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Safety and immunogenicity of a replication-deficient H5N1 influenza virus vaccine lacking NS1

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ABSTRACT

Background: Traditional inactivated influenza vaccines are the type of vaccines that were most frequently developed for immunization against the highly pathogenic avian H5N1 influenza virus. However, clinical trials with inactivated influenza vaccines for H5N1 indicated that high doses and at least two immunizations are required for an effective immune response (Nicholson et al., 2001; Treanor, Campbell et al., 2006; Treanor, Schiff et al., 2006; Ehrlich et al., 2008). We investigated the safety and immunogenicity of a live attenuated H5N1 vaccine (delNS1-H5N1) lacking the interferon antagonist nonstructural protein 1 (NS1).
Methods: We conducted a double-blind, placebo-controlled, phase 1 study in healthy adult participants who were randomly assigned at a 2:1 ratio to receive two immunizations of delNS1-H5N1 vaccine at 6.8 log10 50% tissue culture infectious doses ($TCID_{50}$)/subject or 7.5 log10 $TCID_{50}$/subject, or placebo.
Results: Intranasal vaccination with the live attenuated delNS1-H5N1 vaccine was safe and well tolerated. The most common adverse events identified were symptoms associated with mild influenza infections, such as increased body temperature (>37.0 °C), pharyngeal erythema, rhinitis and throat irritation, and were reported within 7 days after the first immunization. delNS1-H5N1 was able to induce significant vaccine-specific serum antibody titers even at the lower dose level of 6.8 log10 $TCID_{50}$/subject. Seroconversion occurred in 75% of study participants after only one immunization with 7.5 log10 $TCID_{50}$/subject. Vaccine-specific local IgA responses were observed in 41.7% of individuals that showed serum antibody responses after 2nd immunization.
Conclusions: We show that vaccination with a live attenuated H5N1 influenza vaccine lacking NS1 is safe and induces significant levels of vaccine-specific antibodies even after one immunization. The safety and immunogenicity data indicate that delNS1-H5N1 has the potential to fulfil the unmet need for an effective influenza vaccine in pandemic situations. (ClinicalTrials.gov identifier NCT03745274).

1. Introduction

Avian influenza is a highly contagious disease caused by infections with influenza A viruses that primarily circulate in domestic and wild birds but can also infect humans. Avian influenza has continued to occur since the first recorded direct bird-to-human transmission of a highly pathogenic avian influenza A(H5N1) virus in Hong Kong in 1997 [2]. Although isolated human-to-human transmission has been reported in several countries and H5N1 continuously evolves, creating new potentially pathogenic drift variants [3], these viruses have not yet acquired efficient transmissibility among humans [4–6]. The number of human infections with H5N1 has been declining over the past years [7], however, the high pathogenicity of the virus, the observed high mortality of infected patients, and the unprecedented spread of H5N1 viruses have raised concerns that conditions are developing for the generation of a new pandemic virus.

In recent years, live attenuated influenza vaccines (LAIVs) have become increasingly attractive, following the WHO's recognition of LAIVs to increase the production capacity for pandemic vaccines [8]. LAIVs are attractive pandemic vaccine candidates because they mimic natural infection by replicating primarily in the upper respiratory tract and inducing mucosal IgA antibody responses [9–11]. LAIV technologies using vaccine strains have the potential to

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reassort with circulating influenza viruses, which must be considered. A LAIV that is replication-deficient would mitigate the potential for reassortment while offering the advantages of the LAIV approach.

We have developed a novel type of live attenuated influenza vaccine by deleting the interferon antagonist NS1 from influenza virus IVR-116. The 5 internal genes from IVR-116 were co-transfected with the HA, NA and M gene from the pandemic H5N1 virus (A/Vietnam/1203/04), resulting in a live attenuated vaccine virus termed delNS1-H5N1.

Here, we report the safety and immunogenicity after one and two immunizations of a live attenuated delNS1-H5N1 influenza vaccine in healthy male and female humans.

2. Material and methods

2.1. Study design

We performed a randomized, double-blind, placebo-controlled phase 1 study in healthy males and females aged 18–50 to assess the safety and immunogenicity of a monovalent delNS1-H5N1 vaccine candidate containing an A/Vietnam/1203/04(H5N1) delNS1 reassortant. On study days 1 and 29, all participants received one intranasal dose of the monovalent delNS1-H5N1 vaccine candidate at one of two dose levels, or placebo.

delNS1-H5N1 was escalated according to a fixed dose-escalation scheme comprising two dose levels, 6.8 and 7.5 log10 $TCID_{50}$/volunteer. Cohorts of eighteen healthy volunteers per dose level were randomized at a ratio of 2:1 for delNS1-H5N1 or placebo. The volunteers in both cohorts were hospitalized for 7 days after the vaccine was first administered. After discharge on day 7, study participants were asked to record all symptoms and concomitant medications on a diary card which was reviewed and transferred to the case report form (CRF) at the participant's next follow-up visits (day 29 [day of the second vaccination], day 35 and 57 [end of study]). A data safety monitoring board (DSMB) decided upon administration of the second dose and escalation to the higher dose level in a two-step procedure: (i) if the first vaccination of 6.8 log10 was tolerated, participants received the second vaccination or placebo and the study was escalated to the high dose level, (ii) if the first vaccination of 7.5 log10 was tolerated, participants received the second vaccination or placebo.

