MAJOR ARTICLE

A Novel Type of Influenza Vaccine: Safety and Immunogenicity of Replication-Deficient Influenza Virus Created by Deletion of the Interferon Antagonist NS1

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Background. The nonstructural protein NS1 of influenza virus counteracts the interferon-mediated immune response of the host. By deleting the open reading frame of NS1, we have generated a novel type of influenza vaccine. We studied the safety and immunogenicity of an influenza strain lacking the NS1 gene (ΔNS1-H1N1) in healthy volunteers.

Methods. Healthy seronegative adult volunteers were randomized to receive either a single intranasal dose of the ΔNS1-H1N1 A/New Caledonia vaccine at 1 of 5 dose levels (6.4, 6.7, 7.0, 7.4, and 7.7 $\log_{10}$ median tissue culture infective dose) ($n = 36$ recipients) or placebo ($n = 12$ recipients).

Results. Intranasal vaccination with the replication-deficient ΔNS1-H1N1 vaccine was well tolerated. Rhinitis-like symptoms and headache were the most common adverse events identified during the 28-day observation period. Adverse events were similarly distributed between the treatment and placebo groups. Vaccine-specific local and serum antibodies were induced in a dose-dependent manner. In the highest dose group, vaccine-specific antibodies were detected in 10 of 12 volunteers. Importantly, the vaccine also induced neutralizing antibodies against heterologous drift variants.

Conclusions. We show that vaccination with an influenza virus strain lacking the viral interferon antagonist NS1 induces statistically significant levels of strain-specific and cross-neutralizing antibodies despite the highly attenuated replication-deficient phenotype. Further studies are warranted to determine whether these results translate into protection from influenza virus infection.

Trial registration. ClinicalTrials.gov identifier: NCT00724997.

Despite preventive efforts, influenza epidemics are responsible for substantial morbidity and mortality every year [1]. Even among people who are vaccinated, some may not be adequately protected by the vaccine [2–4]. We have developed a novel type of intranasal influenza vaccine by deleting the interferon antagonist NS1 from the virus [5, 6]. In several animal models, vaccine strains lacking the NS1 gene showed 2 properties that

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are highly desirable for safe and immunogenic vaccines. First, ΔNS1 viruses, although capable of infecting nasal epithelial cells and expressing viral antigens, fail to form viral progeny. Therefore, vaccinated animals do not shed vaccine virus. Second, because the ΔNS1 virus has lost its ability to counteract the interferon response of the host, it elicits high levels of interferon, which is known to promote strong B and T cell–mediated immune responses [7–13]. Animals immunized with ΔNS1 strains belonging to the influenza virus A and B subtypes are protected against wild-type virus challenge [7, 14, 15]. We performed, to our knowledge, the first proof-of-concept study assessing the safety, virus shedding, and immunogenicity of a ΔNS1-based vaccine strain in humans.

PARTICIPANTS, MATERIALS, AND METHODS

Vaccine and placebo. The vaccine seed virus, ΔNS1-H1N1, was generated by reverse genetics as described elsewhere, with modifications [6, 14, 16, 17]. ΔNS1-H1N1 contains the surface glycoproteins from A/NC/20/99, whereas the remaining gene segments are from the influenza virus strain IVR-116 (World Health Organization) [18]. In addition, ΔNS1-H1N1 lacks the complete NS1 open reading frame.

The vaccine was produced under good manufacturing practice conditions in Vero cells cultured under serum-free conditions. The harvest was subjected to 2 consecutive chromatographic purification steps that yielded a highly purified virus formulated in a sucrose-phosphate-glutamate stabilizing buffer. The stabilizing buffer was given as placebo. Both vaccine and placebo were stored at −70°C or below and transferred into the nasal spray device (Baby Nasal GPI spray pump; Erich Pfeiffer; Drug Master File no. 6350; dose volume accuracy [tested with water], ±15% per stroke [mean, ±10%]).

