MAJOR ARTICLE

Wide Prevalence of Heterosubtypic Broadly Neutralizing Human Anti–Influenza A Antibodies

Jianhua Sui,[1,2] Jared Sheehan,[1] William C. Hwang,[4] Laurie A. Bankston,[4] Sandra K. Burchett,[4,3] Chiung-Yu Huang,[5] Robert C. Liddington,[4] John H. Beigel,[6] and Wayne A. Marasco[1,2]

[1]Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, [2]Department of Medicine, Harvard Medical School, and [3]Division of Infectious Diseases, Children's Hospital Boston, Boston, Massachusetts; [4]Infectious and Inflammatory Disease Center, Sanford-Burnham Medical Research Institute, La Jolla, California; [5]National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, and [6]Laboratory of Immunoregulation, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, SAIC-Frederick, Inc., NCI-Frederick, Frederick, Maryland

(See the editorial commentary by Donis and Cox, on pages 1010–1012.)

Background. Lack of life-long immunity against influenza viruses represents a major global health care problem with profound medical and economic consequences. A greater understanding of the broad-spectrum "heterosubtypic" neutralizing human antibody (BnAb) response to influenza should bring us closer toward a universal influenza vaccine.

Methods. Serum samples obtained from 77 volunteers in an H5N1 vaccine study were analyzed for cross-reactive antibodies (Abs) against both subtype hemagglutinins (HAs) and a highly conserved pocket on the HA stem of Group 1 viruses. Cross-reactive Abs in commercial intravenous immunoglobulin were affinity purified using H5-coupled beads followed by step-wise monoclonal antibody competition or acid elution. Enzyme-linked immunosorbent assays were used to quantify cross-binding, and neutralization activity was determined with HA-pseudotyped viruses.

Results. Prevaccination serum samples have detectable levels of heterosubtypic HA binding activity to both Group 1 and 2 influenza A viruses, including subtypes H5 and H7, respectively, to which study subjects had not been vaccinated. Two different populations of Broadly neutralizing Abs (BnAbs) were purified from intravenous immunoglobulin by H5 beads: ~0.01% of total immunoglobulin G can bind to HAs from both Group 1 and 2 and neutralize H1N1 and H5N1 viruses; ~0.001% is F10-like Abs directed against the HA stem pocket on Group 1 viruses.

Conclusions. These data—to our knowledge, for the first time—quantitatively show the presence, albeit at low levels, of two populations of heterosubtypic BnAbs against influenza A in human serum. These observations warrant further investigation to determine their origin, host polymorphism(s) that may affect their expression levels and how to boost these BnAb responses by vaccination to reach sustainable protective levels.

Influenza remains a major medical problem and is a constant threat to human health. Of the 3 influenza virus genera (A–C), influenza A is generally associated with more severe disease and is further subtyped by 2 surface proteins, hemagglutinin (HA) and neuraminidase (NA). Various combinations of the 16 HA (2 phylogenetic groups) and 9 NA subtypes define all subtypes of influenza A viruses. The Group 1 HA subtypes are H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16; the Group 2 HA subtypes are H3, H4, H7, H10, H14, and H15. Seasonal viruses, such as influenza A H1N1 (Group 1) and H3N2 (Group 2), and influenza B viruses cause infection in 5%–15% of the population worldwide and 250,000–500,000 deaths annually. In addition to frequent annual epidemics, influenza viruses periodically cause pandemics, the most recent example

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Correspondence: Wayne A. Marasco, MD, PhD, Dept of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, 44 Binney St, JFB 824, Boston, MA 02115 (wayne_marasco@dfci.harvard.edu).

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being the 2009 pandemic caused by the swine-origin H1N1/2009 influenza virus.

