Multiple Infections with Seasonal Influenza A Virus Induce Cross-Protective Immunity against A(H1N1) Pandemic Influenza Virus in a Ferret Model

Karen L. Laurie, Louise A. Carolan, Deborah Middleton, Sue Lowther, Anne Kelso, and Ian G. Barr

Background. An age bias toward children and young adults has been reported for infection and hospitalizations with pandemic H1N1 influenza (A[H1N1]pdm) in the 2009 and 2010 influenza seasons in the Southern and Northern Hemispheres. Serological analysis of prepandemic samples has shown a higher incidence of cross-reactive antibodies to A(H1N1)pdm virus in older populations; conserved T cell epitopes between viruses have been identified. The contribution of preexisting immunity to seasonal influenza to protection against A(H1N1)pdm infection was analyzed in a ferret model.

Methods. Ferrets were pre-infected with influenza A viruses and/or vaccinated with inactivated influenza viruses with adjuvant. Infection after challenge was assessed by measuring shedding virus, transmission to naive animals, and seroconversion.

Results. Homologous vaccination reduced the incidence of infection and delayed transmission. Pre-infection with virus induced sterilizing immunity to homologous challenge. One prior infection with seasonal influenza A virus improved clearance of A(H1N1)pdm virus. Prior infection with A(H1N1)pdm virus reduced shedding after seasonal influenza A challenge. Two infections with seasonal influenza A viruses reduced the incidence of infection, the amount and duration of virus shedding, and the frequency of transmission following A(H1N1)pdm challenge.

Conclusion. These data suggest the reduced incidence and severity of infection with A(H1N1)pdm virus in the adult population during the 2009–2010 influenza season may be a result of previous exposure to seasonal influenza A viruses.

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Potential conflicts of interest: The World Health Organization Collaborating Centre for Reference and Research on Influenza has collaborative agreements with vaccine companies unrelated to this study.


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The first influenza pandemic of the 21st century began in March 2009, and infection with the swine-origin virus (A[H1N1]pdm) rapidly spread worldwide [1]. The burden of disease, as assessed from serosurveys and virological studies, has been largely in children and young adults, with up to 50% of that population showing evidence of infection [2], compared with 10% of the adult population [2, 3]. Severe disease and hospitalization have also been associated with younger age groups [4, 5].

Traditionally, seroconversion to influenza virus infection has been measured by virus neutralization (VN) [6] and hemagglutination inhibition (HI) [7] assays, with the latter correlated with protection from artificial challenge [8, 9]. Serological analyses of prepandemic serum samples has demonstrated that older adults, particularly the very elderly population, had substantial levels of cross-reactive antibodies to A(H1N1)pdm virus, compared with younger adults and children in some [2, 10, 11], but not all, countries [12–14]. Serologic analysis has demonstrated little cross-reactivity between recent seasonal influenza A(H1N1) viruses and A(H1N1)pdm virus [10, 13]. Thus, cross-reactivity to A(H1N1)pdm may be a result of multiple exposures to older viruses with similar B cell epitopes [15]. Furthermore, because there is considerable conservation of T cell epitopes between seasonal influenza A and A(H1N1)pdm viruses [16], cellular immunity may also...
reduce infection and disease severity. The excellent response in adults following a single dose of inactivated A(H1N1)pdm monovalent unadjuvanted vaccine suggests that adults have been primed, whereas children’s responses have been lower, indicating no exposure or lower levels of exposure [14, 17, 18]. These data suggest that past exposure to seasonal influenza virus may protect against A(H1N1)pdm infection.

Guinea pigs, ferrets, and macaques are readily infected with human seasonal influenza viruses, and numerous studies have shown that A(H1N1)pdm viruses also directly infect these species [11, 19–22]. Pathogenicity is similar to seasonal influenza virus infection, with mild disease severity in the ferret model; however, an increased lung pathology was noted, with replication in the lower and upper respiratory tract with some A(H1N1)pdm viruses [11, 19, 20, 22]. Importantly, these disease characteristics are similar to human clinical findings, which indicates that animal models may be useful to analyze human immunity to this new virus.

