A Single-Dose Influenza A (H5N1) Vaccine Safe and Immunogenic in Adult and Elderly Patients: an Approach to Pandemic Vaccine Development[▽]

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With the ongoing pandemic of influenza A (H1N1) virus infection and the threat of high fatality rates for recent human cases of infection with highly pathogenic H5N1 strains, there has been considerable interest in developing pandemic vaccines. Here we report a randomized multicenter dose-finding clinical trial of a whole-virion, inactivated, adjuvanted H5N1 vaccine in adult and elderly volunteers. Four hundred eighty patients were randomly assigned to receive one or two doses of 3.5 µg of the vaccine or one dose of 6 or 12 µg. The subjects were monitored for safety analysis, and serum samples were obtained to assess immunogenicity by hemagglutination inhibition and microneutralization tests. The subjects developed antibody responses against the influenza A (H5N1) virus. Single doses of ≥6 µg fulfilled EU and U.S. licensing criteria for inter-pandemic and pandemic influenza vaccines. Except for occasional injection site pain, malaise, and fever, no adverse events were observed. We found that the present vaccine is safe and immunogenic in healthy adult and elderly subjects and requires low doses and, unlike any other H5N1 vaccines, only one injection to trigger immune responses which comply with licensing criteria. A vaccine using the same methods as those described in this report, but based on a wild-type swine-origin 2009 (H1N1) influenza A virus isolate from the United States (supplied by the CDC), has been developed and is currently being tested by our group.

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With new cases continuing to emerge, as of June 2009, the avian influenza A (H5N1) virus subtype has caused 433 human infections in 15 countries, as confirmed by the World Health Organization (WHO), resulting in severe illness with a high fatality rate (30). Human-to-human spread has been strongly suspected and even evidenced by statistical methods (22, 33). With new human infections continuing to develop, this subtype continues to represent a potential source of an influenza pandemic (33).

Mass vaccination is the most effective approach to reduce illness and death from pandemic influenza. Therefore, vaccine producers are currently developing and assessing vaccines against H5N1 viruses (2, 14, 31). The effects of split, subvirion, and whole-virion H5N1 vaccines have been tested, with various immunogenicity results (31). Three whole-virion vaccines have been tested so far, two of which required two-dose regimens (4, 14), while a one-dose regimen with the present vaccine was found to be immunogenic in 146 adult subjects (24).

The objective of the present study was to determine the safety and immunogenicity of an inactivated whole-virion vaccine against influenza A/Vietnam/1194/2004, using multiple dosing and administration schedules, for adult and elderly subjects. To date, this is the only influenza pandemic prototype vaccine trial examining single-dose regimens in elderly patients.

MATERIALS AND METHODS

Vaccine. The vaccine was produced as described previously (24). Briefly, with the exception of the virus strain, the vaccine was made by the same method as that used for the seasonal influenza vaccine, FluvalAB, for the past 13 years (15, 20). The seasonal vaccine produced by this method has met the requirements of the European Agency for the Evaluation of Medicinal Products (EMA) for inter-pandemic influenza vaccines each year since 1995 and has been administered safely to humans in a total of over 16 million cases (5, 25).

The virus strain (NIBRG-14), a reverse genetics-derived 2:6 reassortant between A/Vietnam/1194/2004 (H5N1) and PR8, was obtained from the National Institute for Biological Standards and Control (NIBSC), London, United Kingdom, in May 2005 and is one of the reference viruses indicated as suitable for use in a mock-up vaccine by the Committee for Medicinal Products for Human Use (CHMP) (6). The seed virus was grown in eggs.

The vaccine, produced by Ominvest LTD (Hungary), contained 3.5, 6, or 12 µg of hemagglutinin (HA)/dose. The HA content was determined before the addition of the aluminum phosphate adjuvant by a single radial immunodiffusion test, using reagents supplied by NIBSC (United Kingdom), as described previously (29).

Purity was evaluated by endotoxin content, which was <0.05 IU/dose, and the amount of ovalbumin, which was <5 ng/dose. Both values are much lower than the concentrations considered acceptable by the European Pharmacopoeia, which are 100 IU and 1,000 ng/human dose, respectively (7). Aluminum phosphate was used as adjuvant, in the amount of 0.31 mg Al/ampoule, and merthio-

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late was added as a preservative (0.1 mg/ml), meeting the requirements of the European Pharmacopoeia (7).