Study design and objectives. This was a randomized, double-blind, placebo-controlled, dose-escalation study of the effects of single-dose intranasal administration of a ΔNS1-H1N1 vaccine in healthy, seronegative volunteers. After signing an initial informed consent form, healthy male volunteers aged 18–50 years were prescreened for titers of antibodies against A/NC/20/99 virus by hemagglutination-inhibition (HAI) assay. Only healthy volunteers with antibody titers of <1:10 were invited for further screening procedures. These volunteers signed a second informed consent form that covered further study procedures, and those who met all eligibility criteria were included in the study. Healthy volunteers were allocated to treatment groups by means of concealed envelopes, according to a computer-generated randomization list. Independent study nurses dispensed either active treatment or placebo. On day 1, volunteers received the study medication by intranasal aerosol application. Adverse events and pharmacokinetic analyses were closely monitored in an inpatient setting for 48 h. After discharge on day 3, healthy volunteers were observed in an outpatient setting by means of follow-up visits on days 4, 5, 8, 15, and 29. During the outpatient period, volunteers were instructed to record all symptoms and medication taken on a diary card. Volunteers who experienced a temperature of >38.0°C were asked to contact the study site for evaluation.

ΔNS1-H1N1 was escalated according to a fixed dose-escalation scheme comprising 5 dose levels. Cohorts of 8 healthy volunteers per dose level were randomized at a 6:2 ratio to receive either ΔNS1-H1N1 at 6.4, 6.7, 7.0, 7.4, and 7.7 $\log_{10}$ median tissue culture infective dose ($TCID_{50}$) per volunteer or placebo. A further 8 volunteers were randomized at a 6:2 ratio to receive the highest dose. The volunteers in each cohort were observed for 1 week after the vaccine was administered. An expert committee then performed an interim safety review. If the committee judged that the dose level had been tolerated, the next step of the dose escalation was performed.

The primary objective of this study was to evaluate the safety and tolerability of ΔNS1-H1N1 administered as single-dose intranasal aerosol for vaccination against influenza A(H1N1) virus. Secondary objectives included the analysis of local and systemic immune responses as well as shedding of ΔNS1-H1N1.

The protocol was approved by the ethics committee at the Department of Clinical Pharmacology, Medical University Vienna, Austria, and was conducted in compliance with good clinical practice guidelines and the Declaration of Helsinki.

Nasal washings. To collect nasal wash samples, a urinary catheter with the tip cut off was placed in the volunteer's nostrils and locked by cuffing. The nasal cavity was then washed 3 times with 6 mL of sucrose-phosphate-glutamate virus-stabilizing buffer.

Vaccine virus recovery. To recover the vaccine virus, 1.5 mL aliquots of nasal washings obtained 12, 24, 48, and 72 h after immunization were analyzed for the presence of viable vaccine virus. Samples were diluted 1:1 and used as inoculum on Vero cells. After 3–5 days of incubation, presence or absence of the cytopathic effect was determined, and positive results were confirmed by immunofluorescence specific for the influenza A nucleoprotein. Recovered viruses were characterized for the absence of the NS1 gene by polymerase chain reaction.

Immunological assays. Analysis of immune responses was performed on serum and nasal wash samples obtained prior to vaccination and on day 29. The increase in the geometric mean titer (GMT) of homologous neutralizing antibodies, compared with the baseline GMT, was assessed by microneutralization assay (MNA) according to standard procedures [19] with A/NC/20/99 wild-type virus. The number of responders, who were defined as having a ⩾4-fold increase in antibody titer, was evaluated.

Serum samples from the highest dose group and from the placebo group were additionally tested by MNA according to standard procedures [19], using reassortant viruses containing

