Safety and Immunogenicity of a Vero Cell Culture-Derived Whole-Virus H5N1 Influenza Vaccine in Chronically Ill and Immunocompromised Patients

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The development of vaccines against H5N1 influenza A viruses is a cornerstone of pandemic preparedness. Clinical trials of H5N1 vaccines have been undertaken in healthy subjects, but studies in risk groups have been lacking. In this study, the immunogenicity and safety of a nonadjuvanted cell culture-derived whole-virus H5N1 vaccine were assessed in chronically ill and immunocompromised adults. Subjects received two priming immunizations with a clade 1 A/Vietnam H5N1 influenza vaccine, and a subset also received a booster immunization with a clade 2.1 A/Indonesia H5N1 vaccine 12 to 24 months later. The antibody responses in the two populations were assessed by virus neutralization and single radial hemolysis assays. The T-cell responses in a subset of immunocompromised patients were assessed by enzyme-linked immunosorbent spot assay (ELISPOT). The priming and booster vaccinations were safe and well tolerated in the two risk populations, and adverse reactions were predominantly mild and transient. The priming immunizations induced neutralizing antibody titers of \( \geq 1:20 \) against the A/Vietnam strain in \( 64.2\% \) of the chronically ill and \( 41.5\% \) of the immunocompromised subjects. After the booster vaccination, neutralizing antibody titers of \( \geq 1:20 \) against the A/Vietnam and A/Indonesia strains were achieved in \( 77.5\% \) and \( 70.8\% \), respectively, of chronically ill subjects and in \( 71.6\% \) and \( 67.5\% \), respectively, of immunocompromised subjects. The T-cell responses against the two H5N1 strains increased significantly over the baseline values. Substantial heterosubtypic T-cell responses were elicited against the 2009 pandemic H1N1 virus and seasonal A(H1N1), A(H3N2), and B subtypes. There was a significant correlation between T-cell responses and neutralizing antibody titers. These data indicate that nonadjuvanted whole-virus cell culture-derived H5N1 influenza vaccines are suitable for immunizing chronically ill and immunocompromised populations. (This study is registered at ClinicalTrials.gov under registration no. NCT00711295.)

Highly pathogenic avian influenza viruses of subtype A(H5N1) continue to cause disease outbreaks in domestic fowl across Africa, Asia, and the Middle East and are enzootic in several countries in these regions (1). To date, evidence of transmission between humans is limited; however, sporadic zoonotic infections continue to occur in regions that are endemic for influenza A(H5N1) virus. At least 650 human H5N1 cases were recorded between 2003 and 2014, with a case fatality rate approaching \( 60\% \) (2). Due to the lack of immunity in the human population, there is concern that the emergence of a highly pathogenic H5N1 strain capable of human-to-human transmission might result in severe pandemic disease. The recent surge in human cases due to infection with a novel A(H7N9) virus in China (3) also illustrates the continuing potential for the emergence and spread of such highly pathogenic avian viruses.

Vaccination is considered to be the most effective intervention for mitigating an influenza pandemic, and as such, the development of candidate pandemic vaccines, such as those against A(H5N1) viruses, is a cornerstone of pandemic preparedness (4). In clinical trials, H5N1 vaccines have been shown to be safe and immunogenic in healthy adults (5–9) and children (10–12). However, few data exist on the use of H5N1 vaccines in populations with chronic diseases and/or congenital or acquired immunodeficiencies, despite the fact that these groups are at risk of developing severe complications from influenza (13, 14). This is a significant knowledge gap considering that there are hundreds of millions of individuals with chronic medical conditions in Europe and the United States alone (13, 15) who would be prioritized for vaccination in the event of a pandemic. Due to increased and prolonged virus shedding (16) and greater potential for the emergence of resistance to antivirals in immunocompromised individuals (17), the vaccination of this risk group is also an important public health consideration for the general population.

Immune dysfunction associated with underlying medical conditions or immunosuppression might reduce vaccine responses, and there has been a perception that the vaccination of some risk populations may be associated with increased side effects (18, 19). Particularly in a pandemic setting, where vaccines may be in short supply, it is crucial that the priority vaccination of specific groups is supported by data demonstrating that vaccination will be well...
tolerated and clinically beneficial (19). We investigated the safety and immunogenicity of a nonadjuvanted cell culture-derived whole-virus A(H5N1) vaccine in chronically ill and immunocompromised adults.

MATERIALS AND METHODS

Study design. An open-label noncontrolled phase III clinical study was conducted at 13 study sites in Austria and Germany between 6 August 2008 and 1 October 2010 in accordance with the International Committee on Harmonisation Guidelines for Good Clinical Practice, the Declaration of Helsinki, Title 21 of the U.S. Code of Federal Regulations, the European Clinical Trial Directive, relevant national laws, and the uniform requirements for manuscripts submitted to biomedical journals. The clinical study protocol and its amendments were approved by the responsible independent ethics committee and institutional review board.

Nonadjuvantedvero cell-derived whole-virus H5N1 vaccines were manufactured as previously described (5, 20) and formulated to contain 7.5 μg hemagglutinin (HA) antigen in 0.5 ml for intramuscular injection into the deltoid muscle of the upper arm using a using a 25-gauge (G) 25-mm needle. Approximately 300 chronically ill and 300 immunocompromised subjects were planned to be enrolled to receive two priming immunizations, 21 days apart, with a clade 1 A/Vietnam/1203/2004 (H5N1) vaccine. The first approximately 100 subjects from each group who agreed to participate were included in the immunogenicity analyses and offered a booster vaccination with the antigenically divergent clade 2.1 A/Indonesia/05/2005 (H5N1) vaccine, approximately 12 to 24 months after the primary immunizations. The booster immunization was not carried out in a randomized manner, as this would have resulted in a substantially extended recruitment period. The subjects were provided with diaries in which to record injection site and systemic reactions, as well as other adverse events, which were graded according to FDA recommendations (21). Blood samples for the assessment of immune responses were drawn immediately before and 21 days after each vaccination, as well as 6 months after the second vaccination. T-cell responses were assessed in all peripheral blood stem cell transplant (PBSCT) recipients enrolled at a single site who had received both priming vaccinations. This study is registered at ClinicalTrials.gov under registration no. NCT00711295.