Vaccination is the principle means of preventing seasonal and pandemic influenza and it's complications. A universal vaccine that induces broad immunity against multiple subtypes of influenza A viruses is a long-sought goal in medical research. Recently, we and others identified a family of human broadly neutralizing "heterosubtypic" antibodies (BnAbs) that bind to a highly conserved pocket on the stem of HA present in all Group 1 influenza viruses [1, 2] and block virus-host cell membrane fusion. These BnAbs were identified by recombinant H5 panning from antibody (Ab)–phage libraries that are constructed from nonimmune B cells [1], immunoglobulin (Ig) M memory B cells of seasonal vaccinees [3], or bone marrow of H5N1-infected "bird-flu" survivors [4]. BnAbs with similar properties have also been recovered from immortalized IgG-expressing memory B cells of seasonal vaccinees [5]. An unexpected finding from these studies is the frequent contribution of *VH1-69* heavy chain genes to these BnAbs, suggesting that a large fraction (up to 10%) of the human naive B cell repertoire has the capability of responding to this conserved epitope [5, 6]. These observations raise additional questions as to whether such BnAbs are present in human serum and at "protective" levels, whether they exist as "natural" Abs, and/or whether are they generated during the immune response to influenza virus infection or vaccination [5, 7]. To explore these questions, we analyzed serum samples from H5N1 vaccinees and commercial intravenous immunoglobulin (IVIG) samples for their cross-reactive binding and neutralization activity against different influenza A subtypes. We also utilized a representative BnAb, F10, for which the precise epitope was determined crystallographically [1], to probe for heterosubtypic BnAbs directed against the highly conserved pocket on the HA stem.

METHODS

Immunization Cohort

Serum samples from 77 healthy volunteers, matched before and after vaccination (1–4 months), had been collected and stored at a single center, from a dose-escalating clinical trial (ClinicalTrials.gov identifier: NCT00383071) on an inactivated H5N1 vaccine, conducted at the National Institute of Allergy and Infectious Diseases, National Institutes of Health [8]. The vaccine (manufactured by Sanofi Pasteur) used in the trial was a monovalent, inactivated subvirion H5N1 vaccine (rgA/Vietnam/1203/04 X A/PR/8/34). The study was conducted in accordance with institutional review board–approved protocol.

Enzyme-linked immunosorbent assays (ELISAs) were performed to test the cross-reactivity of the serum samples against multiple influenza A subtypes. Recombinant HA proteins, including H1 (A/New York/18/2009(H1N1), H1-NY18), H3 (A/Aichi2/68 (H3N2), H3-A2/68), H5 (A/Vietnam/1203/04 (H5N1), H5-VN04), and H7 (A/Netherlands 219/03 (H7N7), H7-NL03), were expressed in insect cells as trimers of HA ectodomains [1]. HA proteins (0.2 μg) were coated onto 96-well Maxisorb ELISA plate (Nunc) at 2 μg/mL in phosphate-buffered saline (PBS) at 4°C overnight. The plate was washed with PBS for 3 times to remove uncoated proteins. Serially diluted serum samples were applied to the HA-coated plates, followed by Horseradish Peroxidase (HRP)-anti-human IgG or IgM (Pierce Biotechnology), to detect the IgGs or IgMs against various HA subtypes in the serum samples. The optical density at 450 nm was measured after incubation of the peroxidase tetramethylbenzidine substrate system.

A competition ELISA assay was conducted to determine the level of F10-like Ab in the serum samples. F10 Ab (human IgG1) was biotinylated with Sulfo-NHS-SS-Biotin (Pierce Biotechnology) in accordance with the manufacturer's instructions. Biotinylated F10 (Bio-F10; 3 ng/mL) was mixed at 1:1 (vol) ratio with serum samples at various dilutions and added to ELISA plates coated with H5-VN04 trimer. The competition of serum samples for the binding of Bio-F10 to H5 was determined by measuring the remaining binding of Bio-F10 using HRP-Streptavidin (BD Bioscience).

IVIG Group

To isolate H5-bound Abs and F10-like Abs from intravenous immunoglobulin (IVIG; 100 mg/mL, Gamunex IVIG; Talecris Biotherapeutic), we first immobilized H5-VN04 proteins on magnetic beads (Dynabeads M-270 Epoxy; Invitrogen) in accordance with the manufacturer's manual, and then used step-wise Bio-F10 elution and acid elution to separate the F10-like Abs and H5-bound Abs present in the IVIG. Specifically, 5×10^8 of H5-beads were incubated with 1.5 mL of IVIG (100 mg/mL) overnight at 4°C, washed extensively with 0.1% BSA/PBS, followed by Bio-F10 elution (100 μg/ml, 0.5ml, total 50 μg) overnight at 4°C. The remaining H5-bound Abs on the H5-beads were then isolated with acid elution (pH = 2.8; buffer, 500 μL/10^9 H5-beads). Next, the Bio-F10 eluents and the acid eluents were incubated with Strep-T1 beads (250 μL of 4×10^8 beads; Invitrogen) at 4°C for 4–8 h each time for 2 and 3 times, respectively. ELISA was performed to verify the Bio-F10 in both samples was completely removed after Strep-T1 beads absorption. The purified H5-bound Ab and F10-like Abs, as well as unpurified IVIG, were quantified by ELISA using known concentrations of F10 monoclonal Ab (mAb) as standards. The aforementioned purification procedure was scaled up proportionally to obtain enough H5-bound Abs and F10-like Abs to test their cross-reactivity against different influenza A subtypes by ELISA and pseudotyped virus neutralization assay, as described elsewhere [1].