We investigated the contributions of variable and conserved immune responses to seasonal influenza A viruses to protection against challenge and subsequent transmission of A(H1N1)pdm virus in the ferret model. The effect of immunity resulting from exposure to A(H1N1)pdm virus on protection from challenge with seasonal influenza A viruses was also investigated. One infection with influenza A(H1N1) or A(H3N2) virus increased clearance after heterologous virus challenge. Importantly, multiple infections with seasonal influenza A viruses could largely protect ferrets from challenge with A(H1N1)pdm, suggesting a mechanism for the lower infection rate and mild disease severity observed in the majority of the adult human population during the 2009–2010 influenza season.

MATERIALS AND METHODS

Ferrets. Male and female ferrets (weight, 500–1500 g) were purchased from independent breeders and housed at CSL, Limited using services provided under a Support Services Agreement with the Victorian Infectious Diseases Reference Laboratory. Serum samples from ferrets were tested by HI assay to ensure seronegativity to current seasonal A(H1N1), A(H3N2), and A(H1N1)pdm influenza strains before use. Experiments were conducted with approval from the CSL Limited/Pfizer Australia and Australian Commonwealth Scientific and Research Organization/Australian Animal Health Laboratory Animal Ethics Committee.

Viruses. Viruses were passaged in embryonated hen’s eggs...
and stored at −80°C. Ferrets were infected with A/Panama/2007/1999 (H3N2), A/Fukushima/141/2006 (H1N1), A/Auckland/1/2009, or A/California/7/2009 ((H1N1)pdm). These and A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2), and ether-split [23] B/Brisbane/3/2007 (a B/Florida/4/2006-like virus) viruses were used in HI assays. An A/California/7/2009 reassortant virus (NYMC-X179A) was purified by sucrose gradient, concentrated, and inactivated with β-propiolactone to create an influenza zonal pool preparation (a gift from CSL Limited) (A/Cal/7/09) for use in HI assays and immunization.

### Vaccine formulation.
Full adult doses of human trivalent influenza vaccine 2008/2009 (Northern Hemisphere) (TIV) (a gift from CSL Limited) were used for immunization. Vaccine contained 15 μg HA of each strain: A/Brisbane/59/2007-like virus, A/Brisbane/10/2007-like virus (A/Uruguay/716/2007), and B/Florida/4/2006-like virus in 0.5 mL. Forty-five micrograms total protein of A/Cal/7/2009, as determined by BCA protein assay (Pierce, Thermo Fisher Scientific), was given per vaccine dose.

### Vaccination and viral challenge of ferrets.
Vaccines were delivered to sedated animals (30 mg/kg Ilium Xylazil-20; Troy Laboratories) intramuscularly in the quadriceps muscles of both hind legs using a 1-mL syringe with a 22-gauge needle and 1 mL per dose, administered 2 weeks apart. Ferrets were anaesthetized (0.2 mL/kg, 1:1 [v/v] Ilium Xylazil-20:Ketamine or ketamine-medetidine anaesthesia [1:1, 1 mL/kg], reversed with atipemazole) and challenged by delivery dropwise into 1 nostril of 10^−3 50% tissue culture infectious doses (TCID_{50}) seasonal A(H1N1) or A(H3N2) virus or 5 × 10^5 TCID_{50} A(H1N1)pdm virus in 0.5 mL. Groups contained 4 directly vaccinated and/or infected ferrets and 2 naive control transmission ferrets unless otherwise indicated.

### Housing, monitoring, and sample collection.
After challenge, 2 ferrets (from the same experimental group) were housed in a high-efficiency particulate air–filtered individual isolation unit. For transmission studies, 2 days after challenge, vaccinated and/or infected ferrets and 2 naive contact transmission ferrets were either euthanized or tested for virus shedding by rapid test analysis of nasal wash specimens (BD Directigen EZ Flu A+B kit; Becton Dickinson). Ferrets deemed noninfectious (ie, those with no virus detected) were regrouped by previous virus infection(s) until challenge.

Animals were visually inspected daily. Body weight was measured at vaccination, on the day of challenge, and daily for 2 weeks after challenge. Temperatures were measured at least daily after challenge using implanted temperature transponders fitted to identification chips (LifeChip Bio-Thermo; Digivet).

Blood samples were collected before and after each vaccination and viral challenge. Nasal washes were collected under sedation on days 1, 3, 5, 7, and 9 after challenge and frozen (−80°C) until analysis.