Participants. Subjects ≥18 years of age who were in stable medical condition and provided written informed consent were eligible for participation. Subjects with chronic cardiovascular, respiratory, renal, or metabolic illness were included in the chronically ill population. Recipients of solid organ transplants (SOT) or PBSCT ≥6 months after transplantation, or individuals with HIV infection with a CD4+ T-cell count of ≥200 x 10⁶/liter were included in the immunocompromised population. Individuals with lower T-cell counts were excluded from the study, as it was considered that such low counts represent a significant medical complication. Subjects were excluded if they had a history of exposure to influenza A(H5N1) virus or vaccine.

Laboratory methods. H5N1 antibodies were assessed by virus neutralization (MN) and single radial hemolysis (SRH) assays, as previously described (5). The seroprotection cutoff of 25 mm² for the SRH assay is based on internationally accepted guidelines for influenza vaccine licensure (22). SRH areas of ≤4 mm² were considered negative. The MN assays were done in a 96-well format, based on the prevention of cytopathic effect (CPE) in a Vero cell monolayer over an incubation period of 5 days at 37°C. The neutralizing titer is expressed as the dilution at which virus growth is inhibited by 50%, calculated by the number of virus-negative wells, whereby the starting dilution is 1:10. The MN titers were calculated by the number of virus-negative wells and the serum dilution according to the method of Reed and Muench (23). On this basis, negative sera were assigned a nominal value of 1:3.9 and considered negative. For the MN assay, no standardized assay and no internationally accepted cutoff for seroprotection exists. The seroprotection MN titer cutoff of 1:20 is based on the previously demonstrated statistically significant correlation with the SRH area of 25 mm² (5) and validation in passive transfer challenge studies in mice (24). Licensure of the whole-virus H5N1 vaccine was granted by the European regulatory authorities based on data obtained using these validated MN and SRH assays (25, 26).

The frequency of peripheral blood mononuclear cells (PBMCs) secreting gamma interferon (IFN-γ) after in vitro stimulation with whole-virus influenza vaccines (10 μg/ml) was assessed by enzyme-linked immunosorbent spot assay (ELISPOT), essentially as previously described (27). The PBMCs were aliquoted, frozen, and stored in liquid nitrogen until used for T-cell response analysis. Samples with ≥800 H5N1-reactive IFN-γ-secreting cells/10⁶ PBMCs were further diluted and restetted using 2 x 10⁶ PBMCs per well. Seasonal influenza virus strains were not restetted due to the limited volume of blood samples available.

The serum samples and PBMCs collected from individuals at baseline, after the first immunization, and after the second immunizations were analyzed simultaneously, as were those collected pre- and postbooster.

Statistical analyses. A sample size of 300 subjects in each risk group provided a 95% probability for detecting an adverse event with an underlying prevalence of 1:100. Point estimates for the rates of subjects with at least one injection site or systemic reaction after each vaccination were calculated separately for the immunocompromised and chronically ill populations.

Point estimates for the rates of subjects with seroprotective antibody titers (MN titer ≥1:20 or SRH area ≥ 25 mm²), who are undergoing seroconversion (≥4-fold increase in MN titer compared to that at baseline, or for SRH, SRH area of ≥25 mm² for subjects seronegative at baseline or ≥50% increase in SRH area for subjects seropositive at baseline), and the geometric mean of the fold increases (GMFI) from baseline for the MN and the SRH titers were calculated separately for the chronically ill and immunocompromised populations.

The increases in MN antibody titers and T-cell counts compared to those at baseline were analyzed using a repeated mixed-model analysis of covariance (ANCOVA), with the change from baseline of the logarithmically transformed titer or T-cell counts as the dependent variable, accounting for the fixed effect of visit, gender, age, and logarithmically transformed baseline titer or T-cell counts. For the repeated visit effect, a spatial covariance structure was assumed. The least-squares mean values (95% confidence interval [CI]) were estimated within this mixed-model framework for each visit and then back transformed into GMFIs by exponentiation. The least-squares mean differences between the study days and their 95% CIs were also computed and back transformed into ratios of geometric means.

In order to assess the relationship between MN antibody titers and T-cell counts in immunocompromised patients, a repeated correlation analysis was performed between the logarithm of the T-cell counts and the logarithm of the MN antibody titers. All pairs (T-cell counts and MN antibody titers) with available data were included in this analysis. Within- and between-subject correlations were estimated as described by Bland and Altman (28). The correlation between the MN titer cutoff of 1:20 and the seroprotective SRH area cutoff of 25 mm² was analyzed according to Cohen’s kappa coefficient (29).