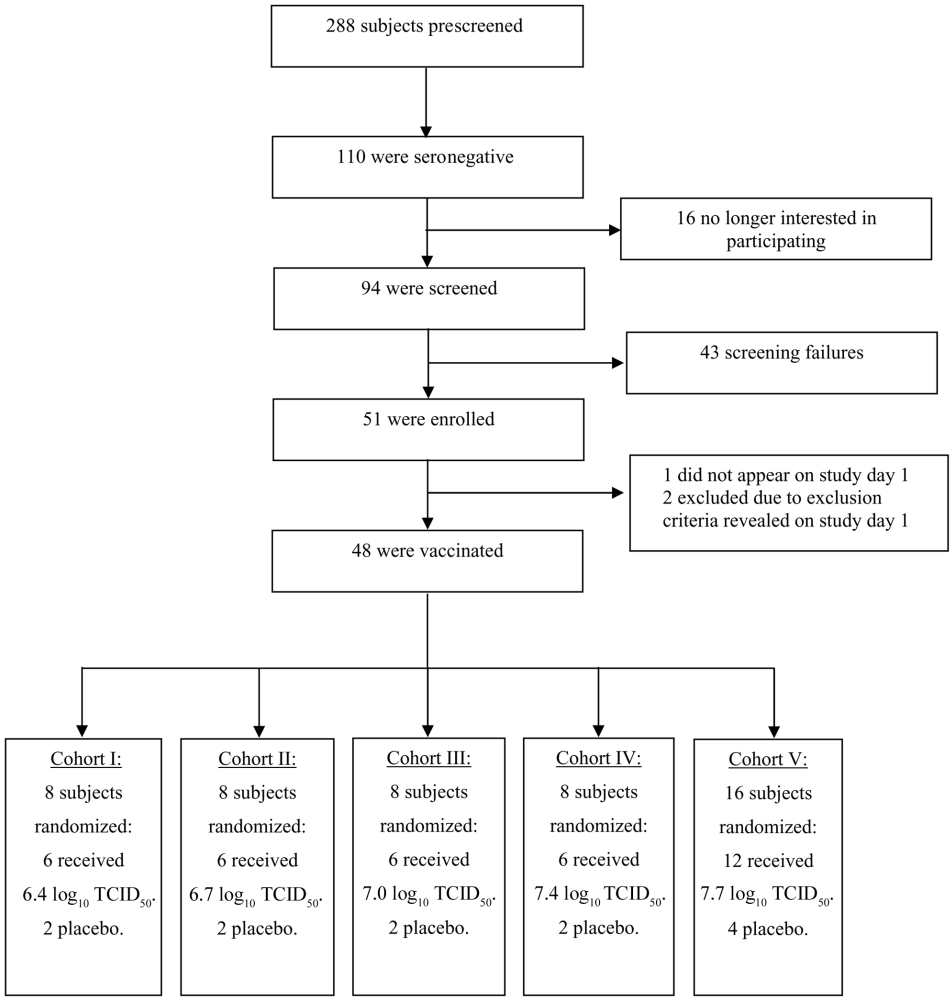

Figure 1. Consolidated Standards of Reporting Trials (CONSORT) flow chart. $TCID_{50}$, median tissue culture infective dose.

the surface glycoproteins of A/NC/20/99, A/Solomon Islands/3/06, and A/Brisbane/59/07 on Vero cells.

HAI antibodies were measured to determine pre- and postvaccination A/NC/20/99-specific titers, according to standard procedures [19]. Serum immunoglobulin G (IgG) and mucosal immunoglobulin A (IgA) were evaluated by enzyme-linked immunosorbent assay (ELISA), with purified A/NC/20/99 hemagglutinin used as coating antigen. The calibration curve for assessment of vaccine-specific IgG and IgA was established using a pool of serum or nasal wash samples with detectable ELISA signals. To minimize IgA concentration differences, vaccine-specific IgA antibodies were normalized to a constant amount of total IgA (1 μg) of each mucosal sample.

Statistical analyses. Statistical analyses were descriptive in nature. Exploratory statistical tests were performed only on the parameters for the secondary objectives. The postvaccination to prevaccination titer or concentration ratio for each volunteer was submitted to logarithmic transformation and tested by analysis of variance. After revealing a significant group effect, each dose group was compared with the placebo group by the Dunnett test. Results for which $P < .05$ were considered to be significant.

RESULTS

From March 2007 through June 2008, 288 male volunteers were prescreened for H1N1 A/NC/20/99–specific antibodies by HAI assay. Of these, 110 (38%) had titers of <1:10, 51 of whom met the eligibility criteria. Three volunteers dropped out before vaccination (Figure 1). From April 2007 onward, a total of 48 healthy volunteers were vaccinated.