### TCID_{50} assay.
Viral titers were quantified by TCID_{50} assay, as described elsewhere [24]. Virus was detected by either the addition of 25 μL 1% turkey red blood cells (RBCs; wells containing fully hemagglutinated RBCs were scored positive) or

<table>
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<tr>
<th>Challenge virus, group</th>
<th>Vaccine</th>
<th>Direct challenge ferrets</th>
<th>Contact ferrets</th>
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<td></td>
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<td>A(H1N1)^a</td>
<td>A(H1N1)pdm^a</td>
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<td>Seasonal A(H1N1)</td>
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<tr>
<td>A</td>
<td>None</td>
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<td>B</td>
<td>PBS plus IFA</td>
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<td>C</td>
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<td>145 (6/7)</td>
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<tr>
<td>D</td>
<td>A/Cal/7/09 plus IFA</td>
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<td>640 (3/3)</td>
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<td>A(H1N1)pdm</td>
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<td>None</td>
<td>&lt;10</td>
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<td>34 (3/4)</td>
<td>&lt;10</td>
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<tr>
<td>H</td>
<td>A/Cal/7/09 plus IFA</td>
<td>&lt;10</td>
<td>1076 (4/4)</td>
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### NOTE.
Data are hemagglutination inhibition (HI) geometric mean titers (no. of ferrets with HI titer/no. of ferrets challenged), unless otherwise indicated. Geometric mean titer was calculated using the value of 5 when titer was below the lower limit of detection (<10).

- ^a Serum samples were collected 1 week after primary vaccination.
- ^b Serum samples were collected 1 week after secondary immunization.
- ^c Serum samples were collected 2 weeks after challenge (terminal).
- ^d HI assays were performed against A/Fukushima/141/2006.
- ^e HI assays were performed against A/California/7/2009.

**Table 1. Serological Responses after Each Immunization and Challenge with Seasonal A(H1N1) or A(H1N1)pdm Virus**
Figure 2. Vaccination with inactivated influenza virus with adjuvant protects against homologous but not heterologous challenge. Ferrets (n = 3–6) were vaccinated as indicated, then challenged with 10^3.5 50% tissue culture infectious doses (TCID_{50}) seasonal A(H1N1) (A–D) or 5 x 10^6 TCID_{50} A(H1N1)pdm (E–H) virus. Virus titer (T) or number of infected animals (N) compared with phosphate-buffered saline (PBS) plus Freund's Incomplete Adjuvant (IFA) groups, *P<.05 and **P<.01. TIV, human trivalent influenza vaccine 2008/2009 (Northern Hemisphere).

by full cytopathic effects. Titers were calculated as described by Reed and Muench [23]. Detection limit was 0.5 TCID_{50} per 100 μL.

HI and VN assays. Reactivity of serum samples was measured using HI [7] and VN assays [6]. HI assays were performed as described elsewhere [3]. For VN assays, serum samples were inactivated at 56°C for 30 min. Heat-treated serum (2-fold dilutions from 1:10 to 1:1280) and 200 TCID_{50} virus (1:1, v/v) were incubated at 35°C for 1 h and then added to Madin-Darby canine kidney cells monolayers in 96-well flat-bottomed plates as for the TCID_{50} assay. Titers were expressed as the reciprocal of the highest dilution of serum for which hemagglutination was prevented.

Genetic sequencing. Sequencing of viruses was described elsewhere [24]. Primer sequences are available upon request. GenBank accession numbers were as follows: A/Fukushima/141/2006, CY056353–57; A/Panama/2007/1999, CY056358–62; and A/Auckland/1/2009, GQ258464–67 and FJ973556.

Statistics. To compare TCID_{50} titers between groups, Mann-Whitney U test (2-tailed) was used. To compare the number of animals shedding virus, Fisher’s exact test was used. P<.05 was considered to be statistically significant.

RESULTS

High levels of antibodies protect against direct challenge with homologous virus and reduce transmission events. Protection from challenge with seasonal and pandemic influenza A virus
after vaccination was examined according to the protocol in Figure 1A. Ferrets were immunized twice with the full adult dose of TIV or an equivalent dose of A/Cal/7/09 in adjuvant (IFA). Infection was measured by (1) the amount and duration of virus shedding in nasal washes of directly challenged and immunized ferrets and (2) the ability of these animals to transmit virus to naive contact ferrets. Seroconversion after infection was also determined.