All analyses were carried out utilizing the statistical software package SAS, version 9.1.3 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Study population. The trial profile is shown in Fig. 1. All subjects who were vaccinated at least once with the A/Vietnam vaccine were included in the safety analysis. The demographic and clinical characteristics of the study population are shown in Table 1. Three hundred chronically ill patients were in the safety data set, the majority of whom had multiorgan disease (59.0%), metabolic disease (21.3%), respiratory disease (14.3%), or cardiovascular disease (5.0%). Of 319 immunocompromised subjects in the safety data set, 187 (58.6%) were HIV-infected patients and 132 (41.4%) were transplant patients. Of the transplant patients in the safety
The immunogenicity data set consisted of 123 chronically ill and 122 immunocompromised subjects who received the A/Vietnam vaccine, as well as 89 chronically ill and 69 immunocompromised subjects who agreed to receive a booster immunization with the A/Indonesia vaccine. The baseline characteristics of the patients included in the immunogenicity data set were very similar to those included in the safety data set, with the exception that 51/63 (88.9%) of the transplant patients in the immunogenicity data set were PBSCT recipients. The larger proportion of PBSCT patients in the immunogenicity data set compared to the safety data set resulted from the fact that a large number of the first 100 participants to enroll in the study (and who were thus included in the immunogenicity data set) were from a single study site that enrolled a majority of the PBSCT patients. Thirty-six PBSCT recipients who received the two priming vaccinations and 8 of these subjects who also received the booster immunization were included in the T-cell analysis. No T-cell analyses were done for chronically ill patients, SOT recipients, or HIV patients.

Safety and tolerability. The Vero-derived whole-virus H5N1 vaccines were found to be safe and well tolerated after the primary and booster immunizations, and any adverse reactions were predominantly mild and transient (Table 2). Pain was the most frequent injection site reaction in the two populations, and fatigue, headache, and malaise were the most common systemic reactions. Fever occurred at a low rate of 2.7% of the chronically ill and 1.9% of the immunocompromised patients after the first priming immunization; fewer cases of fever were reported after the second priming and booster immunizations. Only one serious adverse event (herpes zoster infection in an immunocompromised subject after receipt of the booster immunization, which resolved after treatment) was judged as being possibly related to vaccination.

Antibody responses in chronically ill and immunocompromised subjects. The hemagglutination inhibition (HI) assay is considered the standard assay for assessing antibody responses to the seasonal influenza vaccine, but it is reported to be insensitive for measuring H5 antibodies compared to virus neutralization and SRH assays (5,30). The immunogenicity studies in the present study therefore focused on data obtained using MN and SRH assays.

A substantial neutralizing antibody response was induced after two priming immunizations with the A/Vietnam vaccine, with 64.2% of the chronically ill and 41.5% of the immunocompromised subjects achieving MN titers of 1:20 against the homologous A/Vietnam strain (Table 3). The proportion of subjects with MN titers of 1:20 remained higher than that at baseline at 6 months after priming; this was statistically significant (P < 0.001) for the chronically ill subjects but not for the immunocompromised subjects (P = 0.0667). Seroconversion (≥4-fold increase in MN titer compared to that at baseline) was achieved in 35.0% and 32.2% of the chronically ill and immunocompromised patients, respectively, after the primary immunizations.

A substantial antibody response was also induced in the two populations by a single booster immunization with the heterologous A/Indonesia vaccine administered 12 to 24 months after...
priming. The rates of subjects achieving MN titers of $\geq 1:20$ after the booster immunization were 77.5% and 71.6% against the A/Vietnam strain and 70.8% and 65.7% against the A/Indonesia strain in the chronically ill and immunocompromised patients, respectively. After the booster immunization, seroconversion (compared to baseline) was achieved in 51.7% and 65.2% of the chronically ill and immunocompromised patients, respectively.

ANOVA showed that the baseline MN antibody responses had a significant effect ($P < 0.0001$) on postvaccination responses in the chronically ill and the immunocompromised patients. There was no significant effect of age ($P = 0.0810$) or sex ($P = 0.4052$) on the MN antibody responses in the chronically ill patients. In the immunocompromised patients, there was also no significant effect of sex ($P = 0.6717$), but age did have a significant effect ($P < 0.01$) on the MN antibody titers.

The SRH data (Table 4) were generally supportive of the MN data. Seroprotective SRH responses against the A/Vietnam strain were induced in 42.3% and 53.4% of the chronically ill and immunocompromised subjects, respectively, after two priming immunizations. After boosting with the A/Indonesia vaccine, the corresponding seroprotection rates increased to 64.0% and 64.2% against the A/Vietnam strain, and to 50.6% and 41.8% against the A/Indonesia strain (Table 4) in the two populations, respectively.

Seroconversion (SRH area of $\geq 25$ mm$^2$ for subjects who were seronegative at baseline or $\geq 50$% increase in SRH area for subjects who were seropositive at baseline) was achieved in 33.3% and 35.6% of the chronically ill and immunocompromised patients, respectively, after the primary immunization. After the booster immunization, seroconversion (compared to baseline) was achieved against the A/Vietnam and A/Indonesia strains in 62.9% and 53.9% of the chronically ill patients and in 61.2% and 41.8% of the immunocompromised patients, respectively.

In contrast to previous studies of whole-virus H5N1 vaccines in healthy adults and children (5, 12), the correlation between the seroprotective titer of 1:20 measured by MN and the seroprotective SRH area of 25 mm$^2$ was poor (overall kappa coefficient, 0.361; 95% CI, 0.308 to 0.414). For the MN titer cutoffs of 1:10 or 1:40, this correlation was even poorer (kappa, 0.152; 95% CI, 0.108 to 0.195, and kappa, 0.330; 95% CI, 0.283 to 0.377, respectively). In most cases, however, there was substantial overlap between the 95% CIs of the rates of seroprotection and seroconversion.
A/Vietnam vaccine are shown in Fig. 2A. T-cell responses were tested in PBSCT recipients after two priming immunizations with the whole-virus H5N1 strain. Of interest, systemic reactions to vaccination were low in this group; however, the T-cell responses and antibody responses may be compromised in these individuals (31, 32), we were particularly interested to investigate their T-cell responses to immunization with the whole-virus H5N1 strain.

