Sequential Seasonal H1N1 Influenza Virus Infections Protect Ferrets against Novel 2009 H1N1 Influenza Virus

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Individuals <60 years of age had the lowest incidence of infection, with ~25% of these people having preexisting, cross-reactive antibodies to novel 2009 H1N1 influenza. Many people >60 years old also had preexisting antibodies to novel H1N1. These observations are puzzling because the seasonal H1N1 viruses circulating during the last 60 years were not antigenically similar to novel H1N1. We therefore hypothesized that a sequence of exposures to antigenically different seasonal H1N1 viruses can elicit an antibody response that protects against novel 2009 H1N1. Ferrets were preinfected with seasonal H1N1 viruses and assessed for cross-reactive antibodies to novel H1N1. Serum from infected ferrets was assayed for cross-reactivity to both seasonal and novel 2009 H1N1 strains. These results were compared to those of ferrets that were sequentially infected with H1N1 viruses isolated prior to 1957 or more-recently isolated viruses. Following seroconversion, ferrets were challenged with novel H1N1 influenza virus and assessed for viral titers in the nasal wash, morbidity, and mortality. There was no hemagglutination inhibition (HAI) cross-reactivity in ferrets infected with any single seasonal H1N1 influenza viruses, with limited protection to challenge. However, sequential H1N1 influenza infections reduced the incidence of disease and elicited cross-reactive antibodies to novel H1N1 isolates. The amount and duration of virus shedding and the frequency of transmission following novel H1N1 challenge were reduced. Exposure to multiple seasonal H1N1 influenza viruses, and not to any single H1N1 influenza virus, elicits a breadth of antibodies that neutralize novel H1N1 even though the host was never exposed to the novel H1N1 influenza viruses.

Soon after the novel H1N1 influenza outbreak in 2009, it became apparent that younger people and children were more susceptible to infection than older individuals (1–5). Serological studies revealed that many older and middle-aged adults possessed antibodies that reacted with the novel H1N1 virus prior to the pandemic (6, 7). This preexisting humoral immunity was somewhat surprising because of the differences between the hemagglutinin of the novel H1N1 and those of H1N1 viruses that have circulated in human populations since 1918 (6).

Several lines of evidence suggested antigenic similarity between the novel virus and the 1918 human influenza virus. Monoclonal antibodies derived from survivors of the 1918 pandemic were able to cross-neutralize 2009 H1N1 viruses (8). Exposure of animals to 1918-like viruses elicited antibodies that recognized novel H1N1 influenza isolates, whereas no antibody cross-reactivity or protection was observed following infection with contemporary seasonal influenza viruses (9, 10). There is conservation of antigenic regions between 1918 and 2009 pandemic hemagglutinin (HA) proteins that are not shared with contemporary seasonal H1N1 viruses (9, 11), and the 1918 and 2009 viruses both lack HA glycosylation sites that are found in later seasonal viruses (12–14). It was therefore suggested that exposure to 1918-like virus in the early 20th century may explain the preexisting immunity to the 2009 virus in older adults.

Cross-reactivity with the 1918 virus cannot, however, explain all of the observed preexisting immunity. This immunity was not uncommon in cohorts born decades after 1918, by which time significant antigenic drift had affected circulating viruses (6). Furthermore, although reactivity of human sera to the 2009 virus correlates with reactivity to the 1918 virus, this correlation is not extraordinarily strong (6).

To explain these patterns, we hypothesized that a sequence of

infections with antigenically different H1N1 viruses can elicit antibodies that react with the novel 2009 virus, even if the HAs on the infecting viruses were not antigenically similar to that of the novel H1N1 virus. Older adults would have been exposed to a larger number and diversity of H1N1 viruses and would therefore have possessed greater preexisting immunity to novel virus despite being born well after the era of 1918-like viruses.

To test this hypothesis, we infected ferrets with individual seasonal H1N1 viruses representing the past 75 years of influenza history or infected ferrets in a sequential manner with different seasonal influenza strains. Ferrets infected sequentially with 2 to 3 seasonal H1N1 influenza viruses developed receptor-blocking and virus-neutralizing antibodies that cross-reacted with novel H1N1 influenza. Sequentially infected ferrets were completely protected from morbidity and did not transmit virus to cohoused animals.