Safety. All vaccinated individuals were included in the safety analysis. Intranasal vaccination with ΔNS1-H1N1 was well tolerated in all dose groups. No serious adverse event was observed. The proportions of the most frequent symptoms reported during the first 7 days after vaccination are presented in Table 1. Ninety-six percent of all adverse events in ΔNS1-H1N1–treated subjects were graded as mild. A similar pro-

Table 1. Proportion of Healthy Volunteers with Adverse Events within 7 Days after Vaccination

Adverse event	% (95% confidence interval)						
	Placebo ($n = 12$)	6.4 $\log_{10}$ $TCID_{50}$ ($n = 6$)	6.7 $\log_{10}$ $TCID_{50}$ ($n = 6$)	7.0 $\log_{10}$ $TCID_{50}$ ($n = 6$)	7.4 $\log_{10}$ $TCID_{50}$ ($n = 6$)	7.7 $\log_{10}$ $TCID_{50}$ ($n = 12$)	All doses ($n = 36$)
Any	92 (62–100)	67 (22–95)	100 (54–100)	67 (22–96)	50 (12–88)	100 (74–100)	81 (64–92)
Arthralgia	8 (0–38)	0 (0–46)	17 (0–64)	0 (0–46)	0 (0–46)	25 (5–57)	11 (3–26)
Diarrhea	8 (0–38)	17 (0–64)	0 (0–46)	0 (0–46)	17 (0–64)	25 (5–57)	14 (5–29)
Epistaxis	8 (0–38)	0 (0–46)	17 (0–64)	0 (0–46)	0 (0–46)	0 (0–26)	3 (0–15)
Fatigue	25 (5–57)	0 (0–46)	33 (4–78)	17 (0–64)	0 (0–46)	8 (0–38)	11 (3–26)
Fever[a]	8 (0–38)	17 (0–64)	0 (0–46)	0 (0–46)	0 (0–46)	0 (0–26)	3 (0–15)
Headache	25 (5–57)	50 (12–88)	0 (0–46)	0 (0–46)	17 (0–64)	50 (21–79)	28 (14–45)
Malaise	8 (0–38)	0 (0–46)	0 (0–46)	0 (0–46)	0 (0–46)	0 (0–46)	0 (0–10)
Myalgia	0 (0–26)	0 (0–46)	0 (0–46)	0 (0–46)	0 (0–46)	17 (2–48)	6 (1–19)
Pharyngitis-like symptoms[b]	25 (5–57)	17 (0–64)	17 (0–64)	0 (0–46)	17 (0–64)	33 (10–65)	19 (8–36)
Rhinitis-like symptoms[c]	58 (28–85)	33 (4–78)	50 (12–88)	33 (4–78)	17 (0–64)	33 (10–65)	33 (10–65)
Elevation in transaminase level (ALT grade 1)	25 (5–57)	0 (0–46)	17 (0–64)	50 (12–88)	17 (0–64)	8 (0–38)	17 (6–33)

NOTE. ALT, alanine transaminase; $TCID_{50}$, median tissue culture infective dose.

[a] Fever was defined as an oral temperature of >37.3°C.

[b] Pharyngitis-like symptoms were defined as comprising pharyngitis, nasopharyngitis, pharyngolaryngeal pain, throat irritation, dysphonia, oral leukoplakia, tonsillar disorder, mucosal burning sensation, and dysphagia.

[c] Rhinitis-like symptoms were defined as comprising rhinitis, rhinorrhea, sneezing, nasal congestion, nasal discomfort, and nasal disorder.

portion was noted within the 28-day observation period. The most frequent adverse events were rhinitis-like symptoms (comprising rhinitis, rhinorrhea, sneezing, nasal congestion, nasal discomfort, and nasal disorder), pharyngitis-like symptoms (encompassing pharyngitis, nasopharyngitis, pharyngolaryngeal pain, throat irritation, dysphonia, oral leukoplakia, tonsillar disorder, mucosal burning sensation, and dysphagia), and headache. These symptoms occurred to a similar extent in placebo- and ΔNS1-H1N1–treated subjects. Rhinitis-like symptoms were predominantly observed during the inpatient period, regardless of treatment group (Table 2). Headache episodes were equally distributed over the 28-day observation period in both treatment lines.

