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MAJOR ARTICLE

Surveillance Study of Influenza Occurrence and Immunity in a Wisconsin Cohort During the 2009 Pandemic

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Background. Antibody and T-cell immunity to conserved influenza virus antigens can protect animals against infection with diverse influenza strains. Although immunity against conserved antigens occurs in humans, whether such responses provide cross-protection in humans and could be harnessed as the basis for universal influenza vaccines is controversial. The 2009 pandemic provided an opportunity to investigate whether pre-existing cross-reactive immunity affected susceptibility to infection.

Methods. In 2009, we banked sera and peripheral blood mononuclear cells (PBMC) from blood donors, then monitored them for pandemic influenza infection (pH1N1) by polymerase chain reaction or seroconversion. Antibodies to hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix 2 (M2), and HA-pseudotypes were measured in sera. T-cell inteferon-γ enzyme-linked immunospot responses were measured in PBMC.

Results. There were 13 infections in 117 evaluable donors. Pre-existing T-cell reactivity to pH1N1 was substantial (of 153 donors tested, 146 had >100 spot-forming cells/10^6 cells). Antibodies reactive with pH1N1 were common: anti-NP (all donors) and anti-M2 (44% of donors). Pseudotype-neutralizing antibodies to H1 were detected, but not to highly conserved HA epitopes. Unexpectedly, donors with symptomatic pH1N1 infection had sharp rises in HA pseudotype-neutralizing antibodies, not only pH1N1 but also against multiple seasonal H1s. In addition, an exploratory study of a T-cell marker (response to $NP_{418\text{-}426}$) identified probable infection missed by standard criteria.

Conclusions. Although the number of infections was inadequate for conclusions about mechanisms of protection, this study documents the wide variety of pre-existing, cross-reactive, humoral and cellular immune responses to pandemic influenza virus antigens in humans. These responses can be compared with results of other studies and explored in universal influenza vaccine studies.

Keywords. conserved antigens; cross-protection; heterosubtypic immunity; influenza; pandemic.

Influenza causes widespread morbidity and mortality, especially during pandemics. Current strain-matched vaccines leave public health gaps due to the long lead time for strain selection, vaccine production, and distribution. Universal influenza vaccines inducing broad (heterosubtypic) immunity could protect against diverse strains and even different subtypes of virus, and they could be available off-the-shelf early in an outbreak.

Broad cross-protection to influenza has long been studied in animals (reviewed in [1]). Prior immunity to other strains does not prevent infection, but it greatly reduces morbidity and mortality. Extensive literature documents the ability of conserved antigens including nucleoprotein (NP) and matrix 2 (M2) to induce cross-protection [2–7], and there is evidence that cross-protective vaccination reduces transmission [8]. Nonneutralizing antibodies may also protect [9–11] perhaps by antibody-dependent cell-mediated cytotoxicity [9, 11].

Cross-protection in humans is more controversial. Human T-cell memory responses to conserved influenza antigens are well documented, and they cross-react with virus strains the individuals have never encountered [12–14]. However, there is skepticism about whether T cells protect against disease [15]. Linking immunity to protection requires challenge or surveillance studies, and such evidence has accumulated gradually.

A classic human study found that, in donors lacking neutralizing antibodies, pre-existing cytotoxic T-cell levels against influenza correlated with reduced virus shedding but not reduced symptoms after influenza virus challenge [16]. Epidemiological studies of the 1957 pandemic, an H1N1 to H2N2 shift, suggested

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cross-protection, but these studies were too small for statistical significance [17] or lacked laboratory confirmation of self-reported symptoms [18]. A human challenge study found a correlation of CD4 (but not CD8) memory with reduced illness duration, viral shedding, and symptom severity [19].

The 2009 influenza pandemic provided a rare opportunity to investigate cross-protection in humans, and several studies were performed. Some people >60 years old had neutralizing antibodies to the new virus [20]. In addition, there is evidence for protection by mechanisms other than neutralizing antibodies. A study in Hong Kong showed that previous seasonal influenza infection protected significantly against pandemic influenza infection (pH1N1) despite the absence of pH1N1-neutralizing antibodies [21]. A surveillance study found a correlation of CD8 T-cell memory with reduced symptom severity [22]. The UK Flu Watch study found that T-cell immunity to NP protected against symptomatic, polymerase chain reaction (PCR)-confirmed pH1N1 infection [23].

This report describes a study planned while the pandemic was emerging in spring, 2009, with the hope of evaluating cross-protection. We established a cohort, collected baseline samples, monitored donors for symptoms, and collected follow-up samples. Banked samples were used to characterize pre-existing antibody and T-cell immunity cross-reactive with the pH1N1 virus and responses to infection. The relative mildness of the pandemic was fortunate for public health but resulted in relatively few infections in this cohort. We characterize baseline donor T-cell and antibody responses reactive with pH1N1 plus certain follow-up parameters.

