Effect of Priming with H1N1 Influenza Viruses of Variable Antigenic Distances on Challenge with 2009 Pandemic H1N1 Virus

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Compared to seasonal influenza viruses, the 2009 pandemic H1N1 (pH1N1) virus caused greater morbidity and mortality in children and young adults. People over 60 years of age showed a higher prevalence of cross-reactive pH1N1 antibodies, suggesting that they were previously exposed to an influenza virus or vaccine that was antigenically related to the pH1N1 virus. To define the basis for this cross-reactivity, ferrets were infected with H1N1 viruses of variable antigenic distance that circulated during different decades from the 1930s (Alaska/35), 1940s (Fort Monmouth/47), 1950s (Fort Warren/50), and 1990s (New Caledonia/99) and challenged with 2009 pH1N1 virus 6 weeks later. Ferrets primed with the homologous CA/09 or New Jersey/76 (NJ/76) virus served as a positive control, while the negative control was an influenza B virus that should not cross-protect against influenza A virus infection. Significant protection against challenge virus replication in the respiratory tract was observed in ferrets primed with AK/35, FM/47, and NJ/76; FW/50-primed ferrets showed reduced protection, and NC/99-primed ferrets were not protected. The hemagglutinins (HAs) of AK/35, FM/47, and FW/50 differ in the presence of glycosylation sites. We found that the loss of protective efficacy observed with FW/50 was associated with the presence of a specific glycosylation site. Our results suggest that changes in the HA occurred between 1947 and 1950, such that prior infection could no longer protect against 2009 pH1N1 infection. This provides a mechanistic understanding of the nature of serological cross-protection observed in people over 60 years of age during the 2009 H1N1 pandemic.

Widespread outbreaks of seasonal influenza cause an estimated 20,000 to 36,000 deaths annually in the United States (57). In addition, influenza virus pandemics associated with increased morbidity and mortality occur when novel influenza viruses emerge to which the majority of the human population is immunologically naive (3, 47). Novel influenza viruses can be introduced into humans through antigenic shift, which occurs as a result of genetic reassortment between various influenza virus strains, or the direct transmission of influenza viruses with a novel HA gene from animal influenza viruses, such as avian species or pigs, to humans (3, 15, 22, 32, 55).

The virus responsible for the influenza pandemic of 2009 was a novel H1N1 virus (2009 pandemic H1N1 [pH1N1]) that was antigenically highly divergent from the seasonal H1N1 viruses circulating at the time and to which a large portion of the human population was immunologically naive (17, 22, 24). Phylogenetic analysis of the 2009 pH1N1 virus revealed that it was a reassortant virus with two genes derived from a Eurasian avian-like swine virus and the remaining six genes from a triple-reassortant virus circulating in pigs in North America that in turn had derived from genes from a human H3N2 virus and from North American classical swine and avian lineage influenza viruses (22).

While morbidity and mortality caused by the 2009 pH1N1 virus was not as severe as in previous pandemics, the Centers for Disease Control and Prevention (CDC) reported an estimated 61 million cases of 2009 pH1N1 virus infection in over 206 countries, causing ~274,000 hospitalizations with an ~0.02% case fatality rate (7). In addition, an unusually high frequency of severe disease and death occurred in children and young adults who were otherwise healthy (38, 61). Sixty percent of laboratory-confirmed infections and 32 to 45% of hospitalized cases in the United States occurred in persons under 18 years of age, and cases in persons younger than 65 years of age accounted for ca. 90% of deaths (38,

61). The burden of disease was largely in children and young adults, with up to 50% of this population showing evidence of infection (44), compared to 10% of the adult population (11, 44). Several investigations have attempted to determine why severe disease and hospitalization associated with 2009 pH1N1 infection predominated in younger age groups (5, 8, 24, 27, 37, 40, 42).

Serological analyses of samples collected prior to the 2009 pandemic from humans demonstrated that older adults, particularly the elderly (>65 years old), had substantial levels of cross-reactive antibodies to the 2009 pH1N1 virus compared to younger adults and children in many (24, 27, 44), but not all, countries (10, 56, 64). In contrast, little cross-reactivity was observed between recent H1N1 influenza virus strains and the 2009 pH1N1 virus (24, 56). These data suggest that previous exposure to older seasonal influenza viruses with similar B cell epitopes (39) may have protected against 2009 pH1N1 infection.

The purpose of our study was to develop a better understanding of the nature of the H1N1 viruses that provided serological cross-reactivity and protection against the 2009 pH1N1 virus. We infected ferrets with H1N1 influenza viruses of variable antigenic distance ranging from the 1930s to the present day and determined the effect of prior infection on subsequent challenge with

Received 18 January 2012 Accepted 22 May 2012

Published ahead of print 6 June 2012

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Supplemental material for this article may be found at <http://jvi.asm.org/>.

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doi:10.1128/JVI.00147-12

wild-type 2009 H1N1 influenza virus in terms of challenge virus replication and antibody response.