Participants, the investigator and personnel involved in any study-related analysis were fully blinded. A study nurse responsible for blinding vaccine and placebo for administration was not blinded. This study nurse was not involved in any other study related procedures. The study was conducted at a single center located at the Research Institute of Influenza, Russian Academy of Medical Sciences, St. Petersburg, Russia, between 19 December 2008 and 27 May 2009.

The primary objective of this study was to evaluate the safety and tolerability of two intranasal doses of delNS1-H5N1. Secondary objectives included the analysis of local and systemic immune responses as well as shedding of delNS1-H5N1 vaccine virus.

The study was conducted in compliance with Good Clinical Practice guidelines and the Declaration of Helsinki. It was approved by the responsible independent ethics committee (Ethics Committee under the Federal Service for the Surveillance in Health Care and Social Development of Russian Federation, Russia) and by the Russian regulatory authority.

2.2. Vaccine and placebo

delNS1-H5N1 was generated by reverse genetics as described elsewhere [12–14]. The vaccine candidate lacks the complete NS1 open reading frame and was rescued from cDNA clones by co-transfection of plasmids encoding the hemagglutinin, neuraminidase and matrix protein of influenza A/Vietnam/1203/04 (H5N1), whereas the remaining 5 gene segments are from the influenza virus IVR-116, a reassortant with HA and NA genes from influenza A/New Caledonia/20/99(H1N1), the PB1 gene from A/Texas/1/77(H3N2), and all other genes from the A/Puerto Rico/8/34 (H1N1)(PR8) virus (WHO) [15]. The HA cleavage site of the seed virus was genetically modified to remove the highly pathogenic trait of the H5N1 virus. As a result delNS1-H5N1 was a non-pathogenic 5:3 reassortant virus. The vaccine was produced in Vero cells under serum-free conditions following good manufacturing practice (GMP). The virus harvest was purified in two chromatographic steps and formulated with sucrose/phosphate/glutamate/sodium chloride as a stabilizer. Two dose levels containing 6.8 or 7.5 log10 50% tissue culture infectious doses ($TCID_{50}$) of the monovalent H5N1 reassortant vaccine strain were produced (batch numbers: G070/LC1/070417 and G070/LC1/070523, AVIR Green Hills Biotechnology). The placebo consisted of the formulation buffer (batch number: G070/PL/070417, AVIR Green Hills Biotechnology). Both vaccine and placebo were stored frozen at ≤ -60 °C and transferred into a nasal spray device (St. Gobain, Germany) before intranasal administration. A total of 520 μl of vaccine or placebo was administered into both nostrils of each participant (260 μl/nostril) at each vaccination.

2.3. Study population

After signing an informed consent form, healthy male and females aged 18–50 years were prescreened for titers of antibodies to A/Vietnam/1203/04(H5N1) and A/Brisbane/59/2007(H1N1) virus by hemagglutination-inhibition (HAI) assay. Only healthy volunteers with antibody titers of <1:10 for H5N1 and with titers $\leq$1:20 for influenza A/H1N1 were invited for further screening procedures. Individuals with signs of acute febrile illness (>37.0 °C); acute or chronic upper- or lower-tract respiratory illness (i.e., sneezing, cough, tonsillitis, otitis media); with a medical history of severe atopy, leukemia or cancer; with rhinosurgery within 30 days before immunization; or with seasonal influenza vaccination in 2006/2007 and/or later seasons were excluded. Individuals who had been treated with immunosuppressive drugs, who received antiviral drugs, immunoglobulins or blood transfusions within 4 weeks before immunization, or were seropositive for HIV, hepatitis B or C were also excluded from the clinical study. Females of childbearing potential were only included with negative pregnancy testing prior to immunization. Written informed consent was provided by all participants. The study participants were randomly assigned 2:1 to one of two cohorts (two immunizations of delNS1-H5N1 vaccine at 6.8 log10 $TCID_{50}$/subject or 7.5 log10 $TCID_{50}$/subject or placebo) with the help of a computer-generated randomization schedule.

2.4. Safety

Adverse events (AE) were collected from the day of the first vaccination until study day 57. Serious adverse events were spontaneously collected from day of enrolment until 30 days after the end of the study. The investigator assessed AEs for relationship to study medication based on the causality assessment of the WHO guideline "Adverse Events Following Immunization" (AEFI). After participants were discharged on day 7 after the first immunization, they were asked to record all clinical symptoms, events and concomitant medications in a diary card until study day 57. After discharge healthy volunteers were observed in an outpatient setting at the Research Institute of Influenza. Volunteers presented for follow-up visits on day 29, day 35 and day 57 (end of study). On day 29 the second vaccination was administered. Each day

between day 29 and day 35 volunteers were contacted by telephone to assess their state of health.

