Characterization of an influenza A H5N2 reassortant as a candidate for live-attenuated and inactivated vaccines against highly pathogenic H5N1 viruses with pandemic potential

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Received 2 April 2006; received in revised form 31 May 2006; accepted 14 June 2006
Available online 28 June 2006

Abstract

We generated a high-growth 7:1 reassortant (Len17/H5) that contained the hemagglutinin (HA) gene from non-pathogenic A/Duck/Potsdam/1402-6/86 (H5N2) virus and other genes from the cold-adapted (\textit{ca}) attenuated A/Leningrad/134/17/57 (H2H2) strain. Len17/H5 demonstrated an attenuated phenotype in mice and did not infect chickens. Mice administered Len17/H5 either as a live-attenuated intranasal vaccine or as an inactivated intramuscular vaccine were substantially protected from lethal challenge with highly pathogenic A/Hong Kong/483/97 (H5N1) virus and were protected from pulmonary infection with antigenically distinct A/Hong Kong/213/2003 (H5N1) virus. The cross-protective effect correlated with the levels of virus-specific mucosal IgA and/or serum IgG antibodies. Our results suggest a new strategy of using classical genetic reassortment between a high-growth \textit{ca} H2N2 strain and antigenically related non-pathogenic avian viruses to prepare live-attenuated and inactivated vaccines for influenza pandemic.

Keywords: Avian influenza vaccine; Influenza pandemic; Mouse model

1. Introduction

Repeated outbreaks of H5N1 influenza in Asia continue to pose a pandemic threat to human health. Highly pathogenic avian influenza (HPAI) A (H5N1) viruses were first recognized to cause respiratory disease in humans in 1997 when viruses from infected poultry were transmitted to humans, causing 18 documented cases including six fatalities [1–3]. In 2003, H5N1 virus reemerged in humans to infect two family members in Hong Kong resulting in the death of one person [4]. Since late 2003, unprecedented numbers of HPAI H5N1 outbreaks in poultry have occurred in many Asian, European and African countries, resulting in more than 220 laboratory-confirmed human cases with a fatality rate of greater than 50% [5]. Thus, the development of safe, dose-sparing and effective human vaccines against H5N1 influenza is a high priority for global public health.

Since 1997, HPAI H5N1 viruses from birds have undergone rapid genetic evolution [6–8]. The viruses isolated from humans have reflected this genetic variation with concomitant antigenic variation. H5N1 viruses from 2004 to 2005 comprise two genetically distinct virus clades, both of which are antigenically distinct from the 2003 human isolates, which in turn were antigenically distinct from those isolated from humans in 1997 [9–11]. Once recognized to cause human
disease, new candidate vaccine strains must be generated for each H5N1 antigenic variant. A number of different strategies have been applied to generate vaccine candidates against HPAI H5N1 viruses, including the use of antigenically related non-pathogenic viruses to produce an inactivated influenza vaccine (IIV) and the use of purified recombinant HA protein. Both of these approaches have been evaluated clinically with suboptimal results [12–14]. More recently reverse genetics techniques have been optimized to allow for the generation of vaccine reassortant strains that possess HA with the modified multibasic cleavage site, which is associated with virulence in birds, and internal genes derived from a human vaccine donor strain [15–17]. This approach allows for the inclusion of an HA protein, albeit modified, that is antigenically highly related to that found in the circulating HPAI H5N1 virus.

Development of live-attenuated influenza vaccine (LAIV) for pandemic preparedness has certain advantages over other vaccine strategies. Since LAIV may provide effective protection against a broader range of variants, an exact match between the vaccine strain and circulating viruses may be less critical. As an example, LAIV was shown to provide highly effective protection in healthy pre-school children against a drift variant of influenza A (H3N2) in a pre-licensure study of LAIV in the USA [18]. Similar data were obtained in Russia (reviewed in [19]). The heterotypic efficacy of LAIV may be, at least in part, due to the induction of enhanced IgA antibody responses in the respiratory tract compared with those induced by IIV [20,21]. Furthermore, since vaccine will be in short supply during a pandemic, multiple vaccine production options may be important.