MATERIALS AND METHODS

Viruses. The A/Alaska/35 (AK/35) H1N1 virus and the 2009 pH1N1 virus A/California/07/2009 (CA/09) virus were provided by Alexander Klimov (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/Fort Warren/1/1950 (FW/50) H1N1 virus was provided Jeffrey Taubenberger (National Institutes of Health/National Institute of Allergy and Infectious Diseases [NIH/NIAID]). The A/New Jersey/8/1976 (NJ/76) H1N1 virus was provided Brian Murphy (NIH/NIAID). The H1N1 virus A/New Caledonia/20/1999 (NC/99) and the influenza B virus strain B/Malaysia/2504/2004 (B/Mal) that was used as a control were provided by Zhiping Ye (FDA). Each virus was propagated in the allantoic cavity of 9- to 11-day-old embryonated specific-pathogen-free hen's eggs. 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 infectious dose (TCID_{50}) for each virus was determined by serial titration of virus in MDCK cells and calculated by the method developed by Reed and Muench (50).

Production of pseudotyped virion particles. Pseudotype virion particles were generated as described previously (60). Briefly, 30 to 50% confluent 293T cells seeded in 10-cm dishes were transfected with plasmids expressing the HIV backbone (pCMV Δ 8.2), the luciferase reporter gene (pHR'CMV-Luc), a serine protease (TMPRSS2), and influenza virus hemagglutinin (HA) and neuraminidase (NA) plasmids. 293T cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. Transfection was performed using a Profection mammalian transfection kit (Promega) according to the manufacturer's protocol. After an overnight incubation at 37°C, the cells were washed and replenished with 10 ml of fresh medium, and 48 h later the supernatant was collected and filtered through a 0.45- μm -pore-size syringe filter. The filtrate was divided into aliquots and stored at -80°C until use.

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

Evaluation of clinical signs. Ferrets were evaluated for symptoms and/or clinical signs after both priming and challenge. The infected animals were monitored daily for 14 days for clinical signs of influenza virus infection, including changes in weight and body temperature, following both priming and challenge. Ferrets were evaluated for nasal symptoms including nasal rattling, sneezing, nasal discharge, mouth breathing, and level of activity (52).

Assessment of viral replication in ferrets. Ferrets were lightly anesthetized with isoflurane and inoculated intranasally (i.n.) with 10^7 TCID_{50} of one of the H1N1 or influenza B viruses in a volume of 0.5 ml (0.25 ml per nostril). After 6 weeks, ferrets were challenged with CA/09 virus at a similar concentration and volume as priming (10^7 TCID_{50} i.n. in a volume of 0.5 ml). On days 1 and 3 postchallenge, groups of four ferrets were euthanized, and the nasal turbinates and portions of the right and left lung were harvested for virus titration. Tissues were homogenized and the amount of infectious virus in 10% (wt/vol) tissue homogenates was determined as previously described (30). Infectivity was determined by recording the presence of cytopathic effect (CPE) when an inoculum of 20 μl of the tissue homogenate was applied in quadruplicate on a 96-well tissue culture plate of MDCK cells and was serially diluted. The dilution at which 50% of the wells are infected (TCID_{50}) was computed using the Reed and Muench method (50). Titers are expressed as the $\log_{10}$ $\text{TCID}_{50}/\text{g}$ of tissue.

Evaluation of antibody response in ferrets. Serum samples were collected before and 2, 4, and 6 weeks after priming and 7, 14, 21, and 28 days postchallenge. Nasal washes were collected weekly throughout the duration of the study. At 28 days postchallenge, ferrets were euthanized, terminal bleeds were performed, and the spleens were harvested. Peripheral blood mononuclear cells and splenocytes were purified from blood and spleen samples, respectively.

HAI assay. A previously described protocol was followed (9). Briefly, ferret sera treated with receptor destroying enzyme (RDE) were 2-fold serially 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 sera 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. Hemagglutination inhibition (HAI) titers were recorded as the inverse of the highest antibody dilution that inhibited hemagglutination.

Neutralization assay. A previously described protocol was followed (36). Briefly, influenza viruses diluted to a concentration of 100 TCID_{50} per 50 μl ($10^{3.3}$ $\text{TCID}_{50}/\text{ml}$) in minimal essential medium–0.5% bovine serum albumin (BSA) were added to ferret sera that were 2-fold serially diluted in PBS, and the mixtures were incubated for 1 h at room temperature. After 1 h, the virus-serum mixtures were added to MDCK cells cultured in 96-well plates in quadruplicate. Cells were incubated at 33 or 37°C, and the neutralization titer was scored 4 days later by CPE. The neutralization titer is the inverse of the highest serum dilution where complete neutralization of CPE occurred in 50% of the wells.

Pseudovirion luciferase assay. Pseudovirions were first titrated by serial dilution. Similar amounts of virus were then added to RDE-treated ferret sera 2-fold serially diluted in PBS in 96-well U-bottom plates. The virus and sera were incubated together for 20 min at room temperature and then added in triplicate to 293A cells (10,000 cells/well) cultured on 96-well black-and-white isoplates (Perkin-Elmer), followed by incubation for 1 to 2 h at 37°C. The virus-serum mixture was removed, the plates were washed, 200 μl of fresh medium was added, and the plates were then incubated at 37°C for 48 h. The cells were washed once in PBS, and 20 μl of cell lysis buffer (Promega) was added. Plates were shaken for 10 to 20 min, and 100 μl of luciferase substrate (Promega) was added to each well. The luminescence was measured immediately by using a plate reader (Spectromax). The antibody titers shown are the highest dilution to show at least 50% neutralization.