Statistical analyses were performed using the paired t test for the comparisons of prevaccination and postvaccination

Ab-binding levels and Bio-F10 Ab inhibition activities. The neutralization activity was compared using $\log_2$ microneutralization assay (MN) [8] titers. The proportions of >2-fold increase in Ab level were compared by McNemar's test for paired binary outcomes. All *P* values were 2-sided, and *P* values <.05 were considered to be statistically significant. The correlation between F10-like IgG Abs and MN titer against H5N1 in postvaccination serum samples ($n = 77$) was analyzed by Spearman rank correlation coefficient analysis.

RESULTS

In the H5N1 vaccine group, pre-vaccine IgMs against recombinant H5-VN04 protein were detectable in the majority of study subjects. The IgM binding level increased significantly after vaccination ($P = .006$); however, only 8 (10.4%) of 77 subjects demonstrated a >2-fold increase (as shown by a >2-fold increase in optical density at 450 nm by ELISA) (Figure 1A). All of the study subjects had pre-immune IgG Abs that bound to H5-VN04 (Figure 1B). As expected, the binding to H5-VN04 ($P < .001$) (Figure 1B) and neutralization activity (MN titer) against H5N1 (the vaccine strain; $P < .001$) (Figure 1E) significantly increased after vaccination. IgGs against recombinant H3-A2/68 protein (Figure 1C) and H7-NL03 protein (Figure 1D) were also detected in the pre-immune serum samples. In contrast to H5-binding, the postvaccination IgG binding to H3 did not increase significantly ($P = .11$); however, it increased to reach statistical significance for H7 ($P < .001$), but the proportion of specimens with a >2-fold increase in H7 binding was significantly lower than that for H5 binding (1.3% vs 76.6%; $P < .001$).

To investigate further the presence of heterosubtypic BnAbs in serum, we probed serum samples for the presence of F10-like IgG Abs, as determined by inhibition of the binding of biotinylated F10 (Bio-F10) to H5-VN04 in a competition ELISA assay. We found that F10-like Abs that could compete for Bio-F10 binding were also present in prevaccination serum specimens, with 23 (29.9%) of 77 samples demonstrating >30% inhibition of Bio-F10 binding at 1:90 dilution (Figure 1F). Furthermore, the F10-like IgG titers increased significantly after vaccination ($P < .001$), and the majority of vaccinees (54 of 77) had a 1.5-fold increase. In addition, there is a weak but significant correlation between the F10-like IgGs and the MN titer against H5N1 in post-immune serum samples, as determined by Spearman rank correlation analysis (co-efficient = .44); however, these stem pocket-directed BnAbs do not reach high enough levels to render a strongly significant correlation.

To determine whether these BnAbs were unique to the subject population tested or are present more broadly, we quantified the anti-H5 and F10-like Abs in a commercial IVIG and determined their breadth of heterosubtypic binding and neutralization activity. The IVIG contained pooled IgGs from thousands of donors and is representative of the pre-immune IgG Ab composition in the general population. Although it cannot be formally ruled out that IVIG is truly H5 naive, this was the best representative sample available for this study. H5-immobilized magnetic beads were used to affinity-purify F10-like and anti-H5 Abs from the IVIG. F10-like Abs were isolated by Bio-F10 competition elution from the total H5-beads bound Abs, and the remaining H5-bound Abs on the H5-beads were released with acid elution. By quantitative ELISA, we found that, from 100 mg of IVIG, ~10 μg of Abs bound to the H5-coated plate (~0.01% of the total). Of these, ~10% could be purified using affinity purification with H5-beads by acid elution. The final yields were 1–1.4 μg acid-eluted anti-H5. The Bio-F10 competitive elution yielded ~0.1 μg of F10-like Abs per 100 mg of IVIG (~0.001% of the total Ig level) and with similar or higher efficiency of recovery as compared with acid elution.