2.5. Nasal sample collection

Nasal swabs were collected from each participant 24 h before immunization, and at 24, 48 and 72 h, and study days 4, and 29 after the first immunization, day 35 (6 days after the second vaccination) and day 57, using cotton swabs that were placed into the inferior turbinates of each nostril at the study site. The participants were instructed to gently massage the nose and occasionally rotate the swab for 5–10 min. Both swabs were pooled and placed in 2 ml elution solution (0.1% Tween20, 1% antibiotic/antimycotic mix, 0.1% BSA, 1% protease inhibitor in sterile PBS) in specific specimen collection tubes containing two chambers (Salivette®, Sarstedt) and stored at 2–8 °C for 1 h. The swabs were then vortexed, transferred to the upper chamber of the collection tube and centrifuged at 4 °C in a bench centrifuge. Nasal samples were transferred to Salivette® tubes and stored frozen at −70 °C until analyses.

2.6. Virus recovery

Viable vaccine virus in nasal swab samples obtained 24, 48 and 72 h after the first immunization was determined by limiting dilutions in Vero cells. Serial 10-fold dilutions of nasal swab samples were passaged in Vero cells cultivated at 37 °C and 5% CO2 in Opti-PRO™ medium (Invitrogen) supplemented with 5 mg/ml porcine trypsin (Sigma) and 1% antibiotics mix (penicillin and streptomycin). After 3–5 days of incubation infectious virus titers in $TCID_{50}$/ml were determined and calculated according to Reed and Muench [16]. The lower limit of detection was 1.5 log10 $TCID_{50}$.

2.7. Measurement of immune response

Induction of local and systemic immune responses was evaluated in nasal or serum samples taken prior to vaccination (day 1) and on study days 29, 35 (only mucosal IgA) and 57. The level of neutralizing antibodies compared to baseline was determined by microneutralization assay (MNA) with NIBRG-14, a reassortant prepared by reverse genetics from A/Vietnam/1194/2004 (H5N1) virus (in which the polybasic HA cleavage site has been excised) and A/Puerto Rico/8/34(H1N1) virus according to standard procedures [17].

To perform the MNA, serial twofold dilutions of receptor-destroying enzyme (RDE, Denka-Seiken, Japan) pre-treated sera were prepared in 96-well microtiter plates (Nunc) with 1% antibiotic mix and 50 ml of a standardized viral suspension (NIBRG-14, 100 $TCID_{50}$/50 μl) were added to each well. After an incubation period of 2 h at 37 °C, the MDCK cells were added. The plates were incubated for 48 h, washed and acetone fixed. An influenza A virus NP-specific monoclonal antibody conjugated with horseradish-peroxidase (HRP, 0.125 mg/ml) diluted in a blocking buffer (PBS containing 5% skim milk powder) was added for 1 h. The plates were washed and the substrate (TMB, Sigma) was added. The reaction was stopped with 2 N H2SO4. The average absorption at 450 nm (A450) was determined for the control wells of virus-infected (VC) and uninfected (CC) cells and the neutralizing endpoint (NEP) was determined by using a 50% specific signal calculation.

$$NEP = \frac{[(average\ A450\ of\ VC\ wells) - (average\ A450\ of\ CC\ wells)]}{2} + (average\ A450\ of\ CC\ wells)$$

The hemagglutination inhibition (HAI) antibodies were measured according to standard procedures with modifications. Two-fold serial dilutions (starting from 1:10) of 25 μl of RDE-inactivated sera in U-well microtiter plates were incubated for 45 min at room temperature (RT) with 50 μl of influenza NIBRG-14 virus adjusted to 4 hemagglutination units/50 μl. 100 μl of 0.5–0.6% horse RBCs (red blood cells) were added, and the reaction mixture was incubated for a further 45 min at RT. Wells were examined visually for inhibition of HA, as indicated by the appearance of well-defined RBC "buttons" or teardrop formation upon plate tilting. HAI titers were the reciprocal of the highest dilution of serum that completely prevented HA. HAI and MNA titers of less than 1:10 were rated as negative. Responders were defined as having a ≥4-fold increase in geometric mean titers (GMT) as compared to baseline.

Both the HAI and the MN assay were performed following international standards for influenza diagnosis as published by the WHO [18]. HAI and MN titers below the limit of detection (i.e. <10) were denoted as half of the threshold detection value (i.e. 5) for statistical calculations.

