Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a mechanism for the extinction of seasonal H1N1 viruses

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After the emergence of pandemic influenza viruses in 1957, 1968, and 2009, existing seasonal viruses were observed to be replaced in the human population by the novel pandemic strains. We have previously hypothesized that the replacement of seasonal strains was mediated, in part, by a population-scale boost in antibodies specific for conserved regions of the hemagglutinin stalk and the viral neuraminidase. Numerous recent studies have shown the role of stalk-specific antibodies in neutralization of influenza viruses; the finding that stalk antibodies can effectively neutralize virus alters the existing dogma that influenza virus neutralization is mediated solely by antibodies that react with the globular head of the viral hemagglutinin. The present study explores the possibility that stalk-specific antibodies were boosted by infection with the 2009 H1N1 pandemic virus and that those antibodies could have contributed to the disappearance of existing seasonal H1N1 influenza virus strains. To study stalk-specific antibodies, we have developed chimeric hemagglutinin constructs that enable the measurement of antibodies that bind the hemagglutinin protein and neutralize virus but do not have hemagglutination inhibition activity. Using these chimeric hemagglutinin reagents, we show that infection with the 2009 pandemic H1N1 virus elicited a boost in titer of virus-neutralizing antibodies directed against the hemagglutinin stalk. In addition, we describe assays that can be used to measure influenza virus-neutralizing antibodies that are not detected in the traditional hemagglutination inhibition assay.

cross-reactivity | cross-protection | subtype

Each year, influenza A (H1 and H3 subtypes) and influenza B viruses cause seasonal epidemics that result in significant morbidity and mortality. Illness can contribute to missed school and work days and also places an increased burden on the medical care system. In the United States alone, influenza viruses are thought to contribute to hundreds of thousands of hospitalizations and an average of 30,000 deaths per year (1). In the face of pandemic strains, mortality rates can be quite severe, with an estimation of at least 50 million deaths during the pandemic of 1918 (H1N1; Spanish influenza virus) and several million deaths during the 1957 (H2N2; Asian influenza virus) and 1968 (H3N2; Hong Kong influenza virus) pandemics (2, 3).

In April of 2009, a novel swine influenza H1N1 virus emerged against which most of the general population was immunologically naïve. Rapid spread of this virus resulted in its classification as a pandemic strain (pH1N1) by the World Health Organization in the months after its identification (4). Interestingly, pH1N1 viruses essentially replaced the normally circulating, seasonal (sH1N1) influenza viruses in the subsequent 2010–2011 influenza season. This finding was not particularly surprising given the disappearance of other circulating influenza virus strains after the emergence of a novel pandemic virus in 1957 and 1968 (5, 6). Here, we present data suggesting a mechanism by which the induction of

stalk-specific antibodies results in the elimination of the seasonal H1N1 viruses.

The influenza virus expresses two major glycoproteins on its cell surface: hemagglutinin (HA) and neuraminidase (NA). There are 16 known HA subtypes and 9 NA subtypes. The HA mediates viral entry into the cell and is the main antigenic driver of the adaptive immune response (6). The dominant immune response against influenza HA is thought to be directed to the head of the glycoprotein (amino acids 52–277, H3 numbering), specifically to defined antigenic regions that surround the receptor binding pocket. Antibodies against these sites are known to be quite potent, and act by neutralizing the binding of virus to host substrates. An immune response can also be directed against the stalk of the influenza virus HA, but antibodies of this type are typically less abundant and less potent than are antibodies specific for the globular head. Nonetheless, antistalk antibodies can provide protection through passive transfer in animal models (7–14). Whereas globular head antibodies can neutralize virus by preventing binding to the host cell, antistalk antibodies have been shown to act by preventing the fusion step of virus entry (13–15). Because antistalk antibodies are usually specific for epitopes that are highly conserved, these antibodies can be cross-reactive between HAs of distinct subtypes.

In mice, it has been shown that sequential exposure to antigenically divergent HAs can generate broadly reactive stalk antibodies (13, 16). Vaccination protocols have also been developed that selectively elicit a higher titer of stalk-specific antibody specificities (14). These antibodies have also been found, at low levels, in individuals after influenza virus vaccination or infection (11, 12, 15, 17, 18).

We have previously hypothesized that the immune status of the general population is responsible, at least in part, for the extinction of circulating seasonal influenza viruses after the emergence of novel pandemic strains. Specifically, we speculated that the sudden boost in population immunity mediated by antistalk antibodies generated after pH1N1 infection played a substantial role in the disappearance of the existing seasonal H1 viruses (5).