MATERIALS AND METHODS

Infection of ferrets. Fitch ferrets (*Mustela putorius furo*, female, 6 to 12 months of age), which were determined to be negative for antibody to circulating influenza A (H1N1, H3N2) and influenza B viruses, were descended and purchased from Marshall Farms (Sayre, PA). Ferrets were

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FIG 1 Phylogenetic diversity of H1N1 influenza. (A) Phylogenetic tree inferred from the amino acid sequences of human H1N1 hemagglutinins. The tree was constructed using PhyML (40). The NCBI accession numbers for the HA sequences used in phylogeny inference were obtained through the Influenza Virus Resource (41). (B) Schematic of the infection schedule. Ferrets were infected intranasally (10^6 PFU/ml) with one of 6 seasonal H1N1 influenza viruses. Ferrets were bled at days 14, 28, 42, 56, and 84 postinfection. At day 84, all ferrets were infected with the novel H1N1 influenza virus A/CA/07/09 (10^6 PFU/ml) and observed for 2 weeks for clinical signs of infection. (C) Ferrets were infected with three different seasonal H1N1 strains at 3-month intervals. One set of three viruses represented seasonal H1N1 viruses from 1934 to 1957 (historical), and the second set represented viruses from 1991 to 2007 (modern). Ferrets were bled at the same time intervals following each infection as those described for individually infected ferrets. At day 252, ferrets were infected with novel H1N1 as described for individually infected ferrets.

pair housed in stainless steel cages (Shor-line, Kansas City, KS) containing Sani-Chips laboratory animal bedding (P. J. Murphy Forest Products, Montville, NJ). Ferrets were provided with Teklad Global Ferret Diet (Harlan Teklad, Madison, WI) and fresh water *ad libitum*. Ferrets ($n = 4$) were preinfected with seasonal H1N1 influenza viruses (1×10^6 PFU) intranasally at 12-week intervals (Fig. 1).

Ferrets were sequentially infected with seasonal H1N1 viruses (Fig. 1). Two sets of three viruses were used to infect ferrets at 3-month intervals. One group was infected sequentially with seasonal isolates from 1934, 1947, and 1957 and termed the historical sequential series. A second group of ferrets was infected with isolates from 1991, 1999, and 2007 and termed the modern sequential series. For comparison, additional ferrets were infected with each of the six viruses individually. Animals were monitored weekly during the infection regimen for adverse events, including weight loss, temperature, loss of activity, nasal discharge, sneezing, and diarrhea. Two groups of ferrets were infected with the same virus (1934 or 1947

virus) sequentially at 3-month intervals. Blood was harvested from all anesthetized ferrets via the anterior vena cava subclavian vein at days 14, 28, 56, and 84 after each infection. Serum was transferred to a centrifuge tube. Tubes were centrifuged, and serum was removed and frozen at $-20 \pm 5^\circ\text{C}$.

Four weeks after final preinfection, ferrets were challenged intranasally with 1×10^6 PFU of the novel 2009 H1N1 virus A/California/07/2009 in a volume of 0.5 ml in each nostril for a total infection volume of 1 ml. After infection, ferrets were monitored daily for weight loss, disease signs, and death for 14 days after infection. Individual body weights, sickness scores, and death were recorded for each group on each day after inoculation. Sickness scores were determined by evaluating activity (0 = normal, 1 = alert and active after stimulation, 2 = alert but not active after stimulation, 3 = neither active nor alert after stimulation), nasal discharge (0 = absent, 1 = present), sneezing (0 = absent, 1 = present), decreased food intake (0 = absent, 1 = present), diarrhea (0 = absent, 1 = present),

dyspnea (0 = absent, 1 = present), and neurological symptoms (0 = absent, 1 = present) as previously described (14). Experimental endpoints were defined as >20% weight loss, development of neurological disease, or an activity score of 3 (neither active nor alert after stimulation). Nasal washes were performed by instilling 3 ml of phosphate-buffered saline (PBS) into the nares of anesthetized ferrets each day for 14 days after inoculation. Washes were collected and stored at -80°C until use.

Respiratory droplet and contact transmission experiments were conducted as previously described (15, 16). Briefly, 24 h after inoculation, uninfected ferrets were housed either in adjacent transmission cages (respiratory droplet transmission) or in the same cage (direct-contact transmission). All procedures were in accordance with the NRC Guide for the Care and Use of Laboratory Animals, the Animal Welfare Act, and the CDC/NIH Biosafety in Microbiological and Biomedical Laboratories. An influenza-specific enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition (HAI), neutralization, and viral plaque assays were performed as described previously (13, 17–19). For more details, see the supplemental material.

ELISA. The ELISA was used to assess total antibody titer to the HA. High-binding, half-area 96-well polystyrene plates (Costar, Lowell, MA) were coated overnight at 4°C with 250 ng/well of recombinant HA from A/California/07/2009 (BEI Resources, NIH, Bethesda, MD) in carbonate/bicarbonate buffer (Pierce, Rockford, IL). Plates were washed with PBS-0.05% Tween 20 (PBS-T) buffer and blocked with 5% skimmed milk diluted in PBS for 1 h at 37°C . Serum samples were diluted in dilution buffer (1% skimmed milk in PBS) and added to plates. Serum was 3-fold serially diluted (starting from 1:300) and allowed to incubate for 2 h at 37°C . For experiments aimed to analyze high-avidity antibodies, urea wash was included for 5 min at 37°C as described elsewhere (20). Plates were washed with PBS-T, and biotinylated species-specific antibody against IgG (Rockland Immunochemicals, PA) was diluted in dilution buffer and added to plates. Plates were incubated for 1 h at 37°C , washed with PBS-T, and incubated with horseradish peroxidase (HRP)-linked streptavidin (Rockland Immunochemicals, PA) diluted in dilution buffer. Plates were washed with PBS-T, and HRP was developed with TMB substrate (Pierce, Rockford, IL). Plates were incubated in the dark for 20 min at room temperature, and then the reaction was stopped with 2N H_2SO_4 (20). Optical densities at a wavelength of 450 nm (OD_{450}) were read by a spectrophotometer (BioTek, Winooski, VT), and endpoint dilution titers were determined as the reciprocal dilution of the last well which had an OD_{450} above the mean OD_{450} plus two standard deviations of naive animal sera.