These anti-H5 and F10-like Abs were further tested for their heterosubtypic HA binding and neutralization activity against Group 1 and 2 viruses. The acid-eluted anti-H5 Abs are not H5-subtype specific: they showed cross-binding activity to H1-NY18, to H3-A2/68, and weakly to H7-NL219 (Figure 2A–D); they neutralized both H5N1 (A/Thailand/2-SP-33/2004, H5-TH04) and H1N1 (A/South Carolina/1/1918 (H1N1), H1-SC1918) pseudotyped virus infection (Figure 2E–F). Bio-F10 eluted F10-like Abs, showed Group 1 specificity as expected and bound to H1 and H5 (Group I), but not to H3 and H7 (Group II) (Figure 2A–D); they also neutralized both H1N1 and H5N1 pseudoviruses (Figure 2E–F).

DISCUSSION

In this study, we show that prevaccination serum samples have baseline heterosubtypic HA Ab binding activity to both Group 1 and 2 HA subtypes, including H5 and H7, to which these subjects are most likely unexposed because of their US geographic location. The IgM and IgG Abs to H5 increased significantly after the H5N1 vaccination, whereas this did not happen for IgG Abs to H3. F10-like IgG Abs are also detected in pre-immune serum samples and increased significantly in H5N1 vaccinees. A low level of serum anti-HA Abs that bind and neutralize H5N1 viruses has been reported to be age and influenza exposure dependent [9, 10]. Other investigators have reported enhanced levels of HA-directed anti-H5N1 neutralizing Abs in healthy donors after boosting with unrelated human influenza H1N1/H3N2 seasonal vaccines [5, 11, 12]. These serum Abs may be directed to the Group 1 stem pocket on HA. Indeed, Corti et al [5] showed that a majority of 2007 and 2008 seasonal vaccinees had preexisting neutralizing Ab titers against pseudotyped H5N1 viruses that markedly increased after seasonal vaccination and that the HA stem-pocket directed Abs were present at