The T-cell responses against H5N1 viruses in immunocompromised subjects. Of the immunocompromised patients included in the immunogenicity analysis, 42% were allogeneic PBSCT recipients. Since T-cell responses and antibody responses may be reduced in these individuals (31, 32), we were particularly interested to investigate their T-cell responses to immunization with the whole-virus H5N1 strain.

The frequencies of H5N1-reactive IFN-γ-secreting T cells in PBSCT recipients after two priming immunizations with the A/Vietnam vaccine are shown in Fig. 2A. T-cell responses were low against the A/Vietnam and the A/Indonesia strains at baseline but were significantly increased against the two strains after the first (P < 0.01) and second (P < 0.0001) priming vaccinations, as well as 6 months postvaccination (P < 0.0001). The T-cell responses GMFI after two priming vaccinations were 2.8 and 2.5 against the A/Vietnam and A/Indonesia strains, respectively, and were maintained at similar levels 6 months postvaccination. In the eight patients who received a booster immunization, the T-cell responses were also significantly increased (P < 0.0001) compared to baseline both prebooster (GMFI, 7.2 and 5.7 against the A/Vietnam and A/Indonesia strains, respectively) and postbooster (GMFI, 9.6 against the A/Vietnam strain and 6.9 against the A/Indonesia strain) (data not shown). ANCOVA showed that baseline T-cell response levels had a significant effect (P < 0.0001) on the vaccine-induced T-cell responses to the two H5N1 strains. There was no effect of age or sex on any T-cell responses against either H5N1 strain.

A repeated correlation analysis of the relationship between neutralizing antibody titers and T-cell responses revealed significant positive correlation coefficients, both between- and within-subject, for the A/Vietnam strain (0.473, P < 0.0001, and 0.547, P = 0.005, and 0.547, P = 0.194, respectively), indicating that subjects with higher T-cell responses also had higher neutralizing antibody responses.

### TABLE 2 Injection site and systemic reactions after priming and booster immunizations

| Reaction type | Chronically ill | Immunocompromised |
|---------------|----------------|-------------------|
|               | First dose (300) | Second dose (284) | Booster (89) | First dose (319) | Second dose (311) | Booster (67) |
| Injection site reactions |                   |                   |              |                   |                   |              |
| Any (% of subjects [95% CI]) | 17.0 (12.9–21.7) | 13.4 (9.6–19.7) | 23.6 (15.2–33.8) | 12.5 (9.1–16.7) | 8.4 (5.5–12.0) | 7.5 (2.5–16.6) |
| Severity (no. [%] of subjects) | Mild 46 (15.3) | 35 (12.3) | 19 (21.3) | 33 (10.3) | 17 (5.5) | 5 (7.5) |
| | Moderate 5 (1.7) | 2 (0.7) | 2 (2.2) | 6 (1.9) | 5 (1.6) | 0 (0.0) |
| | Severe 0 (0.0) | 1 (0.4) | 0 (0.0) | 1 (0.3) | 3 (1.0) | 0 (0.0) |
| Type (% of subjects [95% CI]) | Pain 11.3 (8.0–15.5) | 11.3 (7.8–15.5) | 18.0 (10.6–27.5) | 10.0 (7.0–13.9) | 6.4 (4.0–9.8) | 6.0 (1.7–14.6) |
| | Erythema 0.7 (0.1–2.4) | 0.7 (0.1–2.5) | 0.0 (0.0–4.1) | 0.6 (0.1–2.2) | 0.6 (0.1–2.3) | 0.0 (0.0–5.4) |
| | Swelling 1.7 (0.5–3.8) | 0.4 (0.0–1.9) | 1.1 (0.0–6.1) | 1.6 (0.5–3.6) | 1.3 (0.4–3.3) | 0.0 (0.0–5.4) |
| | Induration 1.3 (0.4–3.4) | 0.0 (0.0–1.3) | 2.2 (0.3–7.9) | 0.9 (0.2–2.7) | 1.0 (0.2–2.8) | 0.0 (0.0–5.4) |
| | Ecchymosis 5.0 (2.8–8.1) | 1.1 (0.2–3.1) | 6.7 (2.5–14.1) | 1.9 (0.7–4.0) | 1.9 (0.7–4.2) | 1.5 (0.0–8.0) |
| Systemic reactions | Any (% of subjects [95% CI]) | 36.7 (31.2–42.4) | 20.1 (15.6–25.2) | 20.2 (12.4–30.1) | 28.5 (23.6–33.8) | 16.7 (12.7–21.3) | 11.9 (5.3–22.2) |
| | Severity (no. [%] of subjects) | Mild 80 (26.7) | 45 (15.8) | 13 (14.6) | 68 (21.3) | 33 (10.6) | 5 (7.5) |
| | Moderate 26 (8.7) | 10 (3.5) | 4 (4.5) | 18 (5.6) | 14 (4.5) | 3 (4.5) |
| | Severe 4 (1.3) | 2 (0.7) | 1 (1.1) | 5 (1.6) | 5 (1.6) | 0 (0.0) |

a Reactions occurred within 21 days of each vaccination, unless otherwise indicated. CI, confidence interval.

b Occurred within 7 days after vaccination.


c n = 283.
d n = 317.

e n = 303.
line (GMFI, 2.4; \( P < 0.001 \)), which was maintained at 6 months postvaccination (Fig. 2B).