Typical adverse events (such as malaise, myalgia, or fever) reported from previous studies with live cold-adapted influenza vaccines [20] were noted only rarely (Table 1). Fever was observed in 4 subjects, 1 in the placebo group and 3 in the ΔNS1-H1N1 group. Two of these vaccine recipients, one who received 6.4 $\log_{10}$ $TCID_{50}$ and one who received 6.7 $\log_{10}$ $TCID_{50}$, experienced mild fever (temperature, ≤38.0°C) on day 3 and day 16 after vaccination, respectively, and the third vaccine recipient, who received 7.7 $\log_{10}$ $TCID_{50}$, experienced moderate fever (temperature up to 38.1°C) on day 24 after vaccination. Four subjects, 2 in the placebo group and 2 in the ΔNS1-H1N1 group who received 6.7 $\log_{10}$ $TCID_{50}$, experienced an episode of epistaxis. In the ΔNS1-H1N1–treated subjects, these mild- and moderate-graded episodes occurred on days 7 and 10; in the placebo group, they occurred on days 2 and 13. Ear-nose-throat control examinations after epistaxis and on days 2, 3, 5, 8, and 29 revealed no local adverse events in response to vaccination at any dose level.

Clinical laboratory safety testing of subjects showed no statistically significant abnormalities. Mild elevations in transaminase levels were observed with the same frequency in vaccine- and placebo-treated subjects (grade 1; see Table 1). Transient elevations in bilirubin levels of grade 3 were observed in 2 subjects (dose level, 7.7 $\log_{10}$ $TCID_{50}$): both had entered the trial with known preexisting asymptomatic grade 2 elevations in bilirubin levels. These rises in bilirubin levels did not coincide with any elevation in transaminase levels. There was no indication of a dose dependency with any of the adverse events observed.

Shedding of vaccine virus. To confirm the replication-defective phenotype of the vaccine virus, we analyzed nasal washings collected after 12, 24, 48, and 72 h for the presence of vaccine virus. Vaccine virus was recovered from samples from 2 subjects in the highest dose group (7.7 $\log_{10}$ $TCID_{50}$) at 12 h after immunization. After this point in time, ΔNS1-H1N1 was no longer present in any of the samples.

Immune response. Immunogenicity of ΔNS1-H1N1 was determined on the basis of the presence of vaccine-specific antibodies in nasal washings and serum samples obtained before vaccination and on day 29 after vaccination. In the highest dose group (7.7 $\log_{10}$ $TCID_{50}$), 8 (67%) of 12 volunteers had a 4-fold or higher increase in neutralization titers (Table 3). The increase in the GMT in this group was 6.4-fold (from 22.6 before immunization to 143.7 after immunization) and was significantly different from that in the placebo group ($P <$

Table 2. Time Course of Rhinitis-Like Symptoms after Immunization

	Day after vaccination																												
Treatment group (total subjects in group), subject	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
6.4 $\log_{10}$ $TCID_{50}$ ($n = 6$)																													
1	X	X																											
2	X																												
6.7 $\log_{10}$ $TCID_{50}$ ($n = 6$)																													
1	X																												
2														X															
3	X																												
4				X																									
5																											X		
7.0 $\log_{10}$ $TCID_{50}$ ($n = 6$)																													
1		X																											
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7.4 $\log_{10}$ $TCID_{50}$ ($n = 6$)																													
1		X																											
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3	X																												
4		X		X																									
5		X																											
6	X				X																								
7	X																												

NOTE. *X* indicates an episode of rhinitis-like symptoms, defined as comprising rhinitis, rhinorrhea, sneezing, nasal congestion, nasal discomfort, and nasal disorder. $TCID_{50}$, median tissue culture infective dose.