HAI assay. The HAI assay was used to assess functional antibodies to the HA able to inhibit agglutination of turkey erythrocytes. The protocol was adapted from the CDC laboratory-based influenza surveillance manual (21). To inactivate nonspecific inhibitors, sera were treated with receptor-destroying enzyme (RDE; Denka Seiken, Co., Japan) prior to being tested (18, 19, 22–24). Briefly, three parts RDE was added to one part serum and incubated overnight at 37°C . RDE was inactivated by incubation at 56°C for ~30 min with 6 times the serum volume, and 0.9% saline RDE-treated serum was 2-fold serially diluted in v-bottom microtiter plates. An equal volume of each virus, adjusted to approximately 8 hemagglutinating units (HAU)/50 μl , was added to each well. The plates were covered and incubated at room temperature for 20 min, followed by the addition of 1% turkey erythrocytes (TRBC) (Lampire Biologicals, Pipersville, PA) in PBS. Red blood cells were stored at 4°C and used within 72 h of preparation. The plates were mixed by agitation and covered, and the RBC were allowed to settle for 1 h at room temperature (25). The HAI titer was determined by the reciprocal dilution of the last well which contained nonagglutinated RBC. Positive and negative serum controls were included for each plate. All ferrets were negative (HAI titer $\leq 1:10$) for antibodies to currently circulating human influenza viruses prior to vaccination.

MN assay. Serum neutralizing antibody titers were determined by microneutralization (MN) assays performed on Madin-Darby canine

kidney (MDCK) cells by following the procedure described by Rowe et al. (26). Briefly, individual RDE-treated serum samples were serially diluted 2-fold (starting at a 1:10 dilution) in MDCK diluent buffer in a cell culture plate, followed by the addition of a predetermined amount (100 50% tissue culture infective doses [TCID_{50}]) of each H1N1 virus. Sera and viruses were mixed and incubated at 37°C for 60 min. MDCK cells were added, and the plates were incubated overnight at 37°C in a 5% CO_2 cell culture incubator. The presence of viral protein was detected by ELISA with a monoclonal antibody (A-3) to the influenza A nucleoprotein (NP). The neutralizing antibody titers are expressed as the reciprocal of the highest dilution of serum that gave 50% neutralization of 100 TCID_{50} of virus in MDCK cells. Positive serum control and negative cell controls with no serum were included on each plate. Geometric mean titers of neutralizing antibody were calculated for each group.

Surface plasmon resonance. To assess the binding properties of serum antibodies, surface plasmon resonance (SPR) technology was performed using a Biacore 3000 (GE/Biacore AB, Uppsala, Sweden). Protein A (Pierce, Rockford, IL) was immobilized to the surface of a CM5 sensor chip (GE/Biacore, Inc., Piscataway, NJ) using standard amine-coupling chemistry. The surface of the chip was activated using a 1:1 mixture of *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) (Biacore, Inc.). Protein A (75 $\mu\text{g}/\text{ml}$) was immobilized on experimental and reference (adjacent) flow cells at a high level of density (approximately 5,000 response units [RU]). Remaining active carboxyl groups were inactivated with an injection of ethanolamine. Pooled polyclonal IgG from vaccinated ferrets was diluted in HEPES buffer solution-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20; GE Healthcare/Biacore, Inc., Piscataway, NJ) and captured at approximately 300 RU. After capture of IgG, various concentrations (0.8 to 66 nM, series of 3-fold dilutions) of recombinant HA (rHA) representing novel and seasonal H1N1 strains (Protein Sciences, Meriden, CT) were passed sequentially over both flow cells. A blank (0 nM) injection was also included for double referencing.

Binding isotherms were then analyzed using BIAevaluation 4.1.1 software (Biacore AB). Polyclonal serum appeared to yield monospecific binding, and hence, a 1:1 Langmuir fit was utilized for kinetic determinations. However, since kinetic rates returned using these binding models for polyclonal serum represent only apparent rates of binding due to the multiple specificities inherent to a polyclonal response, we referred to the results as “relative association” and “relative dissociation.”