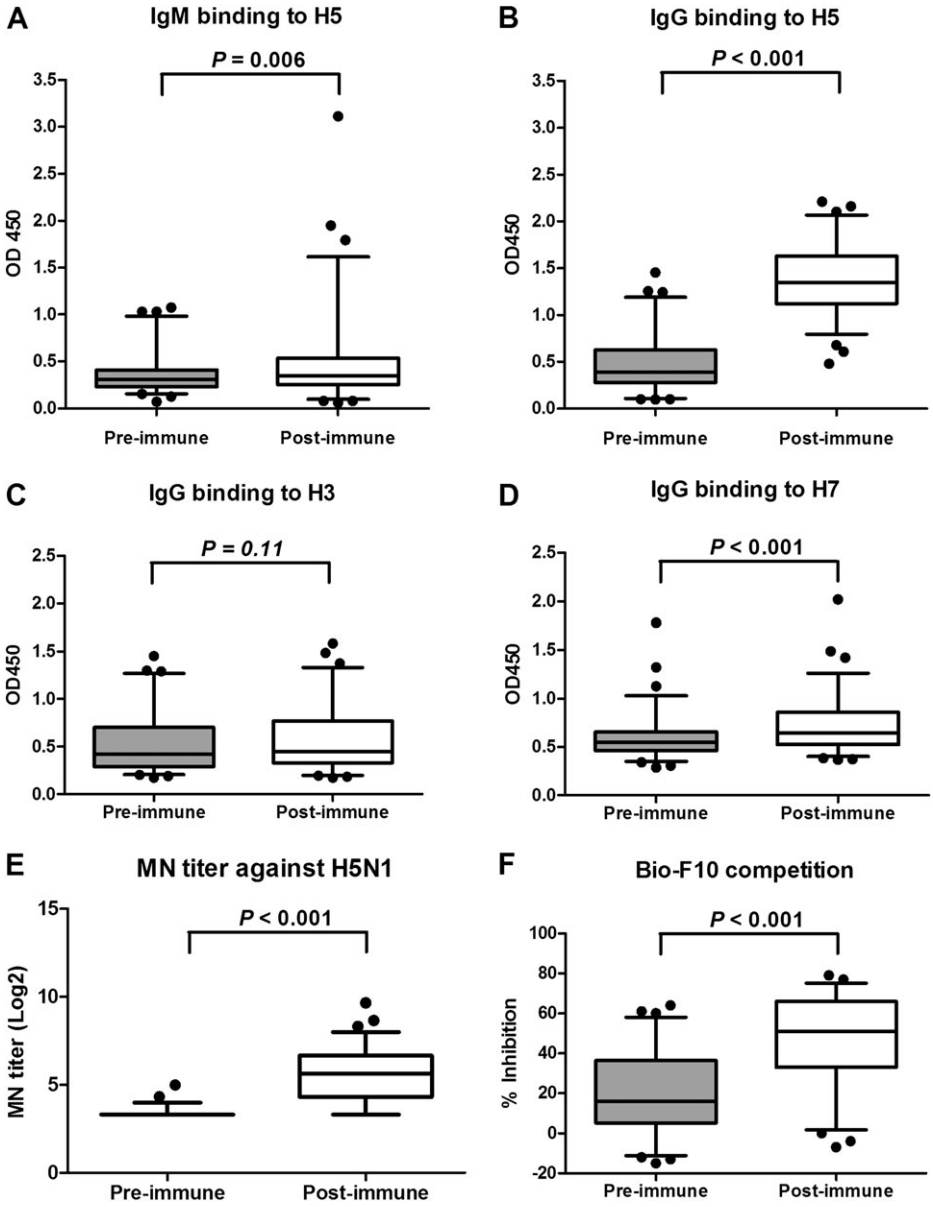

Figure 1. Serological study of 77 paired pre- and postimmune serum samples from H5N1 vaccinees. *A–D,* Enzyme-linked immunosorbent assays (ELISAs). Serum samples were serially diluted and applied to H5-VN04– *(A* and *B),* H3-A2/68– *(C),* or H7-NL03– *(D)* coated ELISA plates and the binding of immunoglobulin (Ig) M or IgG in serum samples to hemagglutinin (HA) proteins were determined using secondary Ab HRP-anti-human IgM *(A)* or IgG *(B – –D).* The binding levels are shown as optical density at 450 nm (OD 450). *E,* microneutralization assay (MN) titer against H5N1 virus; y-axis shows the Log_2 MN titer. *F,* Competition ELISA. Pre- and post-immune serum samples from H5N1 vaccinees were tested for their competition activity against a Group 1-specific BnAb, F10, binding to H5-VN04. Serially diluted serum samples were mixed with 3 ng/mL Bio-F10 and applied to H5-coated ELISA plates. The serum competition for binding of Bio-F10 to H5 was determined by measuring the remaining binding of Bio-F10 using HRP-Streptavidin. The serum competition activity is shown as percentage of inhibition. For all panels except panel E, data at 1 representative serum dilution are shown, as follows: panel A, 1:270; panel B, 1:5120; panels C and D, 1:2430; and panel F, 1:90. For all panels, data are shown in a box and whiskers graph. The box extends from 25th percentile to the 75th percentile, with a line at the median. The whiskers above and below the box indicate the 95th and 5th percentiles, respectively. The dots above and below the whiskers are data points beyond the 95th and 5th percentiles.

relatively low levels, compared with Abs that bind to the globular head of HA.

We also observed an increase of H7 (Group 2) reactivity after H5N1 (Group 1) vaccination (Figure 1D). The precise location of the cross-binding epitope(s) is currently not known. Observations that support the possible presence of hetero-subtypic Abs of the broader type that bind Group 1 and 2 viruses have also been seen in children after primary influenza infection [13] and mucosal vaccination of experimental animals [14, 15]. In one study, a cross-neutralizing murine mAb with

