Comparison of a Live Attenuated 2009 H1N1 Vaccine with Seasonal Influenza Vaccines against 2009 Pandemic H1N1 Virus Infection in Mice and Ferrets

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The role of seasonal influenza vaccination in pandemic influenza A H1N1 disease is important to address, because a large segment of the population is vaccinated annually. We administered 1 or 2 doses of pandemic H1N1 vaccine (CA/7 ca), a seasonal trivalent inactivated (s-TIV), or live attenuated influenza vaccine (s-LAIV) to mice and ferrets and subsequently challenged them with a pandemic H1N1 virus. In both species, CA/7 ca was immunogenic and conferred complete protection against challenge. s-TIV did not confer protection in either animal model, and s-LAIV did not confer any protection in ferrets. In mice, 2 doses of s-LAIV led to complete protection in the upper respiratory tract and partial protection in the lungs. Our data indicate that vaccination with the seasonal influenza vaccines did not confer complete protection in the lower respiratory tract in either animal model, whereas the CA/7 ca vaccine conferred complete protection in both animal models.

The emergence of the 2009 pandemic influenza A H1N1 virus was a surprise on many fronts. The novel 2009 pandemic H1N1 virus is genetically and antigenically distinct from seasonal human H1N1 influenza viruses [1, 2]. However, because an H1N1 influenza subtype has cocirculated with an H3N2 subtype in humans since 1977, many questions regarding the effect of prior exposure to seasonal influenza viruses on the response to the pandemic virus remain unanswered. Unlike seasonal influenza, the highest incidence of pandemic H1N1 infection was observed among children and young adults [2–5]. Individuals aged >50 years appeared to be less susceptible, suggesting that these individuals have been exposed to an influenza strain that has conferred a degree of protection against the current pandemic H1N1 virus.

The role of seasonal influenza vaccination on pandemic H1N1 infection has yet to be clearly delineated and is important to address. The observed effect of prior seasonal influenza vaccine has differed in the clinical studies that have thus far been published. In a small case-controlled study conducted in Mexico [6], more severe clinical outcomes were noted among individuals infected with the 2009 H1N1 virus who had not been vaccinated with seasonal influenza vaccine, compared with individuals who had received the 2008–2009 seasonal trivalent inactivated vaccine. In another retrospective analysis from Mexico [7], the risk of pandemic 2009 H1N1 infection was observed to be lower among individuals who had been vaccinated with 2008–2009 seasonal influenza vaccine. Hancock et al [8] noted a >4-fold increase in the level of cross-reactive antibody
to the pandemic 2009 H1N1 virus in 12%–22% of adults aged 18–64 years after receipt of a seasonal influenza vaccine, suggesting that vaccination may induce a cross-reactive antibody response to the pandemic H1N1 influenza virus in a proportion of adults. In contrast, 2 studies conducted in the United States and Australia showed that seasonal influenza vaccine did not have a significant effect on pandemic influenza infection [9, 10]. Recently, observational studies conducted in Canada have observed an association between seasonal influenza vaccination and an increased incidence of pandemic H1N1 infection [11].

In animal models, the effect of seasonal influenza vaccination on pandemic H1N1 disease has also not been fully explored. In 2 studies in ferrets, neither 1 [12] nor 2 doses [13] of seasonal influenza vaccination elicited protective antibody titers or conferred protection against pandemic H1N1 infection, although 2 doses of adjuvanted seasonal influenza vaccine primed for a robust response to the pandemic H1N1 vaccine [13]. We designed a study to further evaluate the immunogenicity and efficacy of seasonal influenza vaccines in protection against 2009 pandemic H1N1 influenza in mice and ferrets. We included both inactivated and live attenuated seasonal influenza vaccines to compare different forms of the seasonal influenza vaccines, because previous studies have observed broad cross-reactivity elicited by vaccination with live vaccines in naive populations [14–16]. We compared these seasonal influenza vaccines with the live attenuated 2009 pandemic H1N1 influenza vaccine for their ability to protect against 2009 H1N1 virus infection.

