Humans and Ferrets with Prior H1N1 Influenza Virus Infections Do Not Exhibit Evidence of Original Antigenic Sin after Infection or Vaccination with the 2009 Pandemic H1N1 Influenza Virus

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The hypothesis of original antigenic sin (OAS) states that the imprint established by an individual’s first influenza virus infection governs the antibody response thereafter. Subsequent influenza virus infection results in an antibody response against the original infecting virus and an impaired immune response against the newer influenza virus. The purpose of our study was to seek evidence of OAS after infection or vaccination with the 2009 pandemic H1N1 (2009 pH1N1) virus in ferrets and humans previously infected with H1N1 viruses with various antigenic distances from the 2009 pH1N1 virus, including viruses from 1935 through 1999. In ferrets, seasonal H1N1 priming did not diminish the antibody response to infection or vaccination with the 2009 pH1N1 virus, nor did it diminish the T-cell response, indicating the absence of OAS in seasonal H1N1 virus-primed ferrets. Analysis of paired samples of human serum taken before and after vaccination with a monovalent inactivated 2009 pH1N1 vaccine showed a significantly greater-fold rise in the titer of antibody against the 2009 pH1N1 virus than against H1N1 viruses that circulated during the childhood of each subject. Thus, prior experience with H1N1 viruses did not result in an impairment of the antibody response against the 2009 pH1N1 vaccine. Our data from ferrets and humans suggest that prior exposure to H1N1 viruses did not impair the immune response against the 2009 pH1N1 virus.

The hypothesis of original antigenic sin (OAS) states that an individual’s first influenza virus infection leaves a permanent mark upon the immune system, such that throughout his or her lifetime, that person mounts an immune response to the influenza virus with which he or she was first infected, resulting in a diminished antibody response to subsequent antigenically dissimilar influenza viruses (1–3). This phenomenon was first described by Thomas Francis, Jr., in 1960 (3) on the basis of analysis in the late 1940s and 1950s of the antibody responses to an antigenically distinct H1N1 influenza A virus that emerged in 1947 (designated A prime) in individuals previously vaccinated with older H1N1 influenza viruses (designated classical H1N1 viruses) (1–5). Those studies showed that after an A prime H1N1 virus infection, previously vaccinated individuals produced higher titers of antibody against the older, classical H1N1 viruses and a diminished antibody response against the A prime H1N1 virus (1–5).

While OAS has been observed with other pathogens, such as dengue virus and HIV, its role in influenza virus infection remains uncertain (6–8). Several investigators found evidence of OAS during influenza virus infection of various animal models, including ferrets, mice, rats, and rabbits (9–14). However, a number of investigators failed to find evidence of OAS and questioned its role in influenza virus infection (15–17).

The topic of OAS has recently gained more attention since the 2009 H1N1 influenza virus pandemic (18–21). During the 2009 H1N1 pandemic, an unusually high frequency of severe disease and death occurred in children and young adults (22, 23). Serological analyses demonstrated that older adults, particularly the elderly (>65 years old), had substantial levels of antibodies that cross-reacted with the 2009 pH1N1 virus, suggesting that past exposure to older seasonal H1N1 viruses may provide partial protection from 2009 pH1N1 infection (24–26). We and others reported that primary infection with H1N1 viruses isolated in or before 1947 provided protection against 2009 pH1N1 virus infection in animals and that antigenic changes in the hemagglutinin (HA) that emerged between 1947 and 1950 were responsible for a loss of protective efficacy against H1N1 viruses isolated in 1950 or later (27–30).

It has been suggested that the unusual age distribution of severe disease and death during the 2009 H1N1 pandemic, compared to seasonal influenza outbreaks, may be at least partly due to OAS (18, 20, 21). Unlike annual seasonal influenza virus infections, where the elderly are at the greatest risk of suffering from severe disease and death, this age group was protected because of preexisting immunity in the 2009 H1N1 pandemic, while severe disease and death predominated in young adults and children (22–25). It has also been suggested that the elderly, who were exposed during childhood to viruses that were antigenically similar to the 2009 H1N1 virus, generated an antibody response against the viruses of childhood that cross-reacted with the 2009 pH1N1 virus. In contrast, younger individuals produced an antibody response against antigenically dissimilar influenza viruses during childhood that failed to cross-react with the 2009 pH1N1 virus and diminished

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their response to the 2009 pH1N1 virus, resulting in more severe disease and death in this age group during the pandemic. To better understand the nature of the protection conferred by H1N1 viruses against subsequent exposure to the 2009 pH1N1 virus, we asked whether prior infection with older seasonal H1N1 influenza viruses would induce OAS upon subsequent infection with the 2009 pH1N1 virus. Evidence of OAS was sought by using human and ferret serum samples. Ferrets were primed with H1N1 viruses with various antigenic distances from 2009 pH1N1 and challenged 6 weeks later with A/California/07/2009 wild-type (CA/09 WT) pH1N1 virus or vaccinated with a monovalent live attenuated pH1N1 vaccine (CA/09 ca) or monovalent inactivated pH1N1 vaccine (CA/09 iv). Paired human serum samples collected from 38 subjects before and after vaccination with the monovalent inactivated 2009 pH1N1 vaccine were tested against the 2009 pH1N1 virus and seasonal H1N1 viruses that circulated during the first 10 years of each subject’s childhood. This part of the study was designed to ask the following questions. (i) Do humans in different age cohorts show a significant rise in titers of antibodies to influenza viruses that circulated during their childhood following 2009 pH1N1 vaccination? This would be indicative of OAS. (ii) If so, is there evidence that this response diminishes the antibody response to the 2009 pH1N1 vaccine? It is possible that OAS could indirectly lead to more severe disease because a diminished antibody response could leave an individual more susceptible to severe disease associated with the novel virus. Reduced antibody titers could lead to increased viral loads and delayed viral clearance associated with greater disease severity. Our results indicate that increased severity of pH1N1 disease in young adults does not appear to be due to a diminished antibody response to the 2009 pH1N1 virus.