To test this hypothesis, we generated analytical tools in the form of chimeric HA (cHA) proteins and viruses expressing those chimeric proteins. These tools allow us to selectively detect

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stalk-specific antibodies in preparations that also include antibodies that bind the globular head of HA proteins. These HA constructs have a constant H1 subtype stalk with globular head domains from distinct HA subtypes (for example, H1 stalk with H6 head). This was accomplished by taking advantage of a disulfide bond that exists between cysteines 52 and 277 in the HA protein (19) and by exchanging the intervening sequence with that from a different HA subtype. By selecting HA heads against which most of the human population is naïve, we reason that serum antibodies that are reactive with the chimeric constructs are likely directed to the stalk region.

Using the cHA constructs, we have been able to show that a small cohort of humans with confirmed pH1N1 virus infections generated a high titer of stalk-specific neutralizing antibodies compared with uninfected adult and pediatric controls (not infected with pH1N1 viruses). Our findings support the hypothesis that antibodies reactive with the HA stalk, generated in response to pH1N1 infection, likely contributed to the dying out of seasonal H1N1 viruses that were circulating before the influenza pandemic of 2009.

Results

Development of cHA-Based Reagents. cHA constructs were generated to serve as analytical tools to assess the presence of stalk antibodies in human sera. By taking advantage of a disulfide bond that exists between C52 and C277 and delineates the boundary between the HA stalk and head, we engineered expression plasmids that encode the globular head domain of an H6 or H9 HA atop the stalk domain from the HA of PR8 (H1) virus (cH6/1 and cH9/1) (Fig. 1*A* and *SI Materials and Methods*). We hypothesized that human serum samples containing stalk antibodies would likely be negative for hemagglutination inhibition (HI) activity against H6 or H9 viruses (because of lack of prior exposure to these virus subtypes) but would be reactive with the cH6/1 or cH9/1 constructs because of prior exposure to the H1 HA stalk. These tools, recombinantly expressed cHA proteins and viruses expressing the cHAs, could be used to assess relative amounts of stalk antibodies in human serum samples and measure any neutralizing activity mediated by those stalk-specific antibodies.

To generate cH6/1 and cH9/1 proteins for analytical assays, plasmids coding for the cHAs were generated and expressed as soluble proteins in a baculovirus expression system. Coomassie staining of 2 μ g total protein suggests a high degree of purity in our preparations (Fig. S1*A*). The slight delayed migration of cH9/1 is thought to be the result of an increased number of glycosylation sites on the cH9/1 head. We also characterized the cHA proteins by Western blot analysis using antibodies reactive to various parts of the HA. As shown in Fig. S1*B*, only cH6/1, cH9/1, and full-length HA from PR8 reacted with a rabbit polyclonal antiserum specific for the PR8 stalk. Antibodies against the head domains of H6, H9, and PR8 confirmed that our chimeric constructs were expressing exotic heads atop a PR8 stalk. H3 protein was used as a negative control and was only detected when using the pan-H3 antibody 12D1 (13).

Recombinant viruses expressing the chimeric molecules were also rescued for the purpose of detecting neutralizing stalk antibodies (*SI Materials and Methods*). Because all human influenza viruses over the last century have encoded NA of the N1 or N2 subtype, we reasoned that the rescue of the cH9/1 N3 reassortant virus would allow us to assess the neutralizing capability of stalk-specific antibodies, while not measuring any (N1 or N2) neuraminidase antibody activity. The cH9/1 N3 virus (expressing the H1 stalk with H9 globular head HA with an N3 subtype neuraminidase) was rescued using reverse genetics and grown to high titers in embryonated chicken eggs. The plaque assay phenotype of this virus was similar to the phenotype of PR8 WT virus (Fig. S1*C*). To confirm the presence of the H9 head after virus passage, cells were infected with cH9/1 N3 virus.