Participants. Between 10 September 2006 and 11 June 2007, we performed a multicenter, randomized, investigator-blinded, dose-finding trial in four centers in Hungary (National Center for Allergy and Immunology, Budapest; State Health Center, Budapest; Zakanysek State Clinic; and Kenezy Hospital, Debrecen). Patients were recruited by their primary care physicians, and a brief summary of the study, along with a call for patients approved by the National Institute of Pharmacy and the Central Ethics Committee for Clinical Pharmacology of the Medical Research Council, Hungary, was published on the study centers' websites.

A total of 586 healthy volunteers over the age of 18 years were screened, and 480 were enrolled to receive vaccination. Written informed consent was obtained from potential subjects. A negative pregnancy test on day 0 was required for women of childbearing potential, and the use of an acceptable contraception method was required for the duration of the study. Exclusion criteria included immunodeficiency, history of Guillain-Barré syndrome, disease states that may affect immune reactivity (e.g., uncontrolled diabetes, autoimmune disease, or malignancy), use of immunosuppressive medications, conditions that precluded compliance with the protocol, receiving an inactivated vaccine 14 days prior to the study, use of live attenuated vaccines within 60 days of study, use of investigational agents within 30 days, receipt of blood products or immunoglobulins in the past 6 months, acute febrile illness 1 week before vaccination, nursing, and hypersensitivity to vaccine components.

We randomly assigned participants to one of four dose groups. Subjects were stratified according to age. Subjects in group I received 3.5 µg on day 1, repeated 21 days later, while the other groups received only one injection of 3.5 µg (group II), 6 µg (group III), or 12 µg (group IV) of hemagglutinin by intramuscular injection. Each dose group consisted of two age groups: patients aged 18 to 60 years (groups Ia, IIa, IIIa, and IVa) and subjects older than 60 years of age (groups Ib, IIb, IIIb, and IVb).

The study was conducted in compliance with good clinical practice guidelines and the provisions of the Declaration of Helsinki. The protocol was approved by the National Institute of Pharmacy and the Central Ethics Committee for Clinical Pharmacology of the Medical Research Council, Hungary. The study was registered with the European Union Drug Regulatory Authorities, European Medicines Agency (<http://eudract.emea.europa.eu/>), under clinical trial registration number EUDRA CT 2006-003448-40. The study design was presented to and registered with the WHO and can be found at http://www.who.int/vaccine_research/diseases/influenza/flu_trials_tables/en/index.html.

Laboratory tests. Serum antibody titers against the vaccine virus strain were measured by hemagglutination inhibition (HI), using chicken red blood cells and following standard procedures (11, 12).

The microneutralization (MN) assay was performed as previously described (18). Briefly, all assays were performed with Madin-Darby canine kidney (MDCK) cells. The 50% tissue culture infectious dose (TCID₅₀) of the virus was determined by titration in Madin-Darby canine kidney cells, using high-binding 96-well styrene immunoassay plates, and was calculated by the method of Reed and Muench (17). Human sera were heat inactivated for 30 min at 56°C, and twofold serial dilutions were made in a 50-µl volume of viral diluent in immunoassay plates with an initial dilution of 1:2. The diluted sera were mixed with an equal volume of viral diluent containing influenza virus at 2×10^3 TCID₅₀/ml (100 TCID₅₀/50 µl). Four control wells of virus plus viral diluent or viral diluent alone were included on each plate. The plates were gently shaken. After a 2-h incubation at 37°C in a 5% CO₂ humidified atmosphere, 100 µl of Madin-Darby canine kidney cells at 1.5×10^5 /ml was added to each well. The plates were incubated for 20 h at 37°C and 5% CO₂. The monolayers were washed with phosphate-buffered saline (PBS) and fixed in ice-cold 80% acetone for 10 min. The presence of viral protein was detected by enzyme-linked immunosorbent assay (ELISA) as follows. Acetone-fixed plates were dried and washed three times with Tween 20 containing PBS. One hundred microliters of influenza A virus nucleoprotein-specific mouse monoclonal antibody (dilution, 1:3,000; Chemicon) was added to each well. Plates were incubated for 60 min at 37°C. After washing of the plates four times with PBS-Tween 20, 100 µl of horseradish peroxidase (HRP)-labeled goat anti-mouse antibody (dilution, 1:500; Chemicon) was added to each well. After 60 min of incubation at 37°C, the plates were washed, and 100 µl substrate (250 µg/ml *ortho*-phenylenediamine [OPD] and 1 µl/ml concentrated [30%] H₂O₂ diluted in citrate buffer, pH 5) was added to each well. After the proper color development, the reaction was stopped by the addition of 100 µl of 2 M H₂SO₄. Absorbance was measured at a 492-nm wavelength. Virus-neutralizing antibody titers for each serum were calculated according to the optical density (OD) values of serum dilution-containing wells compared to the ODs of virus control (VC; wells without serum) and cell control