MATERIALS AND METHODS

Viruses. The A/Alaska/35 (AK/35) H1N1 virus and the 2009 A/California/07/2009 (CA/09) pH1N1 virus were provided by Alexander Klomov (Centers for Disease Control and Prevention [CDC]). The A/Fort Monmouth/1/1947 (FM/47) H1N1 virus was provided by Suzanne Epstein (U.S. Food and Drug Administration [FDA]). The A/New Caledonia/20/1999 (NC/99) H1N1 virus and the B/Malaysia/2504/2004 (B/Mal) influenza B virus were provided by Zhiping Ye (FDA). The A/Fort Warren/1/1950 (FW/50) and A/Malaysia/1954 (Mal/54) H1N1 viruses were provided by Jeffrey Taubenberger (National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH]). The A/New Jersey/8/1976 (NJ/76) H1N1 virus was provided by Brian Murphy (NIAID, NIH). The A/Hickox/40 (Hic/40), A/Bellamy/42 (Bel/42), and A/Weiss/43 (W/43) H1N1 viruses were provided by Jack Bennink and Jonathon Yewdell (NIAID, NIH). The remaining H1N1 viruses were provided by Catherine Smith (CDC). Each virus was propagated in the allantoic cavity of a 9- to 11-day-old embryonated specific-pathogen-free hen’s egg. The inoculated eggs were incubated at 35°C (H1N1 viruses) or 33°C (B/Mal). The allantoic fluid was harvested 48 to 72 h after inoculation, tested for hemagglutinating activity, and stored at −80°C until use. The 50% tissue culture infective dose (TCID50) of each virus was determined by serial titration of the virus in MDCK cells and calculated by the method developed by Reed and Muench (31).

Animals. Eight- to 12-week-old male and female ferrets were used in groups of four (Triple F Farms, Sayre, PA). Ferret serum samples were prescreened for the presence of antibodies against H1N1, H3N2, and influenza B viruses with a hemagglutination inhibition (HAI) assay. All ferret experiments were performed at the NIH with the approval of and in compliance with the guidelines of the NIAID, NIH, Institutional Animal Care and Use Committee.

Human serum samples. Paired pre- and postvaccination serum samples from consenting subjects vaccinated with the monovalent inactivated 2009 pH1N1 vaccine from the 2009-2010 season were provided by John Treanor (University of Rochester). The purpose of the vaccine study was to evaluate the impact of age on the antibody response to the 2009 pH1N1 inactivated-virus vaccine (32). Subjects reported no prior infection with the 2009 pH1N1 virus before vaccination. The study protocol was approved by the University of Rochester Research Subjects Review Board.

Evaluation of antibody response in ferrets. Groups of four ferrets were lightly anesthetized with isoflurane and inoculated intranasally (i.n.) with 106 TCID50 of one of the H1N1 or influenza B viruses in a volume of 0.5 ml (0.25 ml per nostril). After 6 weeks, the ferrets were challenged with CA/09 virus at a concentration and volume similar to those used for priming (104 TCID50 i.n. in a volume of 0.5 ml) or vaccinated with either the cold-adapted CA/09 (CA/09 ca) virus (104 TCID50 i.n.) or 15 μg of the monovalent inactivated CA/09 (CA/09 iv) vaccine from the 2009-2010 season. Serum samples were collected before and 6 weeks after priming and 28 days postchallenge. At 28 days postchallenge, ferrets were euthanized and terminal bleeding was performed. Peripheral blood mononuclear cells (PBMCs) were purified from the blood samples.

HAI assay. A previously described protocol was used for the HAI assay (33). Briefly, serum samples treated with receptor-destroying enzyme (RDE) were serially 2-fold diluted in 96-well V-bottom plates starting at a dilution of 1:10, and 4 HA units of virus was added. Control wells received phosphate-buffered saline (PBS) alone or PBS with virus in the absence of antibody. Virus and serum samples were incubated together for 30 min at room temperature. Next, 50 μl of a 0.5% (vol/vol) suspension of turkey erythrocytes was added. The antibody, virus, and erythrocytes were gently mixed, and the results were recorded after incubation for 45 to 60 min at room temperature. HAI antibody titers were recorded as the inverse of the highest antibody dilution that inhibited hemagglutination. By convention, antibody titers are considered significantly different only if they differ by more than 4- to 8-fold.

Neutralization assay. A previously described protocol was used for the neutralization assay (34). Briefly, influenza viruses diluted to a concentration of 100 TCID50/50 μl (10−3 TCID50/ml) in minimal essential medium supplemented with 0.5% bovine serum albumin (BSA) were added to ferret serum samples that were serially 2-fold diluted in PBS, and the mixtures were incubated for 1 h at room temperature. After 1 h, the virus-serum mixtures were added in quadruplicate to MDCK cells cultured in 96-well plates. Cells were incubated at 33 or 37°C, and the neutralization titer was scored 4 days later by cytopathic effect (CPE). The neutralization titer is the inverse of the highest serum dilution where complete CPE neutralization occurred in 50% of the wells.