Table 3. Systemic and Mucosal Antibody Response 28 Days after Immunization

Treatment group	Microneutralization			HAI			Mucosal IgA			Serum IgG			No. (%) of volunteers with any response
	Before, GMT (95% CI)	After, GMT (95% CI)	Responders,[a] no. (%)	Before, GMT (95% CI)	After, GMT (95% CI)	Responders,[a] no. (%)	Before, GMC (95% CI)	After, GMC (95% CI)	Responders,[b] no. (%)	Before, GMC (95% CI)	After, GMC (95% CI)	Responders,[b] no. (%)	
6.4 $\log_{10}$ $TCID_{50}$ ($n = 6$)	16.0 (6.4–40.2)	28.5 (7.5–108.3)	1 (17)	5.0 (5.0)	5.0 (5.0)	0	1.5 (0.9–2.3)	1.2 (0.8–1.8)	0	44.7 (20.5–97.3)	50.0 (24.2–103.7)	0	1 (17)
6.7 $\log_{10}$ $TCID_{50}$ ($n = 6$)	12.7 (3.3–49.2)	16.0 (3.7–68.5)	0	5.0 (5.0)	5.6 (4.2–7.6)	0	1.6 (1.0–2.4)	1.8 (1.0–3.3)	1 (17)	31.7 (13.9–72.4)	34.2 (15.8–74.2)	0	1 (17)
7.0 $\log_{10}$ $TCID_{50}$ ($n = 6$)	20.2 (4.5–90.6)	32.0 (4.5–225.3)	2 (33)	5.0 (5.0)	6.3 (3.5–11.4)	1 (17)	3.1 (1.1–8.7)	2.3 (1.0–5.4)	0	32.3 (10.4–100.3)	36.1 (11.1–118.0)	0	2 (33)
7.4 $\log_{10}$ $TCID_{50}$ ($n = 6$)	9.0 (3.8–21.0)	20.2 (7.5–54.5)	3 (50)	5.0 (5.0)	5.0 (5.0)	0	2.8 (1.4–5.6)	2.6 (1.4–4.6)	1 (17)	21.9 (9.7–49.6)	29.3 (16.4–52.4)	1 (17)	3 (50)
7.7 $\log_{10}$ $TCID_{50}$ ($n = 12$)	22.6 (13.1–39.1)	143.7[d] (45.3–456.1)	8 (67)	5.3[c] (4.7–6.0)	17.8[d] (9.3–34.0)	6 (50)	1.7 (0.9–3.5)	4.3[e] (2.1–8.6)	5 (42)	26.7 (14.6–48.8)	68.1[d] (42.5–109.0)	7 (58)	10 (83)
Placebo ($n = 12$)	32.0 (11.2–91.0)	30.2 (11.1–82.2)	0	5.3[c] (4.7–6.0)	5.3 (4.7–6.0)	0	1.8 (1.4–2.3)	1.8 (1.2–2.6)	1 (8.3)	41.7 (19.2–90.3)	42.1 (19.3–91.6)	0	1 (8.3)

NOTE. CI, confidence interval; GMC, geometric mean concentration; GMT, geometric mean titer; IgG, immunoglobulin G; $TCID_{50}$, median tissue culture infective dose.

[a] Responders were defined as having a ⩾4-fold increase from the baseline level.

[b] Responders were defined as having a ⩾2-fold increase from the baseline level.

[c] One subject exhibited a titer of 1:10 on day 1, although prescreening values were <1:10.

[d] $P < .001$ for an increase from the baseline level to the postvaccination level compared with placebo (Dunnett test).

[e] $P < .05$ for an increase from the baseline level to the postvaccination level compared with placebo (Dunnett test).

.001). Although several subjects in the lower dose groups were classified as responders, the increase in the GMT in these groups was not significant in comparison with the placebo group.

Similarly, 6 (50%) of 12 volunteers receiving the highest dose had a 4-fold or higher increase in HAI antibody titer after immunization (Table 3). At this dose level, a 3.4-fold increase in the GMT was obtained (from 5.3 to 17.8). This increase was significantly different from that in the placebo group ($P <$ .001), whereas the rise in GMT in the other dose groups did not significantly differ from that in the placebo group.