(CC; wells without virus) wells. The end-point titer was expressed as the reciprocal of the highest serum dilution with an OD value of less than x , where $x = [(average\ OD\ of\ VC\ wells - average\ OD\ of\ CC\ wells)/2] + average\ OD\ of\ CC\ wells$ (28).

All serological tests were performed at a central laboratory (Department of Virology, National Center for Epidemiology, Budapest, Hungary). In addition, six sera were also tested independently by the Health Protection Agency, United Kingdom, where the levels of neutralizing antibody were confirmed.

Procedures. Baseline evaluations included demographic data, a medical history, and physical examination. Blood samples were drawn for HI and microneutralization assays. A total of 0.5 ml of the vaccine was injected into the deltoid muscle. For group I, the injection was repeated on day 21, whereas the injection was not repeated for any other groups. Since group I participants received two injections, the blinding was maintained by all vaccinations being performed by specific study personnel who did not take part in the assessment of safety or immunogenicity. On day 21 (and day 42 for group I), a medical history and a list of medications used during the days since the last visit were taken, physical examination was performed, and blood samples were obtained for HI and microneutralization assays. With the exception of the blood draw, the procedures listed for day 21 were repeated on days 90 and 180. Safety variable data were collected at the follow-up visits by taking histories and performing physical examinations. In addition, subjects were asked to take their temperatures on days 1, 2, and 3, using thermometers supplied by the study centers. Diary cards were supplied, and patients were asked to call if they experienced any side effects.

Statistical analysis. All data analyses were carried out according to a preestablished analysis plan. Safety and immunogenicity were prospectively identified as coprimary objectives. The sample size was chosen to exceed the requirements of 50 patients per group of the European guidelines for yearly influenza vaccine trials (5). Our objectives were to describe the immune response to and safety profile of the vaccine 21 days after each vaccination. We summarized results with point estimates and 95% confidence intervals (CI). We report safety data in terms of the number and proportion of individuals who had reactions in each group, and we used Fisher's exact test to compare groups when relevant. In particular, we assessed the occurrence of the following reactions in the 3 days after vaccination, in accordance with guidance for interpanemic vaccines: injection site induration of more than 5 cm for more than 3 days, injection site ecchymosis, body temperature of more than 38.0°C for 24 h or more, malaise, and shivering (19).

We gave hemagglutinin inhibition and microneutralization titers below the limit of detection (1:4) an arbitrary intermediate value of 1:2. The geometric mean of duplicate results for each specified time was used for the calculation. The hemagglutinin inhibition end point was the geometric mean titer (GMT) at each time point, as well as the following variables recommended for interpanemic and prepanemic influenza vaccines: postvaccination seropositivity rate (% of subjects with titers of ≥ 64 , which is higher than the titer of 1:40 required by CHMP, the European Centre for Disease Protection and Control [ECDC], and the U.S. Food and Drug Administration [FDA]); the postvaccination-to-prevaccination GMT ratio; and the proportion of people seroconverting, meaning those displaying at least a fourfold titer increase postvaccination and having postvaccination titers of at least 1:64, which again is higher than the CHMP, ECDC, and FDA requirement of 1:40 (5, 10, 23). The hemagglutinin inhibition titer distributions are described with reverse cumulative distribution curves and were tested with the nonparametric Kruskal-Wallis test, as appropriate, to test for differences between groups. A P value of <0.05 was considered significant. Since there are no guidelines for microneutralization, we applied the same conventional criteria as those described above for HI.