Nine of 11 ferrets responded serologically after 1 vaccination with TIV plus IFA, and all ferrets responded after 2 vaccinations. All ferrets (n = 7) responded after vaccination with A/Cal/7/09 plus IFA (Table 1). Immunization of naive ferrets requires adjuvant ([25–28] and data not shown) or priming via prior infection [29] for consistent seroconversion to split seasonal or prepandemic influenza vaccines. No cross-reactive antibodies were detected to seasonal A(H1N1) or A(H1N1)pdm after vaccination by HI assay (Table 1) or VN assay (data not shown). Vaccination with adjuvant alone (phosphate-buffered saline [PBS] plus IFA) did not induce specific antibodies to influenza viruses as measured by HI assay (Table 1) or VN assay (data not shown). Vaccination with PBS plus IFA enhanced virus replication on day 3 (P < .05) but did not affect the incidence of infection, transmission, or seroconversion (Figure 2B and Table 1). Vaccination with TIV plus IFA reduced the incidence of infection, transmission, or seroconversion (Figure 2A).

Minimal clinical signs were detected after challenge of naive ferrets with a seasonal A/Brisbane/59/2007-like A(H1N1) virus (A/Fukushima/141/2006). Two of 4 ferrets had fever (mean change in temperature ± standard deviation [SD], +0.7 °C ± 0.7 °C). Virus was shed for 5 days and transmitted to naive contact recipients (Figure 2A). All challenged animals experienced seroconversion (Table 1). Vaccination with PBS plus IFA enhanced virus replication on day 3 (P < .05) but did not affect the incidence of infection, transmission, or seroconversion (Figure 2B and Table 1). Vaccination with TIV plus IFA reduced

| Table 2. Serological Responses to Pre-Infection and Vaccine before and after Challenge with Seasonal Influenza A or A(H1N1)pdm Virus |
|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Pre-infection strain/challenge strain | A(H1N1) | A(H3N2) | B | A(H1N1)pdm | A(H1N1) | A(H3N2) | B | A(H1N1)pdm |
| Direct challenge ferrets | Before challenge | After challenge | Contact ferrets |
| Pre-infected | <10 | <10 | <10 | 1076 (4/4) | <10 | <10 | <10 | 761 (4/4) | <10 |
| Naive | <10 | <10 | <10 | 1076 (4/4) | <10 | <10 | <10 | 5120 (4/4) | 7241 (2/2) |
| A(H1N1)/A(H3N2) | Pre-infected | 202 (4/4) | <10 | <10 | <10 | 260 (4/4) | <10 | <10 | 905 (2/2) |
| Naive | <10 | <10 | <10 | 1076 (4/4) | <10 | <10 | <10 | 5120 (4/4) | 7241 (2/2) |
| A(H3N2)/A(H1N1)pdm | Pre-infected | <10 | 190 (4/4) | <10 | <10 | <10 | <10 | 3044 (4/4) | 7241 (2/2) |
| Naive | <10 | <10 | <10 | 1076 (4/4) | <10 | <10 | <10 | 3044 (4/4) | 7241 (2/2) |
| A(H1N1)/A(H1N1)pdm | Pre-infected | 127 (3/3) | <10 | <10 | <10 | 320 (3/3) | <10 | <10 | 3620 (2/2) |
| Naive | <10 | <10 | <10 | 1076 (4/4) | <10 | <10 | <10 | 3044 (4/4) | 7241 (2/2) |
| A(H1N1)pdm/A(H3N2) | Pre-infected | <10 | <10 | <10 | 1522 (4/4) | <10 | 1076 (4/4) | <10 | 1522 (4/4) | 5120 (2/2) |
| Naive | <10 | <10 | <10 | 1076 (4/4) | <10 | <10 | <10 | 3044 (4/4) | 7241 (2/2) |
| A(H1N1)pdm/A(H1N1) | Pre-infected | <10 | <10 | <10 | 1810 (4/4) | <10 | 1076 (4/4) | <10 | 1522 (4/4) | 5120 (2/2) |
| Naive | <10 | <10 | <10 | 1076 (4/4) | <10 | <10 | <10 | 3044 (4/4) | 7241 (2/2) |
| TIV plus IFA/A(H1N1)pdm | Pre-infected | 1810 (4/4) | <10 | 3620 (4/4) | <10 | 1076 (4/4) | <10 | 113 (3/3) | 1810 (4/4) | 80 (1/2) |
| Naive | <10 | <10 | <10 | 1076 (4/4) | <10 | <10 | <10 | 3044 (4/4) | 7241 (2/2) |