The mean baseline T-cell responses against the H1N1 A/Brisbane/59/2007 and H3N2 A/Uruguay/716/2007 seasonal influenza viruses, which had circulated widely and were recommended components of the seasonal influenza vaccine in the Northern Hemisphere for the 2008 to 2010 influenza seasons, were higher than those against the H5N1 and pandemic H1N1 strains (Fig. 2B). The T-cell responses compared to those at baseline were significantly increased against the two seasonal influenza subtypes after a single immunization, and they increased further after the second immunization (GMFI, 1.6; \( P < 0.001 \)).

The baseline responses against B/Brisbane/60/2008, which was not a recommended vaccine strain until the 2009-2010 season, were considerably lower than those against the seasonal A/Influenza subtype strains. The T-cell responses to the B strain increased after the two priming vaccinations, but these increases were not statistically significant. Six months after the primary immunization schedule, however, the T-cell responses to all influenza virus strains increased and were considerably lower than those against the seasonal A/H1N1/Brisbane, A/H3N2/Uruguay, and B/Brisbane, respectively (data not shown).

### DISCUSSION

A Vero cell culture-derived whole-virus H5N1 influenza vaccine is well tolerated and immunogenic in chronically ill and immunocompromised patients, inducing antibody and T-cell memory responses that were effectively boosted by a heterologous vaccine up to 2 years after priming. Adverse reactions were predominantly mild and transient in nature and occurred at a frequency similar to that previously reported for the nonadjuvanted whole-virus H5N1 vaccines in healthy adult and older subjects (5, 6, 33). The standard criteria for determining the immunogenicity of influenza vaccines are based on HI assay measurements. However, it has been reported that this assay is insensitive for measuring H5 antibodies (5, 30), although the use of novel adjuvants has been reported to enhance the induction of this type of antibody (7, 34–37). Previous studies with the nonadjuvanted whole-virus vaccine (5) in healthy subjects had, however, demonstrated it was a poor inducer of HI antibodies, although high titers of neutralizing antibodies were induced (5, 6, 12, 33, 38). For this reason, the immunogenicity determinations focused on the use of neutralizing and SRH antibody measurements to determine functional antibody responses. In the present study, the rate of chronically ill subjects achieving MN titers of \( \geq 1:20 \) after two priming immunizations with the A/Vietnam vaccine was similar to that previously reported in healthy subjects (5, 6, 33). However, as expected, the

### TABLE 3 Neutralizing-antibody responses to A/Vietnam and A/Indonesia viruses after two A/Vietnam priming immunizations and a 12- to 24-month A/Indonesia booster immunization

| Data by patient group (n) | Seroprotection (% [95% CI])a | Seroconversion (% [95% CI])b | GMFI (mean [95% CI])c |
|---------------------------|-----------------------------|-----------------------------|----------------------|
| **Chronically ill patients** |                             |                             |                      |
| A/Vietnam                 |                             |                             |                      |
| Day 0 (123)               | 6.5 (2.8–12.4)              | NA                          | NA                   |
| Day 21 (122)              | 44.3 (35.3–53.5)            | 17.2 (11.0–25.1)            | 2.3 (2.0–2.6)        |
| Day 42 (123)              | 64.2 (55.1–72.7)            | 35.0 (26.6–44.1)            | 3.0 (2.6–3.5)        |
| Day 201 (123)             | 31.7 (23.6–40.7)            | ND                          | 1.6 (1.4–1.8)        |
| Prebooster (89)           | 24.7 (16.2–35.0)            | NA                          | NA                   |
| 21 days postbooster (89)  | 77.5 (67.4–85.7)            | 51.7 (40.8–62.4)/31.5 (22.0–42.2)d | 4.7 (3.8–5.9)/3.0 (2.5–3.6)d |
| A/Indonesia               |                             |                             |                      |
| Prebooster (89)           | 2.2 (0.3–7.9)               | NA                          | NA                   |
| 21 days postbooster (89)  | 70.8 (60.2–79.9)            | 65.2 (54.3–75.0)            | 8.4 (6.3–11.2)       |
| **Immunocompromised patients** |                         |                             |                      |
| A/Vietnam                 |                             |                             |                      |
| Day 0 (122)               | 4.9 (1.8–10.4)              | NA                          | NA                   |
| Day 21 (121)              | 24.8 (17.4–33.3)            | 9.1 (4.6–15.7)              | 1.6 (1.4–1.8)        |
| Day 42 (118)              | 41.5 (32.5–51.0)            | 32.2 (23.9–41.4)            | 2.5 (2.2–2.9)        |
| Day 201 (113)             | 11.5 (6.3–18.9)             | ND                          | 1.1 (1.0–1.3)        |
| Prebooster (67)           | 10.4 (4.3–20.3)             | NA                          | NA                   |
| 21 days postbooster (67)  | 71.6 (59.3–82.0)            | 49.3 (36.8–61.8)/37.3 (25.8–50.0)d | 4.9 (3.5–6.8)/3.8 (3.0–4.9)d |
| A/Indonesia               |                             |                             |                      |
| Prebooster (67)           | 1.5 (0.0–8.0)               | NA                          | NA                   |
| 21 days postbooster (67)  | 65.7 (53.1–76.8)            | 61.2 (48.5–72.9)d           | 7.7 (5.2–11.4)d      |

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*a Defined as an MN titer of \( \geq 1:20 \).

*b A \( \geq 4 \)-fold titer increase compared to baseline. ND, not done; NA, not applicable.

c Geometric mean of fold increases from baseline.

d Compared to prebooster titers.
antibody responses in immunocompromised subjects were lower than those previously reported in healthy subjects (5, 6, 33).