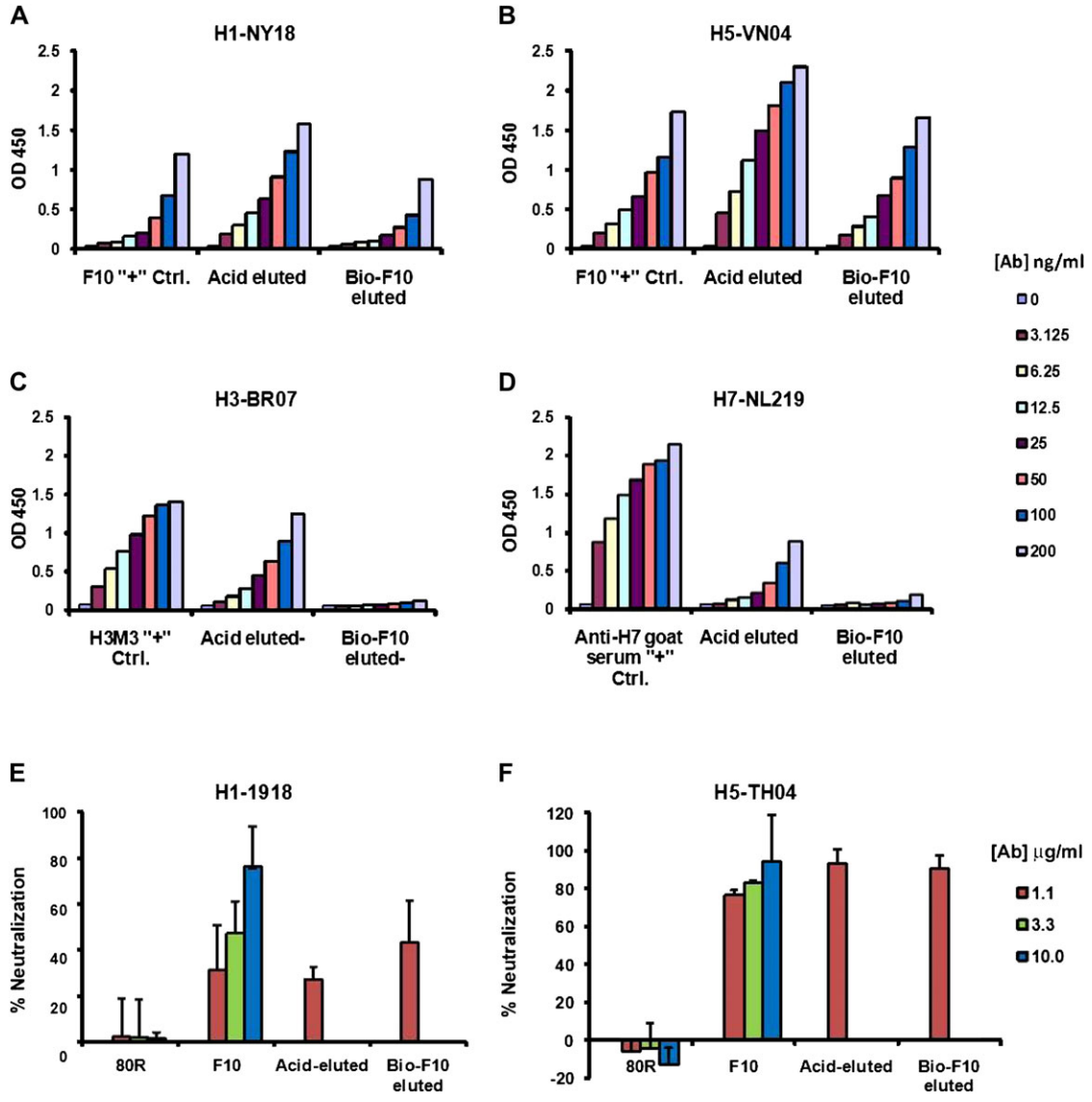

Figure 2. Heterosubtypic antibodies against influenza A viruses in intravenous immunoglobulin (IVIG). H5-VN04 was immobilized on magnetic beads (H5-beads) and the beads were used to affinity purify antibodies (Abs) from the IVIG sample. F10-like Abs and the remaining H5-bound Abs were purified separately. F10-like Abs were purified by Bio-F10 competition elution followed by multiple steps of streptavidin-beads absorption to eliminate Bio-F10 completely. The remaining H5-bound Abs on the H5 beads were eluted with standard acid elution method followed by a complete absorption of Bio-F10 using Streptavidin-beads as well.An enzyme-linked immunosorbent assay (ELISA) detecting Bio-F10 was used to confirm that no residual Bio-F10 remained in either Bio-F10-eluted or the acid-eluted samples. The binding activity of these purified Abs to H1-NY18 *(A),* H5-VN04 *(B),* H3-A2/68 *(C),* and H7-NL03 *(D)* was measured by ELISA at different serially diluted concentration. The neutralization activity of these samples was measured using neutralization assay with pseudotyped viruses of H1-1918 *(E)* and H5-TH04 *(F);* 80R was used as a negative control mAb that is specifically against the spike protein of severe acute respiratory syndrome coronavirus [24]. OD 450, optical density at 450 nm.

hemagglutination inhibition activity against Group 1 (H1, H2, H5, H9, and H13) and Group 2 (H3) was recovered from an intranasally vaccinated mouse. In contrast to the F10-like Abs, this mAb was directed to the globular head, and escape mutants were readily obtained [16]. Other animal vaccine studies have shown that antibodies to the fusion peptide and an HA2 linear peptide can have broad reactivity to Group 1 and 2 HAs [17–19] and neutralizing activity [20, 21], respectively. Whether different quantitative amounts of Group 1–specific BnAbs and/or Group 1 and 2–directed BnAbs are elicited by vaccination or natural infection remains an important but unanswered question of our study.