NOTE. Data are hemagglutination inhibition (HI) geometric mean titers (no. of ferrets with HI titer ≥40/no. of ferrets challenged), unless otherwise indicated. Ferrets were pre-infected with virus then rested for 8 weeks before challenge. Geometric mean titer was calculated using the value of 5 when titer was below the lower limit of detection (<10). IFA, Freund's Incomplete Adjuvant; TIV, human trivalent influenza vaccine 2008/2009 (Northern Hemisphere).

a Serum samples were collected 1 week before challenge.
b Serum samples were collected 2 weeks after challenge.
c HI titer against A/Fukushima/141/2006.
d HI titer against A/Panama/2007/1999.
e HI titer against B/Brisbane/3/2007.
f HI titer against A/California/7/2009.
g P < .05, compared with naive control animals.
h Exposure but no detectable infection.
i Vaccine contained A/Brisbane/10/2007 with limited cross-reactivity to A/Panama/2007/1999.
Figure 3. Pre-infection with influenza A virus reduces virus shedding upon heterologous challenge. Ferrets (n = 3–4) were pre-infected with seasonal influenza A or A(H1N1)pdm influenza A virus (□) then challenged with a second influenza A virus (italics) along with naive (no pre-infection) control ferrets (■). One group of animals was vaccinated twice with human trivalent influenza vaccine 2008/2009 (Northern Hemisphere) (TIV) plus Freund’s Incomplete Adjuvant (IFA) before the first infection (G, from Figure 2C). Virus titer (*) or number of infected animals (%) compared with phosphate-buffered saline (PBS) plus Freund’s Incomplete Adjuvant (IFA) groups, *P < .05. Underlined text indicates exposure but no detectable infection.

the infection rate (P < .01, days 3 and 5), amount of virus shed (P < .01, days 3 and 5), and duration of shedding in animals challenged with seasonal A(H1N1) virus. Transmission to naive contact animals was reduced. Two of 7 vaccinated animals and 1 of 4 contact animals shed virus (Figure 2C). The contact ferret had a low HI titer (20) to A(H1N1), suggestive of a very low level infection (Table 1). Immunization with A/Cal/7/09 plus IFA significantly reduced the amount of virus shed (P < .05, day 3) and delayed shedding in 1 ferret (Figure 2D and Table 1). TIV alone did not protect from A(H1N1) challenge (data not shown).

Fever was detected on day 2 after A(H1N1)pdm (A/Auckland/1/2009) challenge in naive ferrets (mean temperature change ± SD, +1.1°C ± 0.90°C). Virus shedding was detected for 5–7 days and transmitted to naive ferrets (Figure 2E). Vaccination with PBS plus IFA or TIV plus IFA had no effect on A(H1N1)pdm infection (Figure 2F and 2G and Table 1). A(H1N1)pdm infection was almost completely prevented by vaccination with A/Cal/7/09 plus IFA. The infection rate (P < .05, day 5), amount of virus shed (P < .05, days 1, 3, and 5) and duration of shedding were significantly reduced (Figure 2H). Similarly, no fever was detected (mean change in temperature ± SD, +0.35°C ± 0.60°C; mean change in temperature ± SD for PBS plus IFA group, +1.85°C ± 0.39°C; P < .05). Transmission to naive recipients was delayed by 2 days (Figure 2H), and both contact ferrets experienced seroconver-
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sulted in sterilizing immunity to homologous challenge (Figure 3A and Table 2). Pre-infection with A(H1N1) enhanced clearance following heterosubtypic A(H3N2) challenge (Figure 3B). Compared with naïve control animals, pre-infected animals cleared virus more rapidly and shed significantly less virus on day 5 (P<.05). A significantly lower HI titer to A(H3N2) virus was also detected in A(H1N1) pre-infected ferrets, compared with control ferrets (P<.05; Table 2) which suggest a milder infection. Transmission to naïve contact animals was not reduced by pre-infection (Figure 3B). Pre-infection with seasonal influenza A(H3N2) or A(H1N1) virus improved clearance of A(H1N1)pdm virus and reduced shedding (P<.05), compared with shedding in naïve control animals, but did not affect the infection rate. Transmission of A(H1N1)pdm virus was not reduced (Figure 3C and 3D). Prior infection with A(H1N1)pdm improved clearance of A(H3N2) virus and reduced virus shedding (P<.05) (Figure 3E). Prior infection with A(H1N1)pdm virus did not significantly improve clearance or transmission after A(H1N1) infection (Figure 3F). All directly challenged and contact ferrets also experienced seroconversion (Table 2). To ensure that pre-infection with A(H1N1) was responsible for improved clearance of A(H1N1)pdm virus, ferrets were vaccinated with TIV plus IFA before seasonal A(H1N1) challenge, resulting in the prevention of infection due to seasonal A(H1N1) virus (Figure 2C). A low level of cross-reactive antibodies to A(H1N1)pdm virus was detected in this group immediately before challenge with A(H1N1)pdm (Table 1). Upon challenge with A(H1N1)pdm, no difference in virus shedding was detected, compared with shedding in naïve ferrets (Figure 3G). Seasonal A(H1N1) pre-infection and A(H1N1)pdm challenge with and without TIV plus IFA vaccination were repeated with essentially the same result (data not shown). These data suggest that pre-infection, rather than vaccination, is responsible for improved clearance of virus.