Here we evaluate an H5 pandemic vaccine candidate created using classical reassortment techniques from an antigenically related non-pathogenic avian influenza H5N2 and an influenza cold-adapted (ca) donor strain A/Leningrad134/17/57 (H2N2; Len17) [22] for its protective efficacy against antigenically heterologous HPAI H5N1 strains. The H5 pandemic vaccine candidate (Len17/H5) possesses the HA from non-pathogenic A/Duck/Potsdam/1402-6/86 (H5N2; Pot/86) virus and all other genes from Len17 (7:1 genome composition) [23]. Len17/H5 demonstrated ca and ts phenotypes in vitro similar to those of the Len17 ca donor strain, grew to high titers in embryonated eggs and shared antigenic similarity with the H5N1 viruses isolated from humans in 1997 [23]. We demonstrate that the reassortant Len17/H5 virus is attenuated in mice and non-infectious for chickens, and effectively protects mice against heterologous HPAI H5N1 infection when used as either an LAIV or IIV. These results suggest a pandemic vaccine strategy that does not require reverse genetics technology, a heightened bio-safety level, or a precise antigenic match for vaccine strain generation, yet may offer protection against a heterologous virus in the early phase of a pandemic.

2. Materials and methods

2.1. Viruses

The high-growth reassortant Len17/H5 that contained the HA gene from the non-pathogenic A/Duck/Potsdam/1402-6/86 (H5N2) virus (Pot/86) and other genes from the ca attenuated A/Leningrad/134/17/57 (H2H2) strain (Len17) was obtained in accordance with Ghendon et al, [24]. Wildtype H5N1 viruses used in this study were A/Hong Kong/156/97 (HK/156), A/Hong Kong/483/97 (HK/483), and A/Hong Kong/213/03 (HK/213). Viruses were propagated in the allantoic cavity of 10-day-old embryonated hens’ eggs at 34 °C for 2 days (Len17/H5, Len17, and Pot/86) or at 37 °C for 26–28 h (HK/156, HK/483, and HK/213). Virus stocks were aliquoted and stored at −70 °C until use. Fifty percent egg infectious dose (EID50) titers were determined by serial titration of virus in eggs and calculated by the method of Reed and Muench [25]. The titer of Len17/H5 was 1280 HAU or 10⁶ EID50/ml.

2.2. Laboratory facility

All experiments with infectious wild-type avian H5 and human H2 viruses and the reassortant H5 virus were conducted using bio-safety level (BSL) 3 enhanced containment procedures.

2.3. Pathogenicity and infectivity in chickens

For the determination of pathogenicity, eight chickens per group were inoculated intravenously (i.v.) with 0.2 ml of a 10⁻¹ dilution of each virus and observed daily for 14 days for clinical signs and death. To determine infectivity, five chickens were inoculated intranasally (i.n.) with 10⁶ EID50 of each virus in 0.1 ml. On day 3 post-inoculation (p.i.), oropharyngeal and cloacal swabs were collected from each chicken and virus replication was assessed in embryonated chicken eggs. The chickens were observed for clinical signs of disease and death for 21 days, at which time serum samples were harvested and tested for presence of antibodies by agar gel immunodiffusion (AGID) test.

2.4. Pathogenicity and infectivity in mice

Ten-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME, USA) were lightly anesthetized with CO₂, and 50 μl of 10¹ to 10⁷ EID50 of Len17/H5, Len17, or Pot/86 diluted in phosphate-buffered saline (PBS) was inoculated i.n. for determining infectivity (50% mouse infectious dose (MID50)) and pathogenicity (50% lethal dose (LD50)) as previously described [26]. To evaluate the replication of Len17/H5 and two parent viruses, mice were infected i.n. with 10⁶ EID50 of these viruses. The organs were collected on day 3 (lung and nose) and day 6 (brain) p.i. and titrated for infectious virus in eggs [26].
2.5. Vaccine preparation and immunization of mice

Groups of 8-week-old female BALB/c mice were immunized i.n. with one dose of 300 MID50 of Len17/H5 (=10^7 EID50) or Len17 (=10^7.3 EID50) LAIV. Mice were infected i.n. with either 300 MID50 of Pot/86 (=10^5.7 EID50) or 100 MID50 of HK/213 virus (=10^3.8 EID50) as positive controls and received PBS as a negative control. The high-growth Len17/H5 virus was concentrated from allantoic fluid and purified on a sucrose gradient as previously described [27] and prepared as IIV by treating purified virus with 0.25% formalin at 4 °C for 3 days. A group of mice was injected intramuscularly (i.m.) with one dose of 10 μg of IIV (≈3 μg HA protein) in a volume of 0.1 ml.

