

Serum Samples From Middle-aged Adults Vaccinated Annually with Seasonal Influenza Vaccines Cross-neutralize Some Potential Pandemic Influenza Viruses

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We examined serum samples from adults ages 48–64 who received multiple seasonal influenza vaccines from 2004 to 2009 for cross-neutralizing antibodies to potential pandemic strains. Using pseudoviruses bearing various hemagglutinins (HA-pseudoviruses), we found serum neutralization titers (≥ 160) in 100% against A/Japan/305/1957 (H2N2), 53% against A/Hong Kong/1073/99 (H9N2), 56% against the H3N2 variant A/Indiana/08/11 (H3N2v), 11% against A/Hong Kong/G9/97 (H9N2), and 36% A/chicken/Hong Kong/SF4/01 (H6N1). None had titers >160 to A/Shanghai/2/13 (H7N9) or A/Netherlands/219/03 (H7N7). Thirty-six percent to 0% had neutralization titers to various H5N1 strains. Titers to H9, H6, and H5 HA-pseudoviruses correlated with each other, but not with H3N2v, suggesting group-specific cross-neutralization.

Keywords. cross-neutralization; influenza vaccines; neutralizing antibodies; pandemic influenza; stem antibodies.

Humoral immune responses against the influenza hemagglutinin protein (HA), the principal antigen in inactivated influenza vaccines, correlate with protection against influenza. Vaccination therefore provides an important public health strategy. HA antibodies can last for decades in humans, but the frequent emergence of influenza variants with mutations in HA that changes antigenicity requires annual reformulation of influenza vaccines to cover the dominant circulating strains.

Major antigenic shifts can lead to influenza pandemics. In the past 100 years, there were 4 major pandemics, including the Spanish Flu (1918–1920, H1N1), the Asian Flu (1957–1958, H2N2), the Hong Kong Flu (1968–1969, H3N2), and the

Swine Flu (2009–2010, A[H1N1]pdm09). Recently, non-circulating H3N2v, H5N1, H6N1, H7N7, H7N9, and H9N2 viruses have caused infections in small numbers of humans. The potential spread of these viruses and the possible reemergence of H2N2 strains raise questions as to whether prior influenza infections and vaccinations could confer any degree of protection.

HA is a noncovalently associated homotrimer, with each monomer composed of 2 disulfide-linked subunits, HA1 and HA2. The trimeric HA contains a membrane-distal, globular head domain formed only by HA1, and an elongated membrane-proximal stem domain composed of the HA2 ectodomain together with the N- and C-terminal segments of HA1. HA-neutralizing antibodies primarily target immunodominant epitopes in the receptor-binding domain within the globular head. Although these antibodies can be protective, they are often strain-specific because of the high variability of such epitopes.

Cross-neutralizing antibodies to various HAs have been identified in some persons with prior histories of influenza infections or vaccinations [1–3]. Recently, broadly neutralizing monoclonal antibodies (mAbs) against HA head and stem epitopes were extensively characterized (comprehensively reviewed in [4, 5]). Phylogenetically, the 18 HA subtypes characterized so far are divided into 2 groups: group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18) and group 2 (H3, H4, H7, H10, H14, and H15). Some of these broadly neutralizing antibodies neutralize group 1 viruses, and others are specific for group 2 viruses. A few monoclonal antibodies, including C05, S139/1, 2G1, F045-092, F026-427, 39.29, 81.39, PN-SIA28, Uni-1, CR9114, and FI6, were reported to neutralize both groups 1 and 2 viruses (see reviews [4, 5]).