HA and NA purification. A previously described protocol was used for HA and neuraminidase (NA) purification (35, 36). Briefly, 9- to 11-day-old embryonated chicken eggs inoculated with influenza viruses were incubated at 35°C (H1N1 viruses) or 33°C (B/Mal) for 48 to 72 h. The allantoic fluid was harvested and centrifuged to remove cellular debris (2,000 rpm for 10 min), and virus was pelleted by centrifugation at 10,000 rpm overnight at 4°C (1.26 XP centrifuge). Virus was resuspended in PBS, purified with a linear 30 to 60% sucrose gradient in 10 mM Tris (pH 7.4), and centrifuged at 24,000 rpm for 2 h at 4°C. The virus band at the 30-to-60% sucrose interface was collected, diluted in PBS, and pelleted by centrifugation at 24,000 rpm for 2 h at 4°C. The virus suspension was resuspended in 1 ml of sodium acetate buffer (0.05 M sodium acetate, 2 mM NaCl, 0.2 mM EDTA [pH 7.0]), and an equal volume (1 ml) of 15% octylglucoside (1-O-α-β-D-glucopyranoside; Sigma) in sodium acetate buffer was added with mild agitation. The HA and NA proteins were separated from the internal core proteins by centrifugation at 19,000 rpm for 1 h at 4°C. The supernatant containing purified HA and NA was collected.

ELISA. Purified HA-NA preparations were diluted in carbonate buffer (pH 9.8), added to 96-well enzyme-linked immunosorbent assay (ELISA) plates at a concentration of 50 HA units, and incubated overnight at 4°C. As a control, some wells received carbonate buffer alone. The following
control antigen. Next, 50 (PMA; 1 mg/ml) plus ionophore (1 mg/ml) diluted 1:500 was used as a
icillin-streptomycin was added to the plates. Phorbol myristate acetate
(OD) of
nm was measured with a spectrophotometer, and wells with an optical density
stopped with 1% sodium dodecyl sulfate (SDS). The absorbance at 405
thiazolinesulfonic acid) ELISA reagent was added, and the reaction was
bated with the purified HA overnight at 4°C. The next day, the plates were
ples were transferred to the ELISA plates. The serum samples were incu-
ated ferret serum samples were diluted 1:10 in 1% BSA carbonate buffer
ate buffer and then incubated at room temperature for 2 h. Heat-inacti-
vated ferret serum samples were diluted 1:10 in 1% BSA carbonate buffer
serially diluted 2-fold in separate U-bottom 96-well plates. The blocking
solution was removed from the ELISA plates, the plates were washed three times with PBS–0.05% Tween (PBST), and the diluted serum samples were transferred to the ELISA plates. The serum samples were incubated with the purified HA overnight at 4°C. The next day, the plates were washed three times in PBST, and 100 µl of horseradish peroxidase (HRP)-conjugated goat anti-ferret IgG (Immunology Consultants Laboratory, Inc.) diluted 1:1,000 in 1% BSA carbonate buffer was added. The plates were incubated for 2 h at room temperature and washed three times in PBS without Tween. Next, 100 µl of ABTS (2,2’-azinobis(3-ethylbenz-thiazolinesulfonic acid)) ELISA reagent was added, and the reaction was stopped with 1% sodium dodecyl sulfate (SDS). The absorbance at 405 nm was measured with a spectrometer, and wells with an optical density (OD) of >0.2 were considered positive.

Ferret IFN-γ ELISpot assay. Brieﬂy, for the ferret IFN-γ enzyme-linked immunospot (ELISpot) assay, 96-well polystyrene difluoride plates (Millipore catalog no. MSIPS4W10) were coated with 20 µg/ml of mouse anti-canine gamma interferon (IFN-γ) antibody (R&D catalog no. 142529). After overnight incubation at 4°C in a humidified chamber, the plates were washed and blocked with RPMI 1640 medium with 10% fetal bovine serum for 2 h at 37°C. Next, 50 µl of whole β-propiolactone (BPL)-inactivated virus (500 HA units/ml) diluted in CTL test medium (CTL catalog no. CTLT-010) with 1% l-glutamine (2 mM) and 1% penicillin-streptomycin was added to the plates. Phorbol myristate acetate (PMA; 1 mg/ml) plus ionophore (1 mg/ml) diluted 1:500 was used as a control antigen. Next, 50 µl of ferret PBMCs at a concentration of 500,000 cells/well was added to the plates and incubated at 37°C for 48 h. The cells were washed six times with PBS plus 0.05% Tween 20, and 2.0 µg/ml of biotinylated anti-canine IFN-γ antibody (R&D catalog no. BAFT81) diluted in PBS plus 1% BSA was added. After overnight incubation at 4°C, the cells were washed six times with PBS plus 0.05% Tween 20, and a 1:100 dilution of streptavidin–HRP in PBS plus 1% BSA was added for 45 min of incubation at 37°C. After the cells were washed six times with PBS plus 0.05% Tween 20 twice, 3-amino-9-ethylcarbazole (AEC) substrate was added to the wells and 20 min was allowed for spot development. The plates were then washed with PBS, rinsed with deionized water, and left to dry for 24 h in the dark. Spots were counted with an ImmunoSpot Analyzer.

RESULTS

Testing for evidence of OAS in serum samples from ferrets primed with seasonal H1N1 viruses. Ferrets were primed with
the A/Alaska/35 (AK/35), A/Fort Monmouth/1/1947 (FM/47), A/Fort Warren/1/1950 (FW/50), or A/New Caledonia/20/1999 (NC/99) H1N1 virus. As positive controls, groups of ferrets were primed with the CA/09 WT virus or the closely related swine virus A/New Jersey/8/1976 (NJ/76), which was previously shown to protect against 2009 pH1N1 virus infection (27, 28, 30), and as a negative control, ferrets were primed with influenza B virus B/Malaysia/2504/2004 (B/Mal). No evidence of OAS has been observed upon sequential infection with influenza A and B viruses, so the antibody response against CA/09 in the B/Mal-primed ferrets should be unimpaired.