HA-NA purification. A previously described protocol was followed (2, 21). Briefly, 9- to 11-day-old embryonated chicken eggs inoculated with one of the 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 (J-26 XP centrifuge). Virus was resuspended in PBS, purified using 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 and 60% sucrose interface was collected, diluted in PBS, and pelleted by centrifugation at 24,000 rpm for 2 h at 4°C. The virus 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*- n - 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 the purified HA and NA was collected.

ELISA. Purified HA-NA preparations were diluted in carbonate buffer (pH 9.8) and added to 96-well enzyme-linked immunosorbent assay (ELISA) plates at a concentration of 50 HA units, followed by incubation overnight at 4°C. As a control, some wells received carbonate buffer alone. The following day, the plates were blocked by adding 200 μl of 1% BSA carbonate buffer, followed by incubation at room temperature for 2 h.

Heat-inactivated ferret sera were diluted 1:10 in 1% BSA carbonate buffer and 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 sera were transferred to the ELISA plates. The sera 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 either goat anti-ferret horseradish peroxidase (HRP)-IgG (Immunology Consultants Laboratory, Inc.), anti-ferret HRP-IgM (Alpha Diagnostics), or anti-ferret HRP-IgA (Alpha Diagnostics; Rockland Immunochemicals) 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'-azino-bis(3-ethylbenzthiazolinesulfonic acid)] ELISA reagent was added, and the reaction was stopped by using 1% sodium dodecyl sulfate (SDS). The absorbance was measured at 405 nm using a spectrometer, and wells with an optical density (OD) of >0.2 were considered positive.

Statistical analysis. The significance of difference between any two different groups was assessed by a Mann-Whitney test using Prism, version 5 (GraphPad Software, CA). The Mann-Whitney test lacks the power to detect significance at a *P* value of 0.05 in small samples. Therefore, in selected experiments, where the sample size of each group was <5, an unpaired *t* test was used with the assumption that the data fit a normal distribution. *P* values of <0.05 are considered significantly different.

RESULTS

Effect of H1N1 priming on subsequent infection with CA/09 virus. The effect of prior H1N1 virus infection on replication of the CA/09 virus was evaluated by priming ferrets with one of several H1N1 viruses of variable antigenic distance from the 2009 pH1N1 virus and subsequently challenging them with CA/09 virus 6 weeks later. The selected H1N1 viruses circulated during different decades from the 1930s (AK/35), 1940s (FM/47), 1950s (FW/50), and 1990s (NC/99). We selected one virus from each decade of H1N1 virus circulation for the present study, having considered the results from previous studies using mice that were infected or vaccinated with viruses isolated in 1943, 1947, and 1957 (39, 54). Ferrets primed with the homologous CA/09 or NJ/76 virus served as a positive control, while the negative control was an influenza B virus (B/Mal) that would not cross-protect against influenza A virus infection (20). NJ/76, which caused limited infections in humans and never circulated among the general population, is a genetically and antigenically related swine origin H1N1 virus previously shown to protect against CA/09 virus infection (31, 45).

The ferrets did not show any clinical signs of influenza virus infection after priming or challenge. No significant decrease in body weight was observed in any of the groups, and body temperatures remained within the normal range (data not shown). In addition, nasal symptoms such as sneezing or nasal discharge were not observed. This was consistent with the results from previous studies using ferrets of a similar age (45).

Ferret nasal turbinates and lungs were harvested 1 or 3 days after challenge with the CA/09 virus to evaluate viral replication in the upper and lower respiratory tract, respectively. As expected, priming with the CA/09 virus provided robust protection from homologous challenge compared to priming with B/Mal (Fig. 1). In the lungs, ferrets primed with NJ/76 showed significantly reduced virus titers that were statistically similar to those in ferrets primed with the homologous CA/09 virus (Fig. 1A). In contrast, ferrets primed with NC/99 showed lung virus titers similar to those primed with B/Mal, suggesting that prior infection with this virus is unable to protect against CA/09 virus infection.

Ferrets primed with AK/35, FM/47, or FW/50 showed a step-

wise increase in lung virus titer that correlated inversely with the time since these viruses circulated in humans. Interestingly, ferrets primed with AK/35 or FM/47 showed marked differences in lung virus titer compared to ferrets primed with FW/50 (Fig. 1A). Priming with AK/35 or FM/47 resulted in an ~170- to ~400-fold reduction in titer compared to those primed with B/Mal, while priming with FW/50 resulted in only an ~10- to ~12-fold reduction (Fig. 1A). Significantly higher lung virus titers (~14- to ~30-fold) were seen in ferrets primed with FW/50 compared to those primed with AK/35 or FM/47. However, ferrets primed with any of these three viruses showed statistically higher lung virus titers compared to ferrets primed with the NJ/76 or CA/09 viruses (*P* < 0.05) (Fig. 1A).

In the nasal turbinates, similar titers were measured in ferrets primed with NJ/76 or CA/09 viruses, while ferrets primed with NC/99 showed virus titers similar to those primed with B/Mal (Fig. 1B). Ferrets primed with AK/35, FM/47, or FW/50 did not show a stepwise increase in virus titer as in the lungs, however, ferrets primed with FW/50 still showed significantly higher titers than ferrets primed with AK/35 or FM/47 (Fig. 1B). Ferrets primed with AK/35 or FM/47 showed similar virus titers, with an ~200- to ~850-fold lower titer, while priming with FW/50 resulted in only a 36-fold lower titer than priming with B/Mal (Fig. 1B). Titers in ferrets primed with FW/50 were ~6- to ~25-fold higher than in ferrets primed with the AK/35 or FM/47 viruses (Fig. 1B). However, as in the lungs, these titers were still significantly higher than those in ferrets primed with the CA/09 virus. Overall, the protective efficacy of each priming virus was similar in the upper and lower respiratory tracts of ferrets against challenge with the CA/09 virus.