2.6. Antibody sample collection and assays

Six weeks after i.n. or i.m. immunization, blood, lung and nasal wash samples were collected from five mice per group as previously described [28]. Sera were treated with receptor-destroying enzyme from Vibrio cholerae (Denka-Seiken, Tokyo, Japan) before testing for the presence of H5-specific antibodies [29]. Titers of neutralizing antibody were determined using a microneutralization assay as previously described [30]. Neutralizing antibody titers are expressed as the reciprocal of the highest dilution of serum that gave 50% neutralization of 100 TCID50 of virus in Madin Darby Canine Kidney cells. Influenza H5-specific IgG and IgA antibodies were detected by an enzyme-linked immunosorbent assay (ELISA) as previously described [28] except that 2 μg/ml of a purified baculovirus-expressed H5 (HK/156) recombinant HA protein (Protein Sciences Corporation, Meriden, CT, USA) was used to coat plates. The end-point ELISA titers were expressed as the highest dilution that yielded an optical density (OD) greater than twice the mean OD plus standard deviation (S.D.) of similarly diluted control samples.

2.7. Virus challenge

Six weeks after i.n. or i.m. immunization, vaccinated mice were challenged i.n. with 50 μl of 100 MID50 of HK/213 or 50 LD50 of HK/483. Three or 6 days after challenge, five animals per group were euthanized and the tissues were collected and stored at −70 °C. Thawed tissues were homogenized in 1 ml of cold PBS and titrated for virus infectivity in 10-day-old embryonated eggs as previously described [26]. Virus end-point titers are expressed as the mean log_{10} EID50/ml ± S.D. The eight mice in each group that were challenged i.n. with the highly pathogenic (HP) HK/483 virus were observed daily for signs of disease, weight loss and death for 14 days after challenge.

2.8. Statistical analysis

Statistical significance of the data was determined by using two-tailed Student’s t-test.

3. Results

3.1. Pathotyping and replication of Len17/H5 vaccine in chickens

The two parent and reassortant Len17/H5 viruses were administered to specific pathogen free (SPF) chickens to determine their potential risk for animal agriculture which included assessment of the ability to cause morbidity and mortality following i.v. inoculation (pathogenicity) and the level of tissue-specific replication following simulated natural exposure (i.n. inoculation). With i.v. or i.n. inoculation, no clinical disease signs or deaths were observed in the chickens with any of the three viruses over the 14 or 21 days observation period, respectively (Table 1). For the i.n. inoculated group on day 3 p.i., which is the peak replication time for low pathogenic (LP) avian influenza viruses, virus was not isolated from respiratory (oropharyngeal swab) or intestinal (cloacal swab) tracts, but antibodies to avian influenza viral proteins were detected in chickens inoculated with the avian Pot/86 parent virus. The combined data from the two experiments suggests that the two parent and reassortant Len17/H5 viruses were not HP for chickens. Following simulated natural exposure, the Pot/86 parent virus apparently replicated poorly in chickens; evidence of infection was only detected by presence of antibodies and not by detection of virus in respiratory or intestinal tracts. A similar resistance to infection has been reported following inoculation of chickens with