Analyses of the serological data did not show a statistically significant correlation between an MN titer cutoff of 1:20 and the seroprotection SRH area cutoff of 25 mm² that was reported for previous studies of whole-virus H5N1 vaccines (5, 12). This might be due to the relatively small sample size included in the current study compared to the larger data sets used for previous correlation analyses. However, this correlation was even poorer for the lower MN titer cutoff of 1:40, indicating that the 1:20 cutoff provides the best agreement with the seroprotective SRH area of 25 mm².

Moreover, the rates of seroprotection and seroconversion as determined by the MN and SRH assays were generally similar, as indicated by overlapping 95% CIs in almost all cases.

A substantial proportion (42%) of the immunocompromised patients included in the immunogenicity data set who had undergone bone marrow ablation and T-cell depletion before their transplantation procedure. The depletion of B and T cells both contribute to reduced immune responses in these patients, particularly as a consequence of the loss of CD4⁺ T helper cells, which have a central role in coordinating a number of immune components, including the activation, clonal expansion, and longevity of B cells (31, 32). Our data indicate that two priming vaccinations with the whole-virus H5N1 vaccine can effectively reconstitute the influenza-specific T-cell compartment in immunocompromised patients to levels previously reported in healthy adults (39). Although the ELISPOT used in our study does not differentiate between CD4⁺ and CD8⁺ T cells, and whole-virus vaccines may also effectively induce cytotoxic T-cell responses, it is likely that CD4⁺ cells account for the majority of the IFN-γ responses to whole-virus H5N1 vaccines; in similar studies undertaken in healthy adults, 85% of the IFN-γ-secreting T cells were CD4⁺ positive (39).

H5N1-reactive and heterosubtypic and heterotypic influenza-specific T-cell responses remained elevated 6 months after priming and were further increased by a late heterologous booster immunization with the A/Indonesia vaccine. The effective reconstitution of influenza-specific T helper functions is reflected in the similarly robust antibody responses in chronically ill and immunocompromised patients after boosting, with 71% to 78% and 66% to 72% of the subjects achieving MN titers ≥1:20 against the clade 1 and clade 2.1 A(H5N1) strains, respectively.

Notably, there was a statistically significant correlation between T-cell responses and MN antibody titers; in similar studies undertaken in healthy adults, 85% of the IFN-γ-secreting T cells were CD4⁺ positive (39).

The conclusions of our study are subject to a number of limitations. Because the immunogenicity data set was restricted to the first approximately 100 subjects from each group who agreed to participate, the immunogenicity data set was not randomly selected. In general, the demographic characteristics of the safety and immunogenicity data sets are very similar, as are the clinical characteristics of the safety and immunogenicity data sets for the chronically ill patients. However, because many of the first 100 immunocompromised patients were enrolled at a single site, where a large number of PBSC patients were enrolled, the im-

### Table 4

| Patient group and data type (no. of patients) | Seroprotection (% [95% CI])ᵃ | Seroconversion (% [95% CI])ᵇ | GMFI (mean [95% CI])ᶜ |
|---------------------------------------------|-------------------------------|-----------------------------|-----------------------|
| **Chronically ill patients**                |                               |                             |                       |
| A/Vietnam                                   |                               |                             |                       |
| Day 0 (123)                                 | 8.9 (4.5–15.4)                | NA                          | NA                    |
| Day 21 (122)                                | 28.7 (20.9–37.6)              | 20.5 (13.7–28.7)            | 1.5 (1.3–1.8)         |
| Day 42 (123)                                | 42.3 (33.4–51.5)              | 33.3 (25.1–42.4)            | 2.0 (1.7–2.5)         |
| Day 201 (123)                               | 37.4 (28.8–46.6)              | ND                          | 1.8 (1.5–2.1)         |
| Prebooster (89)                             | 31.5 (22.0–42.2)              | NA                          | NA                    |
| 21 days postbooster (89)                    | 64.0 (53.2–73.9)              | 62.9 (52.0–72.9)/43.8 (33.3–54.7)ᵈ | 3.7 (2.9–4.7)/2.0 (1.7–2.4)ᵈ |
| A/Indonesia                                 |                               |                             |                       |
| Prebooster (89)                             | 2.2 (0.3–7.9)                 | NA                          | NA                    |
| 21 days postbooster (89)                    | 50.6 (39.8–61.3)              | 53.9 (43.0–64.6)ᵈ           | 3.3 (2.6–4.2)ᵈ        |
| **Immunocompromised patients**              |                               |                             |                       |
| A/Vietnam                                   |                               |                             |                       |
| Day 0 (122)                                 | 16.4 (10.3–24.2)              | NA                          | NA                    |
| Day 21 (121)                                | 37.2 (28.6–46.4)              | 19.8 (13.1–28.1)            | 1.4 (1.3–1.6)         |
| Day 42 (118)                                | 53.4 (44.0–62.6)              | 35.6 (27.0–44.9)            | 1.9 (1.6–2.2)         |
| Day 201 (113)                               | 45.1 (35.8–54.8)              | ND                          | 1.7 (1.4–1.9)         |
| Prebooster (67)                             | 43.3 (31.2–56.0)              | NA                          | NA                    |
| 21 days postbooster (67)                    | 64.2 (51.5–75.3)              | 61.2 (48.5–72.9)/43.3 (31.2–56.0)ᵈ | 2.8 (2.1–3.6)/1.8 (1.5–2.1)ᵈ |
| A/Indonesia                                 |                               |                             |                       |
| Prebooster (67)                             | 3.0 (0.4–10.4)                | NA                          | NA                    |
| 21 days postbooster (67)                    | 41.8 (29.8–54.5)              | 41.8 (29.8–54.5)ᵈ           | 3.0 (2.3–3.9)ᵈ        |

ᵃ ≥25 mm² SRH area.
ᵇ ≥25 mm² SRH area for subjects seronegative (≤4 mm²) at baseline or ≥50% increase in SRH area for subjects seropositive at baseline. NA, not applicable; ND, not done.
ᶜ Geometric mean of fold increases from baseline.
ᵈ Compared to prebooster titers.
This is the first report of a vaccine against H5N1 influenza A virus in chronically ill and immunocompromised patients. The results of this study demonstrate that nonadjuvanted whole-virus H5N1 vaccines are safe and immunogenic in chronically ill and immunocompromised patients, further supporting the use of influenza vaccines for prepanedemic and pandemic vaccination of these highly vulnerable risk groups.