Secretory immunoglobulin A (sIgA) was evaluated by enzyme-linked immunosorbent assay (ELISA), with purified A/Vietnam/1203/04 (H5N1) hemagglutinin used as coating antigen (SinoBiological, China). 96-well Nunc MaxiSorp™ were coated with 0.5 mg/ml (100 ml/well) of the recombinant hemagglutinin of the A/VN/1203/04(H5N1) influenza virus coupled to mouse Ig Fc receptor part HA-mFc (Sinobiological Ltd, China) at 4 °C overnight. The plates were washed (PBS containing 0.1% Tween-20) and blocked with assay buffer (PBS containing 0.5% I-Block [Tropix] and 0.1% Tween-20). Serially twofold diluted nasal swab samples were added and incubated at RT for 1–2 h. On each plate, the reference standards for the respective target antibody, appropriately diluted in the assay buffer, were included. The standard curve for the assessment of H5-specific IgA was established by utilizing a pool of nasal swab samples (IgA reference standard; commercially not available) exhibiting a detectable signal that was determined in a preliminary endpoint ELISA. A 1:40 dilution of the IgA reference standard was defined as 100 arbitrary units (AU) of H5-specific IgA per ml. After washing, H5-specific IgA antibodies were detected with goat anti-ferret IgA conjugated with HRP (1.0 μg/ml; Zymed). The plates were incubated for 1 h, washed again, and Ultra TMB-ELISA substrate (Thermo) was added for secretory IgA ELISA (Aureon Biosystems). The luminescence signal in the sIgA ELISA was measured with a luminometer (Mediators PHL, Austria) after incubating the plates for 60 min in the dark. The concentration of sIgA in the individual samples was expressed in AU/ml based on the IgA reference standard calibration curve by the 4-parameter non-linear logistic curve fit (Gen5 software).

In the mucosal samples H5-specific sIgA antibodies were normalized. A standard quantitative ELISA was performed by using affinity purified goat anti-human IgA (1 μg/ml; Zymed) and commercially available human IgA from colostrum (Sigma). The IgA concentration in each sample was calculated based on the IgA reference standard curve by a 4-parameter non-linear logistic fit. A dilution of 1:40 of the IgA reference standard was defined as 100 AU of the total IgA/ml. The final normalized results were expressed in H5-specific AU/ml of the total IgA for each individual mucosal sample (μg/ml).

2.8. Statistical analyses

Statistical analyses were descriptive and exploratory in nature with no confirmatory proof of hypotheses. Analysis of Variance (ANOVA) was used to calculate increases in geometric mean titers with baseline values as covariate. Individual pairwise comparisons were calculated using the Tukey-Kramer multiple comparison test to control the error rate under any complete or partial null hypothesis. Fisher's exact or Chi-square tests were applied for comparing response rates between treatment groups. For these tests, p values

<0.05 were considered to indicate significance. All participants who were randomized and had received study medication were included in the full-analysis set. Participants who completed the study without major protocol violations were included in the per-protocol analysis set.

3. Results

91 female and male volunteers were screened for H5N1 A/Vietnam/1203/04 and H1N1-specific A/Brisbane/59/2007 antibody titers by HAI assay. Of these, 55 volunteers dropped out before vaccination (Fig. 1). All 36 participants eligible for the study were randomized in one of two cohorts. The male and female participants were almost equally distributed within the study (18 female and 18 male) and the cohorts (6.8log10: 6 males and 6 females, 7.5log10: 7 males and 5 females, placebo: 5 males and 7 females). Study medication was administered to all of the 36 participants. All of them completed the study according to protocol.

3.1. Safety

All immunized participants were included in the safety analyses. Intranasal vaccination with delNS1-H5N1 was well tolerated in both dose groups. No serious adverse events were observed. The proportions of most frequent symptoms reported during the first 7 days after the first and second vaccination are presented in Fig. 2.

Within 7 days after the first vaccination, adverse events were reported to a larger extent than after the second vaccination. The most frequent adverse events after the first vaccination were increased body temperature (>37.0 °C), pharyngeal erythema, rhinitis and throat irritation, and occurred to a similar extent in placebo- and delNS1-H5N1-treated individuals. Other AEs typically expected for live attenuated influenza vaccines such as coughing, myalgia or fever were noted only rarely (Fig. 2). Fever (temperature ≥ 38.0 °C) was observed in one participant treated with the first dose of 7.5 log10 $TCID_{50}$ delNS1-H5N1.

Within 7 days after the second vaccination, increased body temperature and rhinitis were the only adverse events observed, which involved only a few study participants treated with delNS1-H5N1 (2 and 1 subject, respectively) or placebo (2 subjects) (Fig. 2). There was no indication of a dose dependency with any of the adverse events observed.

3.2. Vaccine virus shedding

To confirm the replication-deficient phenotype of the vaccine virus, we analyzed nasal swabs collected 24, 48 and 72 h after the first immunization for the presence of vaccine virus by $TCID_{50}$ assay [16]. No vaccine virus was recovered from any sample at any time point.

3.3. Immune response

The HAI assay was used to detect serum antibody levels against influenza H5N1 A/Vietnam/1203/04 as contained in the vaccine. At both dose levels, the seroconversion rate was higher after two vaccinations (day 57) than after one vaccination (day 29) (Table 1). In the lower dose group using 6.8 log10 $TCID_{50}$, a significant increase was reached in 5 (42%) out of 12 subjects after the first immunization (HAI GMT increase from 5.0 to 11.9), and in 11 (92%) subjects after the second immunization (HAI GMT increase to 40.0) compared to placebo.