Plaque assay. For ferret infections, nasal wash virus titers were used to assess viral burden. Nasal wash virus titers were determined using a plaque assay (27, 28). Briefly, nasal washes from infected ferrets were harvested postinfection, snap-frozen, and stored at -80°C until use. Samples were thawed and diluted in an appropriate volume of Dulbecco’s modified Eagle medium (DMEM) supplemented with penicillin-streptomycin (iDMEM).

Madin-Darby canine kidney (MDCK) cells were plated (5×10^5) in each well of a 6-well plate. Samples (nasal washes) were diluted (dilution factors of 1×10^1 to 10^6) and overlaid onto the cells in 100 μl of iDMEM and incubated for 1 h. Virus-containing medium was removed and replaced with 2 ml of L15 medium plus 0.8% agarose (Cambrex, East Rutherford, NJ), and the new medium was incubated for 96 h at 37°C with 5% CO_2 . Agarose was removed and discarded. Cells were fixed with 10% buffered formalin and then stained with 1% crystal violet for 15 min. Following thorough washing in distilled water (dH_2O) to remove excess crystal violet, plates were allowed to dry, plaques were counted, and the numbers of PFU or PFU/ml for nasal washes were calculated.

Statistical analysis. Statistical significance of the antibody titers was determined using Student’s paired *t* test with a significance *P* value of <0.05 .

RESULTS

Ferrets were sequentially infected with seasonal H1N1 viruses (Fig. 1). One group was infected sequentially with seasonal isolates

FIG 2 Protection from novel H1N1 virus challenge. Ferrets were infected with novel H1N1 A/California/07/2009 and monitored for 2 weeks. (A and B) Ferrets were evaluated daily for weight loss. (C and D) Viral titers were determined from nasal washes collected at days 1, 2, 3, 5, 7, and 9 postinfection. Bars indicate mean virus titers ($\pm$ standard deviations).

from 1934, 1947, and 1957 (historical sequential group). A second group was infected with isolates from 1991, 1999, and 2007 (modern sequential group). For comparison, additional ferrets were infected with each of the six viruses individually (see Table S1 in the supplemental material). To assess protection, all animals were then infected with novel H1N1 virus. In addition, the collected serum harvested after each infection was characterized to determine the antibody response.

Sequential preinfection with seasonal H1N1 viruses protects ferrets from novel H1N1. To determine the protective efficacy of preimmune H1N1 ferrets, all animals were challenged with 1×10^6 PFU/ml of the novel H1N1 influenza virus A/California/07/2009 (Fig. 1). Naïve ferrets challenged with novel H1N1 influenza had a rapid drop in weight, losing $\sim 15\%$ of their body weight by day 7 postinfection, that was maintained for the 14 days of observation. These ferrets showed signs of morbidity, including lethargy, sneezing, and nasal discharge, as previously described for novel H1N1 infection (29). Naïve ferrets that were not challenged with influenza virus remained healthy and gained weight throughout the study. Ferrets infected with one of three historical strains of seasonal H1N1 influenza had various degrees of morbidity and weight loss (Fig. 2A). Ferrets preinfected with 1957 virus and then challenged 3 months later with novel H1N1 influenza had a pattern of weight loss and morbidity similar to that of uninfected ferrets challenged with novel H1N1 influenza. In contrast, ferrets preinfected with either the 1934 or 1947 influenza virus lost $\sim 5\%$ of their body weight over the 14-day period and had only mild symptoms of disease. Weight loss correlated with the amount of virus recovered from nasal washes (Fig. 2B). Naïve animals or ferrets preimmune to the 1934 or 1957 virus had a peak viral titer between 1×10^5 and 1×10^8 PFU/ml at day 2 postinfection that corresponded with how rapidly virus returned to undetectable

levels postinfection. In contrast, ferrets preimmune to the 1947 virus had low viral titer at day 1 postinfection in 1 out of 4 ferrets (Fig. 2B). Ferrets sequentially infected with the three historical H1N1 influenza viruses had no signs of disease and gained weight following challenge with the novel H1N1 influenza virus. In contrast, ferrets which were preinfected with a single seasonal H1N1 virus (Fig. 2A) had no detectable virus at any point postinfection (Fig. 2B).

Preinfection with one of the three modern seasonal H1N1 influenza viruses did not protect ferrets from morbidity or weight loss following challenge with novel H1N1 influenza (Fig. 2C). In contrast, ferrets sequentially infected with three modern seasonal H1N1 influenza viruses were protected against disease and gained weight over the 14 days of observation. High viral titers were detected in the nasal washes in all ferrets except ferrets preinfected sequentially with the modern H1N1 influenza viruses (Fig. 2D).