Nasal wash samples were analyzed for vaccine-specific local IgA induction (Table 3). Five (42%) of 12 subjects in the 7.7 $\log_{10}$ $TCID_{50}$ dose group showed a 2-fold or higher increase and were classified as responders. The increase in the geometric mean concentration in this dose group was 2.5-fold and was significantly different from that in the placebo group ($P < .05$). No significant increase was observed in any of the lower dose groups.

Volunteers who experienced a ⩾2-fold increase in IgG serum concentrations of antibodies against purified hemagglutinin derived from A/New Caledonia/20/99 virus were classified as responders (Table 3). In line with the neutralization and HAI antibody titers, the highest rate of responders (7/12 [58%]) was observed in the 7.7 $\log_{10}$ $TCID_{50}$ dose group, and the increase in the geometric mean concentration in this group was significant in comparison with the placebo group ($P <$ .001).

The number of overall responders (volunteers classified as responders in any of the 4 categories) was dose dependent, with 10 (83%) of 12 subjects in the highest dose group, 3 (50%) of 6 in the 7.4 $\log_{10}$ $TCID_{50}$ dose group, 2 (33%) of 6 in the 7.0 $\log_{10}$ $TCID_{50}$ dose group, and 1 responder each in the 2 lowest dose groups (Table 3).

Serum samples from the highest dose group were also analyzed for their cross-neutralizing activity, by employing reassortant strains containing the surface glycoproteins from influenza A/NC/20/99, A/Solomon Islands/3/06, or A/Brisbane/59/07 in the MNA. Whereas 8 (67%) of 12 subjects experienced a ⩾4-fold increase in neutralization titers against the homologous A/NC strain, 7 (58%) of 12 experienced a ⩾4-fold increase in titer against A/Solomon Islands, and 6 (50%) of 12 showed an increase in titer against A/Brisbane (Figure 2). The corresponding increases in GMTs from before to after vaccination were 4.5 (from 16.4 to 74.5), 3.3 (from 13.4 to 44.9), and 3.3 (from 16.0 to 53.1), respectively.

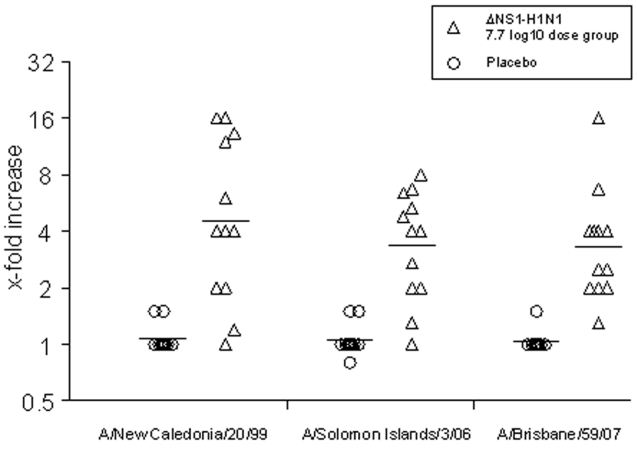

Figure 2. Cross-neutralizing antibody response after ΔNS1-H1N1 vaccination. Volunteers were immunized with either 7.7 $\log_{10}$ median tissue culture infective dose ($TCID_{50}$) of ΔNS1-H1N1 vaccine (A/NC/20/99-like) or placebo. Titers of serum neutralizing antibodies against reassortant viruses containing the hemagglutinin and neuraminidase from A/NC/20/99-like, A/Solomon Islands/3/06–like, and A/Brisbane/59/07-like vaccines were determined. Shown are the *x*-fold increases in pre- to postvaccination titers; horizontal lines indicate the geometric mean fold increases.

DISCUSSION

To our knowledge, this study provides the first safety and immunogenicity data in humans for a replication-deficient influenza vaccine lacking NS1 (ΔNS1-H1N1). Intranasal vaccination with ΔNS1-H1N1 was well tolerated. The most frequent symptoms were rhinitis-like symptoms and mild headache. However, because these symptoms were seen to a similar extent in placebo- and ΔNS1-H1N1–treated subjects (with largely overlapping 95% confidence intervals), a drug-effect relationship is unlikely.

