An open-label phase I trial of a live attenuated H2N2 influenza virus vaccine in healthy adults

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Background  Live attenuated influenza vaccines (LAIV) against a variety of strains of pandemic potential are being developed and tested. We describe the results of an open-label phase I trial of a live attenuated H2N2 virus vaccine.

Objectives  To evaluate the safety, infectivity, and immunogenicity of a live attenuated H2N2 influenza virus vaccine.

Participants/methods  The A/Ann Arbor/6/60 (H2N2) virus used in this study is the attenuated, cold-adapted, temperature-sensitive strain that provides the genetic backbone of seasonal LAIV (MedImmune). We evaluated the safety, infectivity, and immunogenicity of two doses of 10⁷ TCID₅₀ of this vaccine administered by nasal spray 4 weeks apart to normal healthy seronegative adults.

Results  Twenty-one participants received a first dose of the vaccine; 18 participants received a second dose. No serious adverse events occurred during the trial. The most common adverse events after vaccination were headache and musculoskeletal pain. The vaccine was restricted in replication: 24% and 17% had virus detectable by culture or rRT-PCR after the first and second dose, respectively. Antibody responses to the vaccine were also restricted: 24% of participants developed an antibody response as measured by either hemagglutination-inhibition assay (10%), or ELISA for H2 HA-specific serum IgG (24%) or IgA (16%) after either one or two doses. None of the participants had a neutralizing antibody response. Vaccine-specific IgG-secreting cells as measured by enzyme-linked immunospot increased from a mean of 0 to 0.5 x 10⁶ peripheral blood mononuclear cells (PBMCs); vaccine-specific IgA-secreting cells increased from 0 to 0.5 x 10⁶ PBMCs.

Conclusions  The live attenuated H2N2 1960 AA ca vaccine demonstrated a safety profile consistent with seasonal trivalent LAIV but was restricted in replication and minimally immunogenic in healthy seronegative adults.

Keywords  H2N2, human, influenza, influenza vaccine, live attenuated influenza vaccines, neuraminidase inhibition.

Introduction

Influenza A viruses cause periodic pandemics in addition to the yearly outbreaks and epidemics. H1N1, H2N2, and H3N2 influenza viruses entered the human population and led to pandemics in the 20th century; the novel 2009 H1N1 influenza virus is the first pandemic influenza virus of the 21st century.¹² The H1N1 2009 pandemic, as well as repeated introductions of avian influenza strains into human populations, underscore the importance of developing vaccines for influenza viruses of pandemic potential.

H2N2 influenza viruses emerged in the 1950s through reassortment of the circulating H1N1 human influenza virus with an avian H2N2 virus.³⁴ The first H2N2 influenza cases occurred in China in 1956, and the disease became widespread in 1957–1958, resulting in the ‘Asian Influenza’ pandemic that was responsible for more than one million deaths worldwide.³⁵ H2N2 viruses have not circulated in humans since 1968, when they were replaced by H3N2 influenza viruses, and consequently, a large proportion of the population is now susceptible to infection with H2 influenza viruses.

H2N2 viruses continue to circulate in birds, leading to the potential reintroduction of these viruses into susceptible human populations. There was a small, unsustained outbreak of H2N2 in Leningrad in 1980.⁷ In 2004–2005,
influenza A H2N2 virus (A/Japan/305/57) was inadvertently included in an influenza proficiency testing panel sent to 3748 laboratories in 18 countries.8,9 There were no cases of infection reported in persons who handled the proficiency panel. Given the waning immunity to H2N2 in the human population as the proportion of individuals born after 1968 increases, the development of a vaccine against this subtype that is a proven cause of illness and death in humans is an important priority in the development of a library of vaccines against potential pandemic strains of influenza.

In 1967, Maassab10 attenuated an A/Ann Arbor/6/60 H2N2 influenza virus strain by serial passage at successively lower temperatures in specific pathogen-free primary chick kidney cells (23 passages followed by seven plaque purifications) followed by amplification in embryonated eggs (three passages). The resulting virus was shown to be attenuated (att) in ferrets, cold adapted (ca), and temperature sensitive (ts). The virus was evaluated in 13 human subjects who had low to moderate levels of pre-existing antibody to A/AA/6/60 influenza.11 A dose of $3 \times 10^6$ TCID$_{50}$ of the virus was immunogenic in seven of eight individuals with low levels of pre-existing antibody.