An at least 4-fold increase in antibody titers against H5N1 was detected in 9 (75%) out of 12 subjects treated with 7.5 log10 $TCID_{50}$ after the first immunization (HAI GMT increase from 5.3 to 28.3) and in 11 (92%) subjects after the second immunization (HAI GMT increase to 50.4) compared to placebo.

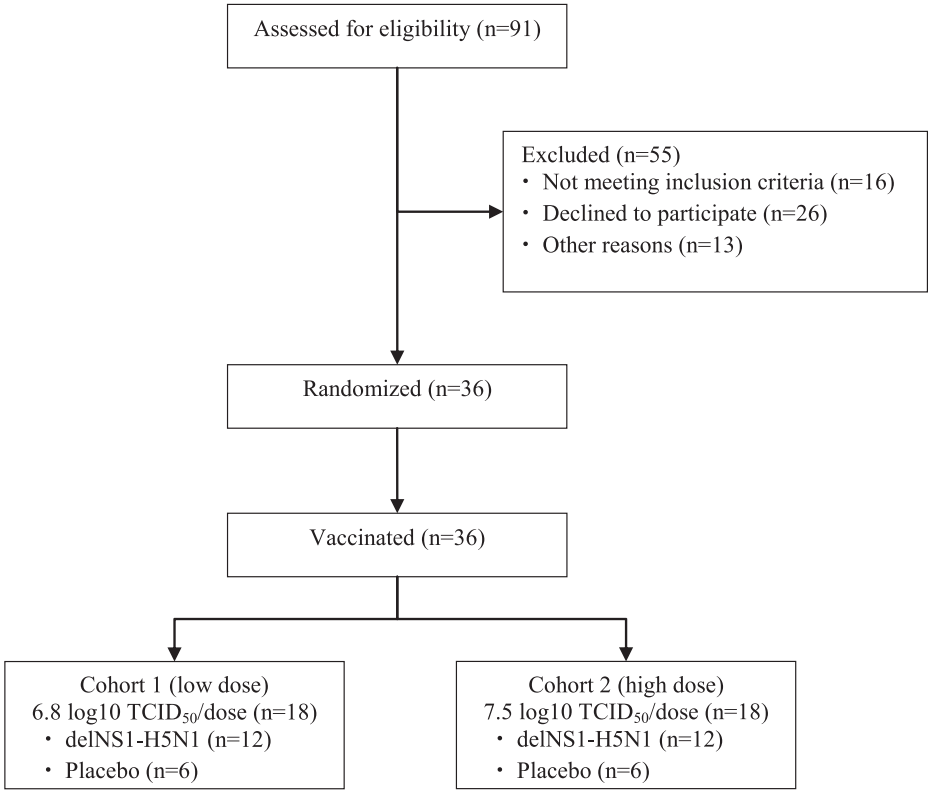

Fig. 1. Flow chart.