METHODS

Donor Cohort, Sample Banking, and Surveillance

The study was approved by the BloodCenter of Wisconsin Institutional Review Board and the US Food and Drug Administration Research in Human Subjects Committee. Healthy blood donors were enrolled and consented to the research study. At baseline, a blood donation and questionnaire about influenza vaccination or symptoms were collected. Peripheral blood mononuclear cells (PBMC) and sera were frozen. Donors were instructed to contact study personnel immediately if they experienced influenza-like symptoms. Influenza-like illness (ILI) was defined by fever ≥37.8°C and 2 other influenza symptoms listed by the Centers for Disease Control and Prevention ([CDC] http://www.cdc.gov/h1n1flu/symptoms.htm). Donors reporting ILI symptoms were visited by a study nurse the same day or the next day to collect nasal swabs, 50 mL of blood, vital signs, and a questionnaire about influenza vaccination and symptoms. The donor was asked to donate 50 mL of blood 7 and 14 days later. At subsequent blood donations, sample banking and questionnaires were repeated.

Real-Time, Polymerase Chain Reaction

Viral ribonucleic acid (RNA) was extracted from nasal swabs using the MagNA Pure Compact RNA isolation kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Polymerase chain reaction was performed using the CDC real-time reverse transcriptase-PCR Swine Flu Panel for detection and characterization of pH1N1 influenza viruses from donor specimens.

Hemagglutination Inhibition and Neuraminidase Inhibition Antibody Testing

Hemagglutination inhibition (HI) [24] was performed in batches at the City of Milwaukee Health Department using pH1N1 strain A/CA/04/09, later replaced with A/CA/07/09 NYMC X-179A, and guinea pig red blood cells (RBC). Additional HI testing was performed at the Center for Biologics Evaluation and Research on available sera using A/CA/07/09 NYMC X-179A and turkey RBC.

Neuraminidase inhibition (NI) was measured by enzyme-linked lectin assay [25]. To eliminate effects of hemagglutinin (HA) cross-reactivity, a reassortant with H6 from A/turkey/Massachusetts/3740/1965 (H6N2), 6 internal genes from PR8, and neuraminidase (NA) from A/CA/04/09 (same NA as in X-179A) was used.

Postseason antibody testing was performed on samples collected at the next blood donation visit, and only donors with such samples were considered evaluable for infection. Seroconversion was defined as ≥4-fold increase in HI or NI titer. We followed standard criteria for vaccine seroconversion [26] also used in some studies of infection [27], under which an increase from <1:10 to 20 does not qualify as 4-fold.

Peptides

17-mer peptides spanning all proteins of pH1N1 were prepared using a consensus of GenBank sequences of Wisconsin pandemic viruses isolated from June to December 2009 and used as 25 pools (Supplementary Table 1). 17-mers were chosen to permit detection of both CD4 and CD8 responses. The peptides for the 25 pools and pH1N1 $NP_{418-426}$ variant LPFERATVM were synthesized by GenScript (Piscataway, NJ). Human immunodeficiency virus-Gag SLYNTVATL was obtained from New England Peptide (Gardner, MA). A mixture of cytomegalovirus, Epstein-Barr virus, and influenza peptides ([CEF] Mabtech, Mariemont, OH) served as a control.

Interferon-γ Enzyme-Linked Immunospot

Peripheral blood mononuclear cells were cultured in triplicate for 24 hours with stimulating peptide pools containing 2 μg/mL of each peptide, in anti-interferon (IFN)-γ-coated plates. Spots were developed with biotinylated anti-IFN-γ antibody, streptavidin-alkaline phosphatase, and substrate. Results were calculated as spot-forming cells (SFC) per 10^6 above background for cells with medium alone. The standardized enzyme-linked immunospot (ELISPOT) protocol always included responses of the same 2 reference donors to CEF control peptides. These PBMC reference samples were banked frozen cells from a moderate responder and a low responder, thus

representing a robust response and a response with sensitivity near the detection limit. Results demonstrate a high degree of consistency among assays and stability of frozen PBMC (Supplementary Figure 1).

M2 Protein Antibody Testing

The cell surface flow cytometric assay (M2-CFA) [28] was used to measure antibodies to cell surface M2. 293FT transfectants expressing M2 of A/Panama/2007/99 (H3N2) (termed HM2) and A/CA/04/09 (pH1N1) (termed SWM2) were generously provided by Weimin Zhong (Centers for Disease Control and Prevention, Atlanta, GA). Positive control human monoclonal antibody (mAb) Ab1-10 was made at University of Toyama. Results are expressed as units (normalized % M2+ cells) = [(percentage with test serum sample on transfected cells – percentage with sample on untransfected cells)/(percentage with positive mAb on transfected cells – percentage with positive mAb on untransfected cells)] × 100.