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REFERENCES

1. WHO. 2013. Areas with confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003–2013. World Health Organization, Geneva, Switzerland. http://gamapserver.who.int/mapLibrary/Files/Maps/2003_AvianInfluenza_GlobalMap_01Feb13.png

2. WHO. 2014. Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO, World Health Organization, Geneva, Switzerland. http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/index.html

3. Center for Infectious Disease Research and Policy (CIDRAP). 2014. Ten new H7N9 cases push outbreak total past 300. Center for Infectious Disease Research and Policy, University of Minnesota, Minneapolis, MN. http://www.cidrap.umn.edu/news-perspective/2014/02/ten-new-h7n9-cases-push-outbreak-total-past-300

4. Ferguson NM, Cummings DA, Fraser C, Cajka JC, Cooley PC, Burke DS. 2006. Strategies for mitigating an influenza pandemic. Nature 442: 448–452. http://dx.doi.org/10.1038/nature04795

5. Ehrlich HJ, Müller M, Oh HM, Tambyah PA, Joukhadar C, Montomoli E, Fisher D, Berezuk G, Fritsch S, Low-Baselli A, Vartian N, Bobrovsky R, Pavlova BG, Pollabauer EM, Kistner O, Barrett PN, Baxter H5N1 Pandemic Influenza Vaccine Clinical Study Team. 2008. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. N. Engl. J. Med. 358:2573–2584. http://dx.doi.org/10.1056/NEJMoa073121

6. Ehrlich HJ, Müller M, Fritsch S, Zeitlinger M, Berezuk G, Low-Baselli A, van der Velden MV, Pollabauer EM, Maritsch F, Pavlova BG, Tambyah PA, Oh HM, Montomoli E, Kistner O, Noel Barrett P. 2009. A cell culture (Vero)-derived H5N1 whole-virus vaccine induces cross-reactive memory responses. J. Infect. Dis. 200:1113–1118. http://dx.doi.org/10.1086/605608

7. Leroux-Roels I, Borkowski A, Vanwolleghem T, Dramé M, Clement F, Hons E, Devaster JM, Leroux-Roels G. 2007. Antigen sparing and cross-reactive immunity with an adjuvanted H5N1 prototype pandemic influenza vaccine: a randomised, placebo-controlled trial. Lancet 370:580–589. http://dx.doi.org/10.1016/S0140-6736(07)61297-5

8. Levie K, Leroux-Roels I, Hoppenbrouwers K, Kervyn AD, Vandermeulen C, Forgus S, Leroux-Roels G, Pichon S, Kusters I. 2008. An adjuvanted, low-dose, pandemic influenza A (H5N1) vaccine candidate is safe, immunogenic, and induces cross-reactive immune responses in healthy adults. J. Infect. Dis. 198:642–649. http://dx.doi.org/10.1086/590913.
9. Wu J, Fang HH, Chen JT, Zhou JC, Fong ZJ, Li QG, Qiu YZ, Liu Y, Lu M, Liu LY, Dong SS, Gao Q, Zhang WM, Wang N, Yin WD, Dong XP. 2009. Immunogenicity, safety, and cross-reactivity of an inactivated, adjuvanted, prototype pandemic influenza (H5N1) vaccine: a phase II, double-blind, randomized trial. Clin. Infect. Dis. 48:1087–1095. http://dx.doi.org/10.1086/597926.

10. Diez-Domingo J, Garcés-Sánchez M, Baldó JM, Planelles MV, Ubeda I, Jußert A, Mares J, Moris P, Garcia-Corpeira P, Dramé M, Gillard P. 2010. Immunogenicity and Safety of H5N1 A/Vietnam/1194/2004 (Clade 1) AS03-adjuvanted pre-pandemic candidate influenza vaccines in children aged 3 to 9 years: a phase II, randomized, open, controlled study. Pediatr. Infect. Dis. J. 29:e35–e6. http://dx.doi.org/10.1097/INF.0b013e3181da921.

11. Vesikari T, Karvonen A, Törmänen S, Borkowski A, Montomoli E, Banzhof A, Clemens R. 2010. Immunogenicity and safety of MF59-adjuvanted H5N1 influenza vaccine from infancy to adolescence. Pediatrics 126:e762–e770. http://dx.doi.org/10.1542/peds.2009-2628.

12. van der Velden MV, Fritz R, Pollabauer EM, Portsmouth D, Howard MK, Kreil TR, Dvorak T, Fritsch S, Vesikari T, Diez-Domingo J, Richmond P, Lee BW, Kistner O, Ehrlich HJ, Barrett PN, Aichinger G. 2014. Safety and immunogenicity of a Vero cell culture-derived whole-virus influenza A(H5N1) vaccine in a pediatric population. J. Infect. Dis. 209:12–23. http://dx.doi.org/10.1093/infdis/jit498.