Table 1

<table>
<thead>
<tr>
<th>Viruses</th>
<th>i.v. pathogenicitya</th>
<th>i.n. pathogenicity and infectivityb</th>
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<tbody>
<tr>
<td></td>
<td>Morbidity (sick/total)</td>
<td>Mortality (dead/total)</td>
</tr>
<tr>
<td>Len17/H5</td>
<td>0/8</td>
<td>0/8</td>
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<tr>
<td>Len17</td>
<td>0/8</td>
<td>0/8</td>
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<tr>
<td>Pot/86</td>
<td>0/5</td>
<td>0/5</td>
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</table>

a Groups of eight chickens were infected i.v. with 0.2 ml 1:10 dilution of each virus and observed for 14 days for clinical signs and death.
b Groups of five chickens were infected i.n. with 0.1 ml of 10^6 EID50 of each virus. The oropharyngeal and cloacal swabs were collected 3 days p.i. and titrated in eggs for assessing viral replication. The chickens were observed for clinical signs of disease and death for 21 days. To determine infectivity, sera were collected 21 days p.i. and tested for the presence of antibodies by agar gel immunodiffusion (AGID) test.
viruses isolated from wild waterfowl [31]. Furthermore, reassortant Len17/H5 failed to replicate in chickens following simulated natural exposure; i.e. i.n. inoculation. These observations suggest that the use of the reassortant Len17/H5 in the manufacturing human vaccines will not pose a threat to the poultry industry.

3.2. Pathotyping and replication of Len17/H5 virus in mice

As shown in Table 2, reassortant Len17/H5 and two parent viruses were all non-lethal for mice (LD$_{50}$ > $10^7$ EID$_{50}$). Like the Len17 ca H2N2 donor strain, Len17/H5 virus had 10-fold higher MID$_{50}$ compared with the parent Pot/86 virus. Replication of the reassortant Len17/H5 virus in the upper and lower respiratory tract of mice was evaluated as a measure of attenuation. Mice were infected i.n. with $10^6$ EID$_{50}$ of each virus. Replication of the parent and reassortant viruses and the titers of virus present in the nose and lungs were determined 3 days p.i. The parental Pot/86 virus replicated efficiently in mouse lungs but poorly in the nose. In contrast, the Len17/H5 reassortant replicated well in the nose but poorly in the lungs, as did the Len17 ca strain for which virus was recovered from only one of three mouse lungs (titer = $10^{3.3}$ EID$_{50}$/ml). None of the viruses were detected in the brains of any infected mouse on day 6 p.i. (data not shown). These results indicated that reassortant Len17/H5 virus replicated predominantly in the upper respiratory tract and was attenuated in mice.