Nucleoprotein Antibody Testing

Enzyme-linked immunosorbent assays (ELISA) were performed on plates coated with recombinant NP (rNP) of the Wisconsin 2009 pH1N1 consensus sequence, A/Brisbane/10/2007(H3N2), or A/PR/8/34(H1N1). Optical density (OD) was measured at 405 nm. Endpoint titers were defined as the greatest dilution with OD ≥0.1 after background subtraction.

Hemagglutinin-Pseudotype Neutralization Assay

The luciferase reporter assay using constructs expressing H1 HA from H1N1 viruses A/Mexico/4108/2009 (pH1N1), A/Solomon Islands/3/2006 (SI/06), A/New Caledonia/20/1999 (NC/99), and A/Brisbane/59/2007 (BR/07), and H5 HA from A/Vietnam/1203/2004 (VN/04), has been described previously [29]. Titers were expressed as relative luminescence units/mL supernatant (RLU/mL). Neutralization was tested as described [30] using duplicates. The 95% inhibitory titer (IC95) = dilution causing 95% reduction of RLU compared with control, and this was calculated using GraphPad Prism.

Statistical Analysis

Two-tailed Student *t* test, Pearson correlation, and linear regression were performed in SigmaPlot 13. Pseudovirus neutralization titer changes between groups were evaluated by Mann-Whitney *U* test using GraphPad Prism. *P* values <.05 were considered statistically significant. Discriminant analysis and principal component analysis were used to explore multivariate associations between immunologic parameters and disease outcomes.

For additional details of all methods, see Supplementary Methods.

RESULTS

Surveillance Results

The cohort enrolled 182 people, 117 of whom were evaluable for infection (HI and NI antibody results for paired baseline and follow-up sera available). Table 1 shows donor age, gender, and infections. Pandemic (pH1N1) infections were defined by real-time PCR detecting pH1N1 in swabs of donors with ILI or by HI or NI seroconversion as defined in Methods, without vaccination against pH1N1. Of 36 donors reporting symptoms, 21 met the definition of ILI and had swabs collected the day of reporting symptoms or the next day, and these were tested by PCR. Of those, 5 had PCR-confirmed pH1N1. Seroconversion detected 2 additional pH1N1 infections in PCR-negative donors with ILI symptoms; presumably, viral shedding was low or missed by swab timing. These 7 cases are termed "pH1N1+ILI." Donors with ILI symptoms but negative PCR and no seroconversion are termed "non-pH1N1 febrile illness." Six pH1N1 infections were detected by seroconversion in donors asymptomatic or reporting symptoms milder than ILI. They are termed "mild/asymptomatic."

Pre-existing T-Cell Immunity to the Pandemic Strain

Responses of 153 donors are shown in Figure 1A; other donors had inadequate PBMC samples for complete T-cell testing. The rank order of response to the 25 peptide pools and average response are shown in Supplementary Table 1. Every influenza protein elicited responses from some donors. For individual donors, summed responses to all 25 pools ranged from 47 to 5004 SFC/10^6 cells. Total response did not correlate with age (data not shown).

To assess linkage to reduction in symptoms, baseline T-cell results were compared for donors later infected with pH1N1 who had ILI (pH1N1+ILI; Figure 1B) versus those with mild/asymptomatic infections (Figure 1C). There was a trend toward greater T-cell responses in the mild/asymptomatic group, but the difference was not statistically significant. We also compared our 2 small groups of infected donors for IFN-γ responses to the peptides with which other investigators found T-cell differences: NP pools only rather than total ELISPOT in the case of Hayward et al [23], and NP, M, PB1 9-mer peptides from the Biodefense

Table 1. Donor Population Demographics[a]

Age	Female (Infected With pH1N1)	Male (Infected With pH1N1)	Total (Infected With pH1N1)
Number in Age Range			
21–30	11 (1)	2 (0)	13 (1)
31–40	18 (1)	8 (1)	26 (2)
41–50	47 (4)	19 (2)	66 (6)
51–60	9 (2)	2 (1)	11 (3)
61+	0	1 (1)	1 (1)
Median age	45	46.5	45
Total number	85 (8)	32 (5)	117 (13)
% infected	9.4	15.6	11.1

Abbreviations: HI, hemagglutination inhibition; NI, neuraminidase inhibition; pH1N1, pandemic influenza.

[a]Table includes only donors evaluable for seroconversion (HI and/or NI results available for both baseline and follow-up serum samples). Donors with either a baseline serum or a follow-up serum but not both were considered not evaluable for infection and excluded.