The extent to which broadly cross-neutralizing antibodies can be found in serum samples of persons who have received seasonal influenza vaccines for multiple years remains unknown. The degree to which different strains within potential pandemic subtypes vary in their susceptibilities to these cross-neutralizing antibodies is also unclear. In this report, we investigated whether middle-aged adults immunized annually with seasonal influenza vaccines (during 2004/05 to 2008/09 seasons) harbor neutralizing antibodies to a variety of potential pandemic viruses, including H2N2 (A/Japan/305/1957), H3N2v (A/Indiana/08/11), and several strains from H5N1, H6N1, H7N7, H7N9, and H9N2 subtype viruses. Our data suggest that cross-neutralizing antibodies to potential pandemic influenza viruses can be found in some middle-aged adults who received multiple annual seasonal influenza vaccines. However, the magnitude of neutralization by these broadly neutralizing antibodies

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varied widely among the different potential pandemic strains, even among strains within the same subtype.

METHODS

Plasmids and Cell Lines

The plasmids expressing HAs of A/Vietnam/1203/2004, A/Indonesia/5/2005, NA of A/California/04/2009, and HAT (pCAGGS-HATcop, HATcop) were described previously [3, 6]. Other HA genes (sequences shown in [Supplementary Table 1](#)) were chemically synthesized (GenScript, Piscataway, New Jersey) and placed into the pCMV/R expression plasmid [6]. pCMV/R, as well as HIV gag/pol (pCMV ΔR8.2) and Luciferase reporter (pHR'CMV-Luc) plasmids [6], were obtained from Gary J. Nabel (NIH, Bethesda, Maryland). As described previously [3], 293T cells were cultured.

Ethics Statement

Serum samples were obtained with informed consent and ethics approval by the Research Involving Human Subjects Committee (RIHSC) at the US Food and Drug Administration (RIHSC Protocol #09-110B).

Serum Samples

Serum samples were described previously [3], and collected in September–December 2009 from 45 volunteers aged 48–64 years, without a self-reported history of influenza symptoms, exposures, or vaccinations in 2009 prior to serum sample collection. All subjects received all seasonal influenza vaccines during the 2004/05 to 2008/09 seasons. Twenty-three subjects also received the A/New Jersey/1976 swine influenza vaccine. Sixty-five prevaccination serum samples from A/New Jersey/1976 swine influenza vaccine trials conducted in 1976 were used as controls.

Production of HA-pseudoviruses and Neutralization Assay

HA-pseudoviruses carrying a luciferase (Luc) reporter gene were produced in 293T cells as described previously [6]. HA-pseudoviruses containing approximately 15 ng/mL p24 antigen and 12 ng/mL HA were incubated with heat-inactivated serum samples for 1 hour at 37°C, prior to inoculating mixtures onto 293T, as described previously [3]. The serum dilution causing a 95% reduction of luciferase activity compared to control (IC_{95}), calculated using Graphpad Prism software, was used as the neutralization titer [7]. Neutralization titers over 160 that inhibited 95% infectivity were considered clinically significant based on comparisons with microneutralization assays [7, 8].

Statistical Analysis

Titers were analyzed with nonlinear regression using GraphPad Prism software. The correlation of neutralization titers was evaluated with Spearman's r , a test for nonparametric correlation, using GraphPad Prism software. P values $<.05$ were considered statistically significant.

RESULTS

Cross-neutralization of Pseudoviruses Bearing HA From Noncirculating Influenza A Strains

Forty-five serum samples from 48–64-year-old subjects who had received multiple seasonal influenza vaccines during 2004–2009 were assessed for neutralizing activity (Figure 1) against potential pandemic type A viruses using pseudoviruses bearing HA (HA-pseudoviruses) from the H9, H7, H6, H5, H3N2v, and H2 subtype influenza ([Supplementary Table 1](#)). Previously, we showed that HA-pseudovirus neutralization titers using 95% inhibitory concentration (IC_{95}) correlate well