Samples were collected 6 weeks after priming, before the ferrets were challenged or vaccinated, and again 4 weeks postchallenge or -vaccination. Influenza virus–specific antibody titers were measured with paired pre- and postchallenge or -vaccination serum samples by HAI (Table 1) and neutralization assays (Table 2) and by ELISA (Table 3) against both the priming virus and the CA/09 WT virus. Ferrets were seronegative by HAI prior to priming (data not shown). All of the ferrets developed a robust antibody response to

| TABLE 1 Ferret serum HAI titer of antibodies against priming and challenge (CA/09 wt) viruses |
|-----------------------------------------------|
| Serum sample from ferrets primed with virus indicated | HAI titer before challenge | HAI titer after challenge | Fold increase in HAI titer vs: |
| Self CA/09 | Self CA/09 | Self CA/09 |
| AK/35 | 5,120 640 1,600 0 160 |
| FM/47 | 640 10 1,920 1,600 3 160 |
| FW/50 | 2,560 10 7,680 1,280 3 128 |
| NC/99 | 640 10 320 960 0 96 |
| CA/09 | 1,280 1,280 3,200 3,200 2.5 2.5 |
| NJ/76 | 1,280 640 2,560 2,560 2 4 |
| B/Mal | 960 10 480 2,560 0 256 |
| ^a The lower limit of detection of this assay was a titer of 20. Samples with undetectable antibodies were assigned a value of 10.

| TABLE 2 Ferret serum titers of neutralizing antibodies against priming and challenge (CA/09 wt) viruses |
|-----------------------------------------------|
| Serum sample from ferrets primed with virus indicated | NtAb titer before challenge | NtAb titer after challenge | Fold increase in NtAb titer vs: |
| Self CA/09 | Self CA/09 | Self CA/09 |
| AK/35 | 5,120 640 1,600 0 160 |
| FM/47 | 640 10 1,920 1,600 3 160 |
| FW/50 | 2,560 10 7,680 1,280 3 128 |
| NC/99 | 640 10 320 960 0 96 |
| CA/09 | 1,280 1,280 3,200 3,200 2.5 2.5 |
| NJ/76 | 1,280 640 2,560 2,560 2 4 |
| B/Mal | 960 10 480 2,560 0 256 |
| ^a The lower limit of detection of this assay was a titer of 20. Samples with undetectable antibodies were assigned a value of 10.

| TABLE 3 Ferret serum titers of IgG antibodies against priming and challenge (CA/09 wt) viruses detected by ELISA |
|-----------------------------------------------|
| Serum sample from ferrets primed with virus indicated | ELISA titer before challenge | ELISA titer after challenge | Fold increase in ELISA titer vs: |
| Self CA/09 | Self CA/09 | Self CA/09 |
| AK/35 | 2,560 160 10,240 2,560 4 16 |
| FM/47 | 2,560 160 10,240 2,560 4 16 |
| FW/50 | 2,560 40 10,240 640 4 16 |
| NC/99 | 640 10 640 640 0 64 |
| CA/09 | 2,560 2,560 10,240 10,240 4 4 |
| NJ/76 | 2,560 640 10,240 10,240 4 16 |
| B/Mal | 1,280 10 640 1,280 0 128 |
| ^a Absorbance at 405 nm was measured with a spectrophotometer, and wells with an OD of >0.2 were considered positive.

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the homologous priming virus (Tables 1 to 3). Priming with FW/50, NC/99, or B/Mal did not induce significant cross-reactive antibodies against CA/09 WT virus (Tables 1 to 3). In contrast, priming with AK/35 and FM/47 generated cross-reactive ELISA antibodies, and FM/47 elicited cross-reactive HAI antibodies (Tables 1 and 3) but not neutralizing antibodies (Table 2). As expected, priming with the NJ/76 virus elicited significant titers of cross-reactive antibodies against CA/09 (Tables 1 to 3).

Following a CA/09 WT virus challenge, there was a modest (≤4-fold) increase in the titers of antibodies against some of the priming viruses (Tables 1 to 3). However, a 16-fold or greater rise in CA/09-reactive antibody titers was detected in all groups except the ferrets previously infected with the CA/09 or NJ/76 virus (Tables 1 to 3). A secondary antibody response to the CA/09 WT virus was observed in ferrets primed with these two antigenically related viruses, while all other groups showed a primary antibody response to the CA/09 virus, with a 16- to 256-fold antibody titer increase (Tables 1 to 3).

The rise in the titers of antibodies (0- to 4-fold) to some of the priming viruses after a challenge with the 2009 pH1N1 virus was significantly lower than the 16- to 160-fold rise in the titer of antibody against the CA/09 WT virus postchallenge. Importantly, we did not observe an impairment of the antibody response to the CA/09 WT virus that would be expected with OAS. The postchallenge antibody titers in the H1N1-primed ferrets were within 3-fold of the titers achieved in the B/Mal-primed ferrets. This modest difference is not considered to be significant and could be due to preexisting immunity in the H1N1-primed ferrets, such as cross-reactive memory CD8+ T cells or antibodies against conserved viral epitopes that can enhance viral clearance, thereby decreasing antigen exposure (37, 38). Our data suggest that priming with H1N1 viruses with various antigenic distances from the 1930s to the 1950s, and from the late 1990s, did not diminish the antibody response against subsequent CA/09 WT virus infection, and thus, OAS was not observed.