Local symptoms (rhinitis- and pharyngitis-like symptoms) occurred predominantly during the first 4 days after vaccination. Because these symptoms were equally frequent in the placebo and vaccine groups, the air conditioning at the study site during hospitalization and nasal wash procedures might be the cause of these. One subject from the highest dose group reported a temperature of 38.1°C on day 24. On the same day, this person also had sunburn, and the fever lasted for only 1 day. Nevertheless, a relationship between the elevated temperature and the study medication could not be entirely excluded and thus was judged to be possibly related.

The only adverse event graded as moderate and probably related to the study medication was an episode of epistaxis on day 10 in a subject vaccinated at a dose level of 6.7 $\log_{10}$ $TCID_{50}$. However, because episodes of epistaxis were equally distributed among placebo and vaccine groups, it is possible that nasal wash procedures for pharmacokinetic evaluations might have provoked the bleeding. All other moderate adverse events ($n = 29$; 12 in the placebo group and 17 in the vaccine group) were judged by the investigators to be not related or only possibly related to the study medication.

An important safety aspect of live attenuated influenza vaccines is the potential to replicate and shed vaccine virus. For example, current live cold-adapted vaccines can replicate in the upper respiratory tract, resulting in viral shedding for up to 21

days [20]. Viral shedding may lead to transmission to persons in close contact with vaccine recipients and bears the risk of reversion of the replicating vaccine virus. Results from earlier studies in animals suggest that the lack of NS1 increases the production of interferon, which can block viral replication. Consistent with these observations, only 2 subjects were found to have virus present in nasal washings and only at the earliest time point of testing, indicating that the ΔNS1-H1N1 vaccine undergoes abortive replication.

Despite the replication-deficient phenotype of the ΔNS1-H1N1 vaccine, local and systemic antibodies were induced in a dose-dependent manner. In the highest dose group, 10 of 12 volunteers responded to the vaccine. Even if the sample size of a phase 1 study is small by nature, it is noteworthy to put the immunogenicity results observed in the context of what is known for current live attenuated influenza vaccines. Subjects who tested negative for HAI antibodies against H1N1 live cold-adapted vaccines showed an up to 2.0-fold increase in the GMT of HAI antibodies, with a ⩾4-fold increase observed in ~20% of the vaccine recipients [21–23]. Remarkably, despite the relatively low number of HAI responders, an overall clinical efficacy of 85%–93% was reached in these trials [23, 24]. Given all the limitations of comparisons with historic controls, in our study the group receiving the highest dose of ΔNS1-H1N1 showed a 3.4-fold increase in the GMT of HAI antibodies, and a ⩾4-fold increase was observed in 50% of all subjects. Even though a limited number of volunteers were vaccinated with ΔNS1-H1N1, the increases in the GMTs of vaccine-specific antibodies in the highest dose group were statistically significant. It should be noted that the number of responders was different in the different assays, which most likely was due to different sensitivities of the assays.

Cross protection against drift variants is an important factor for the development of effective influenza vaccines. We found that neutralizing antibodies induced by the A/NC/20/99-like ΔNS1-H1N1 vaccine were also active against drift variants such as A/Solomon Islands/3/06 and A/Brisbane/59/07, which have appeared during the last 2 years. These results give hope that ΔNS1-H1N1 vaccines will more efficiently protect vaccinated individuals against drift variants that evolve between the time at which the vaccine composition was recommended by authorities and the actual epidemics.

In summary, this randomized, double-blind, placebo-controlled, proof-of-concept study demonstrated for the first time to our knowledge that an influenza virus strain lacking the NS1 protein is a safe and well-tolerated vaccine for humans. The ΔNS1 vaccine strain displayed a statistically significant immunogenic potential, and we confirmed the replication-deficient phenotype of ΔNS-H1N1. This encouraging proof of concept warrants further clinical development of a trivalent influenza vaccine.

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