Fig. 1. Chimeric HA (cHA) proteins and recombinant chimeric virus. (*A*) Schematic representation of cHAs. The globular head domain is defined as the intervening amino acid sequence between residues C52 and C277 (H3 numbering). Using this disulfide bond as the demarcation between head and stalk, exotic HA heads were introduced atop heterologous stalks. The stalk domain is defined as the remaining portions of HA1 and HA2 subunits. CT, cytoplasmic tail; SP, signal peptide; TM, transmembrane domain. The full-length HA structures were downloaded from the Protein Database (PDB): PR8 (H1) HA (PDB ID 1RU7) and A/guinea fowl/Hong Kong/WF10/99 HA [represented by A/swine/Hong Kong/9/98 (H9; PDB ID 1JSD)]. Final images were generated with PyMol (Delano Scientific). Because no structure of an H6 HA has been published, the image of the head-folding of the PR8 HA is used for the cH6/1 construct. (*B*) Immunofluorescence to confirm expression of cHA. MDCK cells were infected with either WT PR8 or cH9/1 N3 virus, or they were mock-infected. Antibodies specific for the head and stalk of PR8 virus as well as an antibody with H9 reactivity were used to confirm cHA expression. (Magnification bar: 40 $\times$.)

Infected cells were then probed with mouse mAb G1-26, an antibody specific for H9 subtype HA proteins. A pan-H1 stalk-specific antibody, 6F12, was used to detect both WT PR8 and cH9/1 N3 virus-infected cells (Fig. 1*B*).

Stalk-Specific Antibodies Bind and Neutralize cHA. To confirm that cH6/1 and cH9/1 proteins could be used as tools to detect stem antibodies, we first validated the use of these cHAs with an antibody known to react with the HA stalk. Indeed, mouse mAb C179, an antibody reactive with the stalk of H1 HA (20), bound to baculovirus-expressed cH6/1 and cH9/1 proteins by ELISA in a dose-dependent manner (*SI Materials and Methods* and Fig. S2*A* and *B*).

We next wanted to ascertain whether replication of the cH9/1 N3 virus could be inhibited by monoclonal antibody 6F12, which has neutralizing activity against H1 influenza viruses. Antibody 6F12 was able to bind and neutralize cH9/1 N3 virus in a plaque

samples from infected and uninfected adults were pooled, and total IgG was purified to remove nonspecific inhibitors (e.g., sialic acid containing molecules and lectins) that would bind to the HA head. Using these pure IgG preparations, we were able to completely inhibit plaque formation at antibody concentrations above 55.5 $\mu\text{g}/\text{mL}$ total serum IgG (Fig. 3*A* and *B*). We observed an ~ 30 -fold difference in neutralizing capability when comparing sera from pH1N1-infected adults with sera from uninfected adults. Using mAb 6F12 as a standard, we were able to compare neutralizing activities mediated by 6F12 and the polyclonal human IgG preparation. By comparing the concentrations of 6F12 and human IgGs that yielded 50% neutralization of cHA virus, we estimated that 7% of total human IgG from serum comprised neutralizing stalk antibodies.

Finally, we evaluated the neutralizing capability of stalk-reactive antibodies using a pseudotype particle infection assay that has a readout of luciferase activity generated following virus entry into host cells. Pseudotyped particles expressing the cH9/1 protein were incubated with purified human IgG, and neutralizing activity was measured by inhibition of particle entry, resulting in absence of luciferase enzymatic activity in cell supernatants (*Materials and Methods*). Consistent with the plaque reduction assay, the pseudotyped particle assay also showed 100% neutralization of particles at total IgG concentrations of greater than 10 $\mu\text{g}/\text{mL}$ (Fig. 3*C*).

Fig. 3. Adult patients infected with pandemic H1N1 virus have high titers of neutralizing antibodies that are specific for the HA stalk. (*A* and *B*) Sera from pH1N1-infected ($n = 14$) and adults not infected with pH1N1 ($n = 5$) were pooled separately, and total IgG from both pools was purified. Neutralizing capability of stalk antibodies was assessed by plaque reduction assay using cH9/1 N3 virus. Data points represent the mean and SE of two experiments. Plaques were immunostained with anti-H9 antibody G1-26. *B* shows plaque reduction of the four dilutions of sera shown along the top. (*C*) Pseudotype particle neutralization assay measures neutralizing antibody activity of the human-purified IgG preparations (sera from pH1N1-infected adults and adults not infected with pH1N1). Total IgG concentrations were 50, 10, and 2 $\mu\text{g}/\text{mL}$. As a positive control, the stalk-specific monoclonal antibody 6F12 was used.

Discussion

For decades, it was thought that neutralizing antibodies against influenza viruses bind the globular head of HA and act solely by preventing binding of virus to host cell sialic acids. These antibodies are typically measured using the classical HI assay (22). Numerous recent studies, including the present study, extend the definition of influenza virus neutralizing antibodies to include those antibodies that bind HA stalk epitopes (8, 10–16, 18, 21, 23). In this report, we introduce reagents and assays that enable measurement of this second basic type of neutralizing antibody.