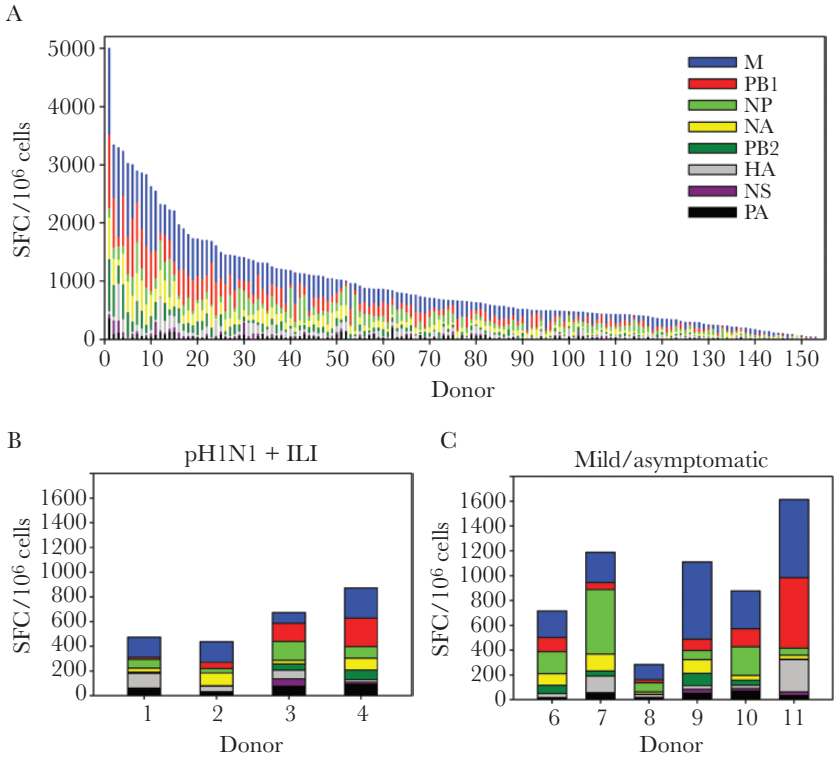


Figure 1. Cellular immunity to pandemic influenza virus (pH1N1) antigens in donor baseline peripheral blood mononuclear cells. Responses were measured by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT). Stimulation used 25 pH1N1 2009 peptide pools (see Methods and Supplementary Table 1), at 2 μg/mL final concentration of each peptide in the culture. A standard CEF peptide pool and phytohemagglutinin were used as positive controls, and media alone was used as negative control. Spots were counted 24 hours later. Bars show responses for individual donors as IFN-γ spot-forming cells (SFC)/10^6 cells above background. Colored segments of bars indicate antigen recognized. (A) Responses at the baseline time point for 153 donors (not all of whom were evaluable for infection because some lacked follow-up serum samples). (B and C) Responses at the baseline time point in those donors later infected with pH1N1, grouped for symptoms of differing severity. Due to limited availability of cells, certain infected donors were not tested with the 25 peptide pools and thus are not shown. (B) Infected donors with influenza-like illness (ILI) symptoms. (C) Donors with milder or no symptoms. Total IFN-γ ELISPOT response did not differ significantly between the ILI and mild/asymptomatic groups, $P = .127$ by 2-tailed Student t test.

and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases of National Institutes of Health in the case of Sridhar et al [22]. Differences between the pH1N1+ILI and mild/asymptomatic groups were not significant for these measures (data not shown).

Baseline Antibody Reactivity to Highly Conserved Antigens

We analyzed pre-existing antibodies to highly conserved antigens NP and M2, which are known to provide cross-protection in animal models. Nucleoprotein is highly conserved among viral strains. Some antibodies can distinguish pH1N1 NP from earlier strains [31], but polyclonal sera like ours mainly detect shared epitopes. Enzyme-linked immunosorbent assay was performed on rNP proteins of A/Brisbane/10/2007(H3N2), A/PR/8/34(H1N1), and pH1N1. The distribution of baseline endpoint titers is shown in Figure 2. Most donors had titers of 1280 or 5120. Titers on the 3 NP strains for individual sera were identical or differed by only 1 dilution.

Antibodies recognizing cell surface conformational epitopes of M2 [32] are more broadly cross-reactive and protective in passive protection than those recognizing linear epitopes [33]. For that reason, we used the M2-CFA and transfectants of Zhong et al [28] to measure antibodies to cell surface "human M2" (HM2) from A/Panama/2007/99 (H3N2) or "swine M2" (SWM2) from A/CA/04/09 (pH1N1). Human mAb Ab1-10, which recognizes M2 of seasonal, pandemic, and avian viruses and inhibits pandemic virus plaques [34], was used as a positive control.