**MATERIALS AND METHODS**

**Viruses**

The wild-type (wt) pandemic 2009 H1N1 virus, A/California/7/2009 (CA/7 wt), was kindly provided by Drs. Ruben Donis and Alexander Klimov from the Influenza Division, Centers for Disease Control and Prevention (CDC). The live attenuated cold-adapted (ca) reassortant virus, A/California/7/2009 (CA/7 ca) H1N1 virus, was generated by reverse genetics, as described elsewhere [17]. The H1 HA and N1 NA of the reassortant H1N1 virus, was generated by reverse genetics, as described in ferrets, we administered 200 L of L-15 media (mock-immunized virus) intranasally to lightly anesthetized ferrets. On days 2, 3, 4, and 7 after inoculation, lungs, nasal turbinates and brains (including olfactory bulbs) were harvested from 4 mice in each group. Organs were homogenized in L-15 medium containing antibiotic-antimycotic (Invitrogen-GIBCO) to make 5% tissue homogenates. Tissue homogenates were clarified by centrifugation and titrated in 24 and 96-well tissue culture plates containing MDCK cell monolayers, as described elsewhere [21]. Titters are expressed as log_{10} TCID_{50}/g of tissue.

To determine the replication kinetics of the CA/7 ca vaccine in ferrets, we administered 200 L containing 1 × 10^{6} TCID_{50} of the CA/7 ca vaccine intranasally to lightly anesthetized ferrets. On days 1, 3, and 5 after inoculation, lungs, nasal turbinates, and brains were harvested from 3 ferrets in each group. Viral titers were determined as described above.

**Evaluation of the Vaccines**

To compare the immunogenicity and efficacy of the CA/7 ca vaccine with seasonal influenza vaccine against 2009 pandemic H1N1 infection in mice, we administered 1 or 2 doses of virus or vaccine to groups of 8 mice lightly anesthetized with isoﬂurane. The mice received 50 L of L-15 media (mock-immunized
played in Figure 1A. In the lower respiratory tract, the CA/7 virus is detected in the lungs of ferrets as early as day 1 postinfection, and peak viral replication occurs on day 5 after inoculation. Viral titers were determined as described above. The kinetic of replication of the CA/7 wt virus replicated to high titer in the respiratory tract. To determine efficacy against challenge in mice, we harvested lungs and nasal turbinates from 4 mice in each group on days 2 and 4 after challenge. Viral titers were determined as described above.

Assessment of Immunogenicity
Neutralizing antibody titers in pre- and postvaccination mouse and ferret serum samples were determined using a micro-neutralization assay. Serial 2-fold dilutions of heat-inactivated serum were prepared starting from a 1:20 dilution. Equal volumes of serum and virus were mixed and incubated for 60 min at room temperature. The residual infectivity of the virus-serum mixture was determined in MDCK cells in 4 replicates for each dilution. Neutralizing antibody titers were defined as the reciprocal of the highest dilution of serum that completely neutralized the infectivity of 100 TCID$_{50}$ of the virus, indicated by the absence of viral cytopathic effect at day 4.

Assessment of Efficacy against CA/7 wt Challenge
To determine efficacy against challenge in mice, we harvested lungs and nasal turbinates from 4 mice in each group on days 2 and 4 after challenge. Viral titers were determined as described above.

To determine efficacy against challenge in ferrets, we harvested nasal turbinates and portions of the right and left lower lobes of the lung on days 1 and 5 after challenge. We chose these time points based on previous observations in our laboratory that replication of CA/7 wt virus is detected in the lungs of ferrets as early as day 1 after infection, and peak viral replication occurs on day 5 after infection. Viral titers were determined as described above.

RESULTS

Evaluation of Viral Replication of the CA/7 ca Vaccine in Mice and Ferrets
The kinetics of replication of the CA/7 ca vaccine in mice is displayed in Figure 1A. In the lower respiratory tract, the CA/7 ca virus replicated to a mean peak titer on day 2 after inoculation of $1 \times 10^{1.9}$ TCID$_{50}$/g and was not detected at later time points; in the upper respiratory tract, the CA/7 ca virus replicated to a mean peak titer on day 2 after inoculation of $1 \times 10^{2.1}$ TCID$_{50}$/g and was not detected at later time points. Virus was not detected in the brain of any of the mice that received the CA/7 ca virus.

The kinetics of replication of the CA/7 ca vaccine in ferrets is displayed in Figure 1B. In ferrets, the CA/7 ca vaccine achieved titers of $1 \times 10^{2.7}$ to $1 \times 10^{4.5}$ TCID$_{50}$/g in the upper respiratory tract and was not detected in the lower respiratory tract of 8 of 9 ferrets or in the brains of any of the ferrets.

In contrast, the CA/7 wt virus replicated to high titer in the entire respiratory tract of mice and ferrets and was detected at all
time points tested. The CA/7 ca vaccine was therefore restricted in replication in both mice and ferrets, compared with the CA/7 wt virus.