We also examined the response of H1N1 virus-primed ferrets to immunization with the live attenuated CA/09 ca (Tables 4 to 6) or inactivated CA/09 iav (Tables 7 to 9) vaccine. Ferrets primed with CA/09 or NJ/76 virus exhibited a secondary response to the vaccines, while ferrets primed with the other viruses exhibited a primary response (Tables 4 to 9). Ferrets primed with B/Mal failed to seroconvert after CA/09 iav vaccination (Table 7); this is not unexpected because previous studies have shown that a single dose of an inactivated influenza vaccine does not elicit a protective immune response in unprimed ferrets (39, 40). Moreover, the antibody responses induced by the CA/09 vaccines were not impaired by prior priming with any of the seasonal H1N1 viruses (compared to ferrets primed with B/Mal), indicating that OAS did not occur after immunization with live attenuated or inactivated CA/09 vaccines. In fact, the postvaccination titers of antibody to CA/09 appear to have been boosted in ferrets primed with antigenically distant H1N1 viruses. A notable finding was the modest increase in the titer of antibody against the priming virus observed following a challenge with the live-virus vaccine (Tables 4 to 6) but not in ferrets that received the inactivated-virus vaccine (Tables 7 to 9). This suggests that the CA09 iav-vaccinated ferrets generated a more strain-specific antibody response than those infected with live WT or attenuated virus. Similar results have also been observed in previous H1N1 and H3N2 influenza virus studies (13, 41, 42).

### TABLE 4 Ferret serum titers of HAI antibodies against priming and live attenuated vaccine (CA/09 ca) viruses

| Serum sample from ferrets primed with virus indicated | HAI titer before vaccination* vs: | HAI titer after vaccination vs: | Fold increase in HAI titer vs: |
|-----------------------------------------------------|----------------------------------|---------------------------------|-----------------------------|
|                                                      | Self CA/09                        | Self CA/09                       |                             |
| AK/35                                               | 1,920 5                         | 2,560 480                        | 1.33 48                      |
| FM/47                                               | 960 5                            | 1,920 320                        | 2 32                        |
| FW/50                                               | 1,280 5                          | 2,560 240                        | 2 24                        |
| NC/99                                               | 800 5                            | 480 160                          | 0 16                        |
| CA/09                                               | 640 640                          | 1,920 1,920                      | 3 3                          |
| NJ/76                                               | 960 320                          | 2,240 960                        | 2.33 3                       |
| B/Mal                                               | 640 5                            | 320 160                          | 0 16                        |

* HAI titers were recorded as the inverse of the highest antibody dilution that inhibited the agglutination of red blood cells by 4 HA units of virus.

### TABLE 5 Ferret serum titers of neutralizing antibodies (NtAb) against priming and live attenuated vaccine (CA/09 ca) viruses

| Serum sample from ferrets primed with virus indicated | NtAb titer before vaccination* vs: | NtAb titer after vaccination vs: | Fold increase in NtAb titer vs: |
|-----------------------------------------------------|----------------------------------|---------------------------------|-----------------------------|
|                                                      | Self CA/09                        | Self CA/09                       |                             |
| AK/35                                               | 3,840 100                        | 3,840 640                       | 0 64                        |
| FM/47                                               | 1,920 10                          | 2,560 560                       | 1.33 56                      |
| FW/50                                               | 2,240 10                          | 4,480 480                       | 2 48                        |
| NC/99                                               | 1,600 10                          | 960 280                          | 0 28                        |
| CA/09                                               | 1,920 1,920                       | 3,840 3,840                      | 2 2                          |
| NJ/76                                               | 1,920 640                         | 2,240 2,240                      | 2 3.5                       |
| B/Mal                                               | 960 10                           | 560 280                          | 0 28                        |

* The neutralization titer is the inverse of the highest serum dilution to neutralize the infectivity of 100 TCD50 of virus.

### TABLE 6 Ferret serum titers of IgG antibodies against priming and live attenuated vaccine (CA/09 ca) viruses detected by ELISA

| Serum sample from ferrets primed with virus indicated | ELISA titer before vaccination* vs: | ELISA titer after vaccination vs: | Fold increase in ELISA titer vs: |
|-----------------------------------------------------|----------------------------------|---------------------------------|-----------------------------|
|                                                      | Self CA/09                        | Self CA/09                       |                             |
| AK/35                                               | 2,560 160                         | 2,560 2,560                      | 0 8                         |
| FM/47                                               | 2,560 160                         | 10,240 2,560                     | 4 8                         |
| FW/50                                               | 2,560 40                          | 2,560 640                        | 0 8                         |
| NC/99                                               | 2,560 10                          | 640 640                          | 0 64                        |
| CA/09                                               | 640 640                          | 2,560 2,560                      | 4 4                         |
| NJ/76                                               | 2,560 640                         | 2,560 2,560                      | 0 4                         |
| B/Mal                                               | 640 10                           | 640 640                          | 0 64                        |

* Absorbance at 405 nm was measured with a spectrophotometer, and wells with an OD of >0.2 were considered positive.

* The lower limit of detection of this assay was a titer of 10. Samples with undetectable antibodies were assigned a value of 10.
TABLE 7 Ferret serum titers of HAI antibodies against priming viruses and inactivated vaccine (CA/09 iav)

| Serum sample from ferrets primed with virus indicated | HAI titer before vaccination* vs: | HAI titer after vaccination vs: | Fold increase in HAI titer vs: |
|------------------------------------------------------|---------------------------------|---------------------------------|------------------------------|
|                                                      | Self CA/09                      | Self CA/09                      | Self CA/09                   |
| AK/35                                                | 2,560 5                        | 640 5                           | 0 16                         |
| FM/47                                                | 640 5                           | 320 5                           | 0 16                         |
| FW/50                                                | 1,280 5                         | 640 320                         | 0 32                         |
| NC/99                                                | 960 5                           | 480 60                          | 0 6                          |
| CA/09                                                | 640 640                         | 1,600 1,600                     | 2.5 2.5                      |
| NJ/76                                                | 800 160                         | 1,920 640                       | 2.4 4                        |
| B/Mal                                                | 320 5                           | 160 5                           | 0 0                          |

a HAI titers were recorded as the inverse of the highest antibody dilution that inhibited the agglutination of red blood cells by 4 HA units of virus.
b The lower limit of detection of this assay was a titer of 10. Samples with undetectable antibodies were assigned a value of 5.