Antibody cross-reactivity following priming with H1N1 viruses. Protection against influenza virus infection is most efficiently mediated by neutralizing antibodies (14, 19, 48). Analyses of human sera have shown a correlation between protection against 2009 pH1N1 infection and the presence of cross-reactive antibodies against the 2009 pH1N1 virus (24, 27). We tested antibodies generated in response to H1N1 priming in sera collected 4 weeks after priming for their ability to cross-react with the CA/09 virus in ELISA, HAI, and neutralization assays to identify a correlation between cross-reactive antibody titers against CA/09 virus and the protective efficacy of the priming viruses against CA/09 virus replication in the ferret respiratory tract.

Results from HAI and neutralization assays are presented in Tables 1 and 2, respectively, while the ELISA results are presented in Table S1 in the supplemental material. For each group of ferrets, the four individual sera were pooled together after it was determined that the homologous titers were within 2-fold of each other (data not shown). Ferret sera collected immediately prior to influenza virus priming (preprime) showed no detectable antibodies in any of the assays, demonstrating that the ferrets were seronegative to influenza prior to priming. Fold increases in antibody titer were based on a comparison of antibody titers after priming compared to the preprime samples.

Ferrets achieved high antibody titers against the homologous influenza virus with which they were primed (Tables 1 and 2; see also Table S1 in the supplemental material). However, cross-reactive HAI antibodies against CA/09 virus were only detected in sera from ferrets primed with AK/35, FM/47, or NJ/76 (Table 1). Anti-NJ/76 sera showed the highest cross-reactive HAI antibody levels against CA/09 virus, with titers within 4-fold of the titers seen in

FIG 1 Replication of CA/09 after priming with various influenza viruses. The lungs (A) and nasal turbinates (B) from groups of four ferrets primed with various influenza viruses, and subsequently challenged with CA/09, were harvested at day 1 (d1) and d3 postinfection. At necropsy, portions of the right and left lung were harvested for virus titration. Thus, there are two data points for each ferret (one for the right lobe and one for the left lobe) and, with four ferrets per group, there are eight data points for each group at d1 and d3 postinfection. Titters are expressed as the log₁₀ TCID₅₀/g tissue. Each data point represents the titer from an individual animal, and the mean is indicated by a bar. The limit of detection for this assay was 1.8 log₁₀ TCID₅₀/g tissue and is highlighted by the broken black bar in each graph. Virus titers were compared to B/Mal for statistical significance (*, *P* < 0.05).

ferrets previously primed with the homologous CA/09 virus (Table 1). Lower levels of cross-reactive HAI antibodies against the CA/09 virus were detected in the anti-AK/35 and anti-FM/47 sera, while cross-reactive HAI antibodies were not detected in the sera from ferrets primed with FW/50, NC/99, or B/Mal (Table 1). In

the neutralization assay, only the anti-AK/35 and anti-NJ/76 sera showed detectable neutralizing cross-reactive antibodies against the CA/09 virus (Table 2). The presence of cross-reactive HAI and/or neutralizing antibodies in sera from ferrets primed with AK/35, FM/47, and NJ/76 explains why priming with these viruses

TABLE 1 HAI antibody titers in sera from ferrets primed with selected viruses

Virus	HAI antibody titer in ferrets primed with the indicated virus						
	AK/35	FM/47	FW/50	NJ/76	NC/99	CA/09	B/Mal
AK/35	2,560^a	40	20	40	80	20	5
FM/47	5 ^b	1,280	40	5	5	5	5
FW/50	5	80	1,280	5	5	5	5
NJ/76	5	40	5	640	5	320	5
NC/99	5	5	5	5	640	5	5
CA/09	20	40	5	320	5	1,280	5
B/Mal	5	5	5	5	5	5	1,280

^a Homologous titers are indicated in boldface.

^b The lower limit of detection for this assay was an HAI titer of 10. Samples with undetectable antibodies were assigned a value of 5.

TABLE 2 Cross-neutralizing antibody titers in sera from ferrets primed with selected viruses

Virus	Neutralizing antibody titer in ferrets primed with the indicated virus						
	AK/35	FM/47	FW/50	NJ/76	NC/99	CA/09	B/Mal
AK/35	5,120^a	10	20	60	10	20	10
FM/47	10 ^b	1,280	10	10	10	10	10
FW/50	10	40	3,840	10	10	10	10
NJ/76	10	40	10	1,280	10	640	10
NC/99	10	10	10	10	640	10	10
CA/09	30	10	10	640	10	2,560	10
B/Mal	10	10	10	10	10	10	960

^a Homologous titers are indicated in boldface.^b The lower limit of detection for this assay was a neutralization titer of 20. Samples with undetectable antibodies were assigned a value of 10.

provided significantly greater protection against CA/09 virus replication in the ferret respiratory tract compared to the other priming viruses.