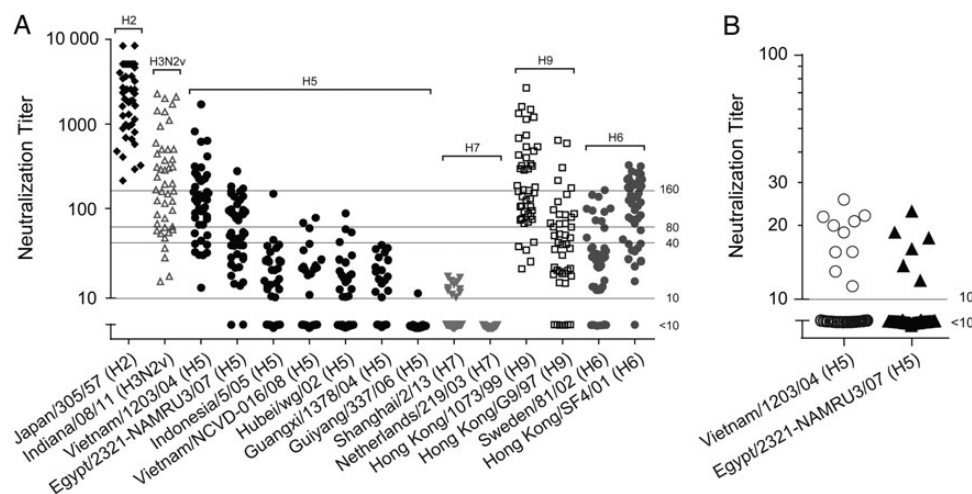


Figure 1. Middle-aged adults immunized with recent seasonal influenza vaccines harbor neutralizing antibodies to noncirculating influenza viruses. *A*, Neutralization titers to H2, H3N2v, H5, H6, H7, and H9 HA-pseudoviruses in the subjects aged 48–64 years who had received seasonal influenza vaccinations that included A/New Caledonia/20/1999 (H1N1), A/Solomon Islands/3/2006 (H1N1), A/Brisbane/59/2007 (H1N1), A/Fujian/411/2002 (H3N2), A/California/7/2004 (H3N2), A/Wisconsin/67/2005 (H3N2), and A/Brisbane/10/2007 (H3N2). *B*, Neutralization titers to A/Vietnam/1203/04 (H5) and A/Egypt/2321-NAMRU3/07 (H5) in subjects before they received the A/NJ/76 (H1N1) clinical trial vaccination in 1976. Abbreviation: HA-pseudoviruses, pseudoviruses bearing various hemagglutinins.

with microneutralization titers using replicating influenza virus [7]. Microneutralization titers >160 have been proposed as correlates of seroprotection [8], but protective titers based on HA-pseudoviruses neutralization have not been determined.

Against Japan/305/57 (H2N2) HA-pseudoviruses, we found that 100% of serum samples have neutralization titers ≥ 160 , consistent with past exposures as H2N2 circulated between 1957 and 1968. For some viruses that have never circulated, we unexpectedly found neutralizing activity in a significant proportion of serum samples. In particular, 53% had titers ≥ 160 against Hong Kong/1073/99 (H9N2), 11% against Hong Kong/G9/97 (H9N2), 36% against Hong Kong/SF4/01 (H6N1), and 56% against Indiana/08/11 (H3N2v). For subtype H5N1 strains, 36% had titers ≥ 160 against Vietnam/1203/04 (H5N1), 9% against Egypt/2321-NAMRU3/07 (H5N1), and none had titers ≥ 160 against Indonesia/5/05 (H5N1), Vietnam/NCVD-016/08 (H5N1), Hubei/wg/02 (H5N1), GuangXi/1378/04 (H5N1), and Guiyang/337/06 (H5N1). Neutralization titers were also undetectable for Shanghai/2/13 (H7N9) and Netherlands/219/03 (H7N7). Many additional serum samples had neutralization titers between 40 and 160 against several of these noncirculating strains (Figure 1A). We note that for the strains that never circulated in the human population (all except for the H2N2 strain), the neutralization titers are generally much lower than those that we reported previously for H1N1 and H3N2 strains that have circulated in the human population [3, 6].