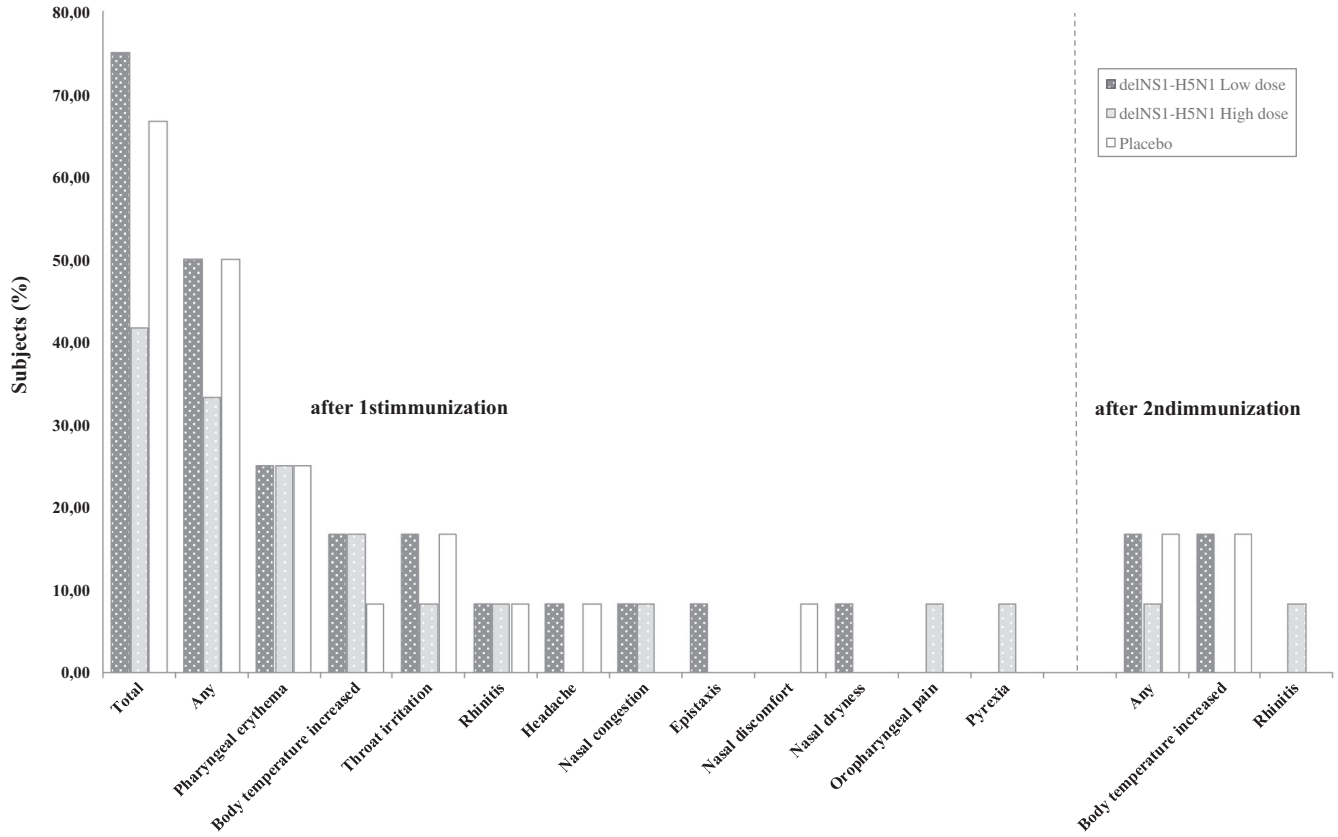

Fig. 2. Proportion of volunteers with Adverse Events within 7 days after first and second vaccination.

Overall, the number of responders was significantly different from placebo after the first and second immunization in both dose groups. The geometric mean titer increases were significantly different from placebo already after the first vaccination with the higher dose and further increased after the second immunization. At the lower dose level, geometric mean increases were significant after the second immunization (Table 1).

A 4-fold increase in neutralizing antibodies was detected in subjects vaccinated with 6.8 log10 $TCID_{50}$ in 6 (50%) of 12 subjects after the first immunization (MNA GMT increase from 7.5 to 31.8) and 10 (83%) subjects after the second immunization (MNA GMT increase to 80.0) (Table 1). In the higher dose group using 7.5 log10 $TCID_{50}$ an at least 4-fold increase in neutralization titers was detected in 4 (33%) of 12 participants after the first (MNA GMT increase from 5.6 to 12.6) and in 9 (75%) participants after the second immunization (MNA GMT increase to 33.6) (Table 1). Though a 4-fold increase was detected in a larger number of subjects vaccinated with 6.8 log10 $TCID_{50}$ this difference was not statistically significant compared to the response rate of the higher dose level after one ($p = 0.680$) or two immunizations ($p = 1.000$, Fisher's exact test).

Overall, the number of responders in the lower dose group was significantly different from placebo after the first and second immunizations while in the higher dose group the number of responders was significant after the second immunization. Geometric mean titer increases were significantly different from placebo for the lower dose group (Table 1).

Nasal secretion samples were analyzed for the induction of vaccine-specific sIgA normalized to total IgA (Table 2). Volunteers who experienced an $\geq$2-fold increase in IgA mucosal concentrations of antibodies against purified hemagglutinin derived from A/Vietnam/1203/04 virus were significant and classified as responders. After the first immunization, two (17%) of 12 subjects in the 7.5 log10 $TCID_{50}$ dose group showed a 2-fold or higher increase and were classified as responders. No subject treated with 6.8 log10 was classified as a responder. The increase in the geometric mean concentration was not statistically significant to the placebo group (Table 2). After the second dose the number of responders was significantly higher in both dose groups. An at least 2-fold increase in antibody concentration was observed in 5 (42%) subjects in both dose groups. The increases in the geometric mean concentration (GMC) were not significantly different from placebo (Table 2).

The number of responders in any assay of the four categories was dose-dependent after one vaccination, with 11 (91.7%) of 12 subjects in the high dose group and 8 (66.7%) of 12 subjects in the lower dose group. After two immunizations all (100%) subjects treated with one of the two dose levels responded to delNS1-H5N1 in at least one assay (Table 3). This was statistically significant ($p < 0.05$) after one and two immunizations as compared to placebo.

4. Discussion

This study provides the first safety and immunogenicity data in humans for an avian H5N1 live attenuated influenza vaccine based on the deletion of the NS1 gene. No serious adverse event was observed in study participants treated with the vaccine. The majority of adverse events reported are typical symptoms associated with influenza infections, such as respiratory illnesses, pharyngitis and rhinorrhea [19]. Within 7 days after the first vaccination, adverse events were reported to a larger extent than after the second vaccination, indicating that two doses of delNS1-H5N1 were able to sufficiently protect from typical symptoms induced by LAIVs. The most frequent symptoms (pharyngeal erythema, increased body temperature and throat irritation) were distributed

Table 1
Serum HAI and neutralizing antibody response after first and second immunization.