3.3. Immunogenicity and cross-reactive antibody responses of the reassortant Len17/H5 vaccine in mice

Next, we evaluated the immunogenicity of Len17/H5 inoculated i.n. as an LAIV at a single dose of 300 MID$_{50}$ or i.m. as an IIV (one dose of 10 µg whole virus) in mice. Six weeks after immunization, sera, lung and nasal washes were collected and tested for H5 virus-specific antibodies by microneutralization assay or ELISA [28,30]. As shown in Table 3, neutralizing antibodies against the homologous Pot/86 virus were detected in serum of mice receiving LAIV Len17/H5, but cross-reactive neutralizing antibodies against HPAI H5N1 HK/156 or HK/213 virus were not detected. However, substantial levels of H5N1 virus-specific serum IgG and respiratory tract IgA were detected by ELISA (Fig. 1). As expected, the Len17 ca H2N2 parent virus did not induce any detectable cross-reactive neutralizing antibodies against the H5 viruses, but a low level of serum IgG cross-reactive with H5 HA that was 20- to 100-fold less (p < 0.01) than the subtype-specific IgG response induced by Len17/H5 as a live or killed vaccine, respectively, was detected. Interestingly, the Len17 ca parent virus induced titers of H5-cross-reactive nasal IgA that were not significantly different to that induced by Len17/H5 LAIV suggesting that the local IgA response was generally more subtype-cross-reactive than the serum IgG antibody response (Fig. 1). When used as a formalin-inactivated vaccine, Len17/H5 elicited similar neutralizing antibody titers (160 and 80, respectively) to the homologous Pot/86 virus and antigenically related HK/156 influenza vaccines.
was evaluated in mice challenged with H5N1 virus in mice infected i.n. with 50 LD_{50} of HP HK/483. Eight mice per group were monitored daily for weight loss and death for 14 days. The remaining mice in each group were euthanized on day 6 p.i. to determine the levels of viral replication in the lower (lung) and upper (nose) respiratory tract, brain, and thymus. Day 6 was chosen to evaluate cross-protection because naive mice were shown previously to have substantial titers of HK/483 virus in lung and nose, and have peak of viral replication in brain and thymus at this time point [32]. As shown in Table 4, all unvaccinated control mice that received PBS died 5–9 days after a challenge with HK/483, having a mean maximum weight loss of 22% and high titers of virus in the lung, nose, brain, and thymus on day 6 p.i. In contrast, mice that were inoculated i.n. with the wild-type parental Pot/86 virus exhibited no disease signs over the entire experimental period and no virus was detected in any organ on day 6 p.i. Mice receiving the *ca* parent Len17 (H2N2) virus showed severe disease with a mean maximum weight loss of 19%, but demonstrated a modest increase in survival compared with the unvaccinated group. Consistent with this observation was a modest, but not significant reduction in HK/483 lung viral titers in these mice. On the other hand, viral titers in the upper respiratory tract, brain and thymus were significantly lower in mice that received the parent Len17 (H2N2) virus compared with those that received PBS only. Similar heterosubtypic protection against H5N1 viruses has been observed previously [33]. In contrast, all mice receiving the Len17/H5 LAIV survived a lethal challenge with HP HK/483 virus, but exhibited mild disease as measured by a modest weight loss observed between day 3 and 5 p.i. (data not shown). Only low titers of virus were detected in lungs of two of five Len17/H5 LAIV vaccinated mice (10^{2.3} and 10^{2.5} EID_{50}/ml) on day 6 p.i., and no virus was detected in any other organs tested, indicating that these mice were effectively protected from the HP HK/483 challenge. When delivered as an IIV Len17/H5 protected seven of eight mice from lethal HK/483 virus disease, although the mice experienced modest weight loss. While no virus was detected in the lungs or thymus of mice vaccinated with Len17/H5 IIV, low titers of virus were isolated from the nose of one of five mice (10^{1.6} EID_{50}/ml), and the brains of two of five mice (10^{1.6} and 10^{1.3} EID_{50}/ml) on day 6 p.i.

In a second experiment, five to ten mice in each vaccine group were challenged i.n. with 100 MID_{50} of HK/213 2003 virus and viral lung titers on day 3 p.i. were determined. Mice administered only PBS had high titers of virus in the lungs on day 3 p.i. The lung viral titers in the Len17-immunized mice were slightly lower than those of unvaccinated PBS mice but the difference was not significant. As observed with the HK/483 challenge, no virus was detected in the lungs of any

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**Fig. 1.** Anti-HK/156 HA-specific antibody responses in H5 vaccine immunized mice. Mice were infected i.n. with one dose of 300 MID_{50} of Len17/H5 LAIV or injected i.m. with one dose of 10 µg of Len17/H5 IIV. Two groups of mice were infected i.n. with either 300 MID_{50} of Pot/86 wild-type or 100 MID_{50} of HK/213 virus as positive controls. Mice received PBS as a negative control. Serum (A), lung (B), and nasal washes (C) were collected 6 weeks after vaccination or infection and were tested by ELISA for the presence of IgG and IgA antibody using a purified HK/156 recombinant HA protein as an antigen. Values are the mean (log_{10}) + S.D. of reciprocal end-point titers of five mice per group. *p < 0.05 compared with Len17/H5 IIV group or †p < 0.05 compared with Len17/H5 LAIV group.