The A/Ann Arbor/6/60 Master Donor Virus (MDV-A) (H2N2 1960 AA ca) provides the genetic backbone [i.e. genes encoding proteins other than the hemagglutinin (HA) and neuraminidase (NA) proteins] for seasonal live attenuated influenza vaccines (LAIV). As such, its genetic backbone has been combined with many different human H1 and H3 HA genes and human N1 and N2 NA genes to produce reassortant LAIV strains that have been extensively evaluated in support of licensure in several countries for protection against seasonal and pandemic influenza.12–14 In addition, we have prepared and evaluated several reassortant vaccine viruses bearing avian HA and NA genes and the internal protein genes of the H2N2 1960 AA ca virus, including candidate vaccines for H9, H5, and H7 influenza. In clinical trials, these vaccines were similar to seasonal LAIV in safety and were attenuated but varied in their capacity to induce antibody responses.15–18 Although there is extensive clinical experience with reassortant vaccine strains containing the A/Ann Arbor/6/60 backbone, the H2N2 1960 AA ca virus itself has been subjected to limited clinical testing. Here, we report the phase I evaluation of the safety, infectivity, and immunogenicity of the H2N2 1960 AA ca virus in H2-naïve adults.

**Participants, materials and methods**

**Vaccine virus**

H2N2 1960 AA ca is a live attenuated, cold-adapted, temperature-sensitive influenza virus derived from the A/Ann Arbor/6/60 ca (H2N2) Master Donor Virus (MedImmune, Mountain View, CA, USA) that contains all eight gene segments from the MDV-A virus. The HA and NA from this vaccine share 97.5% and 98.1% amino acid homology with the HA and NA of the 1957 pandemic virus, A/Japan/57 (H2N2).

The H2N2 1960 AA ca vaccine virus was manufactured at MedImmune using plasmid-based reverse genetics as previously described.15–17 The bulk virus was subjected to characterization, and lot release testing, including virus titration, was formulated with sucrose phosphate buffer plus the stabilizing agent gelatin-arginine-glutamate (SP + GAG) and was filled into Accuspray™ devices (Becton-Dickinson, Franklin Lakes, NJ, USA) at a volume of 0.2 ml per sprayer. Filled sprayers were stored frozen at –60°C or below.

**Study population**

This phase 1 clinical trial was conducted during the summer of 2008 at the Center for Immunization Research (CIR) outpatient clinic and at the CIR isolation unit at the Johns Hopkins Bayview Medical Center as previously described.15,16 The clinical protocol was reviewed and approved by the Western Institutional Review Board (WIRB). Informed written consent was obtained from each participant. Healthy adult men and non-pregnant women between 18 and 39 years of age who were seronegative to H2N2 viruses were enrolled in the clinical trial if they met eligibility criteria and were willing to remain on the isolation unit for the duration of the inpatient portion of the trial. Key exclusion criteria included immunosuppression, a history of anaphylaxis, Guillain-Barre Syndrome, recent asthma, significant systemic disease, previous receipt of a LAIV or an H2N2 vaccine, a positive test for HIV, Hepatitis B or C, current narcotic use, allergy to egg, and recent receipt of another vaccine.

**Study design**

This study was conducted as an open-label phase 1 inpatient trial with all participants receiving vaccine. Participants were screened to establish health status with a thorough medical history, physical examination, and laboratory work including hematology, chemistries, urine, and serological tests for HIV, Hepatitis B, and C. If eligible, participants were given vaccine as a nasal spray using the Accuspray™ (Becton-Dickinson) device and examined daily while on the isolation unit by a health care provider (physician or physician’s assistant). Four weeks after the first admission, participants were re-admitted to the isolation unit and received a second dose of vaccine. Participants were isolated for 12 days (from days –2 to 9 after vaccination) for each dose of the vaccine.

The isolation unit, study design, and study procedures have been previously described.15–17 Participants were discharged...
from the isolation unit on study day 9 if rRT-PCR assays for influenza virus were negative on study days 7 and 8.