Treatment group	Hemagglutination inhibition						Microneutralization					
	First immunization			Second immunization			First immunization			Second immunization		
	Pre-, GMT (95%CI)	Post-, GMT (95%CI)	Increase	Responders[a] (%)	Post-, GMT (95%CI)	Increase	Responders[a] (%)	Pre-, GMT (95%CI)	Post-, GMT (95%CI)	Increase	Responders[a] (%)	Post-, GMT (95%CI)	Increase	Responders[a] (%)
6.8 log10TCID50 (*n* = 12)	5.00 (5.00–5.00)	11.89 (8.53–16.57)	2.38	5[c] (41.7)	40.00 (25.25–63.36)	8.00[b]	11[c] (91.7)	7.49 (5.04–11.14)	31.75 (15.59–64.64)	4.24[b]	6[c] (50.0)	80.00 (39.62–161.53)	10.68[b]	10[c] (83.3)[c]
7.5 log10TCID50 (*n* = 12)	5.30 (4.66–6.02)	28.28 (13.84–57.82)	5.34[b]	9[c] (75.0)	50.40 (26.78–94.83)	9.51[b]	11[c] (91.7)	5.61 (4.73–6.66)	12.60 (6.70–23.71)	2.24	4 (33.3)	33.64 (15.82–71.50)	5.99	9[c] (75.0)
Placebo (*n* = 12)	7.49 (4.08–13.75)	7.49 (4.08–13.75)	1.00	0 (0.0)	7.49 (4.08–13.75)	1.00	0 (0.0)	8.91 (4.12–19.25)	10.59 (5.06–22.17)	1.19	0 (0.0)	10.59 (4.53–24.77)	1.19	0 (0.0)

[a] Number of volunteers having a ≥4-fold increase from baseline.
[b] $p < 0.05$ for an increase from the baseline level to the post vaccination level compared with placebo by ANOVA followed by Tukey-Kramer multiple comparison test.
[c] $p < 0.05$ compared with placebo by Fisher's exact test.

to a similar extent in both vaccine cohorts and placebo. Most adverse events were of mild intensity. Other adverse reactions reported for LAIVs, such as fever, headache or coughing [20,21], were noted only rarely. The development of cardiovascular or respiratory disorders (chronic pulmonary diseases, asthma or wheezing) is a major concern of intranasally administered influenza vaccines. In clinical trials with a cold-adapted LAIV the risk of wheezing and asthma was increased in children younger than 2 years of age [22,23]. Though these safety signals were not observed with delNS1-influenza vaccine candidates [24,25], these potential risks needs to be further investigated in clinical trials with larger treatment groups.

Shedding of the vaccine virus is an important aspect for live vaccines in regard to potential reassortment with circulating influenza viruses or unintended transmission. Though LAIVs are attractive candidates for pandemic vaccines, there is a concern of reversion to virulence in pandemic situations. In our clinical study we confirm that delNS1-H5N1 possesses a replication-deficient phenotype. No vaccine virus was re-isolated in the nasal washings of any study participant immunized with delNS1-H5N1 vaccine. The replication-deficient phenotype of delNS1 viruses was previously confirmed in a phase 1 clinical trial with a monovalent delNS1-H1N1 vaccine [24] and a phase 1/2 trial with a trivalent delNS1 candidate [25]. We believe that delNS1-H5N1 might be an attractive option for prevention of a potentially pandemic disease, however a certain risk of a human-to-human transmission and subsequent reassortment with a current circulating seasonal influenza strain cannot be fully excluded. Importantly, since the delNS1 vaccine strain is replication-deficient, no shedding occurs, and no viral RNA is detectable post infection, the likelihood of reassortment is negligible. In the unlikely event that reassortment did take place, reassortant viruses usually are more attenuated as compared to the parental viruses [26–28]. This observation is also very likely in the case with potential H5-delNS1 reassortants, since in addition to the attenuating deletions in the NS1 gene and the H5 HA cleavage site, the other internal genes are derived from A/PR8 virus. The PR8 backbone was shown to be attenuated in humans [29], and PR8-based live vaccines were shown to be safe in several thousand volunteers in multiple clinical trials [29–31]. In summary, while it may not be recommended to use non-circulating pandemic delNS1 strains in a pre-pandemic setting, the concept of using pandemic delNS1 strains during the pandemic appears reasonably safe.