Using the cHA constructs as analytical tools, we have been able to show that relatively high titers of stalk-specific IgG antibodies can be found in the sera of individuals who were infected with pH1N1 influenza virus compared with adults or children that were not. By engineering constructs with exotic HA heads, specifically using subtypes against which reactivity is not typically seen in the human population, we have shown that antibodies are reacting with the stalk domain. Importantly, these antibodies are capable of reducing virus replication.

We have previously proposed the hypothesis that stalk antibodies generated during pH1N1 infection may have played a role in the suppression of circulating sH1N1 influenza viruses in humans (5). The pace of the spread of pH1N1 virus was much more rapid than the spread of sH1N1 virus in 2009, and we believe that the sudden boost in antistalk titer that occurred with pH1N1 virus infection may have generated herd immunity against the seasonal virus, ultimately resulting in its disappearance. Because the titers of stalk antibodies wane after infection, it is likely that people previously infected with pH1N1 virus would be susceptible to infection with seasonal variants of the 2009 H1 virus. Antistalk titers may not be boosted to the same degree by subsequent infection with a seasonal variant, because the globular head domain would be similar to the pH1N1 virus; therefore, it would elicit the majority of an anamnestic response, because it is the immunodominant portion of the HA protein.

A similar event may have occurred in 1957, when the emergence of an H2N2 pandemic strain likely boosted the antistalk titer to a degree that caused the elimination of existing seasonal H1N1 viruses. In contrast, the replacement of seasonal H2N2 strains by the pandemic H3N2 virus of 1968 was likely mediated, in part, by antibodies specific for the retained N2 subtype neuraminidase. H3 and H2 subtype HAs fall into distinct phylogenetic groups and have dissimilar stalk structures; although stalk antibodies that neutralize HAs from phylogenetically distinct groups have been identified (9), it is more likely that boosted neuraminidase antibodies, which can have protective activity in vivo, were the major force behind the disappearance of H2N2 strains in 1968. Antibodies against the N1 may similarly have contributed to the elimination of seasonal H1N1 strains after the emergence of the pH1N1 virus of 2009 (5).

On infection or vaccination with seasonal influenza virus strains, an adaptive immune response is primarily generated to the immunodominant head of the HA rather than the HA stalk. On subsequent infections by a similar influenza virus, memory B cells are stimulated, and produce antibodies that bind to the head of the HA and block virus attachment to host cells; this finding is the principle on which annual influenza virus vaccination is based. We speculate, however, that a different process occurs when individuals are infected with a virus expressing an HA with an immunologically foreign head atop an immunologically familiar stalk, such as was seen with the pH1N1 relative to the sH1N1. We reason that, because the head of the pH1N1 HA is so dissimilar compared with the head of sH1N1 viruses, memory B cells against the head were not stimulated to the extent that they would be after infection with a relatively similar seasonal H1 virus. Instead, memory B cells specific for conserved regions of the stalk of the HA of the sH1N1 virus were selectively boosted. The data

presented by Kashyap et al. (17, 23) and the identification of broadly reactive antibodies isolated from individuals that survived H5 virus infection also support this hypothesis.

Evaluation of reactivity of human sera with H3 HA (Fig. 2E) showed that there is no significant difference between anti-H3 serum titers in pH1N1-infected vs. uninfected subjects. In contrast, reactivity with H5 HA is increased in pH1N1-infected patients compared with uninfected controls (Fig. 2D). Because of the structural similarities that exist between the H1 and H5 stalk regions, we consider that this boost in stalk antibody titer is specific for group 1 HA stalks, and it is not a reflection of an overall stimulation of the adaptive immune response.

In this manuscript, we compare sera from infected patients with sera from adults and children who were not infected with pH1N1 virus. It is possible that recent infection with a pre-pandemic seasonal influenza virus could yield similar results (boosting antistalk titer). We have previously shown that antibodies elicited by vaccination of adults with the inactivated seasonal influenza vaccine are not reactive with the LAH component of the HA stalk (21); in contrast, we show here that pH1N1 infection does elicit LAH-specific serum antibody. This finding may be a result of enhanced antistalk titer generated specifically by infection with the pandemic virus that expressed an antigenically different globular head, or it could be related to the observation that infection/vaccination with live-attenuated strains results in distinct and broader patterns of immunity compared with exposure to inactivated influenza antigens (24–28).