RESULTS

A total of 431 patients completed the trial, and 430 were included in the statistical analyses (Fig. 1). There were no significant differences between the demographic characteristics of the study groups at baseline (Table 1).

Immunogenicity. The proportions of subjects with different postvaccination HI titers are shown in Fig. 2. The increases in geometric mean post- versus prevaccination titers for HI and MN 21 days after the final vaccination (i.e., day 42 for group II and day 21 for all other groups) are shown in Table 2. As expected, none of the individuals displayed measurable levels of HI antibodies before immunization (these titers were con-

FIG. 1. Study flow chart.

sidered 1:2 for calculation). The postvaccination-to-prevaccination GMT ratio (increase) exceeded the CHMP and ECDC criterion for all dose and age groups for HI (Table 2) (5, 10).

On day 21, seroconversion (i.e., a fourfold increase in the HI or MN titer) occurred in all groups, with the exception of HI titers in the adult population and MN titers in the elderly population receiving the smallest (3.5 µg) dose (groups IIa and b) (Table 2). On day 21, the required percentage of subjects with titers of >1:40 for HI was present in all dose and age groups, except the two groups receiving the smallest dose (groups IIa and b) (Table 2) (5, 10).

For groups IIa and IIb, smaller GMT ratio increases ($P < 0.001$), less frequent seroconversion, and smaller percentages of subjects with titers of >1:40 were detected than those for the other groups. Nevertheless, both of these groups still fulfilled at least one independent CHMP, ECDC, and FDA criterion for immunogenicity (Table 2). Otherwise, no significant differences in the end points were found between the study groups.

According to the CHMP interpandemic vaccine require-

ments, the ECDC prepandemic vaccine requirements, and the FDA pandemic vaccine requirements, all groups met at least one independent immunogenicity criterion for HI (Table 2) (5, 10, 23). Even the lowest total dose of one dose of 3.5 µg triggered an immune response, fulfilling one independent efficacy criterion for HI in the adult population and two HI criteria in the elderly population. All other doses met all CHMP, ECDC, and FDA criteria for HI for all age groups (5, 10, 23) (Table 2). Again, in addition, multiple sera were also tested independently by the Health Protection Agency (United Kingdom), where the results were confirmed.

Safety. All subjects included in the immunogenicity analysis were monitored for 180 days to provide safety data. In general, side effects were rare and mild. Injection site pain occurred in four cases (0.93%) and disappeared within 1 day. No other local reactions, such as injection site induration, erythema, swelling, or warmth, were noted. As for systemic reactions, two cases of malaise (0.47%) and two cases of fever (0.47%) were detected. No medical intervention was necessary. No vaccine-related serious adverse events were observed in any of the

TABLE 1. Patient demographics^a

Parameter	Value for group							
	Subjects of 18 to 60 yr				Subjects of >60 yr			
	Ia (2 doses of 3.5 µg)	IIa (1 dose of 3.5 µg)	IIIa (1 dose of 6 µg)	IVa (1 dose of 12 µg)	Ib (2 doses of 3.5 µg)	IIb (1 dose of 3.5 µg)	IIIb (1 dose of 6 µg)	IVb (1 dose of 12 µg)
No. of subjects	58	61	60	61	28	54	54	54
Age (yr) (mean ± SD)	39.9 ± 11.6	41.4 ± 11.1	40.5 ± 13.4	40.0 ± 12.7	69.3 ± 7.6	71.3 ± 7.3	68.1 ± 6.4	69.2 ± 7.1
Age range (yr)	20–59	18–60	19–60	19–60	60–87	60–88	60–83	60–90
% Male/% female	70.7/29.3	59.0/41.0	60.0/40.0	62.3/37.7	50.0/50.0	40.7/59.3	57.4/42.6	55.6/44.4

^a One hundred percent of patients had a white ethnic background.