Testing for evidence of OAS in H1N1-primed ferret T-cell response. In addition to the antibody response, previous studies have also observed OAS in the cytotoxic T lymphocyte response (43). We isolated PBMCs from blood samples at day 28 postchallenge and used a previously described ferret IFN-γ ELISpot assay protocol (40) to measure the T-cell response after the ferret PBMCs were stimulated with either BPL-inactivated CA/09 WT virus or the priming BPL-inactivated H1N1 virus. A greater T-cell response against the primary H1N1 virus would be suggestive of OAS, while a greater response against the CA/09 WT virus would suggest that OAS did not occur.

Results from the ELISpot assay showed that for each of the viruses tested, the T-cell response to the BPL-inactivated CA/09 virus was greater than that to the primary H1N1 virus. Stimulation with BPL-inactivated CA/09 virus resulted in greater IFN-γ production, observed as a significantly greater number of spots (Fig. 1). The response to the BPL-inactivated CA/09 virus may be enhanced because it represents a combination of a boosted cross-reactive T-cell response induced by the primary infection with a specific response to the CA/09 virus. The BPL-inactivated FW/50 and B/Mal viruses did not elicit a T-cell response or IFN-γ release in the ferret PBMC samples and thus are not included in Fig. 1. Because the ferret study was primarily designed to focus on the antibody response, PBMC samples were not collected at time points that would be optimal for the detection of T-cell responses, following priming but before a CA/09 WT virus challenge. However, the results do correlate with a previous influenza virus study that also failed to find evidence of OAS in the T-cell response after sequential influenza virus infection (44).

Overall, definitive evidence of OAS was not observed in either the antibody or the T-cell response of ferrets that were infected with seasonal H1N1 viruses with various antigenic distances and subsequently challenged or vaccinated with the 2009 pH1N1 virus and vaccines, respectively. While there was a modest increase in the titers of antibodies against the priming viruses after challenge, the antibody responses generated against the CA/09 virus were not diminished as a result of priming.

TABLE 8 Ferret serum titers of neutralizing antibodies (NtAb) against priming viruses and inactivated vaccine (CA/09 iav)

| Serum sample from ferrets primed with virus indicated | NtAb titer before vaccination* vs: | NtAb titer after vaccination vs: | Fold increase in NtAb titer vs: |
|------------------------------------------------------|---------------------------------|---------------------------------|------------------------------|
|                                                      | Self CA/09                      | Self CA/09                      | Self CA/09                   |
| AK/35                                                | 6,400 10                        | 2,300 320                      | 0 32                         |
| FM/47                                                | 640 10                          | 2,300 280                      | 0 28                         |
| FW/50                                                | 3,200 10                        | 4,480 480                      | 1.4 48                       |
| NC/99                                                | 960 10                          | 960 80                         | 0 8                          |
| CA/09                                                | 960 960                         | 3,200 3,200                    | 3.33 3.33                    |
| NJ/76                                                | 1,280 320                       | 2,560 1,600                    | 2 5                          |
| B/Mal                                                | 480 10                          | 320 10                         | 0 0                          |

a The neutralization titer is the inverse of the highest serum dilution that neutralized the infectivity of 100 TCID50 of virus.
b The lower limit of detection of this assay was a titer of 10. Samples with undetectable antibodies were assigned a value of 10.

c TABLE 9 Ferret serum titers of IgG antibodies against priming viruses and inactivated vaccine (CA/09 iav) detected by ELISA

| Serum sample from ferrets primed with virus indicated | ELISA titer before vaccination* vs: | ELISA titer after vaccination vs: | Fold increase in ELISA titer vs: |
|------------------------------------------------------|---------------------------------|---------------------------------|------------------------------|
|                                                      | Self CA/09                      | Self CA/09                      | Self CA/09                   |
| AK/35                                                | 2,560 160                       | 640 640                        | 0 4                          |
| FM/47                                                | 2,560 160                       | 2,560 640                      | 0 4                          |
| FW/50                                                | 2,560 40                        | 2,560 640                      | 0 8                          |
| NC/99                                                | 2,560 40                        | 640 160                        | 0 4                          |
| CA/09                                                | 2,560 2,560                     | 10,240 10,240                  | 4 4                          |
| NJ/76                                                | 640 640                         | 2,560 2,560                    | 4 4                          |
| B/Mal                                                | 1,280 10                        | 640 80                         | 0 8                          |

Absence of OAS after 2009 pH1N1 Infection

Overall, definitive evidence of OAS was not observed in either the antibody or the T-cell response of ferrets that were infected with seasonal H1N1 viruses with various antigenic distances and subsequently challenged or vaccinated with the 2009 pH1N1 virus and vaccines, respectively. While there was a modest increase in the titers of antibodies against the priming viruses after challenge, the antibody responses generated against the CA/09 virus were not diminished as a result of priming.

Testing for evidence of OAS in serum samples from humans vaccinated with CA/09 iav. To explore the potential role for OAS in the age-related response in humans to the 2009 pH1N1 virus, paired serum samples collected in 2009 from human subjects before and 28 days after vaccination with the monovalent inactivated 2009 pH1N1 vaccine were tested for evidence of OAS.