Sequential preinfection with seasonal H1N1 viruses prevents transmission of novel H1N1. In order to determine if preinfection with seasonal H1N1 viruses elicits immune responses that reduce or prevent transmission of novel H1N1 influenza following challenge, preimmune ferrets were cohoused with a naïve ferret during the novel H1N1 challenge. Ferrets sequentially infected with historical or modern seasonal H1N1 viruses did not transmit novel H1N1 to naïve ferrets (Fig. 3). Naïve ferrets that were challenged with novel H1N1 easily transmitted virus to the cohoused ferret. The only ferrets preinfected with a single seasonal H1N1 influenza virus that did not transmit virus to a naïve ferret were those preinfected with the 1947 virus (Fig. 3C), whereas all the other ferrets infected with a single seasonal H1N1 virus transmitted virus to the cohoused partners, as indicated by the high virus titers isolated in the nasal wash.

FIG 3 Prevention of transmission of novel H1N1 virus to cohoused naïve ferrets. Ferrets were infected with novel H1N1 A/California/07/2009. Each directly infected ferret was cohoused with an immunologically influenza-naïve ferret and monitored for 2 weeks. (A and B) Ferrets were evaluated daily for weight loss. (C and D) Viral titers were determined from nasal washes collected at days 1, 2, 3, 5, 7, and 9 postinfection. Bars indicate mean virus titers ($\pm$ standard deviations).

Ferrets sequentially infected with seasonal H1N1 viruses develop antibodies to novel H1N1 HA. To determine whether the H1N1 infections elicited antibodies that bind to novel 2009 H1N1 virus, sera were analyzed by ELISA using the A/California/07/2009 hemagglutinin. Ferrets infected with A/FM/1/1947 produced antibodies with high titer that bound to the novel H1N1 HA with high avidity (Table 1; see also Tables S2 to S5 in the supplemental material). These antibodies bound to the novel H1N1 HA slightly less efficiently than antibodies produced by ferrets infected with the homologous A/CA/07/09 novel H1N1 strain. The addition of urea, an agent that disrupts hydrogen bonds and, consequently, enables the distinction of low- and high-avidity antibodies, did not substantially diminish binding of these antibodies to novel H1N1 HA. In contrast, ferrets infected with any of the other 4 H1N1 viruses had antibodies with significantly lower titers ($P < 0.01$) than those in ferrets infected with the novel H1N1 HA, with endpoint dilution titers 1 to 1.5 logs lower than those in sera from A/CA/07/09-infected ferrets.

Sequential infections elicited increasing antibody reactivity toward novel H1N1. Ferrets infected with the first historical virus,

A/PR/8/1934, had low titers to the novel H1N1 HA, but these titers dramatically rose after the second virus infection and remained high after the third virus infection (Table 2). Similarly, ferrets infected sequentially with the modern set of seasonal H1N1 viruses that bound to the novel H1N1 HA had low titers following the first infection, but titers rose after each subsequent infection (Table 2).

To evaluate the relative binding kinetic profile of the antibody elicited by each infection, the sera were evaluated via surface plasmon resonance using recombinant HA protein representing the novel and seasonal H1N1 isolates as detailed in the supplemental material (Fig. 4). Serum samples were diluted, polyclonal IgG was captured, and binding experiments were carried out as previously described with minor modifications (30–32). Sensograms demonstrating the specifics of the binding response for the ferret sera are shown in Fig. S1 in the supplemental material. Sera from ferrets infected individually with the novel H1N1 influenza strain A/CA/07/09 had an apparent single population of antibody that bound specifically to the novel H1N1 HA at a relative association rate (k_a) of 8.00×10^5 . An approximately 1- to 2- $\log_{10}$ difference in relative dissociation rates (k_d) was observed between sera collected from seasonal-influenza-infected ferrets and sera from ferrets infected with novel H1N1 influenza. However, the association rate of the same antiserum showed that it was able to bind to its respective homologous rHA with high affinity, thereby indicating

TABLE 1 Antibodies elicited by infection

Virus	Endpoint dilution titer	Endpoint dilution titer with urea
A/PR/8/1934	24,300 ^a	24,300 ^b
A/FM/1/1947	72,900	72,900
A/Den/1/1957	2,700	300
A/TX/36/1991	2,700	900
A/NC/20/1999	2,700	900
A/Bris/59/2007	2,700	900
A/CA/07/2009	72,900	24,300

^a Average endpoint dilution titer of anti-rHA, A/CA/07/2009, from sera collected 3 months after each infection.

^b Average endpoint dilution titer against rHA in an HA-specific urea displacement ELISA.

TABLE 2 Antibodies elicited by sequential infection

Virus set	Endpoint dilution titer after:		
	1st infection	2nd infection	3rd infection
Historical	8,100 ^a	72,900	72,900
Modern	8,100	24,300	72,900

^a Average endpoint dilution titer of anti-rHA, A/CA/07/2009, from sera collected 3 months after each infection.