Cross-reactive IgG antibody titers against purified CA/09 HA were measured by ELISA (see Table S1 in the supplemental material). At least a 4-fold rise in cross-reactive antibody titers against purified CA/09 HA was detected in sera from ferrets primed with all of the H1N1 viruses except NC/99 (see Table S1 in the supplemental material). Cross-reactive IgG antibody titers in sera from ferrets primed with AK/35 or FM/47 were 4-fold higher against CA/09 HA compared to anti-FW/50 sera; this may explain the differences seen in the protective efficacy of these viruses against challenge with the CA/09 virus (see Table S1 in the supplemental material). Although an ELISA cannot distinguish between neutralizing and non-neutralizing antibodies, a strong correlation was observed between the levels of IgG cross-reactive antibodies against CA/09 HA and the protective efficacy of the priming viruses, further demonstrating the importance of these antibodies generated in response to priming.

Antigenic relatedness of seasonal H1N1 and 2009 pH1N1 influenza viruses. Antigenic relatedness was assessed based on the titers of cross-reactive antibodies detected in the HAI and neutralization assays (23, 29, 63). By convention, two viruses are considered antigenically related if the difference in antibody titer against the homologous virus and the heterologous virus is ≤ 4 -fold (1, 23). Based on these results, only NJ/76 is antigenically related to the CA/09 virus since the anti-NJ/76 sera showed cross-reactive antibody titers against CA/09 virus that were within 4-fold of the homologous anti-CA/09 sera in reciprocal HAI and neutralization assays (Tables 1 and 2). AK/35, FM/47, and FW/50 provided cross-protection even though they were antigenically distinct from the CA/09 virus.

Role of IgM antibody in the protection against CA/09 challenge. IgA and IgM antibodies were also measured by ELISA. Na-

sal washes to measure mucosal IgA titers were collected weekly in 1 ml of PBS after priming and challenge. The limit of detection was 10 for this assay because an initial dilution of 1:10 was used. Both influenza-specific and total IgA were assayed but neither was detected, suggesting that the IgA may have been diluted too much in the nasal wash. Although IgA was not detected in the ferret nasal wash samples, high levels of IgM antibodies were detected in response to influenza virus infection. IgM, which appears early in the course of infection and is often referred to as a “natural antibody,” is typically the first antibody isotype to appear in response to a primary exposure with an antigen such as an influenza virus (4, 6, 13, 28). These biological properties of IgM are often used to distinguish between primary and recurrent infection. Previous studies have shown that humans undergoing a primary infection with an influenza virus to which they are immunologically naive generate a substantial IgM response to the infecting virus, while the IgM response is absent in humans undergoing a secondary infection after they have been primed with the homologous virus or an antigenically similar virus (4, 6, 13). By examining IgM titers after both priming and challenge, we sought to determine whether the ferret response to the CA/09 virus infection was more characteristic of a primary or secondary infection.

IgM titers were measured by ELISA 2 weeks after priming and again at 7 and 14 days after challenge with the CA/09 virus. High levels of homologous IgM antibody were detected in the sera 2 weeks after priming (Table 3). Compared to the preprime sera, at least a 4-fold rise in cross-reactive IgM antibody titer against CA/09 virus was detected 2 weeks after priming in sera from ferrets primed with AK/35, FM/47, and NJ/76, but not in the sera from ferrets primed with FW/50, NC/99, or B/Mal (Table 3). However, at 7 days after CA/09 virus challenge, a significant boost in IgM titer against CA/09 virus was seen in ferrets primed with FW/50, NC/99, or B/Mal; this response was similar to the IgM response to primary infection with the CA/09 virus (6- to 10-fold

TABLE 3 Cross-reactive IgM antibody titers in sera from ferrets primed with selected viruses

Time point of serum collection	IgM antibody titer in ferrets primed with indicated viruses tested against purified HA													
	AK/35 vs:		FM/47 vs:		FW/50 vs:		NJ/76 vs:		NC/99 vs:		CA/09 vs:		B/Mal vs:	
	Self	CA/09	Self	CA/09	Self	CA/09	Self	CA/09	Self	CA/09	Self	CA/09	self	CA/09
Preprime	10 ^a	10	10	10	10	20	10	10	10	20	10	10	10	10
2 weeks postprime	160	40	160	80	160	20	160	80	160	10	160	160	80	10
7 days postchallenge	40	40	20	40	80	160	20	10	40	160	10	10	40	320
14 days postchallenge	20	20	20	40	40	80	20	10	40	80	10	10	20	160

^a The lower limit of detection for this assay was an IgM antibody titer of 20. Samples with undetectable antibodies were assigned a value of 10.

TABLE 4 Cross-reactive antibody titers in sera from ferrets primed with selected viruses against luciferase-encoding pseudovirions

Virus	Glycosylation site(s)	Antibody titers against pseudovirus with indicated glycosylation (G) site(s) ^a			
		1918 wt [none]	1918 1G 142 [142]	1918 1G 177 [177]	1918 2G [142/177]
AK/35	None	2,560	640	160	160
FM/47	144	640	640	160	80
FW/50	172	320	160	640	160
NJ/76	None	2,560	640	320	80
NC/99	142/177	20	40	20	20
CA/09	None	5,120	2,560	640	320
B/Mal		10 ^b	10	10	10

^a The column subheadings indicate the virus [glycosylation site(s)].

