antibody titers against a new vaccine strain sometimes respond more robustly (in fold-increase and absolute final titer) to immunization than do those who had detectable baseline HI titers (78, 79). OAS is not the only form of immune interference with immunization. For example, in an influenza-experienced subject, pre-existing humoral or cell-mediated immunity could interfere with the replication of an attenuated influenza virus, diminishing the 'take' of LAIV. Such an effect would not necessarily result in a stronger response to a previously encountered influenza strain than to a vaccine strain, and, therefore, would not be an example of an OAS response. Documentation of OAS requires comparison of immune responses to past and currently encountered strains.

OAS can be explained as the dominance of pre-existing cross-reactive antibodies and MBCs that rapidly sequester antigens and $CD4^+$ T-cell help that would otherwise be available to trigger the expansion of naive B-cell clones that recognize the new epitopes. According to this model, impact of this dominance on vaccine-elicited antibody titers varies with the number of pre-existing cross-reactive MBCs, and, more importantly, depending on whether the HA epitopes that the MBCs recognize contribute to neutralization of the new virus strain.

OAS does not preclude effective immunization with new influenza variants. Annual influenza immunization with updated seasonal vaccines elicits protective titers to new variants despite pre-existing antibodies and MBCs elicited by exposures to old strains. In children or adults, antibody

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Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00009

responses to new antigenic variants can be dominated by ASCs and antibodies of the switched memory IgG class as early as 1 week after a single dose (78, 80, 81). This response is too rapid to result only from priming of naive B cells, specific for new epitopes. Studies of H5N1 immunization indicate that primary responses require two vaccine doses before substantial numbers of new IgG memory B cells and HI antibodies start appearing in the blood (20, 22). Presumably, MBCs primed by exposure to previous variants, contribute to the 1-week IgG response to drifted strains.

Rapid IgG responses to immunization with new variants could result from expansion of ASCs that produce antibodies that cross-react between the old and new strains. In addition, the B-cell response to influenza vaccines may have considerable plasticity. Activation of B lymphocytes with help from cognate $CD4^+$ T cells can result in new germinal center reactions, during which randomly occurring somatic hypermutation can substantially reshape the specificity and affinity of pre-existing HA-specific MBCs and naive B cells. Presence of abundant cognate help from HA-specific $CD4^+$ T cells will lessen the stringency of competition for antigen binding and favor reshaping of the HA-specific B-cell repertoire.

Role of $CD4^+$ T-cell help in influenza infection and immunization

Antigen-specific T lymphocytes orchestrate the immune response to immunization and infection. The primary role of $CD4^+$ T lymphocytes is to provide help for the optimal activation of $CD8^+$ T cells and early clonal expansion of B lymphocytes (82). $CD4^+$ T cell help promotes the initiation and maintenance of germinal center reactions, somatic hypermutation, the redirection of the pre-existing MBC repertoire required to overcome OAS, and the generation of antigen-specific long-lived plasma cells and MBCs (83, 84). In addition, preclinical studies suggest that influenza-specific $CD4^+$ T cells not only provide help but also accelerate recovery from infection via direct effector functions, mediated by the production of IFN-γ and perforin and the activation of innate responses in infected tissues (85–87).

Although the central role of $CD4^+$ T cells in 'helping' immune responses is widely accepted, few studies have analyzed the expansion of these cells after influenza vaccination and the impact of $CD4^+$ T cells on the human antibody response. $CD4^+$ T-cell responses are less sensitive than B-cell responses to mutations in the virus. Recognition of conserved T-cell epitopes can provide sufficient activation of $CD4^+$ T cells upon exposure to hetero-subtypic strains. Because of previous exposure to infection and/or immunization, $CD4^+$ T cells specific for seasonal influenza are detectable in all adults and rapidly increase after a single dose of vaccine, making immunization against H5N1 avian influenza a cleaner model to study the role of $CD4^+$ T-cell help in vaccination. Antigen-specific $CD4^+$ T-cell activation precedes a neutralizing antibody response to H5N1 immunization: T-cell activation is detected after a single dose of an MF59-adjuvanted vaccine; neutralizing antibodies are detected after two doses. More importantly, the early expansion of $CD4^+$ T cells predicts the subsequent rise of neutralizing antibodies and their persistence months after vaccination (22). Un-adjuvanted H5N1 vaccines do not induce a robust expansion of $CD4^+$ T cells and fail to increase specific antibody titers (88). An increased ability of MF59 to promote $CD4^+$ T-cell help may be responsible for the broadened cross-neutralization of drifted H5N1 variants elicited by adjuvanted vaccines (22, 61, 89, 90).

Epitope spreading elicited by adjuvanted influenza vaccines

Further mechanistic insight into the broadening of the antibody response elicited by adjuvanted vaccines has been provided by recent studies in which whole genome fragment phage display libraries (GFPDL) were used to assess the repertoire and distribution of epitopes recognized by antibodies after human immunization with adjuvanted and un-adjuvanted H5N1 vaccines. In these studies, phage display libraries of peptides encoded by fragments of influenza genome segments are 'panned' with sera. By culturing the phages captured by the antibodies and sequencing their inserts, the distribution of phage-displayed epitopes recognized by the sera can be assessed (90, 91).