**Isolation, quantitation, and identification of the H2N2 virus**
Nasal washes were obtained prior to vaccination and then daily from the day of vaccination until the day of discharge. Specimens were tested for the presence of vaccine virus by quantitative viral culture and by rRT-PCR amplification of a portion of the influenza A M2 gene. The limit of detection of vaccine virus was 50 copies/ml.

**Immunologic assays**

**Hemagglutination inhibition and microneutralization**
Sera were tested for hemagglutination-inhibiting (HI) antibodies to H2N2 as previously described. The sera were tested for neutralizing antibodies using a modified version of a previously described microneutralization assay. Our assay differed from the previously published microneutralization assay as previously described. In addition, our test virus was not the wild-type A/AA/6/60 H2N2 influenza virus, but rather the vaccine virus, H2N2 1960 AA ca. HI and MN were performed at baseline and day 28 after each vaccination.

**ELISA**
Sera were also tested for IgA and IgG antibody to the H2 HA by ELISA. Immulon 2 plates were coated with 30 μg/well of recombinant baculovirus-expressed H2 HA (from the H2N2 1960 AA ca vaccine virus) produced in insect cells (Protein Sciences, Meriden, CT, USA), and the ELISA was performed using endpoint titration. Nasal wash specimens were concentrated and were tested using the same antigen to measure vaccine-specific IgA, expressed as a percent of total IgA, as previously described. ELISA for IgG and IgA was performed at baseline and day 28 after each vaccination.

**Antibody-secreting cells**
Ten milliliters of heparinized whole blood was diluted with 30 ml of Hank’s Balanced Salt Solution (Invitrogen, Grand Island, NY, USA), and peripheral blood mononuclear cell (PBMCs) were isolated using Ficoll gradient centrifugation followed by re-suspension in RPMI 1640 (containing 10% fetal bovine serum, L-glutamine and 1 μg penicillin/streptomycin). Total and influenza vaccine-specific IgG- and IgA antibody-secreting cells (ASCs) were measured using an enzyme-linked immunospot (ELISPOT) assay based on an assay by Sasaki, modified as previously described. For enumeration of total and influenza vaccine-specific ASCs, 3–33 × 10⁶ cells and 1 × 10⁹ PBMC, respectively, were added to each well. Briefly, our assay differed from the published assay in which the wells were coated with betampropiolactone (BPL)-treated H2N2 1960 AA ca virus stock diluted to 5000 HA/ml in Dulbecco’s phosphate-buffered saline (D-PBS; Invitrogen) or purified goat anti-human IgA plus IgG plus IgM (Kierkegaard & Perry Laboratories, Gaithersburg, MD, USA) at a concentration of 5 μg/ml in D-PBS. PBS alone and human CCRC-FEM cells (ATCC, Manassas, VA, USA) were used as negative controls; human IM9 cells (ATCC) were used as a positive control. Plate images were recorded and counted using ImmunSpot 4 software (Cellular Technologies, Ltd., Shaker Heights, OH, USA). Human IgA ASCs were visualized as red spots, and IgG ASC were visualized as blue spots. The number of vaccine-specific IgG and IgA ASC was expressed as the percentage of total IgG and IgA ASC. ELISPOT for ASCs was performed at baseline and day 7 after each vaccination.

**Neuraminidase inhibition assay**
NA-specific antibodies were measured by a previously described miniaturized neuraminidase inhibition (NAI) assay. Measurement of NAI titers requires the use of a virus with an irrelevant hemagglutinin so that anti-hemagglutinin antibodies do not interfere with the detection of anti-NA antibodies. Therefore, two H6N2 reassortant viruses were generated, containing the HA from an A/Teal/W312/HK/97 (H6N1) virus and the N2 NA of the A/Uruguay/716/2007 (H3N2) virus that was representative of viruses circulating at the time of the clinical trial or the N2 NA of the H2N2 1960 AA ca virus. The A/Uruguay/716/2007 (H3N2) strain was the A/Brisbane 10/2007-like H3N2 component of the seasonal LAIV in 2008–2009. Briefly, the NA activity of each virus was standardized by colorimetric analysis of sialic acid released from the substrate fetuin. NAI activity in the sera was determined by comparing the NA activity of the virus alone with the activity measured following incubation of the virus with serially diluted sera. The dilution of serum that resulted in a 50% reduction in NA activity of the virus without serum was recorded as the NAI titer.