The concept of original antigenic sin (or antigenic imprinting; first postulated by Thomas Francis) argues that, after an initial influenza virus exposure, subsequent infections stimulate immune responses biased to the virus of primary exposure (29). This theory has been validated in human and animal models, although debate still exists in the field over a mechanism governing the phenomenon. It is possible that the immunological mechanisms that bias one's response to early exposures to virus may be, in part, explained by the induction of stalk-specific antibodies (30, 31).

In summary, we describe cHA proteins that enable the measurement of stalk-specific antibodies. Using these tools, we have shown that individuals infected with pH1N1 generated virus-neutralizing serum antibodies specific for the stalk of the HA protein. Similar cHAs could be generated that would enable measurement of antibodies specific for the stalks of phylogenetically different HA proteins (28). Our findings support the idea that infection with a virus expressing an immunologically novel HA drives the specificity of the immune response to conserved epitopes, such as those epitopes in the HA stalk, and that these antibodies, at the population level, likely play a role in the extinction of existing seasonal influenza virus strains.

Materials and Methods

Cells and Plasmids. 293T and Madin–Darby Canine Kidney (MDCK) cells were obtained from ATCC and maintained in DMEM and MEM (both from Gibco),

respectively, each supplemented with 10% FCS (HyClone), 100 units/mL penicillin, and 100 µg/mL streptomycin (Pen/Strep; Gibco). *Trichoplusia ni* media–formulation Hink (TNM-FH) media (Gemini Bioproducts) supplemented with 10% FCS and HyClone SFX insect culture media (Thermo-Scientific) were used for Sf9 and BTI-TN5B1-4 (High Five) cell culture.

cHA constructs with the stalk of A/Puerto Rico/8/1934 (PR8) containing the globular head domain from either A/mallard/Sweden/81/02 (cH6/1) virus or A/guinea fowl/Hong Kong/WF10/99 (cH9/1) viruses were generated using methods previously described (32, 33). Briefly, different components of the cHA were amplified by PCR with primers containing Sap I sites, digested with Sap I, and cloned into the Sap I sites of the pDZ plasmid (34). For generation of the baculovirus transfer plasmids, cH6/1 and cH9/1 were amplified by PCR, cut with BamHI and NotI, and cloned in frame into a modified pFastBac (Invitrogen) baculovirus transfer vector that harbors a C-terminal T4 phage fold-on and a 6-his tag (35). The sequences of all plasmids were confirmed by Sanger sequencing.

Human Serum Samples. Human sera were collected from three patient cohorts: adults not infected with pH1N1 virus, children not infected with pH1N1 virus, and pH1N1 virus infected adults. Samples were collected and used in accordance with the institutional review boards of Emory University and Mount Sinai School of Medicine (Emory Institutional Review Board 22371 and 555–2000 and Mount Sinai School of Medicine Grants and Contracts Office (GCO) # 04–0015 and 06–0218). All research studies involving the use of human specimens from Saint Louis University were reviewed and approved by the institutional review boards of Saint Louis University School of Medicine and Mount Sinai School of Medicine. Pre-vaccination human sera samples were obtained from children who were enrolled in clinical trials to test the safety and immunogenicity of an inactivated 2009 H1N1 influenza vaccine at the National Institute of Allergy and Infectious Disease Vaccine and Treatment Evaluation Unit at Saint Louis University. Confirmation of infection was performed by RT-PCR and/or serological assays.

Pseudotype Particle Neutralization Assay. The procedure for pseudotype particle production was adapted from previous studies (36). Briefly, 293T cells were cotransfected with four plasmids encoding (i) a provirus containing the desired reporter (V1-GLuc), (ii) HIV Gag-Pol, (iii) the chimeric cH9/1 HA protein, and (iv) influenza B/Yamagata/16/88 neuraminidase (37). The V1-GLuc plasmid encodes a luciferase protein that is secreted from cells and can be detected in the cell supernatant. Supernatants were collected 48 h post-transfection and subsequently filtered (0.45-µm pore size) to purify the cH9/1 particle preparations. Particles were then incubated (at quantity determined to give luciferase activity within the linear range after infection) with different concentrations (50, 10, and 2 µg/mL) of purified human IgGs and added to MDCK cells. Infections proceeded for 6 h before cells were washed, and fresh supernatant was placed over cells. All infections using pseudotype particles were performed in the presence of 1 µg/mL polybrene (Sigma) (37). Forty-eight hours postinfection, luciferase assays were performed.

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