FIG 4 Kinetics of ferret-elicited antisera following seasonal H1N1 infection(s) binding to rHA antigens. Kinetics of antisera binding to homologous rHA or heterologous rHA proteins was determined. Antisera from ferrets infected with seasonal H1N1 influenza viruses individually (day 84) or sequentially (day 168 and day 252). Values on the x axis represent the apparent association rates of antibody binding to HA, and the values on the y axis represent the apparent disassociation rates of antibody from the HA antigen.

that the antiserum that was capable of high-affinity binding to its homologous protein was not able to bind to the heterologous novel H1N1 HA. Sera collected from ferrets infected with a single modern H1N1 strain had a relative dissociation rate of 1.00×10^3 , and sera from ferrets infected with historical strains had a dissociation rate of 1.03×10^2 to 1.00×10^3 . Similar results were observed in sera collected from ferrets after each sequential infection.

HAI responses. To determine the ability of the anti-HA antisera to block HA receptor binding, sera collected at days 14 and 84 after the final preinfection were tested in an HAI assay. As expected, all ferrets infected with a single seasonal H1N1 virus had HAI activity against that virus (Fig. 5). In some cases, the elicited antibodies also had HAI activity against other seasonal H1N1 isolates tested. For example, ferrets infected with A/Texas/36/1991 exhibited HAI activity against not only the 1991 virus but also the 1999 virus (Fig. 5E). However, only ferrets infected with the novel H1N1 virus had HAI activity against A/CA/07/09 (Fig. 5G); in no case did infection with a single seasonal H1N1 virus produce detectable HAI activity against the novel 2009 H1N1.

Interestingly, HAI activity against novel H1N1 was detected in ferrets infected sequentially with the first two historical seasonal H1N1 viruses (Fig. 6A). A/PR/8/34 influenza-infected ferrets had high HAI titers to the 1934 virus (green line) 14 days after infection (average HAI titer, 1:17,920). These titers declined over the next 3 months but remained high. As observed previously, the elicited antisera did not have HAI activity against any of the other H1N1 influenza viruses, including the novel 2009 virus. Following infection of these same ferrets with A/FM/1/1947, at day 98 (14 days postinfection), these ferrets had HAI activity against the 1947 virus (green line), had boosted the HAI titer against the 1934 virus, and, notably, had HAI activity against the novel H1N1 2009 virus (red line). Once again, 3 months after this second preinfection (day 168), these ferrets were infected with A/Denver/1/1957 and HAI activity was detected at day 182 against the 1957 virus and declined by day 252. HAI titers were not boosted but were maintained against the 1934, 1947, and novel 2009 H1N1 viruses through day 252.

Sequential infection with the modern viruses also elicited detectable activity toward novel H1N1. Fourteen days after the ini-

tial infection with A/Texas/36/1991, all ferrets had high HAI activity against the 1991 virus and also against the 1999 virus (Fig. 6 and Table 1). Three months later, at day 84, these ferrets were infected with the A/New Caledonia/20/1999 influenza virus. HAI activity was boosted against the 1991 (black line) and 1999 (green line) viruses, but no HAI activity was detected against A/Brisbane/59/2007 (blue line). One of the 4 ferrets had a detectable HAI titer (1:40) against the novel 2009 H1N1 influenza virus (red line). Three months after the second preinfection (day 168), these same ferrets were infected again with a third seasonal H1N1 influenza virus, A/Brisbane/59/2007. While HAI activity was boosted against the 1999 virus, there were lower titers against the 2007 virus which were similar to the HAI titers against the novel H1N1 virus (Fig. 6).

As a control, ferrets were infected and then boosted with the same seasonal H1N1 virus. Ferrets infected twice with the 1934 virus and then challenged 3 months later with novel H1N1 influenza virus lost ~10% of their body weight and transmitted virus to contact ferrets. These results were similar to those for ferrets prechallenged with a single 1934 virus infection and infected 3 months later by novel H1N1 influenza virus. Ferrets infected twice with the 1947 virus had weight loss similar to that of ferrets infected with a single 1947 virus infection followed by novel H1N1 influenza virus challenge. In both cases, there were no cross-reactive HAI titers to novel H1N1 influenza virus. In contrast, ferrets infected with the 1934 virus and then infected 3 months later with the 1947 virus had an average HAI titer 14 days postinfection of 1:640. Additional HAI titer data are listed in Table S5 in the supplemental material. Therefore, only when heterologous sequential infection was administered to the ferrets did cross-reactive antibodies to the novel H1N1 influenza virus develop.

mVN titers. Sera were also analyzed with microneutralization (mVN) assays, which can detect some anti-influenza antibodies that do not display HAI activity (Fig. 7). Ferrets infected with any of the seasonal H1N1 viruses had low or no detectable microneutralization titers to the novel H1N1 influenza virus (Fig. 7A). In contrast, ferrets infected with A/CA/07/2009 had high mVN titers at day 14 postchallenge. Interestingly, ferrets infected sequentially with either the historical or modern set of viruses had increasing mVN titers to novel H1N1 after each successive seasonal H1N1 preinfection (Fig. 7B). In fact, ferrets infected with the three historical H1N1 viruses sequentially had mVN titers statistically similar to those of ferrets infected with novel H1N1 only.