**Rhinovirus and enterovirus rRT-PCR**
RNA was extracted from nasal washes using the Nuclisens MiniMAG system (bioMerieux, Hazelwood, MO, USA). rRT-PCR was used to detect rhinovirus, and a modified version of a previously published rRT-PCR assay was used to detect enterovirus.

**Data analysis**
Infection after immunization with the H2N2 1960 AA ca vaccine virus was defined as: (i) shedding of vaccine virus detected by culture and/or (ii) shedding of vaccine virus detected by rRT-PCR any time after study day 1 and/or (iii) a ≥fourfold rise in serum HI antibody, neutralizing
antibody, or H2-specific IgG or IgA serum antibodies as measured by ELISA. Participants whose nasal washes were rRT-PCR positive on study day 1 but had no other evidence of infection were not considered infected because we could not exclude the possibility that input virus, rather than replicating virus, was being detected. To calculate mean titers, HI antibody, neutralizing antibody, and ELISA reciprocal titers were log2 transformed.

**Results**

**Study participants**

Fifty-nine potential participants were screened for the H2N2 1960 AA ca virus vaccine trial, and 21 eligible individuals were enrolled and vaccinated in August 2008. The age of the participants ranged from 20 to 39 years (mean age 28.8 years, SD 4.6); 18 were Black and 11 were men. Eighteen of the 21 participants received a second dose of vaccine 4 weeks later. Three participants chose not to return for the second dose for personal reasons unrelated to the vaccine, but they returned for their days 28 and 61 follow-up visits. One subject who received both doses left the country prior to her day 28 follow-up visit after the vaccine 4 weeks later. Three participants chose not to return for the second dose for personal reasons unrelated to the vaccine, but they returned for their days 28 and 61 follow-up visits. One subject who received both doses left the country prior to her day 28 follow-up visit after the second dose. No participant withdrew because of vaccine-related events.

**Reactogenicity**

The vaccine was generally well tolerated with the exception of a few reported minor illnesses. After the first dose, five participants complained of headache, the most commonly reported symptom. One of these subject also complained of sore throat and nasal congestion on day 2 (Table 1). Vaccine virus was detected by rRT-PCR in two of the participants who reported headache, including the subject with complaints of sore throat and nasal congestion. A middle ear effusion (without evidence of otitis media) was detected in a subject who had reported decreased hearing in the left ear, but this finding was not associated with the detection of vaccine virus by culture or rRT-PCR in nasal wash specimens. Other illnesses reported following the first dose of vaccine included nausea and vomiting (two individuals); and localized musculoskeletal complaints including backache (three individuals), shoulder pain (two individuals), and muscle soreness (two individuals). The detection of vaccine virus by culture or rRT-PCR in the remaining symptomatic participants did not coincide with symptoms. Adventitious viruses were not detected by culture or rRT-PCR in nasal washes obtained from any participants after the first dose.

Following the second dose of vaccine, one subject reported headache, and one additional subject described having an ‘itchy’ throat that did not meet criteria for a diagnosis of pharyngitis (Table 1). Vaccine virus was not detected in either subject. Two participants reported gastrointestinal symptoms, one of whom had moderate (grade 2) nausea, vomiting, and diarrhea for several days starting on day 2. Of note, a staff member had similar gastrointestinal symptoms preceding the participants’ symptoms. The

| Subjects | No. of subjects | % Infected* | Virus detection (culture) (nasal wash) | Virus detection (rRT-PCR) (nasal wash) | Symptoms reported |
|----------|----------------|-------------|----------------------------------------|----------------------------------------|------------------|
|          |                |             | No. shedding virus**                  | No. pos. on day 1                      | No. pos. after day 1 | Duration of shedding mean days | No. pos. on day 1 | No. pos. after day 1 | Duration of shedding mean days | Fever | URI | LRI | Headache | Reactogenicity event† |
| First dose | 21             | 29          | 4                                       | 8                                      | 2                 | 2.3                        | 0             | 1             | 0             | 5         | 5         |
| Second dose | 33             | 0           | 0                                       | 0                                      | 0                 | 0                         | 0             | 0             | 0             | 1         | 1         |

No., number; pos., positive; URI, upper respiratory tract symptoms include rhinorrhea, nasal congestion, cough, pharyngitis; LRI, lower respiratory tract symptoms include pneumonia, wheezing, rhonchi

*Infection is defined as shedding of vaccine virus and/or a fourfold or greater rise in HI antibody titer or in serum microneutralization or ELISA response.