FIG. 2. Reverse cumulative distribution curves for titers of neutralizing antibodies against avian influenza virus in four study groups. The data shown are percentages of subjects with specific HI titers after the first dose (day 21) and second dose (day 42; groups Ia and b only) of the vaccine.

groups. One patient in group IV died of an unrelated event (myocardial infarction 31 days after vaccination). There was no significant difference in the occurrence of adverse events between any of the groups. Notably, these results agree with a previous clinical trial of the present vaccine and with the 13-year experience of more than 16 million vaccinations in humans, using the seasonal influenza vaccine produced by the same method (24, 25).

DISCUSSION

Influenza-associated morbidity and mortality increase with age, especially for individuals with high-risk conditions, and it has been shown that influenza vaccination is associated with a reduced risk of mortality in community-dwelling elderly individuals (27). Nevertheless, only a few trials have examined elderly subjects, and some trials even excluded patients over 40 or 45 years of age (1, 4, 16).

The present vaccine is the only one with single-dose regimens. Most other trials used a larger total amount of antigen, and all of them required two injections, from 21 to 28 days apart, to achieve a response (1, 4, 13, 14, 16, 21). Application of the smallest possible amount of antigen and fewer injections are important in dose-sparing strategies, thereby increasing

production capacity (2, 13). This might become important during a pandemic, when vaccines will be in short supply. Moreover, applying one instead of two injections will shorten the time to develop immunity by 3 to 4 weeks. Patient compliance is also likely to be better with one injection. In our study, more patients in the group scheduled to receive two injections did not complete the trial.

The use of a whole-virus vaccine and an aluminum phosphate adjuvant system may at least partially explain the lower dose and fewer injections required to trigger the immune response in the present study. Whole-virus vaccines are considered to be more immunogenic (2), which can usually be enhanced further by use of adjuvants. Treanor et al. studied a subvirion vaccine without adjuvants (21), Bresson et al. studied a split-virion vaccine with and without aluminum hydroxide adjuvant, which was added to the vaccine at the bedside (1), and Lin et al. (14) and Ehrlich et al. (4) also used aluminum hydroxide, without reporting the preparation method. Leroux-Roels et al. utilized an oil-in-water emulsion, which was added just before vaccination (13). We used aluminum phosphate gel, which was added at the time of the production of the vaccine, thus complying with all principles and detailed guidelines of the CHMP for adjuvants in vaccines for human use (3, 8).

We detected a lower rate of side effects than those in other trials (1, 4, 13, 16, 21). Whole-virus vaccines are thought to be more reactogenic, but the few side effects seen in the present study may be explained at least in part by the smaller dose used in this study than in other trials (1, 4, 13, 16, 21). Furthermore, the endotoxin content of 0.05 IU/dose in the present vaccine was much smaller than the allowed amount of 100 IU/dose accepted by standards (7). The safety results of this study agree with a previous clinical trial of the present vaccine and with the 13-year experience of more than 16 million vaccinations in humans, using the seasonal influenza vaccine produced by the same method (24, 31). However, it should be noted that reporting systems and characteristics of subjects differ among various studies.

A single-dose schedule of 6 or 12 μ g of the studied vaccine showed an encouraging immunogenicity profile, as even the 6- μ g dose fulfilled all HI and microneutralization criteria for both age groups. The ideal dose seems to be 6 μ g, as immune responses seen were similar in patients receiving two 3.5- μ g doses or one 6- or 12- μ g dose. Thus, doses exceeding 6 μ g or two injections seem to be unnecessary. Our findings agree with those of a previous trial of the present vaccine, which found it to be immunogenic after a single dose of 6 μ g in 146 adults (24). We are now extending these studies to children, as a trial with a 6- μ g dose of the present vaccine in pediatric subjects was recently completed (26). Also, studies of cross-reactive immunity in a subset of the present study population were recently published (9).

A potential weakness of the study is that it enrolled white patients only. Since most fatal cases of H5N1 infections occurred in Asian and African countries, studies involving subjects of different ethnic backgrounds are highly desired (30). Thus, we are currently working on trials including African and Asian participants.