The mixture of sera from ferrets individually infected with seasonal H1N1 recognizes novel H1N1. To determine whether mixing sera from ferrets infected individually with a single H1N1 virus can mimic the results observed from sequentially infected ferrets, antisera from ferrets infected with the three historical or three modern H1N1 viruses were mixed at equal ratios with similar HAI titers (within 2-fold) and tested in HAI and mVN assays. Sera mixed from ferrets infected individually with modern H1N1 influenza viruses had HAI activity against all three modern strains but not against the historical strains or against novel H1N1 (Fig. 8A). In contrast, sera mixed from ferrets infected individually with historical H1N1 influenza viruses had HAI activity not only against all three historical strains but also against novel H1N1. Similar results were observed with these same mixed sera in mVN assays (Fig. 8B). When sera from ferrets infected with the 1934 virus were mixed with sera from ferrets infected with the 1957 virus, the HAI titers to novel H1N1 were low but were still detect-

FIG 5 Hemagglutination inhibition (HAI) serum antibody titers induced by single H1N1 infection of ferrets. HAI titers were determined for each group of seasonal H1N1-infected ferrets against a panel of H1N1 influenza viruses. (A) Bars indicate the log₂-transformed geometric mean titers ($\pm$ the standard errors of the mean [SEM]) from antisera collected at day 14 or day 84.

able (Fig. 8C). However, when 1934 virus-induced antiserum was mixed with 2007 virus-induced sera, there was no HAI activity against novel H1N1 influenza.

DISCUSSION

The presence in older people of preexisting humoral immunity to the novel 2009 H1N1 virus presents a puzzle. The exposure to 1918-like viruses many years ago may be responsible for this immunity (33, 34). Some previous studies indeed found evidence for great antigenic similarity between the 1918 virus and the pandemic virus (19, 22, 26). However, preexisting immunity was not limited to those old enough to have been infected with the 1918 virus or similar viruses of that era. Rather, people born much later, when the circulating H1N1 strains were antigenically distinct from the 1918 virus due to decades of antigenic drift, also had

preexisting immunity to novel H1N1 (6). Furthermore, the reactivities of human sera toward the 1918 virus and the 2009 virus are not very strongly correlated (17), suggesting a significant antigenic difference between these two viruses.

These considerations led us to hypothesize that a sequence of exposures to diverse antigenic variants would result in a more broadly protective antibody response. This effect would explain the presence of preexisting antibodies to the 2009 virus in middle-aged individuals, who would have been infected with several H1N1 variants over the course of their lifetimes. To test this hypothesis, we infected ferrets with either a single seasonal H1N1 variant or a sequence of three such variants. The results support a role for sequential infections in producing an antibody response that protects against the 2009 virus.

Of the six seasonal strains used in this study, five did not

FIG 6 Hemagglutination inhibition (HAI) serum antibody titers induced by sequential seasonal H1N1 infection of ferrets. HAI serum antibody titers were determined in ferrets infected sequentially with seasonal H1N1 influenza viruses. Antisera were tested against three seasonal H1N1 influenza viruses as well as the novel H1N1 isolate. (A) Each line represents the $\log_2$ -transformed geometric mean titer ($\pm$ SEM) from antisera collected at various time points postinfection against one of the H1N1 influenza viruses.

individually elicit strong protection against the 2009 virus. Serology confirmed a lack of cross-reactive antibodies. This result is not surprising because these seasonal viruses are not closely related to the 2009 virus. The exception was FM/1/1947. Infection with this virus alone provided some protection against the 2009 virus. Furthermore, antibodies elicited following FM/1/1947 virus infection cross-reacted with the novel H1N1 influenza virus. This is perhaps surprising because of the distance between FM/1/1947 and the 1918 virus. It is noteworthy that the sera exhibited no detectable hemagglutination inhibition activity toward the 2009 virus; this suggests that the mechanism of protection did not involve blocking of receptor binding and contrasts with the results for sequential infection with historical viruses (discussed below). It may be relevant that the FM/1/1947 virus used in this study has two fewer glycosylation sites on its hemagglutinin than modern viruses (35).

Despite the fact that none of the modern viruses elicited strong protection against the 2009 virus, a sequence of infections with these three viruses led to almost complete protection. Analysis of sera indicated antibodies that cross-reacted with the 2009 virus by

several measures, including HAI. Strong cross-reactivity appeared only after the third infection in the sequence.