**Number shedding virus are those volunteers who had virus recoverable by culture.

***Viral titers are expressed as log10 TCID50/ml. Geometric Mean peak titers were calculated for all infected subjects for the first dose. Peak titers of 0.6 TCID50/ml were assigned to culture-negative samples.

*Calculated for all infected subjects. One subject had virus recovered days 1 and 2, two subjects had virus cultured day 2 only, and one subject had culturable virus days 2–6.

†Reactogenicity events are defined as fever, nasal congestion, rhinorrhea, pharyngitis, cough, otitis media, pneumonia, headache, myalgia, chills, conjunctivitis, wheezing, rhonchi and epistaxis. Transient abnormalities in blood pressure, pulse and respiratory rate are not included here, nor are adverse events that were thought to be unrelated or not likely to be related.
Antibody responses

None of the participants had a detectable HI response following the first dose of vaccine. Two of the participants (20%) who received two doses of vaccine had fourfold rises in HI titer, three (14%) following the first dose, and three on days 1 and 2 (Table 1). All five participants who had vaccine virus recovered after the first dose also had detectable virus after the first dose.

Vaccine virus replication

The H2N2 1960 AA vaccine virus was restricted in replication in the respiratory tract. Again, three participants had musculoskeletal complaints, only one of whom had virus detected by RT-PCR on days 1 and 2. One subject reported back pain after the first dose, and one subject was positive by rRT-PCR for virus recovery on days 1 and 12. The subject reported back pain from day 2 through 6 (one individual). Vaccine virus was not detected in any of the participants (Table 2). Five participants had fourfold rises in HI titer, three (14%) following the first dose, and three on days 1 and 2 (Table 1). All five participants who had vaccine virus recovered after the second dose only and three on days 1 and 2 (Table 1). All five participants had a fourfold or greater rise in HI antibody titer or in serum microneutralization or ELISA response. Those with a gradual rise of antibody responses over the two doses were designated as infected after dose 2.

Table 2. Antibody responses following two 10^7 TCID50 doses of H2N2 1960 AA ca

| Subjects   | No. of subjects | % Infected* | % With fourfold rise | % With fourfold rise | % With fourfold rise | % With fourfold rise | % With fourfold rise | % With fourfold rise |
|------------|-----------------|-------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
|            |                 |             | HI                   | Serum HA-IgG ELISA   | Serum HA-IgA ELISA   | Nasal wash HA-IgA ELISA |
| First dose | 21              | 29          | 1:1 (0-4) 12 (04) 0  | 2.3 (0) 2.3 (0) 0    | 94 (20) 95 (15) 14*** | 55 (1) 57 (1) 10     |
| Second dose| 17†             | 33          | 1:2 (0-4) 16 (09) 12† | 2.3 (0) 2.4 (0) 2    | 96 (16) 99 (17) 11   | 56 (1) 56 (1) 6      |
| Any dose   | 21              | 38          | 1:1 (0-4) 16 (09) 10 | 2.3 (0) 2.4 (0) 2    | 94 (20) 97 (18) 24   | 55 (1) 57 (1) 16     |