The M2 antibody results are shown in Figure 3. Using 3 units as the threshold of positivity as defined by others [35], 60 of 136 donors (44%) were positive on SWM2 at baseline, and 92 of 136 (68%) were positive on HM2. For SWM2, the median level in positive donors was 6.3 units for age <40 and 10.6 units for age ≥40 (not significantly different). Regarding cross-reactivity of individual sera on HM2 and SWM2, approximately half recognized both forms. Of the rest, more donors recognized only HM2, and a few recognized only SWM2.

Hemagglutinin-Pseudotype Neutralizing Antibodies

Pseudotype neutralization is a sensitive assay for neutralizing antibodies against influenza HA. In some cases, this assay can detect antibodies missed by HI assays, including antibodies to HA stem epitopes. We tested sera using vectors pseudotyped with HA of pH1N1, VN/04 (H5N1), and 3 seasonal H1N1

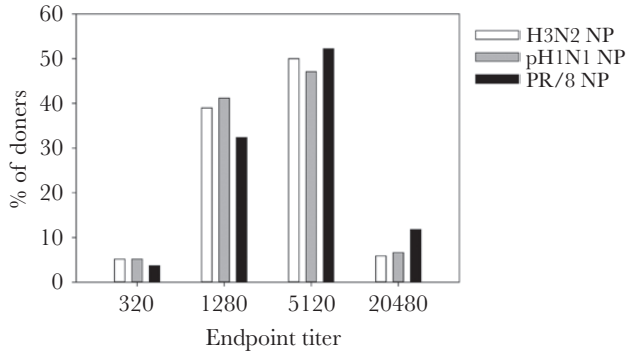


Figure 2. Antibodies to nucleoprotein (NP) in donor baseline sera. Enzyme-linked immunosorbent assay was performed as described under Methods on recombinant NP protein of 3 types: A/Brisbane/10/2007 (H3N2), our consensus sequence of Wisconsin 2009 pandemic influenza virus (pH1N1), and A/PR/8/34. One hundred forty-eight donor sera were tested. Endpoint titer was defined as the greatest dilution with optical density ≥0.1 after subtraction of background (average of secondary antibody alone in 5 antigen-coated wells).

strains. Donor sera had no or only marginal activity on the H5N1 pseudotype, which would detect antibody to conserved HA stem or head epitopes (data not shown).

Testing of baseline and follow-up sera from donors with febrile illnesses revealed a novel relationship. Donors with pH1N1+ILI had increases in titer not only on the pH1N1 construct but also on seasonal H1N1 strains NC/99, SI/06, and BR/07 (Figure 4A), suggesting either bystander memory activation or antibody cross-reactivity. In donors with non-pH1N1 febrile illness (Figure 4B), the changes in titers on NC/99 were variable, whereas the bystander increase on SI/06 and BR/07 was not seen (Figure 4C).

Exploratory Multivariate Analyses

We originally hoped to study T-cell cross-protection that might reduce symptom severity, but we also measured a variety of other parameters of pre-existing immunity (antibodies to NP, M2, and HA pseudotypes, as well as T-cell responses to individual peptide pools). For statistical comparison of so many variables, we performed exploratory principal component and discriminant analyses. These analyses did not reveal any significant relationships of antibody or T-cell parameters at baseline with occurrence of pH1N1 infection or with symptom severity.

Exploratory Study of a T-Cell Marker

We had defined pH1N1 infection by PCR-positive results for pH1N1 or seroconversion as described in Methods, and we did not modify that prospective definition on the basis of this exploration. However, because some infections could be missed by that definition, we explored a T-cell marker of infection.

Human T-cell responses to $NP_{418\text{-}426}$ distinguish the pH1N1 sequence LPFERATVM from earlier variants [36]. In one donor who had neither reported illness nor received pandemic