Because H5 subtype HA-pseudoviruses showed a range of sensitivities to neutralization, we wondered whether neutralization to

Vietnam/1203/04 and Egypt/2321-NAMRU3/07 was due to nonspecific inhibition. We thus tested the neutralization of 65 prevaccination serum samples from A/New Jersey/1976 swine influenza vaccine trials conducted in 1976. No serum samples had neutralization titers ≥ 40 to Vietnam/1203/04 and Egypt/2321-NAMRU3/07 (Figure 1B), indicating that the neutralization seen in our cohort was specific.

Correlation of Cross-neutralization Among Strains Within Influenza A Groups

To further understand the observed cross-neutralization, we investigated potential correlations between neutralization titers among different strains. We found that neutralization titers to group 1 viruses correlated well (Figure 2A and Supplementary Figure 1), but there was no correlation between the group 2 virus H3N2v and group 1 viruses (Figure 2B), with the exception of a slight correlation between H3N2v and H9 virus Hong Kong/1073/99. These results suggest that the neutralizing antibodies target epitopes shared in group 1 viruses, but not with the group 2 viruses. However, different strains in group 1, even within the same subtypes, showed quite different susceptibilities to serum sample neutralization (Figure 1A and Supplementary Figure 1B).

DISCUSSION

The identification of broadly cross-neutralizing monoclonal antibodies to influenza A in persons with a history of influenza infections or seasonal influenza vaccinations raises questions about the seroprevalence of broadly cross-neutralizing antibodies and their potency against diverse, noncirculating influenza A