**Immunogenicity and Efficacy of the CA/7 ca Vaccine against Homologous wt Virus Infection**

**Mice.** Neutralizing antibody titers in mice after immunization are displayed in Table 1. After 1 dose of the CA/7 ca vaccine, mice developed antibodies against the homologous CA/7 wt virus (geometric mean titer [GMT], 53; range, 25–160), and partial protection from replication of the challenge virus was observed in the respiratory tract (Figure 2A and B). The CA/7 wt virus replicated to mean titers of $1 \times 10^{6.2}$ and $1 \times 10^{5.6}$ TCID$_{50}$/g on days 2 and 4 after challenge, respectively, in the nasal turbinates of mock-immunized mice (Figure 2A). The level of replication was reduced in the upper respiratory tract of mice immunized with the CA/7 ca vaccine on day 2 (mean titer of $1 \times 10^{2.5}$ TCID$_{50}$/g) and was not detectable on day 4 after challenge (Figure 2A). The CA/7 wt virus replicated to mean titers of $1 \times 10^{7.6}$ and $1 \times 10^{7}$ TCID$_{50}$/g on days 2 and 4 after challenge, respectively, in the lungs of mock-immunized mice (Figure 2B). The replication of the challenge virus was reduced by 300 to 400-fold in the lower respiratory tract of CA/7 ca immunized mice (mean titer of $1 \times 10^{4}$ and $1 \times 10^{4.5}$ TCID$_{50}$/g on days 2 and 4, respectively; $P < .05$, by Mann-Whitney U test) (Figure 2B).

After 2 doses of CA/7 ca vaccine, mice developed a robust neutralizing antibody response (GMT, 595; range, 160–1810) against the CA/7 wt virus (Table 1) and were completely protected from replication of the CA/7 wt challenge virus in the respiratory tract ($P < .05$, by Mann-Whitney U test) (Figure 2C and D).

**Ferrets.** Neutralizing antibody titers after immunization in ferrets are shown in Table 2. After a single dose of the CA/7 ca vaccine, ferrets developed a robust neutralizing antibody response

Table 1. Serum Neutralizing Antibodies Elicited in Mice after Vaccination

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>CA/7 wt 1 dose</th>
<th>CA/7 wt 2 doses</th>
<th>Brisbane/59/07 wt 1 dose</th>
<th>Brisbane/59/07 wt 2 doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>10</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CA/7 ca</td>
<td>53</td>
<td>595</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>s-TIV</td>
<td>10</td>
<td>12</td>
<td>16</td>
<td>243</td>
</tr>
<tr>
<td>s-LAIV</td>
<td>12</td>
<td>25</td>
<td>64</td>
<td>409</td>
</tr>
</tbody>
</table>

**NOTE.** Homologous antibody titers are expressed in bold. ND, not done.

*Previous serum samples were pooled from groups of mice that received the same immunogen; antibody was not detectable on day 0 before vaccination.

*An undetectable serum neutralizing antibody titer was assigned a value of 10.

Figure 2. Efficacy in mice of 1 or 2 doses of s-TIV, s-LAIV, or monovalent 2009 pandemic H1N1 influenza vaccine against challenge with $10^{6}$ TCID$_{50}$ CA/7 wt virus. Virus titers were determined on days 2 and 4 after challenge; virus titers are expressed as log$_{10}$ TCID$_{50}$/g of tissue. Horizontal bars represent mean titers, and symbols represent titers from individual mice. The dotted lines represent the lower limit of detection. A, Level of replication in the upper respiratory tract after 1 dose of each vaccine. B, Level of replication in the lower respiratory tract after 1 dose of each vaccine. C, Level of replication in the upper respiratory tract after 2 doses of each vaccine. D, Level of replication in the lower respiratory tract after 2 doses of each vaccine.
with a GMT of 854 (range, 320–1613) against the CA/7 wt virus by 28 days after immunization; none of the animals had detectable antibodies before immunization. After 2 doses of the CA/7 ca vaccine, a significantly higher neutralizing antibody titer was noted (GMT, 2428; range, 1810–3620) against the CA/7 wt virus.

One or 2 doses of the CA/7 ca vaccine conferred complete protection against replication of the CA/7 wt challenge virus in ferrets; virus was not detected in the respiratory tract on days 1 and 5 after challenge (Figure 3). The mean titers of the challenge virus in the lungs and nasal turbinates of mock-immunized ferrets were $1 \times 10^{5.6}$ TCID$_{50}$/g and $1 \times 10^{5.3}$ TCID$_{50}$/g, respectively; the reduction in titer in the CA/7 ca vaccinated animals, compared with mock-infected animals, was statistically significant ($P < .05$, Mann-Whitney $U$ test).