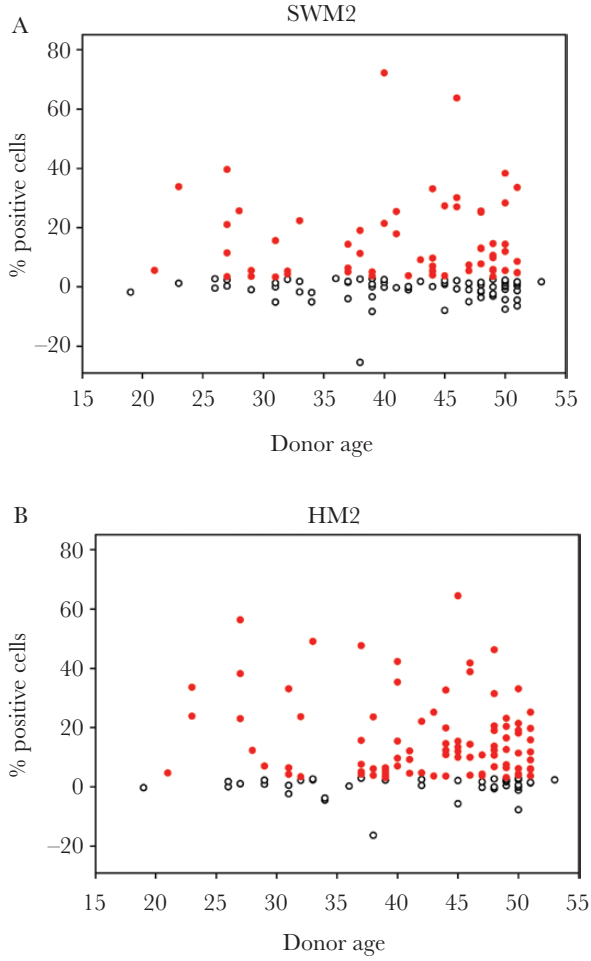


Figure 3. Antibodies to matrix 2 (M2) in donor baseline sera. Antibodies were measured and units calculated as described under Methods using the cell surface flow cytometric assay (M2-CFA) of Zhong et al [28], stable transfectants expressing full-length M2 from A/CA/04/09 (pandemic influenza virus [pH1N1]; SWM2 [A]) and A/Panama/2007/99 (H3N2; HM2 [B]). One hundred thirty-seven donor sera were tested; each circle represents 1 donor. The solid red circles represent values above a 3% threshold considered positive. Open black circles are considered negative. Values <0 reflect more binding to untransfected cells than to transfected cells (see Methods), which is presumably nonspecific.

vaccine, there was low baseline T-cell reactivity to pH1N1 $NP_{418\text{-}426}$, followed by a striking rise and sustained reactivity (Figure 5). At the same time, this donor had increases in total T-cell ELISPOT to pH1N1 (25 pools) and in antibodies to pH1N1 HA pseudotype, pH1N1 NP, and SWM2. Most likely the donor was infected with pH1N1. Thus, in this case, the $NP_{418\text{-}426}$ T-cell assay provided more sensitive detection than HI and NI. Note that donors of a certain human leukocyte antigen (HLA)-B family [36] react to this peptide, but not all donors. Thus, this marker could not be used alone for screening purposes but perhaps as part of a group of markers. We describe it as an interesting case study and proof-of-concept for this type of testing.