Multiple infections due to seasonal influenza A virus reduce the infection rate, duration of shedding, and transmission of A(H1N1)pdm virus. To determine whether 2 infections would enhance viral clearance more rapidly than 1 infection, we pre-infected ferrets with 2 seasonal influenza A strains, then challenged them with A(H1N1)pdm virus (Figure 1C). Infection with seasonal A(H1N1) then A(H3N2) reduced the infection rate (P<.05, days 1 and 5), the amount of virus shed (P<.05), and the duration of shedding in animals challenged with A(H1N1)pdm (A/Auckland/1/2009) virus (Figure 4A). No virus was transmitted to naïve contact animals, and contact animals did not experience seroconversion (Figure 4A and Table 3). Similarly, ferrets that were pre-infected with seasonal A(H3N2) then A(H1N1) had significantly lower levels of virus on all days after A(H1N1)pdm (A/California/7/2009) challenge (P<.05) (Figure 4B). Serological analysis showed that ferrets with multiple pre-infections had significantly lower HI titers

Figure 4. Multiple prior infections with seasonal influenza A viruses protect against challenge with A(H1N1)pdm virus. Ferrets pre-infected twice with seasonal influenza A virus (■) and naïve control ferrets (■) were challenged with A(H1N1)pdm virus. One group of animals was also vaccinated twice with human trivalent influenza vaccine 2008/2009 (Northern Hemisphere) (TIV) plus Freund’s Incomplete Adjuvant (IFA) between the first and second pre-infection (C). Virus titer (T) or number of infected animals (F) compared with phosphate-buffered saline (PBS) plus IFA groups, *P<.05 and **P<.01. Pre-infected ferrets from Figure 3B were used in A. Underlined text indicates exposure but no detectable infection. C, sample contaminated.

A single pre-infection with influenza A virus reduces the duration of shedding following challenge with heterologous influenza A virus. Cross-protective immunity induced by heterologous infection with influenza A was investigated using A/Fukushima/141/2006 (H1N1), A/Panama/2007/1999 (H3N2), and A/Auckland/1/2009 ([H1N1]pdm) viruses (Figure 1B). There was no serological cross-reactivity between viruses by HI or VN assays following virus infection (Table 2). Sequence analysis of internal proteins (NP, PA, PB1, PB2, and M) showed 88%–98% amino acid homology between viruses.

Infection with A(H1N1)pdm or seasonal A(H1N1) virus resulted in sterilizing immunity to homologous challenge (Figure 3A and Table 2). Pre-infection with A(H1N1) enhanced clearance following heterosubtypic A(H3N2) challenge (Figure 3B). Compared with naïve control animals, pre-infected animals cleared virus more rapidly and shed significantly less virus on day 5 (P<.05). A significantly lower HI titer to A(H3N2) virus was also detected in A(H1N1) pre-infected ferrets, compared with control ferrets (P<.05; Table 2) which suggest a milder infection. Transmission to naïve contact animals was not reduced by pre-infection (Figure 3B). Pre-infection with seasonal influenza A(H3N2) or A(H1N1) virus improved clearance of A(H1N1)pdm virus and reduced shedding (P<.05), compared with shedding in naïve control animals, but did not affect the infection rate. Transmission of A(H1N1)pdm virus was not reduced (Figure 3C and 3D). Prior infection with A(H1N1)pdm improved clearance of A(H3N2) virus and reduced virus shedding (P<.05) (Figure 3E). Prior infection with A(H1N1)pdm virus did not significantly improve clearance or transmission after A(H1N1) infection (Figure 3F). All directly challenged and contact ferrets also experienced seroconversion (Table 2). To ensure that pre-infection with A(H1N1) was responsible for improved clearance of A(H1N1)pdm virus, ferrets were vaccinated with TIV plus IFA before seasonal A(H1N1) challenge, resulting in the prevention of infection due to seasonal A(H1N1) virus (Figure 2C). A low level of cross-reactive antibodies to A(H1N1)pdm virus was detected in this group immediately before challenge with A(H1N1)pdm (Table 1). Upon challenge with A(H1N1)pdm, no difference in virus shedding was detected, compared with shedding in naïve ferrets (Figure 3G). Seasonal A(H1N1) pre-infection and A(H1N1)pdm challenge with and without TIV plus IFA vaccination were repeated with essentially the same result (data not shown). These data suggest that pre-infection, rather than vaccination, is responsible for improved clearance of virus.