^b The lower limit of detection for this assay was an antibody titer of 20. Samples with undetectable antibodies were assigned a value of 10.

increase) (Table 3). On the other hand, lower levels of IgM antibodies against the homologous virus were detected in the post-challenge sera than were observed after priming, demonstrating the specificity of the IgM response to the CA/09 virus after challenge.

In contrast, lower levels of IgM antibody were detected against both the homologous virus and CA/09 virus 7 days postchallenge in ferrets primed with AK/35, FM/47, NJ/76, and CA/09 viruses compared to the IgM levels detected 2 weeks after priming. In fact, in the postchallenge sera, IgM antibodies against CA/09 virus were not detected in ferrets primed with NJ/76 and CA/09 virus, and low levels of IgM antibodies were detected in the sera of ferrets primed with AK/35 and FM/47. The response in ferrets primed with AK/35 or FM/47 was significantly diminished compared to that seen in ferrets primed with FW/50, NC/99, and B/Mal. Overall, the low levels of IgM in the postchallenge sera from ferrets primed with AK/35, FM/47, NJ/76, and CA/09 suggests that the response to CA/09 virus challenge was characteristic of a secondary infection. Together, the results show a correlation between the serotype response to CA/09 virus (e.g., primary or secondary response) and the protective efficacy of the priming virus against CA/09 challenge.

Effect of HA glycosylation on serologic cross-reactivity and cross-protection. During the evolution of H1N1 viruses in humans, there has been a tendency toward the accumulation of glycosylation sites on the HA globular head (16, 60). Addition of glycans to the HA globular head has been shown to block neutralization of HA by monoclonal and polyclonal antibodies, as well as, to alter the receptor-binding and fusion properties of the HA (58). The H1N1 viruses used in the present study differ in both the number and the location of glycosylation sites on the HA globular head (60). Figure S1 in the supplemental material shows an alignment of the HA sequences from the H1N1 influenza viruses used here highlighting the location of the glycosylation sites on the HA globular head. The presence and location of these glycosylation sites may affect the ability of the priming viruses to generate an antibody response that can cross-protect against CA/09 virus challenge.

To study this further, ferret sera collected 4 weeks after priming were tested for the ability to cross-react with H1N1 pseudovirions encoding luciferase and bearing 1918 SC HA and NA proteins that were mutated to insert glycosylation sites into the HA globular head (60). The 1918 SC and CA/09 H1N1 viruses share extensive sequence homology in the HA, both have unglycosylated HA globular heads, and previous studies have shown that priming with CA/09 virus can protect against 1918 H1N1 challenge and

vice versa (18, 31, 41, 43, 60). Although pseudovirions bearing either 1918 SC or CA/09 HA and NA have been shown to behave similarly, the 1918 SC pseudovirion was used since it was possible to rescue higher titers of this pseudovirion (60). AK/35 and NJ/76 also have no glycosylation sites on the HA globular head. FM/47 and FW/50 both have one glycosylation each, at amino acid positions 144 and 172, respectively (see Fig. S1 and S2 in the supplemental material) (60). NC/99 has two glycosylation sites at amino acid positions 142 and 177 (see Fig. S1 in the supplemental material) (60). Therefore, the sera were tested against four pseudovirions: 1918 SC wild type (wt), 1918 1G 142, 1918 1G 177, and 1918 2G 142/177. Residues 142 and 177 are in close proximity to residues 144 and 172 (see Fig. S2 in the supplemental material).

As expected, and as has been previously reported, the anti-CA/09 sera cross-reacted well with the 1918 SC wt pseudovirion (Table 4). As glycosylation sites were inserted into the HA globular head of the 1918 SC HA pseudovirion, a significant reduction in antibody binding was observed with antisera from all of the priming groups except NC/99 and B/Mal (Table 4). Specifically, the anti-CA/09 sera showed a 16-fold reduction in antibody binding to the 1918 HA pseudovirion expressing two glycosylation sites (1918 2G 142/177) compared to the 1918 wt pseudovirion. Interestingly, anti-CA/09 sera showed a 4-fold reduction in antibody titer against the 1918 pseudovirion expressing a single glycosylation site at amino acid 177 (1918 1G 177) compared to the 1918 pseudovirion expressing a single glycosylation site at amino acid 142 (1918 1G 142). A similar pattern was also observed with sera from ferrets primed with AK/35, FM/47, or NJ/76 (Table 4).

Together, these data indicate that the addition of glycosylation sites to the HA globular head of H1N1 viruses in or adjacent to the Sa antigenic site (see Fig. S2 in the supplemental material) impairs the ability of the viruses to generate a cross-protective antibody against CA/09 virus challenge. In addition, the differences in cross-reactivity observed against 1918 1G 177 compared to 1918 1G 142 suggests that the specific location of the glycosylation site is also important for cross-protection and for reducing antibody cross-reactivity. This may explain why priming with FM/47, but not FW/50 and NC/99, is able to protect against CA/09 challenge.