Even in the case of H5N1, against which unexposed humans are expected to be naive, prevaccination sera contained antibodies that reacted with the H5N1 GFPDL. These antibodies targeted a region of the HA2 fragment of H5 HA that shows a high degree of conservation with seasonal H1 HA2. Furthermore, following vaccination with unadjuvanted or alum-adjuvanted inactivated H5N1 vaccine, the humoral response was dominated by antibodies directed to epitopes in HA2 (suggesting a recall response) (91). In contrast, in two independent clinical studies, GFPDL analyses demonstrated that MF59 induced spreading of the recognized epitopes to HA1 and to NA. The phage-displayed HA1 fragments recognized included large, conformationally authentic fragments spanning the receptor binding domain. A 20-fold increase in

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Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00009

the ratio of antibodies recognizing targets in HA1 rather than in HA2 was observed in sera after MF59-adjuvanted vaccine administration as well as a two- to threefold increase in the avidity of antibody binding to a properly folded recombinant HA1 globular head. The adjuvant-dependent increased binding to conformational HA1 epitopes correlated with broadening of cross-clade neutralization (90). Strikingly, sera from those immunized with MF59-adjuvanted H5N1 vaccines and from individuals who survived H5N1 infection recognized a greater diversity of HA and NA epitopes.

These data suggest that, following years of influenza exposure and vaccinations, the long term pool of memory B cells that cross-react with a new strain is probably composed of many MBCs targeting the most conserved regions of the HA, mainly within HA2. We hypothesize that vaccines that elicit limited $CD4^+$ T-cell help are more likely to activate these memory cells. Adjuvanted vaccines, which induce more robust $CD4^+$ T-cell help and more prolific germinal center reactions, are better able to activate naive B cells with new specificities, drive somatic hypermutation, and reshape pre-existing MBCs' specificities, so that MBCs that recognize non-cross-reactive sites on the variable HA1 head undergo clonal expansion and affinity maturation.

Increased cross-neutralization between drifted strains after adjuvanted immunization correlates with an increase in the proportion of antibodies recognizing the variable HA1 fragment, not the more conserved HA2 fragment. The highly conserved neutralizing epitope responsible for hetero-subtypic neutralization by a series of monoclonal antibodies is located on the HA stem domains and is formed by amino acid residues from both HA1 and HA2 (64–66). Those residues are widely dispersed in the primary amino acid sequence and form the epitope as a result of the complex folding of HA. Therefore, it is unlikely that the conserved neutralizing stem epitope is displayed by the phage, and these experiments cannot rule out the possibility that the MF59-adjuvanted vaccine elicited an increased proportion of antibodies against this epitope. Although most B cell epitopes in the HA head domain, which consists entirely of residues from the HA1 fragment, are not conserved between subtypes, some of them may be conserved between drifted strains, so that HA1 epitopes can mediate broadened cross-neutralization within a subtype (62, 92).

Conclusions

Eliciting neutralizing antibodies that recognize variable epitopes on the HA head is the dominant means by which influenza vaccines protect individuals from influenza and prevent the spread of influenza through populations. Other mechanisms of immunity may help reduce the severity of infections. T-cell help is required for effective antibody responses. Robust T-cell help allows B-cell responses to mature in sustained germinal center reactions, generating broad and long-lasting neutralizing responses. This help can be primed by immunization, even by vaccines against relatively distant strains. The use of oil-in-water emulsion adjuvants creates a more immunogenic environment at the site of injection, enhancing antigen presentation.

Today, we can examine the results of immunizing with vaccines against viruses with which subjects have different levels of pre-existing immunity: pre-pandemic vaccines target viruses against which the population has no significant pre-existing immunity; the 2009 pandemic vaccine targeted a virus against which most people had a low level of immunity, mainly toward conserved non-neutralizing epitopes; and seasonal vaccines target viruses with neutralization antigens that have drifted modestly from those that most vaccinees have encountered previously through infection or seasonal immunization. The vaccines against viruses for which the population has little if any pre-existing immunity, such as H5, H7, and H9 strains, require two doses and an adjuvant to stimulate an effective immune response in all age groups (20, 21). For pandemic vaccines against viruses with some level of pre-existing immunity, like the 2009 H1N1 virus, one dose of a non-adjuvanted vaccine is sufficient to induce immunity; however, the adjuvant is still necessary to induce good protection in the very young and to permit a lower dose of antigen (18, 19). Finally, seasonal vaccines are administered primarily to people who have both B-and T-cell immunity against the viruses. One dose of seasonal vaccine is sufficient for adults and elderly, but two doses are necessary for influenza-naive infants. Among infants (with immature immune systems and mostly naive B-cell and T-cell repertoires) and the elderly (with senescent immune systems), adjuvants are especially important to increase the magnitude and breadth of elicited immunity (57, 58).

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Merck Sharp & Dohme LLC v. Halozyme Inc.
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Merck Sharp & Dohme LLC v. Halozyme Inc.
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PGR2025-00009