No., number; SD, standard deviation; HI, hemagglutination-inhibiting; Microneut, microneutralizing; Ab, antibody.
*Infection is defined as shedding of vaccine virus by culture on any day and/or by rRT-PCR after day 1 and/or a fourfold or greater rise in HI antibody titer or in serum microneutralization or ELISA response. Those with a gradual rise of antibody responses over the two doses were designated as infected after dose 2.
**Post-values drawn on day 26 for dose 1, day 29 for dose 2.
***One subject who received only one dose had a delayed response by ELISA with the rise in titer occurring during the day 56 visit.
†Data is available on 17 subjects for the second dose. One subject did not return for the follow-up visit.
‡One subject had a gradual rise of antibody response, with a twofold increase after each dose. 
The development of vaccine-specific ASCs was also extremely limited. When measured prior to immunization, six participants had detectable vaccine-specific IgG ASCs, all ≤3 cells/10⁶ PBMCs. Seven days after the first vaccination, the mean (SD) number of vaccine-specific IgG ASCs was 2.0 (3.2), and four of 21 participants had an increase of five or more specific IgG ASCs. By the time of the second dose, vaccine-specific IgG ASCs were nearly undetectable: two more specific IgG ASCs. By the time of the second dose, vaccine-specific IgG ASCs were nearly undetectable: two participants each had one vaccine-specific IgG-secreting cell/10⁶ PBMCs, and specific ASCs were not detected in any of the other participants. Seven days after the second vaccination, the mean (SD) number of vaccine-specific IgG ASCs was 1.2 (2.6-10⁶ PBMCs, and only two of 18 participants (one of whom had also responded to the first dose) had an increase of five or more vaccine-specific IgG ASCs/10⁶ PBMCs. Vaccine-specific IgA responses were of lower magnitude than vaccine-specific IgG responses: none of the participants had an increase of ≥5 vaccine-specific IgA ASCs either after the first or second dose of vaccine (Table 3).

As immunological responses to the vaccine were limited, NAI assays were carried out to see whether pre-existing NA antibodies blocked vaccine replication and immunogenicity (Table 3). Only 1/21 subjects had pre-existing NAI antibodies to the vaccine NA prior to vaccination, and one subject developed a fourfold response to the vaccine NA. In contrast, 18/21 subjects had pre-existing NAI titers to the seasonal N2 NA (Geometric mean titer 1:216; Standard error 36.6), and four subjects had modest increases in titer against this seasonal NA after vaccination; three of the four subjects went from having undetectable to detectable NAI antibodies to the seasonal N2 NA. None of the subjects with a response to the seasonal N2 NA showed a fourfold response to the vaccine N2 NA (Table 3).

Discussion

We have reported results of an open-label phase I study of a pandemic H2N2 influenza A virus vaccine using the H2N2 1960 AA ca virus strain. This vaccine demonstrated a safety profile consistent with current seasonal trivalent LAIV but was restricted in replication and minimally immunogenic in immunologically naive healthy young adults who were born after H2N2 viruses stopped circulating in the human population.

One of the difficulties in evaluating vaccines for novel influenza strains is determining the correlates of vaccine-induced immunity. For seasonal inactivated influenza virus vaccines, the correlate of protection accepted by regulatory agencies is an HI titer of ≥1:40, measured in a standard assay using 4 U of HA. However, seasonal LAIV have been shown to protect against natural infection or challenge with野型 influenza viruses even when HI titers ≥1:40 were not achieved, presumably through the

Table 3. Antibody secreting cells and NAI responses following two 10⁷ TCID₅₀ doses of H2N2 1960 AA ca

| Subjects | No. of | IgG ASC to vaccine virus | IgA ASC to vaccine virus | NAI assay titer (SD) | NAI against A/Uruguay/716.2007 | % With ≥2-fold rise post*** | % With ≥5 cell rise Pre Post*** |
|----------|--------|---------------------------|--------------------------|--------------------|-----------------|-----------------------------|-------------------------------|
| First dose | 21     | 0 (0.9)                   | 0.09 (0.9)               | 0.05 (0.9)         | 0.05 (0.9)      | 0.05 (0.9)                  | 0.05 (0.9)                    |
| Second dose | 17     | 0.0 (0.9)                 | 0.02 (0.9)               | 0.01 (0.9)         | 0.01 (0.9)      | 0.01 (0.9)                  | 0.01 (0.9)                    |
| Any dose  | 21     | 0.03 (0.9)                | 0.03 (0.9)               | 0.02 (0.9)         | 0.02 (0.9)      | 0.02 (0.9)                  | 0.02 (0.9)                    |

Note: *SD, standard deviation; ASC, antibody secreting cell; NAI, neuraminidase inhibition assay. **Post-values for ASC drawn on day 7 after each vaccination. ***Post-values drawn on day 26 for dose 1, day 29 for dose 2.

** Data is available on 17 subjects for the second dose. 1 subject did not return for the follow-up visit.
induction of local mucosal antibody and cellular immunity. Moreover, HI antibody may not be the best measure of protection against infection with H5 and H7 avian influenza viruses, as individuals infected with these viruses often fail to develop substantial HI antibody responses. For this reason, alternative antibody assays, such as the microneutralization assay and ELISA, have been developed.