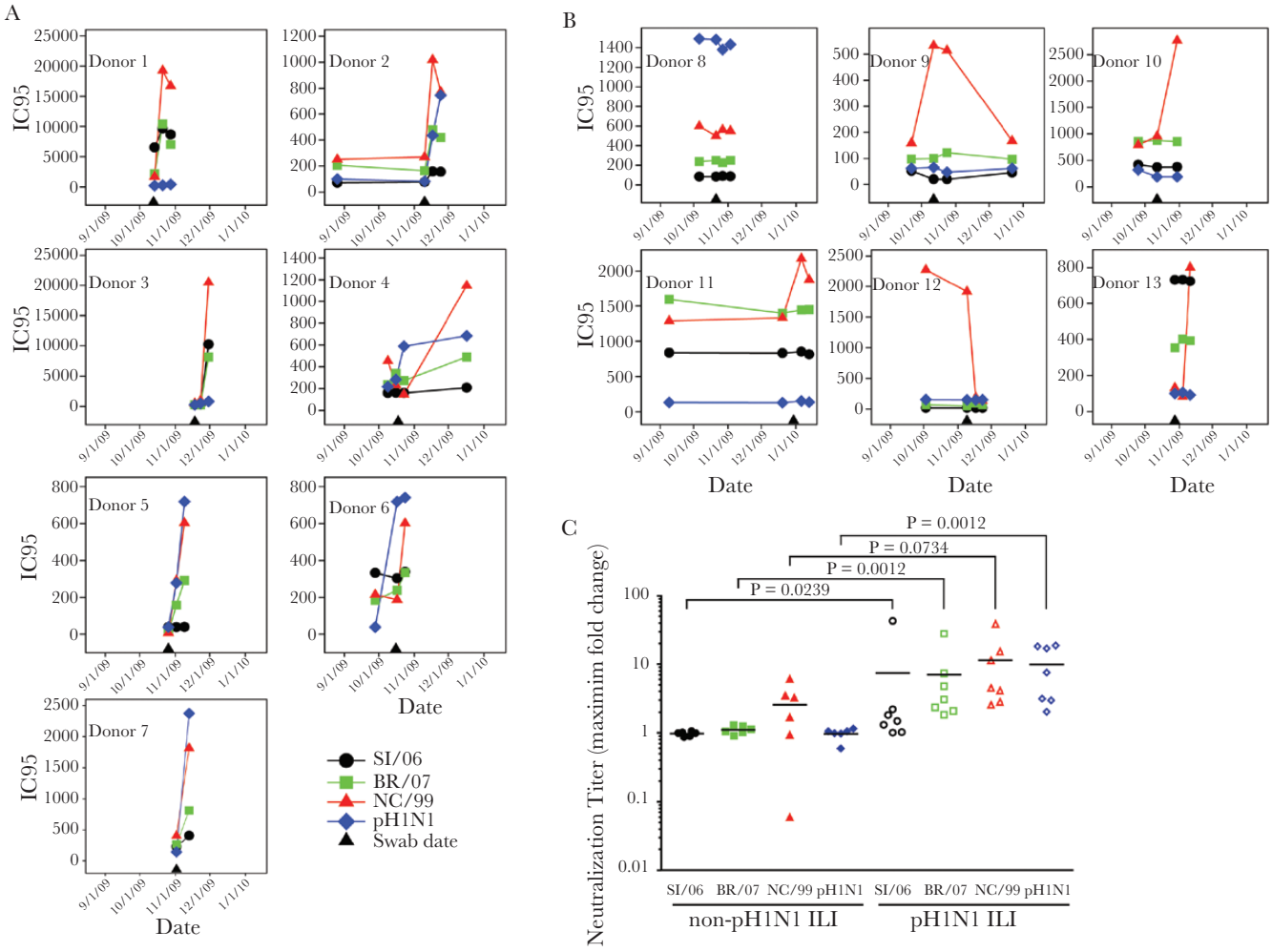


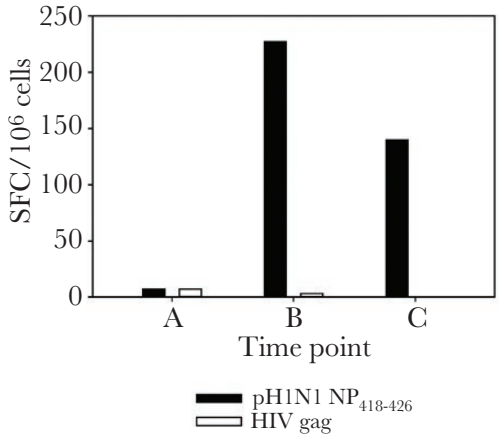
Figure 4. Hemagglutinin (HA) pseudotype-neutralizing antibodies in baseline sera. Hemagglutinin pseudotype-neutralizing antibodies were measured by the reporter assay described under Methods, using constructs with the HAs of A/Solomon Islands/3/2006 (SI/06, black circles), A/Brisbane/59/2007 (BR/07, green squares), A/New Caledonia/20/1999 (NC/99, red triangles), and A/Mexico/4108/2009 (pandemic influenza virus [pH1N1], blue diamonds). Donor numbers shown are arbitrary. Sera were tested at baseline and follow-up time points. The times of infection are indicated by black triangles for swab date. For the time period shown, none of the donors reported seasonal influenza vaccination, and available hemagglutination inhibition data showed no seroconversion to seasonal H1N1 viruses. (A) pH1N1-infected donors. Increases in titer on pH1N1 postinfection are hard to see for some donors because the 95% inhibitory titer (IC95) scale was chosen to cover the range of all pseudovirus strains, but fold increases are shown in C. (B) pH1N1-negative influenza-like illness (ILI) donors. Little change except for NC/99. (C) Comparison of groups for maximum fold titer increases between baseline and follow-up after febrile illness. The titer changes of each tested virus were compared between non-ILI and ILI groups by Mann-Whitney *U* test. *P* values <.05 were considered statistically significant. The horizontal lines represent group means. If the 1 high outlier in the pH1N1+ILI group was excluded, the differences were still significant for SI/06, BR/07, and pH1N1 and remained non-significant for NC/99, as decribed previously.

DISCUSSION

This study was organized to analyze the role of pre-existing immunity to conserved epitopes in protection against a novel influenza virus strain. Blood donors provide the advantage of large PBMC samples permitting extensive T-cell analysis. In addition, we performed testing for antibodies to M2, NP, NA, and conserved regions of HA, which could play roles in cross-protection against new influenza virus strains.