Sequential infection with the three historical viruses also elicited strong protection against the 2009 virus, again accompanied by strong antibody cross-reactivity. In this case, the strong cross-reactivity was apparent after the second infection: sera from ferrets infected with PR/8/1934 and then FM/1/1947 reacted strongly with CA/07/2009. This cross-reactivity extended to HAI, indicating that it was not a simple consequence of FM/1/1947 infection, which by itself did not produce HAI activity against the novel 2009 H1N1 influenza virus. Furthermore, experiments with mixtures of sera from ferrets infected with a single H1N1 virus (discussed below) suggest that infection with PR/8/1934 followed by Den/1/1957, with no exposure to FM/1/1947, would also yield cross-reactivity toward the 2009 virus. In contrast, a sequence of two infections with PR/8/1934, or two infections with FM/1/1947, did not lead to a stronger cross-reactive antibody response toward the 2009 virus than that elicited by a single infection with the same virus. A second infection with identical virus also failed to increase

FIG 7 Virus-neutralizing (mVN) antibody titers. Titers were measured using novel H1N1 influenza viruses. (A) Antisera collected at day 84 from ferrets infected individually with a single H1N1 virus. (B) Antisera collected at day 252 from ferrets infected sequentially with three seasonal H1N1 influenza viruses. Bars indicate the $\log_2$ -transformed geometric mean titers ($\pm$ SEM). A P value of less than 0.05 was considered significant (*, $P < 0.05$).

FIG 8 HAI and mVN titers following mixing of antisera collected from individually infected ferrets. Equal volumes of antisera (with equal titers) from ferrets infected with one of three seasonal H1N1 viruses were mixed together and tested against novel H1N1 influenza virus. (A) One mixture included antisera elicited in ferrets to one of the three historical seasonal H1N1 influenza strains (historical mixed sera). The second mixture included antisera from ferrets infected with one of the three modern H1N1 viruses (modern mixed sera). Both mixtures were tested against seasonal and novel H1N1 viruses. (B) Each mixture of antisera was tested against novel H1N1 influenza virus in an mVN assay. Antiserum collected from ferrets infected with the novel H1N1 isolate A/CA/07/09 was used as a positive control. (C) Mixtures included only two antisera (PR/34-Den/57 or PR/34-Bris/07). Each mixture was tested against the panel of H1N1 influenza viruses in an HAI assay. Bars indicate the log₂-transformed geometric mean titers ($\pm$ SEM).

protection against the 2009 virus. These results confirm the importance of exposure to antigenically different variants.

Two classes of phenomena may contribute to the strong protection against novel H1N1 induced by sequential infection with antigenically diverse viruses. First, due to recall of immunological memory, sequential exposure may lead to production of antibodies that would not be produced in response to any single infection. These may be individually more broadly protective than antibodies elicited by single exposures. Second, even if the response to sequential infection were simply the sum of the responses to the individual viruses, synergy between antibodies might lead to greater-than-additive protection. Experiments with mixed sera (Fig. 6) demonstrate synergy between antibodies elicited by different viruses. Mixtures of sera from individual historical viruses exhibit strong HAI activity toward novel H1N1 (Fig. 8A and C) despite the fact that the individual sera have no detectable HAI activity (Fig. 5). Two mechanisms might contribute to this phenomenon. Binding of antibodies found in different sera might be cooperative. Cooperativity of binding between different anti-HA antibodies has been observed (36, 37). In addition, the effects of bound antibodies on hemagglutination, and on protection *in vivo*, might be synergistic. It is plausible, for example, that antibody molecules bound at distinct sites together form an effective steric barrier to receptor binding whereas either alone would allow receptor binding in some orientations.

Whatever mechanism(s) underlies it, synergy between antibodies cannot be responsible for all of the observed effects of sequential infection. Although modern sequential infection elicited strong protection against, and antibody reactivity toward, novel H1N1, no synergy was detected for the modern viruses in the mixing experiments. Furthermore, even for the historical viruses, little synergy was apparent in the microneutralization assays whereas sequential infection led to high microneutralization titers. Therefore, much of the observed effect appears to involve the production of individual antibodies with broadened specificity, presumably due to recall of immunological memory.

Broadly neutralizing antibodies that recognize the stalk region

of hemagglutinin have been observed (12, 38, 39). These stalk antibodies are boosted in mice by sequential infection with different H1N1 viruses (9). Although antibodies of this type may have contributed to the protection that we observed, our results suggest that other types of broadly neutralizing antibodies were involved. The stalk-recognizing antibodies that have been observed do not exhibit hemagglutination inhibition, whereas the antisera produced in our experiments with sequential infection had significant hemagglutination inhibition activity toward the 2009 virus. Sequential exposure can apparently elicit other types of broadly neutralizing antibodies.

Interactions between responses to sequential exposure are likely complex. Such interactions can help to explain some puzzling epidemiological and serological observations about the novel H1N1 virus. In addition, they point to the possibility of achieving broad vaccine-induced protection against influenza viruses—and other pathogens—by sequential immunization with a series of antigenic variants.

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