### Immunogenicity and Efficacy of Seasonal Influenza Vaccines against CA/7 wt Virus Challenge

**Mice.** After 1 dose of s-TIV, none of the mice developed a detectable neutralizing antibody response against the CA/7 wt virus. After 2 doses of this vaccine, a low neutralizing antibody titer was detected in 3 of 8 mice (GMT, 12; range, 10–20) against the CA/7 wt virus (Table 1). All mice developed a robust antibody response to the seasonal H1N1 virus component in the vaccine. On days 2 and 4 after challenge, no statistically significant difference was noted in the level of replication of the CA/7 wt virus in the respiratory tract of mock-immunized mice and mice that received either 1 or 2 doses of the s-TIV (Figure 2).

After 1 dose of the s-LAIV, all 8 mice developed a robust neutralizing antibody titer to the seasonal H1N1 virus contained in the vaccine (mean titer, 64; range, 28–113). In contrast, only 2 of 7 mice developed detectable neutralizing antibody titers (mean titer, 12; range, 10–20) against the CA/7 wt virus. However, after 2 doses of vaccine, a modest neutralizing antibody titer was detectable in all mice (mean titer, 25; range, 16–32) against the CA/7 wt virus (Table 1). On days 2 and 4 after challenge, no statistically significant difference was noted in the level of replication of the CA/7 wt virus in the respiratory tract of mock-immunized mice and mice that received 1 dose of s-LAIV. After 2 doses of s-LAIV, no virus was detected in the upper respiratory tract on days 2 and 4 after challenge ($P < .05$, by Mann-Whitney $U$ test). A 10- and 100-fold reduction in titer was also observed in the lower respiratory tract of the mice that received 2 doses of s-LAIV (mean titers of $1 \times 10^{6.5}$ and $1 \times 10^{6.2}$ TCID$_{50}$/g, compared with mean titers of $1 \times 10^{7.8}$ and $1 \times 10^{6.6}$ TCID$_{50}$/g in the mock-immunized animals, on days 2 and 4, respectively; $P < .01$, by Mann-Whitney $U$ test) (Figure 2).

**Ferrets.** As shown in Table 2, immunization with 1 or 2 doses of s-TIV did not induce detectable neutralizing antibodies against the CA/7 wt virus in ferrets, although neutralizing antibody was detected against the homologous seasonal H1N1 virus contained in the vaccine (Table 2). On days 1 and 5 after challenge, no statistically significant difference was noted in the level of replication of the CA/7 wt virus in the respiratory tract of mock-immunized ferrets and ferrets that received 1 or 2 doses of s-TIV (Figure 3).

### Table 2. Serum Neutralizing Antibodies Elicited in Ferrets after Vaccination

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>1 dose</th>
<th>2 doses</th>
<th>1 dose</th>
<th>2 doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>10</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CA/7 ca</td>
<td>854</td>
<td>2428</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>s-TIV</td>
<td>10</td>
<td>10</td>
<td>73</td>
<td>82</td>
</tr>
<tr>
<td>s-LAIV</td>
<td>10</td>
<td>ND</td>
<td>174</td>
<td>ND</td>
</tr>
</tbody>
</table>

**NOTE.** Homologous antibody titers are expressed in bold. ND, not done.

* Prevaccination serum samples were pooled from 3 ferrets that received the same immunogen; antibody was not detectable on day 0 before inoculation.

b An undetectable serum neutralizing antibody titer was assigned a value of 10.

Figure 3. Efficacy in ferrets of 1 dose of s-LAIV, 2 doses of s-TIV, or monovalent live attenuated 2009 pandemic H1N1 influenza vaccine against challenge with $10^6$ TCID$_{50}$ CA/7 wt virus. Virus titers were determined on days 1 and 5 after challenge; virus titers are expressed as log$_{10}$TCID$_{50}$/g of tissue. Horizontal bars represent mean titers from individual ferrets, and symbols represent titers from individual ferrets. The dotted lines represent the lower limit of detection. A, Level of replication in the upper respiratory tract after challenge. B, Level of replication in the lower respiratory tract after challenge.
Similarly, after a single dose of s-LAIV, neutralizing antibody against the CA/7 wt virus was not detectable in ferrets, although a robust neutralizing antibody response (GMT, 174; range, 101–320) was detected against the seasonal H1N1 virus contained in the vaccine (Table 2). On days 1 and 5 after challenge, no statistically significant difference was noted in the level of replication of the CA/7 wt virus in the respiratory tract of mock-immunized ferrets or ferrets that received either seasonal influenza vaccine (Figure 3).