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3.4. Cross-protective efficacy of the reassortant Len17/H5 vaccine in mice

The protective efficacy of Len17/H5 as an LAIV or IIV was evaluated in mice challenged with H5N1 viruses isolated from humans in Hong Kong in 1997 (HK/483) and 2003 (HK/213) (Table 4). HK/483 was chosen to represent the 1997 H5N1 viruses as it was previously shown to be highly lethal for naive BALB/c mice [26]; the antigenically variant H5N1 virus, HK/213, was not lethal for mice but replicated to high titers in mouse lungs. In the first experiment, groups of vaccinated mice (n = 13) were infected i.n. with 50 LD_{50} of HP HK/483. Eight mice per group were monitored daily for weight loss and death for 14 days. The remaining mice in each group were euthanized on day 6 p.i. to determine the levels of viral replication in the lower (lung) and upper (nose) respiratory tract, brain, and thymus. Day 6 was chosen to evaluate cross-protection because naive mice were shown previously to have substantial titers of HK/483 virus in lung and nose, and have peak of viral replication in brain and thymus at this time point [32]. As shown in Table 4, all unvaccinated control mice that received PBS died 5–9 days after a challenge with HK/483, having a mean maximum weight loss of 22% and high titers of virus in the lung, nose, brain, and thymus on day 6 p.i. In contrast, mice that were inoculated i.n. with the wild-type parental Pot/86 virus exhibited no disease signs over the entire experimental period and no virus was detected in any organ on day 6 p.i. Mice receiving the *ca* parent Len17 (H2N2) virus showed severe disease with a mean maximum weight loss of 19%, but demonstrated a modest increase in survival compared with the unvaccinated group. Consistent with this observation was a modest, but not significant reduction in HK/483 lung viral titers in these mice. On the other hand, viral titers in the upper respiratory tract, brain and thymus were significantly lower in mice that received the parent Len17 (H2N2) virus compared with those that received PBS only. Similar heterosubtypic protection against H5N1 viruses has been observed previously [33]. In contrast, all mice receiving the Len17/H5 LAIV survived a lethal challenge with HP HK/483 virus, but exhibited mild disease as measured by a modest weight loss observed between day 3 and 5 p.i. (data not shown). Only low titers of virus were detected in lungs of two of five Len17/H5 LAIV vaccinated mice (10^{2.3} and 10^{2.5} EID_{50}/ml) on day 6 p.i., and no virus was detected in any other organs tested, indicating that these mice were effectively protected from the HP HK/483 challenge. When delivered as an IIV Len17/H5 protected seven of eight mice from lethal HK/483 virus disease, although the mice experienced modest weight loss. While no virus was detected in the lungs or thymus of mice vaccinated with Len17/H5 IIV, low titers of virus were isolated from the nose of one of five mice (10^{1.6} EID_{50}/ml), and the brains of two of five mice (10^{1.6} and 10^{1.3} EID_{50}/ml) on day 6 p.i.

In a second experiment, five to ten mice in each vaccine group were challenged i.n. with 100 MID_{50} of HK/213 2003 virus and viral lung titers on day 3 p.i. were determined. Mice administered only PBS had high titers of virus in the lungs on day 3 p.i. The lung viral titers in the Len17-immunized mice were slightly lower than those of unvaccinated PBS mice but the difference was not significant. As observed with the HK/483 challenge, no virus was detected in the lungs of any
or IIV elicited protective immunity in mice against related of Len17/H5 IIV induced better cross-reactive serum neu-
body responses in the respiratory tract, whereas a single dose 
evaluated its utility as an IIV. As an LAIV, a single dose 
eggs that are desirable for the production of IIV, we also 
also possessed the high-growth properties in embryonated 
donor strain Len17. Because the Len17/H5 vaccine candidate 
generated from a non-pathogenic H5N2 strain, antigenically 
genicity and efficacy of a 7:1 reassortant H5 LAIV candidate 
In this proof of concept study, we evaluated the immuno-
interim, a vaccine that is an imperfect antigenic match may 
6 months after the identification of a pandemic strain. In the 
results demonstrated that one dose of Len17/H5 administered 
as either an LAIV or IIV provided substantial protection from 
fection, severe illness and death following challenge with the 
HP HK/483 virus. Additionally, either vaccine protected 
mice against replication of the antigenically variant HK/213 virus.