Essentially all nonvaccinated subjects were in group Ib (elderly patients scheduled for two 3.5- μ g doses). After statistical analysis, the results for group Ib were not different from those

TABLE 2. Immunogenicity findings for subjects in this study^a

Group or agency	HI data or criteria				MN data or criteria	
	Postvaccination GMT (95% CI)	Post/prevaccination GMT ratio (increase)	% of participants who were seropositive	% of participants who seroconverted	% of participants who seroconverted	Post/prevaccination GMT ratio (increase)
Subjects of 18 to 60 yr						
CHMP and ECDC	NA	>2.5	>70	>40	NA	NA
FDA	NA		>70	>40	NA	NA
Ia (2 doses of 3.5 µg) (n = 58)	39.7 (30.2; 52.2)	19.8 (15.1; 26.1)*	72.4 (59.1; 83.3)*	72.4 (59.1; 83.3)*	63.8 (50.1; 76.0)	5.5 (3.8; 7.9)
IIa (1 dose of 3.5 µg) (n = 61)	22.3 (17.4; 28.4)	11.1 (8.7; 14.2)*	37.7 (25.6; 51.0)	37.7 (25.6; 51.0)	42.7 (30.0; 55.9)	2.5 (1.98; 3.18)
IIIa (1 dose of 6 µg) (n = 60)	42.2 (33.0; 54.1)	21.1 (16.5; 27.0)*	71.7 (58.6; 82.6)*	71.7 (58.6; 82.6)*	63.3 (49.9; 75.4)	4.4 (3.3; 6.1)
IVa (1 dose of 12 µg) (n = 61)	48.7 (37.7; 62.9)	22.8 (16.9; 30.2)*	70.5 (57.4; 81.5)*	70.5 (57.4; 81.5)*	63.9 (50.6; 75.8)	5.0 (3.67; 6.87)
Subjects of >60 yr						
CHMP and ECDC	NA	>2	>60	>30	NA	NA
FDA	NA	NA	>60	>30	NA	NA
Ib (2 doses of 3.5 µg) (n = 28)	29.0 (16.4; 51.3)	14.5 (8.2; 25.6)*	60.7 (40.6; 78.5)*	60.7 (40.6; 78.5)*	78.6 (59.1; 91.7)	6.6 (4.2; 10.2)
IIb (1 dose of 3.5 µg) (n = 54)	20.2 (15.2; 26.7)	10.1 (7.6; 13.3)*	35.2 (22.7; 49.4)	35.2 (22.7; 49.4)*	24.1 (13.5; 37.6)	2.4 (1.9; 3.1)
IIIb (1 dose of 6 µg) (n = 54)	29.6 (20.3; 43.2)	14.8 (10.2; 21.6)*	64.8 (50.6; 77.3)*	64.8 (50.6; 77.3)*	64.8 (50.6; 77.3)	5.7 (4.1; 8.0)
IVb (1 dose of 12 µg) (n = 54)	30.4 (21.4; 43.2)	15.2 (10.7; 21.6)*	66.7 (52.5; 78.9)*	66.7 (52.5; 78.9)*	70.4 (56.4; 82.0)	6.0 (4.4; 8.4)

^a *, meets criteria for licensing by the CHMP for inter pandemic, ECDC for pre pandemic, and FDA for pandemic influenza vaccines. CHMP standards for immune responses elicited by inter pandemic influenza vaccines refer to hemagglutinin inhibition responses with a detection limit of 1 in 10 and a threshold of 1 in 40, whereas the results presented summarize hemagglutinin inhibition responses with a detection limit of 1 in 4 and a threshold of 1 in 64. Specimens were obtained 21 days after vaccination, meaning on day 42 for groups Ia and Ib and on day 21 for all other groups. Data are means with standard deviations. CHMP, EU Committee for Human Medicinal Products; ECDC, European Centre for Disease Protection and Control; FDA, U.S. Food and Drug Administration. NA, not applicable.

for groups IIIb and IVb, and all groups, including group Ib, had significantly stronger immune responses than group IIb. Thus, the larger number of patients in group Ib who withdrew consent prior to vaccination did not seem to have an effect on immunogenicity results. It does, however, highlight potential difficulties with compliance among elderly patients receiving multiple injections.