DISCUSSION

Given the limitations on data from retrospective studies, the association between seasonal influenza vaccination and pandemic H1N1 disease may not be readily delineated with clinical studies alone. Although recent studies conducted in guinea pigs and ferrets suggest that partial immunity against 2009 pandemic H1N1 infection may be conferred by exposure to seasonal H1N1 influenza viruses [22, 23], the role of seasonal influenza vaccination has not yet been fully explored in other animal models. We therefore designed a study in mice and ferrets to examine the immunogenicity and efficacy of the seasonal influenza vaccine against infection with the pandemic H1N1 CA/7 wt reference virus. We selected seasonal influenza vaccine from a single season (2008–2009) and evaluated the efficacy of cross-protection shortly after vaccination.

In ferrets, either 1 or 2 doses of the live attenuated CA/7 ca vaccine were immunogenic and provided complete protection against replication of the CA/7 wt virus in the respiratory tract after challenge. The antibody response was robust after either 1 or 2 doses of the CA/7 ca vaccine and was associated with complete protection from challenge. In mice, a single dose of the CA/7 ca vaccine was modestly immunogenic and provided only partial protection against homologous CA/7 wt virus challenge. However, after 2 doses, the CA/7 ca vaccine elicited a robust neutralizing antibody response and provided complete protection against homologous CA/7 wt virus challenge. Thus, in both animal models, the presence of a robust antibody response correlated with protection from challenge with the homologous wt virus.

Although variable protection against heterologous challenge after inactivated influenza vaccine has been reported in humans [24], vaccination with s-TIV did not induce a robust cross-reactive immune response, nor did it provide protection against CA/7 wt virus challenge in either mice or ferrets. Of interest, although s-LAIV was also neither immunogenic nor efficacious against CA/7 wt virus challenge in ferrets, we observed partial protection from 2009 pandemic H1N1 virus infection after vaccination with the s-LAIV in mice. It is possible that the higher body temperature of ferrets, compared with mice (39°C and 37°C, respectively) [25], plays a role in this difference, because mice are semi-permissive for cold-adapted influenza vaccines and the replication of ca vaccines is more restricted in ferrets. Despite a very modest neutralizing antibody response, complete protection from replication of the CA/7 wt virus was observed in the upper respiratory tract of mice after 2 doses of s-LAIV, and there was a 10- and 100-fold reduction in challenge virus titer on days 2 and 4, respectively, in the lower respiratory tract of mice.

The results of our study underscore several characteristics that have previously been observed in the relationship between the immunogenicity and efficacy of influenza vaccines that are areas for future study. First, as has been previously noted, a robust immune response to the live attenuated influenza vaccine is highly predictive of efficacy [15, 21, 26–28]. In our study, the homologous CA/7 ca vaccine was highly immunogenic and conferred complete protection against the 2009 pandemic H1N1 virus. However, as has also been previously noted [21, 26, 28–31], absence of a neutralizing antibody response is not necessarily associated with lack of protective efficacy of live attenuated vaccines. In our study, 2 doses of s-LAIV in mice conferred partial protection against heterologous challenge despite low neutralizing antibody titers against the 2009 pandemic H1N1 virus. The discrepancy in the observed immunogenicity and efficacy of the s-TIV, compared with the s-LAIV, against the CA/7 wt virus suggests that some protective components of the immune response to LAIV are not detected in the serum by neutralizing antibody assays. It is likely that the protection observed in the upper respiratory tract of mice after intranasal administration of s-LAIV is related to the development of mucosal antibodies and innate and cellular immunity. Additional studies are under way to delineate the mucosal antibody and cellular immune response to these vaccines.

Our data confirm several findings reported by others. As noted by Kobinger et al [12], neither s-TIV nor s-LAIV elicited a protective antibody titer or conferred protection against pandemic H1N1 disease in ferrets. However, our ferrets were not assessed beyond 5 days after challenge.

As with any data gathered from animal models, our study design is inherently limited in ability to accurately reflect the human situation. Most individuals are likely to have been exposed to seasonal influenza infection and/or vaccine before the emergence of the 2009 pandemic H1N1 virus. Furthermore, the presence of co-morbidities and individual host response to vaccination and infection in humans confound direct interpretation of our data. However, several meaningful conclusions can be drawn from our observations. First, given the extensive experience with assessment of influenza vaccines in mice and ferrets, the robust immune response and complete protection conferred by the CA/7 ca vaccine suggests that this vaccine will provide protection against the 2009 pandemic H1N1 virus. Our observations also support emerging data that exposure to seasonal influenza vaccines may play a role in...
reducing susceptibility and morbidity associated with pandemic 2009 H1N1 infection [6–8, 32] and suggests the importance of exploring the underlying mechanisms of this phenomenon.

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References