4. Discussion

A vaccine that is a close antigenic match with the circul-
ating pandemic strain is optimal for the control of pandemic 
fluenza, but such a vaccine may not be available for at least 
6 months after the identification of a pandemic strain. In the 
interim, a vaccine that is an imperfect antigenic match may 
still be useful in protecting from severe disease or death. 
In this proof of concept study, we evaluated the immuno-
genicity and efficacy of a 7:1 reassortant H5 LAIV candidate 
generated from a non-pathogenic H5N2 strain, antigenically 
similar to the 1997 H5N1 viruses, and the Russian ca master 
donor strain Len17. Because the Len17/H5 vaccine candidate 
also possessed the high-growth properties in embryonated 
eggs that are desirable for the production of IIV, we also 
evaluated its utility as an IIV. As an LAIV, a single dose 
of Len17/H5 induced superior H5 virus-specific IgA anti-
body responses in the respiratory tract, whereas a single dose 
of Len17/H5 IIV induced better cross-reactive serum neu-
ralizing and IgG antibody responses to HK/156 virus HA. 
Surprisingly, a single dose administered either as an LAIV 
or IIV elicited protective immunity in mice against related 
and antigenically variant H5N1 viruses. The use of a non-
pathogenic H5 virus to generate the Len17/H5 vaccine strain by traditional reassortment methods may be an advantage in countries that have limited containment laboratory capacity 
or access to patented recombinant DNA technology required 
to derive vaccine strains from HPAI H5 viruses. Furthermore, 
the lack of virus replication or induction of virus-specific anti-
body in chickens inoculated with Len17/H5, suggests that the 
large-scale manufacturing of a non-pathogenic H5 reassor-
tant vaccine strain would not pose any threat to the poultry 
industry.

LAIV against H5N1 viruses were first developed using 
reverse genetics technology to modify the HA from the HP 
H5N1 strains isolated from humans in Hong Kong in 1997 
[15]. Two 6:2 reassortants were generated containing modified 
HA genes, lacking the multibasic amino acid cleavage 
site associated with virulence in chickens, the wild type neu-
raminidase (NA) genes from HK/156 and HK/483, and the 
site associated with virulence in chickens, the wild type neu-
raminidase (NA) genes from HK/156 and HK/483, and the 

### Table 4

<table>
<thead>
<tr>
<th>Vaccine group (route)</th>
<th>Challenge with HK/483&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Challenge with HK/213&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum weight loss (%)</td>
<td>Number of death/total number</td>
</tr>
<tr>
<td>Len17/H5 (i.n.)</td>
<td>6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0/8</td>
</tr>
<tr>
<td>Len17/H5 (i.m.)</td>
<td>7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1/8</td>
</tr>
<tr>
<td>Pot/86 (i.n.)</td>
<td>0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0/8</td>
</tr>
<tr>
<td>Len17 (i.n.)</td>
<td>19</td>
<td>6/8</td>
</tr>
<tr>
<td>PBS (i.n.)</td>
<td>22</td>
<td>8/8</td>
</tr>
</tbody>
</table>

<sup>a</sup> BALB/c mice were infected i.n. with one dose of 300 MID<sub>50</sub> of LAIV or injected i.m. with one dose of 10 μg of Len17/H5 IIV. Mice were infected i.n. with 300 MID<sub>50</sub> of Pot/86 wild-type virus as a positive control or received PBS as a negative control.

<sup>b</sup> Mice (n = 13/group) were challenged i.n. 6 weeks later with 50 LD<sub>50</sub> (=1000 MID<sub>50</sub>) of HK/483 virus and eight mice per group were observed daily for weight loss and death for 14 days. Virus titers were determined on day 6 p.i. and represent means log<sub>10</sub>EID<sub>50</sub> ± S.D. of five mice per group. The limit of virus detection was 10<sup>1.5</sup> EID<sub>50</sub>/ml for lungs and 10<sup>0.8</sup> EID<sub>50</sub>/ml for other organs. Tissues in which no virus was detected were given a value of 10<sup>1.5</sup> EID<sub>50</sub>/ml (lung) or 10<sup>0.8</sup> EID<sub>50</sub>/ml (other tissues) for calculation of the mean titer.