DISCUSSION

The purpose of our study was to develop a better understanding of the nature of the H1N1 viruses that provided serological cross-reactivity and protection against the 2009 pH1N1 virus. We primed ferrets with H1N1 influenza viruses of variable antigenic distance isolated from the 1930s to the 1990s and determined the effect of prior infection on subsequent challenge with 2009 H1N1

pandemic influenza virus. We postulated that H1N1 viruses antigenically related to the 2009 pH1N1 virus would protect against challenge with this virus, while those that were antigenically dissimilar would be unable to protect against challenge. We found that prior infection with a similar swine origin virus (NJ/76) or viruses isolated in and prior to 1947 provided significant protection against CA/09 challenge, while significantly lower protective efficacy was observed with viruses isolated in and after 1950. Our data suggest that the addition of glycosylation sites to the HA globular head, and the specific location of those sites, contributed to this loss in antigenic cross-reactivity and protective efficacy.

Soon after the pH1N1 virus appeared, the Sa antigenic site of the CA/09 HA was shown to be similar to that of the 1918 virus. Manicassamy et al. generated inactivated influenza vaccines or VLPs from a panel of 11 H1N1 viruses isolated between 1918 and 2009 and evaluated the ability of these vaccines to protect mice from subsequent challenge with a mouse-lethal 2009 pH1N1 virus (39). 1918 VLPs, inactivated Weiss/43, and inactivated classical swine H1N1 viruses protected mice from lethal infection with the pH1N1 virus, while 1977, 1984, 1991, and 2007 H1N1 viruses offered only partial protection (39). The authors of that study generated and sequenced escape mutants of CA/09 using three monoclonal antibodies that cross-reacted with the 1918 and 2009 H1N1 HAs, confirming that the Sa antigenic site was conserved between the 2009 pH1N1 and 1918 viruses. They concluded that prior exposure to human influenza viruses from 1918–1943, or vaccination with classical swine H1N1 virus (NJ/76), offered significant levels of cross protection against the 2009 pH1N1 virus, thus explaining the lower incidence of disease and infection in persons aged 65 years or older (39). Although Weiss/43-vaccinated mice lost weight after challenge, they recovered; these mice did not have cross-reactive HAI antibodies against the CA/09 virus. Our findings confirm those of Manicassamy et al. and extend them by demonstrating the ability of 1935 and 1947 but not 1950 H1N1 viruses to protect ferrets from infection with the CA/09 virus.

From their determination and analysis of the crystal structure of the CA/09 HA, Xu et al. concluded that the 1918 HA was a remarkably close relative of the CA/09 HA, with only 20% amino acid difference in the antigenic sites, that was mainly restricted to the Ca region, while the CA/09 virus differed substantially from seasonal H1 HAs in all four antigenic regions (62). These researchers also analyzed the presence of glycosylation sites on the HA that can mask the protein surface from antibody recognition and reported that the CA/09 HA, like 1918 HA, does not have glycosylation sites in the Sa region of the globular head, while human H1 HAs have gradually acquired up to three such sites in the relatively conserved Sa region from 1930 to 2007. The lack of glycosylation of the Sa site in the CA/09 HA thus leaves the epitope exposed for antibody recognition, and the authors proposed that Sa-specific antibodies were potentially the major underlying basis of age-related immunity to the 2009 H1N1 virus. They mapped the binding on the 1918 HA of a monoclonal antibody (2D1, isolated from a survivor of the 1918 pandemic) that cross-reacted with the CA/09 HA and found that the footprint of the Fab on the HAs largely coincided with and extended the Sa region and that this site was well conserved in the 1918 and 2009 but not in seasonal H1 HAs. Interestingly, 7 amino acid differences, including three in the Sa site, along with two potential glycosylation sites in the center of the epitope (residues 129 and 163 in the Xu paper correspond to res-

idues 142 and 177 in our paper), explain why 2D1 does not cross-react with seasonal H1 viruses (62). Our findings with the pseudovirion assay confirm these observations of Xu et al. We have also extended their observations by demonstrating antibody cross-reactivity and cross-protection induced by prior infection *in vivo*.

A correlation was observed between the cross-reactivity of antibodies induced by the priming viruses and the protective efficacy against the CA/09 virus. NJ/76, which provided complete protection from CA/09 challenge, was the only H1N1 virus used in the present study that was antigenically related to the CA/09 virus based on HAI and neutralization assays. This is most likely because both are swine origin viruses and both derived their HA from a classical swine virus (31, 39, 42, 45). Our results are consistent with previous studies from our lab and others that have shown that prior exposure to the NJ/76 virus or similar 1976 swine viruses, or prior vaccination with the 1976 “swine flu” vaccine, can protect against 2009 pH1N1 virus infection (31, 39, 42, 45). Individuals who received the 1976 swine flu vaccine had neutralizing antibodies against the 2009 pH1N1 virus (42). Prior infection with AK/35 and FM/47, which provided significant, albeit partial, protection against challenge, generated lower levels of cross-reactive HAI and neutralizing antibodies against CA/09 virus. FW/50, which provided some protection against challenge, induced cross-reactive antibodies against CA/09 virus that were only detectable by ELISA. NC/99 and B/Mal, which provided no protection against challenge, did not induce cross-reactive antibodies against CA/09 virus.