To quantify the baseline levels of anti-influenza heterosubtypic Abs in human serum, we used IVIG as representative of pre-immune IgG Ab composition in the general population, which includes individuals who are likely exposed to seasonal

influenza A virus infection and/or vaccinations (H1N1 and H3N2). Other investigators have shown that a low level of heterotypic anti-HA Abs that bind and neutralize H5N1 viruses is present in IVIG from diverse geographic locations [9, 10]. These investigations further showed that these heterosubtypic anti-H5N1 Abs cross-react with H3N2 and H1N1; however, efforts to purify and characterize these Abs (beyond neutralization titers) have not been reported. Our data show that there are 2 populations of heterosubtypic Abs with different HA binding ability in IVIG: one can bind to HAs from both Group 1 and Group 2 viruses; the other is specifically directed against Group 1 stem pocket. Approximately 0.01% of IVIG (most certainly derived from H5- and H7-naive donors), which is purified by acid elution from H5-beads, has heterosubtypic binding activity to both Group 1 and Group 2 HAs. This fraction of IVIG Abs also demonstrated neutralizing activity against the H1N1 and H5N1 pseudotyped viruses. The F10-like stem pocket-directed Abs were also detected in IVIG and were recovered at ~10% of the levels of H5 binding Abs. As expected, these F10-like Abs displayed similar binding and neutralization profiles among the tested HAs and viruses as the Group 1 specific mAbs that are directed to the stem pocket of HA [1, 3–5]. For both Ab fractions, a broader range of other subtypes were not tested due to limited amount of the Abs that we could purify from IVIG (~1 μg and 0.1 μg/100 mg IVIG, respectively), larger-scale purification of H5 protein and other materials will be required to characterize these heterosubtypic BnAbs in more detail.

Although we quantitatively show that BnAbs that bind to Group 1 and 2 HAs are present at very low levels, as well as that stem pocket-directed F10-like BnAbs exist at even lower levels, the question of whether serum or IVIG has protective levels of either type of heterosubtypic BnAbs is not answered in our study. However, our quantitative data support the notion that the levels of these BnAbs are borderline or below titers that would traditionally be considered protective. For example, there is up to 1 μg of F10-like Abs/100 mg IVIG; assuming 10 mg/mL IgG in normal human serum, then concentrations up to 0.1 μg/ml of F10-like Ab could be present. Likewise, the fraction of acid eluted BnAbs with activities against Groups 1 and 2 could also be in this range. Furthermore, we did observe variability in these levels in our study patients (Figure 1), and it remains possible that host factors, including *VH* polymorphism, may impact the baseline and inducible BnAb levels. In addition, the origins of these 2 populations of heterosubtypic anti-HA Abs are unknown. The possibility that they may be a component of "natural" polyreactive Abs cannot be excluded [22]. However, it is most likely that both our H5N1 vaccine study subjects and the IVIG donors had prior exposure to other Group 1 (H1N1) and Group 2 (H3N2) influenza A viruses, either through seasonal vaccinations and/or natural infection, and this may have given rise to heterosubtypic H5 and H7 binding Abs, respectively.

In summary, our findings show that the human immune system is capable of making BnAbs—not only to the conserved pocket on the HA stem of Group 1 viruses, but also to another unknown epitope(s) that are shared by Group 1 and 2 influenza A viruses. These observations provide the basis for further investigations aimed at obtaining a better understanding of these BnAbs, their origins, and the host genetic factors that restrict or enable their induction [23]. These additional studies should bring us closer to developing a universal influenza vaccine that provides durable protection beyond seasonal vaccines and mitigates that ability of the viruses to undergo neutralization escape. Indeed, a recently reported vaccine regimen—which induced protective level of the Group 1 stem-pocket directed BnAbs in animals—provides experimental evidence that the same may be possible in man [23].

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