13. European Centre for Disease Prevention and Control. 2008. Guidance: priority risk groups for influenza vaccination. European Centre for Disease Prevention and Control, Stockholm, Sweden. http://ecdc.europa.eu/en/publications/Publications/0808_GU_Priority_Risk_Groups_for_Influenza_Vaccination.pdf.

14. CDC. 2013. Prevention and control of seasonal influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices—United States, 2013–14. Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov/flu/professionals/acip/specificpopulations.htm.

15. CDC. 2012. Chronic disease prevention and health promotion. Centers for Disease Control and Prevention, Atlanta, GA. http://www.cdc.gov/chronicdisease/overview/index.htm.

16. Lee N, Chan PK, Hui DS, Rainer TH, Wong E, Choi KW, Lui GC, Wong BC, Wong SY, Lam WY, Chu IM, Lai RW, Cockram CS, Sung JF. 2009. Viral loads and duration of viral shedding in adult patients hospitalized with influenza. J. Infect. Dis. 200:492–500. http://dx.doi.org/10.1086/500383.

17. Meijer A, Jonges M, Abbink F, Ang W, van Beek J, Beersma M, Bloembergen P, Boucher C, Claas E, Donker G, van Gageldonk-Lafeber Reisen L, Iskandar C, van der Jagt J, Kroese A, Leisemehl, R. Reienjager M, Riezebos-Brijman A, Schuttet M, Sebens F, Stelma F, Swaan C, Timen van, v’Veele R, van Venrooij D, ter Wierik M, Verheijen, R, Wido Fritsch S, Benamara K, Kistner O, Müller M, Zeitlinger M, Kollaritsch H, Vesikari T, Ehrlich HJ, Barrett PN. 2012. Safety and cross-reactive memory immune response: homologous or heterologous booster response following two dose or single dose priming. J. Infect. Dis. 206:271–282. http://dx.doi.org/10.1093/infdis/jir390.106.

18. Mir MA, Battachwalla M. 2009. Immune deficits in allogeneic hematopoietic stem cell transplant (HSCT) recipients. Mycopathologia 168:271–282. http://dx.doi.org/10.1007/s11046-009-9181-0.

19. van der Velden MV, Aichinger G, Pollabauer EM, Löw-Baselli A, Fritsch S, Benamara K, Kistner O, Müller M, Zeitlinger M, Kollaritsch H, Vesikari T, Ehrlich HJ, Barrett PN. 2012. Cell culture (Vero cell) derived whole-virus non-adjuvanted H5N1 influenza vaccine induces long-lasting cross-reactive memory immune response: homologous or heterologous booster response following two dose or single dose priming. Vaccine 30:6127–6135. http://dx.doi.org/10.1016/j.vaccine.2012.07.077.

20. Langley JM, Frenette L, Ferguson L, Riff D, Sheldon E, Risi G, Johnson C, Li P, Kenney R, Innis B, Fries L. 2010. Safety and cross-reactive immunogenicity of candidate AS03-adjuvanted pre-pandemic H5N1 influenza vaccines: a randomized controlled phase 1/2 trial in adults. J. Infect. Dis. 201:1644–1653. http://dx.doi.org/10.1086/652701.

21. Langley JM, Risi G, Caldwell M, Gilderman L, Berwald B, Fogarty C, Poling T, Riff D, Baron M, Frenette L, Sheldon E, Collins H, Shepard M, Dionne M, Brune D, Ferguson L, Vaughn D, Li P, Fries L. 2011. Dose-sparing H5N1 A/Indonesia/05/2005 pre-pandemic influenza vaccine in adults and elderly adults: a phase III, placebo-controlled, random-
37. Vesikari T, Forstén A, Herbinger KH, Della Cioppa G, Beygo J, Borkowski A, Groth N, Bennati M, von Sonnenburg F. 2012. Safety and immunogenicity of an MF59-adjuvanted A/H5N1 pre-pandemic influenza vaccine in adults and the elderly. Vaccine 30:1388–1396. http://dx.doi.org/10.1016/j.vaccine.2011.12.009.

38. Tambyah PA, Wilder-Smith A, Pavlova BG, Barrett PN, Oh HM, Hui DS, Yuen KY, Fritsch S, Aichinger G, Loew-Baselli A, van der Velden M, Maritsch F, Kistner O, Ehrlich HJ. 2012. Safety and immunogenicity of two different doses of a Vero cell-derived, whole virus clade 2 H5N1 (A/Indonesia/05/2005) influenza vaccine. Vaccine 30:329–335. http://dx.doi.org/10.1016/j.vaccine.2011.10.088.

39. Crowe BA, Brühl P, Gerencer M, Schwendinger MG, Pilz A, Kistner O, Koelling-Schlebusch K, Aichinger G, Singer J, Zeitlinger M, Müller M, Ehrlich H, Barrett PN. 2010. Evaluation of the cellular immune responses induced by a non-adjuvanted inactivated whole virus A/H5N1/VN/1203 pandemic influenza vaccine in humans. Vaccine 29:166–173. http://dx.doi.org/10.1016/j.vaccine.2010.10.065.

40. Pedersen GK, Madhun AS, Breakwell L, Hoschler K, Sjursen H, Pathirana RD, Goudsmit J, Cox RJ. 2012. T-helper 1 cells elicited by H5N1 vaccination predict seroprotection. J. Infect. Dis. 206:158–166. http://dx.doi.org/10.1093/infdis/jis330.

41. Maini MK, Gilson RJ, Chavda N, Gill S, Fakoya A, Ross EJ, Phillips AN, Weller IV. 1996. Reference ranges and sources of variability of CD4 counts in HIV-seronegative women and men. Genitourin. Med. 72:27–31.