In the last decade, several vaccine manufacturers demonstrated that two doses of vaccine are generally necessary to elicit the level of immunity required to meet licensure criteria [1,32]. Additionally, results from studies with pandemic LAIVs based on cold-adapted strains suggest that this type of vaccine elicits only modest homologous HAI and neutralizing antibody responses even following two doses of 7.5 log10 $TCID_{50}$ administered by nasal spray [33–36]. However, we observed that two immunizations with delNS1-H5N1 were able to sufficiently induce HAI and MNA antibody titers even with a lower dose level of 6.8 log10 $TCID_{50}$. Importantly, seroconversion occurred after one immunization with 7.5 log10 $TCID_{50}$ in 75% of subjects vaccinated. There are several possible explanations why the delNS1-H5N1 candidate is able to seroconvert with a single dose in contrast to other live attenuated and inactivated influenza vaccines. Firstly, the delNS1-H5N1 lacks the entire open reading of the gene encoding NS1, which is an interferon-antagonist. Lacking the ability to counteract the interferon response, delNS1-based vaccines induce high levels of interferon, achieving a natural adjuvant effect that activates and enhances T- and B-cell mediated immune responses [12,37]. Secondly, we observe that mutations occurring after isolating and passaging viruses in eggs affect the stability and immunogenicity of the HA [38]. To avoid mutations that may affect stability or

Table 2
Secretory IgA antibody response after first and second immunization (ratio of H5-specific AU/ml to total IgA level).

	First immunization				Second immunization		
Treatment group	Pre-, GMC [AU/ml] (95%CI)	Post-, GMC [AU/ml] (95%CI)	Increase	Responders[a] (%)	Post-, GMC [AU/ml] (95%CI)	Increase	Responders[a] (%)
6.8 log10TCID50 *(n = 12)*	0.28 (0.16–0.49)	0.32 (0.19–0.54)	1.14	0 (0.0)	0.40 (0.28–0.58)	1.44	5 (41.7)
7.5 log10TCID50 *(n = 12)*	0.25 (0.15–0.43)	0.31 (0.20–0.48)	1.22	2 (17.0)	0.61 (0.25–1.51)	2.41	5 (41.7)
Placebo *(n = 12)*	0.32 (0.22–0.47)	0.25 (0.14–0.43)	0.77	0 (0.0)	0.30 (0.19–0.46)	0.93	0 (0.0)

[a] Number of volunteers having a ≥2-fold increase from baseline.

Table 3
Number of subjects with a response in any assay.

Treatment group	First immunization No. of subjects with any response (%)	Second immunization No. of subjects with any response (%)
6.8 log10TCID50 *(n = 12)*	8[a] (66.7)	12[a] (100.0)
7.5 log10TCID50 *(n = 12)*	11[a] (91.7)	12[a] (100.0)
Placebo *(n = 12)*	0 (0.0)	0 (0.0)

[a] $p < 0.05$ compared with placebo by Fisher's exact test.

immunogenicity, we use sequences derived from primary isolates for generating our vaccine strains. Finally, it should be noted that in the HAI assay we used horse erythrocytes, which show high sensitivity in this assay [39,40]. In addition to the dose level and number of immunizations required to protect from influenza infection, the issue of antibody persistence and responses to booster doses after several months is especially of interest for the development of pandemic influenza vaccines.

The induction of a vaccine virus-specific secretory IgA antibody response in nasal secretions is a major advantage of intranasal influenza virus vaccines that may contribute to protective immunity because they mimic natural infection by replicating primarily in the upper respiratory tract [9–11]. The increased local IgA response after two immunizations versus one immunization demonstrates that delNS1-H5N1-induced sIgA concentrations can be boosted with revaccination; however, the observed increases in sIgA were of moderate magnitude and did not provide statistically significant differences when compared to placebo. In our experience, the quantitative determination of a vaccine virus-specific sIgA antibody response in nasal secretions is a major challenge for nasally administered influenza vaccines because of the high variance in nasal secretion samples and mucosal antibody concentrations within the samples collected [25]. However, sIgA and serum antibody levels correlated, as sIgA responses were only observed in a portion of individuals that showed significant antibody responses in HAI and MNA after the second immunization with the low dose level and after the first and second immunization with 7.8 log10 $TCID_{50}$. Consistent with other studies, it is more likely that measured sIgA responses would be best classified as a relative co-correlate of protection [33].

There are some limitations to the performed clinical study. The number of study participants was limited and only naïve volunteers were included. The potential of delNS1-H5N1 to induce a systemic immune response after one immunization must be confirmed in future studies with larger study populations who are serosusceptible or seropositive to circulating seasonal influenza strains. Furthermore, in view of future pandemic situations, cross protection against viruses of different clades is an important factor for the development of effective pandemic vaccines and should be investigated in future clinical trials. In summary, this randomized, double-blind, placebo-controlled clinical study demonstrated for the first time that a pandemic influenza strain lacking the NS1 protein is safe and well tolerated, and is able to significantly induce serum antibody titers after one and two immunizations. This encouraging proof of concept provides a basis for future clinical studies including the evaluation of delayed boosting strategies and homologous and heterologous immunogenicity after priming and further immunizations.

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Declaration of interest

CN, FG, MW, JS and TM were employed at AVIR Green Hills Biotechnology. TM, JS and MW had stock and share options in the company at the period of this clinical trial. OK was acting as principal investigator for the clinical study and was employed at the Research Institute of Influenza, Russian Academy of Medical Sciences, St. Petersburg, Russia.

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