To assess the antibody response to the H2N2 1960 AA ca vaccine, we used several assays in addition to the standard HI and microneutralization assays. Using the HI assay, we found that only 12% of participants had a 2-fold rise in HI antibody titer after two doses of vaccine, with modest increases achieved (Table 1). We also measured neutralizing antibody, H2-specific IgG and IgA antibody, and production of ASCs against the vaccine virus. Although the ability of each assay to detect a response differed slightly, minimal responses to the vaccine were detected by all assays (Tables 2, 3). Interestingly, unlike other pandemic live attenuated vaccines that have been assessed (H5N1, H7N3), serum IgA was not the most sensitive indicator of an immune response (Table 2). Additionally, measurement of vaccine-specific ASCs detected minimal responses to either dose of the vaccine (Table 3), which is quite different from what was previously observed in children who received seasonal LAIV or in adults administered a live attenuated H7N3 vaccine.

The substantial restriction in replication and poor antibody response to the H2N2 1960 AA ca vaccine were somewhat unexpected because the vaccine was shown to be infectious and immunogenic in mice and ferrets in preclinical studies. Also, our study participants were born after 1968 and should have been susceptible to H2N2 influenza virus infection. However, because of the extensive passage history of the seasonal LAIV MDV in chicken kidney cells and eggs, it is possible that this virus became less infectious for humans. In support of this concept, a previous study of a cold-adapted, temperature-sensitive, attenuated A/Ann Arbor/6/60 (H2N2) virus that had undergone less extensive passage in chicken cells was found to be immunogenic in seven of nine individuals when administered at a 30-fold lower dose (3 × 10^5 TCID50) in 1973. It should be noted that in both the 1973 study and the present study, virus replication could not be detected following nasal administration. There are several possible explanations for these observations. Anti-NA antibodies can restrict the release and spread of influenza viruses. Although only one subject had anti-NA antibody to the vaccine NA, most subjects (18/21) had pre-existing antibody to seasonal NA (Table 3). Therefore, it is possible that pre-existing antibodies against the seasonal NA may have limited the replication and consequently the immunogenicity of the attenuated vaccine virus. However, the NA of the seasonal A/Uruguay/716/2007 H3N2 virus and the NA of the 1960 AA ca vaccine virus share only 84% homology at the amino acid level, and the NAI response was not cross-reactive (Table 3). It appears that the MDV containing this particular H2 and N2 is inherently less infectious and immunogenic than the H1N1 and H3N2 LAIV that have been evaluated in multiple studies. It is also possible that the H2N2 1960 AA ca virus might induce protective immunity in the absence of HI or neutralizing antibodies, as it protected mice against homologous viral challenge after one dose in the absence of a detectable antibodies to the homologous virus and protected mice and ferrets against heterologous viral challenge in the absence of detectable antibodies to heterologous H2N2 viruses. It may be that the vaccine induces immune responses that were not measured in preclinical or clinical studies, including cellular immune responses as has been demonstrated with trivalent seasonal formulations of LAIV. Such responses should be evaluated in future studies.

In summary, we have shown that two doses of a live attenuated cold-adapted H2N2 1960 AA ca vaccine resulted in a low incidence of side effects and were modestly immunogenic when given to healthy adults. Contemporary H2 and N2 genes obtained from low passage human virus isolates should be tested on the same backbone to study the human immune response to a pandemic H2N2 LAIV.

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**Addendum**

K. R. Talaat contributed to protocol development, study conduct, data analysis, and manuscript writing; R. A. Karron involved in the study development and manuscript editing; P. H. Liang contributed to the study conduct, data collection, entry and analysis, and manuscript drafting; B. A. McMahon contributed to the source documents, study logistics, data collection, entry and analysis, and manuscript editing; C. J. Luke contributed to the study design, protocol.
and regulatory document development, and manuscript writing; B. Thumar oversaw laboratory assays, designed assays, and edited the manuscript; G. L. Chen, J.-Y. Min, and E. W. Lamirande involved in assay design and performance and manuscript editing; H. Jin, K. L. Coelingh, G. W. Kemble contributed to the study design, protocol and regulatory document input, and manuscript editing; K. Subbarao contributed to the study design, protocol and regulatory document input, and manuscript writing.

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