Multiple infections due to seasonal influenza A virus reduce the infection rate, duration of shedding, and transmission of A(H1N1)pdm virus. To determine whether 2 infections would enhance viral clearance more rapidly than 1 infection, we pre-infected ferrets with 2 seasonal influenza A strains, then challenged them with A(H1N1)pdm virus (Figure 1C). Infection with seasonal A(H1N1) then A(H3N2) reduced the infection rate (P<.05, days 1 and 5), the amount of virus shed (P<.05), and the duration of shedding in animals challenged with A(H1N1)pdm (A/Auckland/1/2009) virus (Figure 4A). No virus was transmitted to naïve contact animals, and contact animals did not experience seroconversion (Figure 4A and Table 3). Similarly, ferrets that were pre-infected with seasonal A(H3N2) then A(H1N1) had significantly lower levels of virus on all days after A(H1N1)pdm (A/California/7/2009) challenge (P<.05) (Figure 4B). Serological analysis showed that ferrets with multiple pre-infections had significantly lower HI titers
to A(H1N1)pdm virus after challenge, compared with control animals (P<.05), which suggests a very mild infection (Table 3). No significant difference in fever was detected. A final group of ferrets was pre-infected with A(H3N2) and vaccinated twice with TIV plus IFA before challenge with seasonal A(H1N1) virus. Infection with seasonal A(H1N1) virus was prevented (data not shown). A low level of cross-reactive antibodies to A(H1N1)pdm virus was detected in this group immediately before challenge (Table 3). Following challenge with A(H1N1)pdm virus, clearance was enhanced only on day 5, compared with clearance in naive control animals (P<.05) (Figure 4C). Furthermore, all animals experienced seroconversion to A(H1N1)pdm with similar titers, which suggested productive infection. No contact ferrets were included in this group. This experiment was repeated with the same findings (data not shown). Collectively, these data demonstrate that 2 earlier infections with seasonal influenza A induce protection from A(H1N1)pdm challenge in this ferret model.

**DISCUSSION**

We have demonstrated in this study that cross-protective immunity induced by seasonal influenza A virus infections in ferrets can significantly limit infection with newly emergent A(H1N1)pdm virus. Although a single pre-infection with seasonal influenza A virus reduced shedding of A(H1N1)pdm virus, 2 pre-infections reduced both direct infection by A(H1N1)pdm virus and subsequent transmission events. Furthermore, infection with A(H1N1)pdm virus induced sterilizing immunity and reduced viral shedding after subsequent challenge with seasonal influenza virus. This protection was reliant on productive infection, because prevention of infection by vaccination diminished this effect. Because high levels of antibodies to seasonal influenza virus did not reduce A(H1N1)pdm infection, these data suggest that immunity induced to conserved T cell epitopes may contribute to the reduced incidence of A(H1N1)pdm infection in ferrets.

Induction of heterosubtypic immunity in animal models by pre-infection with influenza has been demonstrated by others [21, 26, 28, 30–33]. One prior infection with seasonal influenza A virus reduced the amount of virus shedding after direct A(H1N1)pdm challenge in a guinea pig model of influenza [21]. Contact transmission to naive guinea pigs was also reduced after 1 pre-infection [21]. This is in contrast with our ferret data, in which multiple pre-infections were required to reduce transmission. In both studies, naive recipients were continuously housed with directly infected animals. A difference in receptor specificity and symptoms between species may affect transmission [34]. By limiting the contact time between ferrets or by challenging via natural infection, as has also been used with the guinea pig model [21], subtle differences in immunity may be detected; this was not logistically possible in our study. We attempted to minimize the challenge virus dose in order to maximize any protective effects afforded from vaccination or pre-exposure. Furthermore, use of outbred animals intro-
duced individual variation. Housing constraints limited group sizes, potentially reducing the sensitivity of this ferret model.

