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Journal Title: Proceedings of the National Academy of Sciences
Volume: Volume 109, Number 7
Publisher: NATL ACAD SCIENCES | 2012-02-14, Pages 2573-2578
Type of Work: Article
Publisher DOI: 10.1073/pnas.1200039109
Permanent URL: https://pid.emory.edu/ark:/25593/s5p3m

Final published version: http://dx.doi.org/10.1073/pnas.1200039109

Accessed October 30, 2017 6:13 AM EDT
Hemagglutinin stalk antibodies elicited by the 2009 pandemic influenza virus as a mechanism for the extinction of seasonal H1N1 viruses

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Contributed by Peter Palese, January 3, 2012 (sent for review December 28, 2011)

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. Recent studies have shown the role of stalk-specific antibodies in neutralization of influenza viruses; the finding that stalk antibodies can effectively neutralize viruses 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 22–77, 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...
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 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 stalk/ch6/1 and ch9/1) (Fig. L4 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 μg total protein suggests a high degree of purity in our preparations (Fig. S1A). 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. S1B, 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 subtype, 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: 40x.)

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. 1B).

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. S2A 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
reduction assay (Fig. S2 C and D) in a dose-dependent manner, with 100% inhibition seen at concentrations above 4 μg/mL. These results validated our hypothesis that the chimeric proteins and the recombinant ch9/1 N3 virus could be used to detect stalk antibodies with neutralizing activity.

**Patients Infected with pH1N1 Have High Titers of Antibodies That Bind and Neutralize chHA.** Before using ch6/1 and ch9/1 soluble proteins to quantitate stalk-reactive antibodies in patient blood samples, we tested the sera for HI activity against viruses expressing these two HA subtypes. Utilizing A/duck/303CF/02/76 (H6) and ch9/1 N3 viruses, we confirmed that all adult and pediatric serum samples collected were HI-negative.

We then went on to test the reactivity of the sera with ch6/1 and ch9/1 proteins by ELISA. Sera collected from adult and pediatric subjects not infected with pH1N1 viruses showed little reactivity with either protein. However, sera collected from patients infected with pH1N1 influenza virus showed enhanced binding to both chHA constructs, with a greater than 30-fold difference in IgG reactivity comparing sera from pH1N1-infected adults with sera from uninfected adults and children (comparing dilutions that yield equivalent OD readings) (Fig. 2A and B). We can therefore reason, by taking the negative HI data into account, that reactivity with chHA proteins is occurring in the stalk domain.

Using pooled samples of human sera, we also tested IgG binding to a portion of the HA stem, the long α-helix (LAH), which has been previously shown to mediate protective immunity in mice (21). Sera from patients infected with pH1N1 virus contained antibodies reactive with the H1 LAH, whereas patients unexposed to the pandemic virus had minimal LAH-specific serum antibody (Fig. 2C).

The H5 HA subtype is within the same phylogenetic group as the H1 HA, and it shares a very similar stalk structure (15). Interestingly, patients exposed to the pH1N1 had boosted serum antibody specificities reactive with the H5 protein (Fig. 2D) but did not have any serum HI activity against the homologous H5 subtype virus. This result suggested that exposure to the pH1N1 virus may have conferred a degree of anti-H5 immunity mediated by stalk-specific antibodies.

Importantly, we also noted that patients infected with pH1N1 virus did not have boosted serum antibodies specific for an H3 HA protein (H3 being in a separate phylogenetic group from H1 and H5 HAs) (Fig. 2E). This result shows that the enhanced titer of stalk-specific antibodies in sera from pH1N1-infected patients is not a function of general immune stimulation; rather, the H1 stalk antibody specificities were selectively boosted by infection with the pandemic virus strain.

Next, we wished to determine if stalk reactive antibodies found in these human samples had neutralizing capability. Serum

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**Fig. 2.** Adult patients infected with pandemic H1N1 virus have high titers of neutralizing antibodies that are reactive with the HA stalk. Reactivity of sera of pH1N1-infected adults (n = 9), children not infected with pH1N1 (n = 5), and adults not infected with pH1N1 virus (n = 11) with ch6/1 protein (A), ch9/1 protein (B), the LAH of the HA2 protein (anti-LAH antibody was used as a positive control; C), H5 HA protein (mouse polyclonal serum raised against H5 HA was used as a positive control and a pan-H3 antibody, 12D1, was used as a negative control; D) (13), or H3 HA protein (12D1 was used as a positive control and mouse polyclonal serum raised against H5 HA was used as a negative control; E). All were assessed by ELISA; data points represent average titers with SE or reactivity of pooled samples.
samples from infected and uninfected adults were pooled, and total IgG was purified to remove nonspecific inhibitors (e.g., stialic 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 μg/mL total serum IgG (Fig. 3A 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 μg/mL (Fig. 3C).

**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
We thank Chen Wang for excellent technical assistance. Our measurements of antibodies 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 pandemicseasonal 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; 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 global 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 the immunological response to the stalk for phenotypically 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 nucleopolyhedrosis virus (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/Porto Rico/8/1934 (PR8) containing the globular head domain from either A/mallard/Sweden/81/02 (H6/1') virus or A/guinea fowl/Hong Kong/WF10/99 (H9/1') 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 Sal I, and cloned into the Sal I sites of the pPD2 plasmid (33). For generation of the baculovirus constructs, cH6/1' and cH9/1' were amplified by PCR, cut with BamH I and NotI, and cloned in frame into a modified pFastBac (Invitrogen) baculovirus vector that harbors a C-terminal T4 phase 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 Sinai University were reviewed and approved by the institutional review boards of Saint Louis University School of Medicine and Mount Sinai School of Medicine. Precollection 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 posttransfection 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.

ACKNOWLEDGMENTS. We thank Chen Wang for excellent technical assistance. This research was partially supported by National Institutes of Health Grants U19 AI070469, HHSN266200700010C, U19 AI089987, U54 AI057138–04, and U19-AI057266 with American Recovery and Reinvestment Act Supplement Funding Grants U19 AI057266-0652, HHSN266200700006C, and HHSN272200800003C (Saint Louis University, R. Belshe, PI). N.P. and T.T.W. were supported by Mount Sinai Medical Scientists Training Grant T32 GM007280. F.K. was supported by Erwin Schrödinger Fellowship J 3232 from the Austrian Science Fund (FWF). T.T.W. was supported by National Institutes of Health Training Grant T32 AI007647.