Interestingly, prior infection with AK/35 and FM/47 provided similar levels of protection against CA/09 challenge. Although AK/35 is closely related to swine viruses, FM/47 is not (46, 53). FM/47 was described as an A prime influenza virus that emerged after a major antigenic change occurred in the H1N1 influenza virus in 1947 (34, 35, 49, 53) and would not be expected to provide similar levels of protection as AK/35. Whereas NJ/76 shares significantly more homology (91%) in the total HA protein sequence with the CA/09 virus, the other H1N1 viruses share similar levels of homology: AK/35 (81%), FM/47 (81%), FW/50 (80%), and NC/99 (79%). Thus, homology of the complete protein sequence does not explain the differences in protective efficacy. However, AK/35 and FM/47 share significantly more homology with CA/09 in the Sa antigenic site compared to FW/50, 62% compared to 46%, which may contribute to the differences observed in the protective efficacy of these viruses.

A significant loss in protective efficacy was observed following primary infection with the viruses isolated in 1947 (FM/47) and 1950 (FW/50). Although some studies have shown that viruses isolated prior to 1950 can provide at least partial protection against CA/09 virus challenge, only one H1N1 virus isolated from 1948 to 1957 (Denver/1/1957) has been tested, and this virus did not offer significant protection from CA/09 challenge (31, 39, 45, 54). Our study now provides a much more specific time frame (between 1947 and 1950) as to when H1N1 viruses evolved to the point where they no longer offered effective protection against 2009 pH1N1 infection. This allows us to closely examine the viruses from this period to better understand why these viruses are able or unable to protect against 2009 pH1N1 infection. There was an unusually severe influenza A virus H1N1 epidemic in 1950 and 1951 associated with significant morbidity and mortality, especially in the United Kingdom and Canada; mortality rates in these

countries exceeded those of the 1957 H2N2 and 1968 H3N2 pandemics (26, 46, 59). Interestingly, the 1951 epidemic exhibited geographic disparities in influenza-related deaths, illustrated by high death rates in England and Canada and low death rates in the United States, except in the northeast (12). These disparities are explained in part by laboratory surveillance reports by the World Health Organization (25, 26), indicating that two antigenically distinct influenza virus A/H1N1 strains cocirculated in the northern hemisphere during the 1951 epidemic: the “Scandinavian strain” isolated in northern Europe was associated with mild illness, and the “Liverpool strain” was associated with severe illness and death in Great Britain, Canada, southern Europe, and Mediterranean countries. However, it is not known whether FW/50 was isolated during this epidemic or whether it was associated with clinically severe disease, although antigenic analysis suggests that FW/50 resembled the “Scandinavian” strain (33).

H1N1 influenza viruses underwent a major antigenic change in 1949 that included the addition of several new glycosylation sites that may be responsible for the changes in protective efficacy between FM/47 and FW/50 (51, 60). Before this time, H1N1 viruses typically had one to three glycosylation sites, but later it was common to find eight to ten glycosylation sites on the HA (51, 60). However, the contributions of additional mutations in H1N1 viruses in 1949, independent of glycosylation on the HA globular head, are unknown. Our study shows that the addition of glycosylation sites to the HA globular head and the location of these glycosylation sites can significantly reduce antibody cross-reactivity. FW/50 and NC/99 possess glycosylation sites at amino acids 172 and 177, respectively (60), that are located within the Sa antigenic site on the HA (62) (see also Fig. S2 in the supplemental material). A glycosylation site in this region likely contributed to the loss in protective efficacy shown by these two viruses compared to the other viruses.

Although our results, as well as the results from others (31, 39, 45, 54), have shown that viruses isolated prior to 1950 can protect against 2009 pH1N1 infection, it is possible that not all pre-1950 H1N1 viruses protect equally well against 2009 pH1N1 infection. Several H1N1 isolates from the 1940s, such as Hickox/40, which was shown to only partially protect against 2009 pH1N1 infection (31), are glycosylated at amino acid 178 or 179 (60). Prior infection with mouse-adapted FM/47 or prior vaccination with Weiss/43, neither of which express a glycosylation site at this position, provided significant protection against 2009 pH1N1 infection (39, 54). Thus, the protective efficacy of an H1N1 virus against 2009 pH1N1 infection depends not only on the antigenic distance from the 2009 pH1N1 virus but also on the glycosylation of the HA globular head. Unfortunately, it is difficult to make generalized conclusions from an analysis of a small number of viruses in mice and ferrets, and these studies cannot assess whether glycosylation of the HA played a role in shaping population immunity. However, this was not the intent of this study; rather, it was performed to test the hypothesis that changes in HA glycosylation affected the specificity of the immune response in subjects infected by the 2009 virus. We selected one virus from each decade of H1N1 virus circulation for the study, having considered the results from previous studies using mice that were infected or vaccinated with three viruses isolated in 1943, 1947, and 1957 (39, 54). Despite these limitations, data from experiments in animal models provide useful insights into serologic and epidemiologic observations from the 2009 pandemic.

Overall, we show that H1N1 viruses of variable antigenic distances from the 2009 pH1N1 virus protect to various degrees against subsequent infection with the 2009 pH1N1 virus. A significant reduction in cross-protection conferred by H1N1 viruses was observed between 1947 and 1950, which is likely due to major antigenic changes in H1N1 viruses during this time, specifically associated with the acquisition of glycosylation. This is a likely explanation for the observation that the elderly were protected against 2009 pH1N1 infection while younger adults were more susceptible.

ACKNOWLEDGMENTS

We thank the staff of the Comparative Medicine Branch, NIAID, for technical support for animal studies.

This research was supported by the Intramural Research Program of the NIH, NIAID.

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