It is of interest that the serological responses from vaccinated and/or infected ferrets in this study are similar to findings reported in the pre-pandemic and post-pandemic vaccinated human population. Firstly, low levels of cross-reactive antibodies to A(H1N1)pdm have been detected in pre-pandemic serum samples from older adults [2, 10, 11] and in samples from adults after immunization with TIV [10, 35]. TIV plus IFA immunized ferrets infected with seasonal influenza viruses also had cross-reactive antibodies to A(H1N1)pdm. Pre-pandemic antibodies in older adults have largely been attributed to exposure to older influenza viruses with shared epitopes on hemagglutinin or neuraminidase proteins [15]. As the ferrets had only been exposed to recent seasonal influenza A viruses, it is likely that high baseline titers to seasonal virus may lead to cross-reactivity, as has been suggested following TIV immunization of adults [10]. Importantly, these cross-reactive antibodies did not reduce A(H1N1)pdm infection in ferrets, indicating they may be of low affinity. Second, clinical trials have demonstrated high rates of seroconversion to A(H1N1)pdm hemagglutinin, compared with seasonal and H5N1 vaccines in adults and children [14, 17, 18, 35–37]. Similarly, all ferrets responded after 1 vaccination with A/Cal/7/09 plus IFA, whereas 2 doses of TIV plus IFA were required for 100% seroconversion. A recent study in which ferrets were vaccinated with combinations of MF59-adjuvanted TIV and inactivated A(H1N1)pdm vaccines gave results that were similar to our own [38]. Ferrets were largely protected from challenge with A(H1N1)pdm, but in our study transmission events were still detected. This measure of infection suggests that vaccination per se was not sterilizing in this model and that A(H1N1)pdm virus may still be present, perhaps in the lower respiratory tract, as reported by others [11, 19, 20, 22]. Interestingly, A/Cal/7/09 plus IFA-vaccinated ferrets were partially protected against seasonal A(H1N1), which suggested cross-reactivity between viruses. Additional work is underway to assess whether the vaccine induces cross-protective responses against other subtypes of influenza A virus.

A lack of immunological reagents available for ferrets limited analysis of the innate and adaptive immune responses. By maximizing analysis periods between multiple infections, the contribution of short-term innate immunity was minimized, which is similar to other animal influenza studies [26, 30, 33, 39]. Immunization before challenge to prevent infection attempted to mimic the annual vaccination and exposure patterns in humans, although with a much reduced time frame. Similarly, prevention of prior infection by homologous immunization in mice diminished memory T cell induction and subsequent protection from lethal heterologous influenza challenge [25]. A high proportion of human T cell epitopes are conserved between recent seasonal influenza A strains and A(H1N1)pdm virus [16]. These data suggest that memory T cell responses may be recalled upon infection in this model and may protect against A(H1N1)pdm infection. Use of in vitro cellular assays to identify conserved influenza epitopes recognized by ferret T cells is important, but little progress has been made in this area. Recent work suggests infection(s) with older influenza strains [15] (S. Gras, unpublished data) may also induce cross-protection; therefore, the analysis of older strains would also be of interest to study in this ferret model of multiple infection.

The data in this study demonstrate that immunity induced by prior infection with seasonal influenza A virus may provide significant protection against A(H1N1)pdm challenge and limit disease severity. These findings concur with epidemiological data that show that children and younger adults, who are likely to have had limited exposures to influenza, were more susceptible to A(H1N1)pdm virus in 2009, compared with adults and the elderly population. Furthermore, earlier infection with A(H1N1)pdm virus in the ferret induced sterilizing immunity to homologous challenge and reduced shedding after challenge with seasonal influenza A(H1N1) or A(H3N2) virus. Immunity provided by vaccination with monovalent A(H1N1)pdm vaccine and/or prior infection of seasonal and A(H1N1)pdm viruses may limit the incidence and severity of influenza in the 2010–2011 seasons.

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References