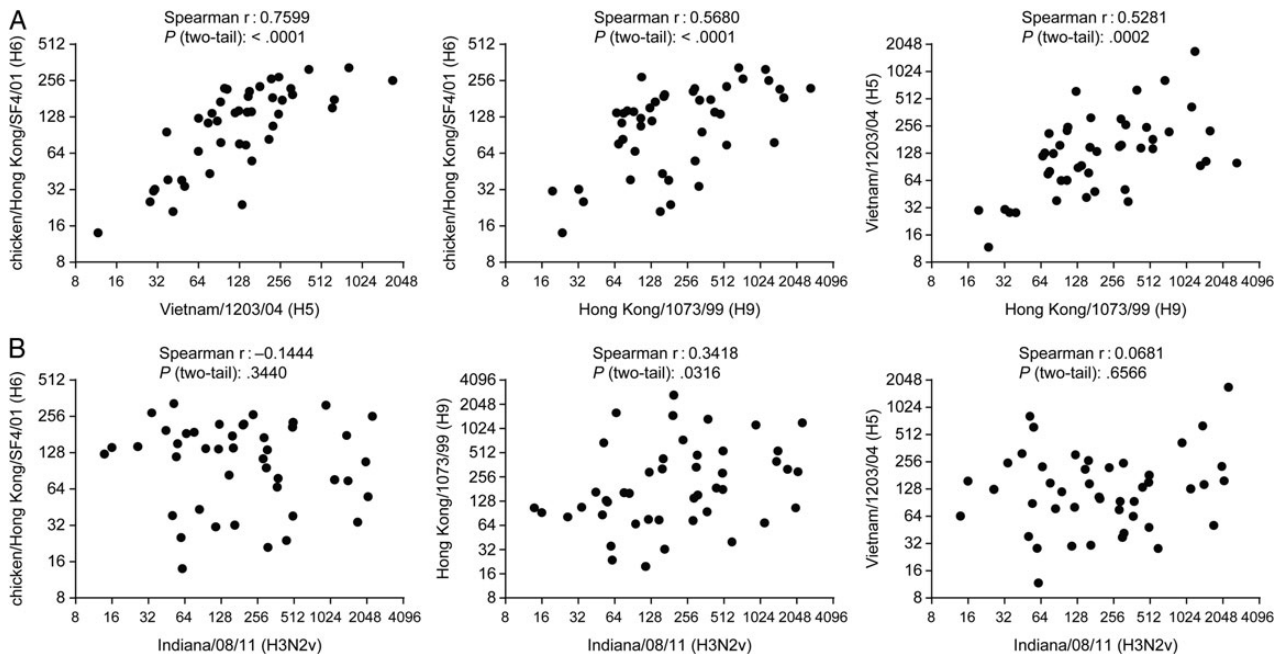


Figure 2. Neutralization titers between different subtype HAs correlate within groups but not between groups. *A*, Correlation of neutralization titers between different subtype HAs in group 1 viruses. *B*, Correlation of neutralization titers between group 1 and group 2 viruses. Abbreviation: HAs, hemagglutinins.

viruses from various subtypes. Our studies show that serum samples from some middle-aged adults who were likely exposed to seasonal H1N1 and H3N2 viruses, as well as past H2N2 viruses, and who have received annual seasonal inactivated influenza vaccines during the 2004/2005–2008/2009 seasons, do have cross-neutralizing antibodies to some noncirculating influenza viruses, including those from H2, H3N2v, H5, H6, and H9, but not H7 subtypes. However, the prevalence and titers of such antibodies varied both among and within subtypes.

Although it has been nearly a half century since the H2N2 influenza viruses have circulated, the threat of a pandemic from an H2 subtype influenza remains because those born after 1968 likely have little immunity to such viruses. Our data show that 100% of serum samples in our cohort of subjects aged 48–64 years still have very high neutralization titers to H2N2, consistent with a previous report [9], but younger individuals were not studied. In mouse and ferret studies, an A/Japan/305/57 (H2N2) virus infection elicited broadly cross-reactive antibody responses against heterosubtypic H2 influenza viruses [10]. Our data indicate that a significant proportion of middle-aged adults may not be highly susceptible to an A/Japan/305/57 (H2N2)-like virus should it reemerge, though younger individuals might be susceptible.

Our study also showed a high prevalence of cross-neutralization titers to H3N2v, probably reflecting cross-reactive antibody responses resulting from exposure to H3N2 viruses, as well as immunizations with seasonal influenza vaccines containing various H3N2 strains over many years. These findings extend previous reports that cross-neutralizing antibodies to H3N2v are frequently detected in the population of adults [1]. In contrast, no cross-neutralizing antibodies to H7 subtype HA-pseudoviruses were seen, suggesting that these subtypes in group 2 do not share relevant epitopes. Thus, some group 2 viruses may not be covered by certain vaccine candidates intended as universal vaccines.

Serum samples from our cohort also contained cross-neutralizing antibodies to H6, H9, and H5 subtype viruses, indicating that there are conserved neutralization epitopes among these viruses. While the stem region between these subtypes is relatively conserved, the presence of broadly neutralizing antibodies against HA head epitopes has been described [11–15]. Additional studies are therefore needed to elucidate the neutralizing determinants for these cross-neutralizing antibodies.

Interestingly, we note that different H5 clades showed dramatically different sensitivities to the serum samples. We note that the HA2 epitopes for broadly neutralizing stem antibodies are identical among the strains that we tested, suggesting that the head region may differentially display determinants for broadly neutralizing antibodies and/or that inter- and intramolecular interactions in the HA heads could affect stem epitopes.

Altogether, these data demonstrate that the serum samples in this cohort contain high-titered neutralizing antibodies to the

previously circulating H2N2 (Japan/305/57) influenza virus, while a significant proportion of subjects also have cross-neutralizing antibodies to noncirculating potential pandemic influenza viruses, including H3N2v, H5, H6, and H9, but not H7, subtype viruses. Because the subjects in our cohort had received multiple influenza vaccines over many seasons, these findings also raise the possibility that annual seasonal influenza vaccination contributes to the generation of cross-neutralizing antibodies to some potential pandemic influenza viruses.

Supplementary Data

Supplementary materials are available at <http://jid.oxfordjournals.org>. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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