<sup>c</sup> Mice (n = 5–10/group) were challenged i.n. 6 weeks later with 100 MID<sub>50</sub> of HK/213 virus. Mean lung virus titers and protection from infection were determined on day 3 p.i. Titers represent mean log<sub>10</sub>EID<sub>50</sub> ± S.D. of five mice per group. The limit of virus detection was 10<sup>1.5</sup> EID<sub>50</sub>/ml for lungs.

<sup>d</sup> p < 0.01 compared with PBS group.
used in this study. Nevertheless, the Len17/H5 vaccines provided effective protection against H5N1 virus induced lethality, severe disease and virus replication. As an LAIV, the Len17/H5 reassortant induced effective protection of mice against a lethal challenge with HK/483 virus, severe illness as measured by weight loss, and reduced lung viral titers by five logs at a time point where unvaccinated control mice succumbed to the lethal infection. At this critical time point, no virus was detected in the upper respiratory tract or in systemic tissues of mice administered Len17/H5 LAIV. The lack of virus in the nose was associated with significant titers of H5-specific IgA in nasal washes. In fact, Len17/H5 LAIV induced nasal and lung wash IgA titers that were comparable to those induced by infection with wild-type Pot/86 or HPAI HK/213 virus, whereas Len17/H5 IIV did not induce respiratory tract IgA responses. In contrast, serum neutralizing and IgG antibody against HK/156 were four-fold higher in mice that received Len17/H5 IIV, compared with those that received LAIV. These results may help explain the complete lack of detectable virus in lungs of mice that received IIV on day 6 p.i. Therefore, although Len17/H5 LAIV or IIV induced optimal responses in different antibody compartments, both vaccines provided substantial cross-protection following challenges with 1997 and 2003 human H5N1 viruses. In a separate study, the Len17/H5 reassortant was shown to provide protection from lethal challenge with recent HP A/Vietnam/1203/2004 (H5N1) virus [34]. While the Len17/H5 reassortant was shown to be immunogenic in ferrets [23], the extent to which it may replicate in and be immunogenic for humans remains unknown.

The NA genes of both parents used for preparation of Len17/H5 were of the N2 subtype. It would require additional effort to select a 6:2 reassortant carrying the NA gene from the wild type parent strain. Because the time can be limited if an urgent preparation of a pandemic reassortant is needed, we studied the 7:1 reassortant vaccine that inherited the NA gene from the ca Len17 parent. Our results have shown that an antigenically related NA was not essential for the protective effect against virulent H5N1 viruses in mice. However, other studies have demonstrated a role for NA-specific antibody in reducing the magnitude of disease in humans [35] or in protecting mice from a lethal challenge with a mouse-adapted human influenza virus [36]. While a LAIV reassortant that possesses both HA and NA related to the circulating pandemic strain is desirable, it may not be appropriate for the N1 NA subtype since some N1 gene products have been shown to enhance trypsin-independent cleavage of the HA molecule [37,38] and thus could, potentially, lessen the attenuation of a live vaccine.

The use of LAIV in a pandemic situation has been considered previously. The generation of a cold-adapted influenza A H9N2 reassortant vaccine strain using classical reassortment techniques has been described [39] and clinical evaluation of such a candidate is ongoing. An important consideration in the use of a live-attenuated vaccine in the event of a pandemic is the potential for reassortment of the vaccine strain with the circulating strain bearing a novel HA. Therefore, LAIV may be best used in a pandemic situation only when the population faces imminent widespread disease due to the novel wild-type pandemic strain.

The results of this study suggest a novel pandemic vaccine strategy that would allow for the stockpiling of an IIV that could be deployed immediately a pandemic strain had been identified. This would presumably be before widespread circulation of the virus, and certainly before a vaccine based on an exact antigenic match is available. If the pandemic strain became established in the population, the use of an LAIV generated from the same seed stock would extend the vaccine availability. An LAIV may have an added advantage of reducing viral shedding from the upper respiratory tract which may be important for reducing transmission in a highly susceptible, immunologically naive population [40]. These results suggest a general strategy of using classical genetic reassortment between a high-growth ca H2N2 strain and antigenically related non-pathogenic avian viruses to prepare live-attenuated and inactivated vaccines against multiple influenza A subtypes with pandemic potential.

References