Subjects from three age groups (18 to 32 years old [n = 20], 60 to 69 years old [n = 20], and ≥70 years old [n = 18]) were enrolled in a study that has been described previously (32). Because the hypothesis of OAS states that a person’s first influenza virus infection during childhood will shape the immune response of that person to all subsequent influenza virus infections, we reconstructed a “history” of reference influenza A H1N1 viruses that each study subject had likely been exposed to in the first decade of life from a list of WHO reference viruses. The pre- and postvaccination serum samples were tested against each of the seasonal H1N1 viruses, as well as the CA/09 WT virus, to determine whether the antibody response to the older seasonal H1N1 viruses resulted in a diminished antibody response to the CA/09 virus. The data are displayed as a heat map, with different colors representing fold rises in the HAI titers of antibodies against the indicated viruses. The year of birth of each subject and the preexisting antibodies were assigned a value of 10.

The 18- to 32-year-old subjects were born between 1977 and...
1991. The viruses that circulated during that time included USSR/77, Brazil/78, Chile/83, Taiwan/86, Texas/91, and NC/99. The pre-vaccination serum samples reacted with one or more of the older seasonal H1N1 viruses tested, suggesting that those individuals had been previously exposed to antigenically related H1N1 viruses. Only 5 of the 20 subjects had preexisting antibodies to the CA/09 WT virus prior to vaccination (Fig. 2A) because the CA/09 WT virus was antigenically dissimilar from the seasonal H1N1 viruses that had been circulating in the past 2 decades (24, 45).

In the postvaccination samples, a majority of the subjects showed a modest (0- to 4-fold) rise in the titers of antibodies against the older H1N1 viruses (Fig. 2A), but all 20 subjects showed a greater-fold rise in the titer of antibody against the CA/09 virus postvaccination than against the seasonal H1N1 viruses (Fig. 2A) and this response was not diminished because of prior H1N1 influenza virus infection.

Similar results were observed in the 60- to 69-year-old and >70-year-old age groups as well (Fig. 2B and C). The pre-vaccination serum samples reacted with one or more of the older H1N1 viruses, suggesting that these individuals were previously exposed to H1N1 influenza viruses. Only subject 24 failed to show detectable titers of antibodies against any of the viruses tested. However, unlike the 18- to 32-year-old subjects, the majority of the subjects in the two older age groups had preexisting immunity to the CA/09 WT virus (Fig. 2B and C). This was not unexpected because previous studies showed that viruses circulating prior to 1947 were antigenically related to the 2009 pH1N1 virus and that viruses isolated in or before 1947 could provide protection against 2009 pH1N1 virus infection (27–30). About 97% of the subjects in these two age groups were born in or before 1947.

Because of the preexisting antibody against CA/09 in the pre-vaccination serum samples of the older age groups, there was not as great a fold rise in the titer of antibody against the CA/09 WT virus in the postvaccination serum samples as in the 18- to 32-year-old group. The subjects with preexisting antibodies in the pre-vaccination serum samples had average rises of 10- and 6-fold in the 60- to 69-year-old and >70-year-old age groups, respectively. However, the six subjects in the 60- to 69-year-old group who did not have detectable antibodies against the CA/09 virus in pre-vaccination serum samples had an average 21-fold increase, and a 27-fold average antibody rise was seen in the five subjects from the >70 year old group who had no detectable pre-vaccination antibodies against the CA/09 virus. In both age groups, a majority of the subjects failed to develop an increase in the titers of antibodies against the older seasonal H1N1 viruses. The lack of a significant rise in the titers of antibodies against the older H1N1 viruses and the fact that the fold rise in the titer of antibody against the CA/09 virus postvaccination exceeded that against the older seasonal H1N1 viruses indicate that OAS did not occur following vaccination with CA/09 iav.

**DISCUSSION**

We hypothesized that if prior seasonal H1N1 infection, followed by subsequent infection or vaccination with the CA/09 virus, leads to OAS, a significant rise in the titer of antibody against the original H1N1 virus would occur and this would result in an impaired antibody response against the CA/09 virus. In both humans and ferrets, we observed only a modest rise in the titer of antibody against the seasonal H1N1 viruses that was significantly lower than the rise in the titer of antibody against the CA/09 virus. This suggests that the antibody response against the CA/09 virus was not diminished because of prior H1N1 virus infection and that OAS was not evident and implies that the age distribution of infection during the 2009 H1N1 pandemic was not due to OAS. Although increased disease severity in the younger population does not appear to be due to a diminished antibody response to the 2009 pH1N1 virus as a result of OAS, preexisting immunity that is unrelated to OAS may still play a role in the unusual age distribution of severe cases observed during the pandemic.
example, enhanced severity of disease could be due to immune complex-mediated disease (46).

Previous studies have suggested that several factors may influence the occurrence of OAS, including the degree of antigenic relatedness between the sequentially administered viruses and the time interval between exposures (6, 11, 19, 47, 48). Several studies have suggested that the two viruses involved in the sequential infection must be of an intermediate relatedness (11, 19, 47, 48). If the two viruses are closely related antigenically, sequential infection will boost the priming response. If the two viruses are too dissimilar antigenically, OAS may not be evident (11, 48). However, a clear definition of antigenic relatedness is lacking. We calculated antigenic relatedness as was conventionally done, from titers of cross-reactive antibodies detected by HAI assays (49). More recently, antigenic relatedness has been assessed by mathematical models that use calculations of binding free energy (47). A standardized definition of antigenic relatedness would be helpful.

In the ferret study, H1N1 viruses with various antigenic distances from the CA/09 WT virus were tested, and a large number of H1N1 viruses from 1931 to 1999 were tested with the human serum samples.