Pre-existing T-cell responses to pH1N1 antigens were detected in most individuals, at widely varying levels. As discussed by Sridhar et al [22], cross-protective T-cell immunity would be expected to reduce severity but not necessarily to completely prevent infection. We compared donors with milder versus more severe symptoms. Although there was a trend toward greater T-cell responses in the mild/asymptomatic group, too few infections had occurred in the cohort for testing of hypotheses about T-cell protection. Our projections of prevalence and protection called for larger numbers of donors, but we could only recruit and bank samples at a certain pace during the outbreak.

Note that the age distribution of our study (mostly over age 40) differs from the Sridhar et al [22] cohort (predominance under age 40), the Hayward et al [23] cohort (household members of all ages), and a study of cellular immunity and seasonal influenza in children [37]. Although age may alter responses and correlates of protection, the most likely reason we did not



	Time point	
	A	B
T cell responses		
$NP_{418-426}$ 2009 pH1N1	7	227
ELISPOT total, 25 pools	350	3177
Antibody responses		
IC95, SF09 pseudotype	157	1140
pH1N1 NP ELISA	1280	20,480
SWM2, flow cytometry	5.46	7.51
NI	<10	20
HI (turkey RBC)	<10	20

Figure 5. Marker of infection in a donor not meeting the pre-established definition of pandemic influenza infection (pH1N1). (A) Time course of enzyme-linked immunospot response to peptide nucleoprotein $(NP)_{418-426}$ with the pandemic sequence, versus human immunodeficiency virus gag control peptide. (B) Summary of changes in other immune parameters between baseline (time point A) and follow up (time point B). ELISA, enzyme-linked immunosorbent assay; HI, hemagglutination inhibition; HIV, human immunodeficiency virus; IC95, 95% inhibitory titer; NI, neuraminidase inhibition; RBC, red blood cells; SFC, spot-forming cells; SF09, swine flu 2009 (pH1N1).

detect similar correlations of T-cell reactivity with outcomes was that we had too few infected donors.

Protective mechanisms in animal models include antibodies to influenza NP and M2. Although NP is highly conserved, occasional antibodies recognize pH1N1 NP but not NP from seasonal viruses [31, 38]. Our ELISA with polyclonal sera mainly detects the common antibodies to shared epitopes. Therefore, it was not surprising that donor baseline sera had substantial, similar anti-NP antibody titers on pandemic and seasonal sequences.

Approximately two thirds of donors had pre-existing antibodies in baseline sera reactive with HM2 and 44% with SWM2. Age dependence of the levels and frequency of M2 antibodies was reported by Zhong et al [35], and the levels in subjects above and below age 40 were different. In our cohort, 65% of donors were 40 or older, and there was a trend towards age dependence, but this was not significant.

Pseudotype-neutralizing antibodies to pandemic HA were detected in baseline sera. Hemagglutinin stem antibodies could provide a mechanism of cross-protection [39], but the sera had no or only marginal activity on an H5N1 pseudotype. Thus, pre-existing antibodies to widely shared HA stem epitopes were marginal by this assay, although they might be detected in binding assays.

Baseline and follow-up sera were also tested for HA-pseudotype neutralizing antibodies to seasonal strains and revealed a novel relationship. pH1N1+ILI was followed by sharp rises in neutralizing titers for most seasonal HA constructs tested. In contrast, other febrile respiratory infections resulted in increased activity, if at all, restricted to NC/99. Perhaps some antibodies to pH1N1 HA cross-react on SI/06 and BR/07 HAs. Alternatively, the innate response to pandemic infection may be more vigorous than to the non-pH1N1 febrile illnesses, resulting in more activation of bystander B-cell memory. Note that the NC/99 strain was prevalent and used in the vaccine for 7 years, rather than 1 year for SI/06 or 2 years for BR/07 [40], so there may be larger memory populations to NC/99.

To detect additional pH1N1 infections missed by standard means, we explored as a T-cell marker the response to $NP_{418-426}$, which distinguishes the pH1N1 sequence (LPFERATVM) from other strains. We identified a donor with results suggesting infection with pH1N1 but who did not meet our predetermined definition. As commonly accepted, a 2-fold rise in HI or NI titer is not reliable enough to score as infection, but such a donor may indeed be infected. The increase in $NP_{418-426}$ T-cell response was dramatic rather than marginal. Although this parameter cannot be used alone for screening purposes due to HLA restriction, it could perhaps serve as part of a panel of markers for confirmatory analysis in specific cases.

CONCLUSIONS

Several studies have provided evidence for human cross-protection against pandemic influenza by pre-existing immunity [21–23]. Those findings reinforce the possibility that conserved antigen vaccines might be effective in humans. Cross-protection may involve multiple influenza virus target antigens and immune mechanisms, both humoral and cellular. The cohort described here documents a wide range of parameters of pre-existing cross-reactive immunity to pandemic virus antigens. Their roles can be explored further in studies of universal vaccine candidates.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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