Some studies have suggested that for OAS to be most evident, the time interval between sequential exposures must be long (6, 11, 19), with most studies waiting at least 4 weeks between exposures (10–14). We infected and challenged ferrets with a 6-week time interval, and for the human subjects, there was presumably an interval of at least several years between the original infection with the seasonal H1N1 virus and CA/09 iav vaccination. An additional confounding factor is single versus multiple prior exposures to influenza viruses. While most animal studies, including our ferret study, are limited to a single prior exposure, humans are likely to be repeatedly exposed to or vaccinated against influenza viruses over multiple seasons before exposure to an antigenically distinct virus. Repeated exposure and continued boosting over
time may be required to observe OAS. However, we did not observe OAS either in ferrets following a single prior exposure or in humans with paired serum samples from subjects who presumably had multiple exposures to influenza viruses during their lifetimes. The data also suggest that continued vaccination or exposure may actually boost prior responses, presumably because of shared epitopes.

Contradictory reports providing evidence that either supports or rejects the existence of OAS during influenza virus infection have made any potential role for OAS in influenza virus infection unclear (9–17). Major obstacles to our understanding of OAS include how OAS is defined and how the results of OAS studies are interpreted. In our study, OAS was considered to have occurred if the fold rise in the titer of antibody against the seasonal H1N1 influenza virus(es) exceeded that against the 2009 pH1N1 virus after infection or vaccination with the CA/09 influenza virus. This would have indicated that the response directed against the original virus resulted in an impaired response to the 2009 pH1N1 virus. Since this was not observed, we concluded that OAS did not occur.

However, the definition of OAS has varied between studies. While we focused on the fold changes in antibody titer in paired serum samples, other studies have focused on the absolute antibody titers (1, 14) and OAS was inferred if, after a sequential infection with two viruses, the absolute titer of antibody against the second virus was lower than that against the first virus. For example, the HAI titers in mice sequentially infected with PR/8/34 and FM/47 were ~4,096 and ~256 at 28 days after FM/47 infection, respectively, so the antibody response against FM/47 infection was considered to be impaired and OAS was reported (13). However, if the fold rise in antibody titer is considered, there was an ~256-fold increase in the titer of antibody against FM/47 and only an ~4-fold rise in the titer of antibody to PR/8/34 (13). We did not consider ≤4-fold antibody titer rises to be significant. However, some investigators interpret even a 2-fold rise in the titer of antibody against the first virus of a sequential infection to be evidence of OAS (50). Furthermore, some studies reported the occurrence of OAS without observing a boost in the response to the first virus in some instances (51) or without evidence of a diminished response to the second virus (50) in sequential infections. It is important to reach a consensus on how antibody responses should be measured to define OAS.

Another area of inconsistency is the distinction between an “impaired” antibody response and a secondary antibody response. It is known that the magnitude of the rise in the antibody titer is inversely correlated with the preexisting antibody titer (52, 53). In our study, individuals with a ≤4-fold rise in antibody titer following vaccination had preexisting antibodies to the CA/09 virus (excluding subject 24, who did not respond to any of the viruses tested). In contrast, some investigators disregard preexisting antibody and use ≤4-fold rises in the titer of antibody to the second virus of a sequential infection to define OAS (1, 51). For example, subjects with preexisting titers of 320 or more to the 2009 pH1N1 virus were classified as having a severely impaired response to the 2009 pH1N1 vaccine (51). Other investigators have performed sequential infections with two related viruses, making it possible that lower fold rises in antibody titer that were attributed to an impaired antibody response were rather a result of a diminished secondary antibody response because of the presence of preexisting antibodies induced by the primary infection (11, 13, 14).

Our study agrees with those of other investigators who failed to find evidence of OAS while analyzing the influenza virus antibody response (15–17). OAS was previously shown not to be responsible for the age-related impairment of the antibody response to influenza vaccines that is seen in the elderly (15). A study using serum from a longitudinal trial of influenza vaccine efficacy to identify correlates of protection by influenza vaccines determined that subjects showed better antibody responses to the vaccine strain than to older influenza virus strains that they had prior exposure to and concluded that while there was no evidence of OAS, there were various degrees of antibody cross-reactivity (16). Analysis of a panel of influenza virus-specific human monoclonal antibodies showed that these antibodies presented the highest affinity for the current vaccine strain and not previously encountered influenza virus strains, suggesting that OAS was not a common occurrence in healthy adults receiving influenza vaccination (17). Li et al. analyzed epitope-specific responses to H1N1 viruses and demonstrated that the ability to detect a response to a novel virus may depend on whether immunogenic epitopes in the HA are conserved or deleted (54). The specificity of responses to the pH1N1 virus in serum samples from 18- to 30-year-old subjects was shifted to a unique site near the receptor-binding domain of the HA because these subjects were previously exposed to seasonal H1N1 viruses possessing a similar epitope and this response was boosted upon exposure to the pH1N1 virus (54). Miller et al. analyzed four sequential serum samples collected between 1997 and 2008 from a cohort of individuals born between 1917 and 1952 by HAI and ELISA against the 1957 H2N2, 1968 H3N2, and 1977 H1N1 pandemic viruses and a few seasonal H1 and H3 influenza viruses (55). They found no evidence of a suboptimal response to the seasonal influenza viruses despite the presence of antibody responses to the pandemic viruses (55).

Overall, these issues highlight a number of variations among OAS studies, including study design, virus selection, data interpretation, and the definition of OAS, that hinder our ability to understand the potential role of OAS during influenza virus infection and must be standardized. The results of our study clearly show that prior infection with a range of seasonal H1N1 viruses did not impair the antibody response to infection or vaccination with the 2009 pH1N1 virus, and our data do not support the suggestion that the age distribution of infection associated with the 2009 H1N1 pandemic was due to OAS.

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