In June 2007, the WHO announced that it is working with vaccine manufacturers to move ahead on plans to create a global stockpile of vaccine for the H5N1 avian influenza virus. The announcement followed a request by the World Health Assembly in May 2007 for WHO to establish an international stockpile of H5N1 vaccine. The vaccine described in the present study has officially been offered to become part of the WHO stockpile (32).

A vaccine using a wild-type influenza A virus H5N1 strain was recently developed and found to be safe and immunogenic in healthy adults (Vajo et al., unpublished observations). In addition, a vaccine using the same methods as those described in this report, but based on a wild-type swine-origin 2009 (H1N1) influenza A virus isolate from the United States (supplied by the CDC), has been developed and tested in preclinical studies, and human trials are currently being completed.

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All authors had access to the data and contributed to data interpretation and to the writing of the manuscript. All authors have read and approved the final version of the manuscript.

REFERENCES

- Bresson, J. L., C. Perronne, O. Launay, C. Gerdil, M. Saville, J. Wood, K. Höschler, and M. C. Zambon. 2006. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. *Lancet* **367**:1657–1664.
- Dennis, C. 2006. Flu-vaccine makers toil to boost supply. *Nature* **440**:1099.
- European Parliament. 28 November 2001. EC guide to good manufacturing practice (GMP), vol. 4. Based upon directive 2001/83/EC of the European Parliament and the Council of 6 November 2001 relating to medicinal products for human use, p. 67–128. European Parliament, Strasbourg, France.
- Ehrlich, H. J., M. Müller, H. M. Oh, P. A. Tambyah, C. Joukadar, E. Montomoli, D. Fisher, G. Berezuk, S. Fritsch, A. Löw-Baselli, N. Vartian, R. Bobrovsky, B. G. Pavlova, E. M. Pöllabauer, O. Kistner, and P. N. Barrett. 2008. Baxter H5N1 Pandemic Influenza Vaccine Clinical Study Team. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. *N. Engl. J. Med.* **358**:2573–2584.
- European Committee for Proprietary Medicinal Products. 12 March 1997. Note for guidance on harmonization of requirements for influenza vaccines, March 1997 (CPMP/BWP/214/96). European Agency for the Evaluation of Medicinal Products, London, United Kingdom.
- European Committee for Proprietary Medicinal Products. 5 April 2004. Guideline on dossier structure and content for pandemic influenza vaccine marketing authorisation application (CPMP/VEG/471/03). European Agency for the Evaluation of Medicinal Products, London, United Kingdom.
- European Directorate for the Quality of Medicines. 2007. European pharmacopeia, p.3406–3407. European Directorate for the Quality of Medicines, Strasbourg, France.
- European Medicines Agency. 2005. Guideline on adjuvants in vaccines for human use (CHMP/VEG/134716/2004). European Medicines Agency, London, United Kingdom. <http://www.emea.europa.eu/pdfs/human/vwp/13471604en.pdf>.
- Fazekas, G., R. Martosne-Mendi, I. Jankovics, I. Szilvasy, and Z. Vajo. 2009.

- Cross-reactive immunity to clade 2 strains of influenza virus A subtype H5N1 induced in adults and elderly patients by Fluvax, a prototype pandemic influenza virus vaccine derived by reverse genetics, formulated with a phosphate adjuvant, and directed to clade 1 strains. *Clin. Vaccine Immunol.* **16**:437–443.
10. Giesecke, J., C. Sellwood, and J. Van-Tam. 2007. Expert advisory groups on human H5N1 vaccines, p.47. European Centre for Disease Control and Prevention, Stockholm, Sweden.
 11. Kendal, A. P., M. S. Pereira, and J. J. Skehel (ed.). 1982. Concepts and procedures for laboratory based influenza surveillance. Centers for Disease Control, Atlanta, GA.
 12. Klimov, A., and N. Cox. 2003. Serologic diagnosis of influenza virus infections by hemagglutination inhibition, p. 7.1–7.5. Centers for Disease Control, Atlanta, GA.
 13. Leroux-Roels, I., A. Borkowski, T. Vanwolleghem, M. Dramé, F. Clement, E. Hons, J. M. Devaster, and G. Leroux-Roels. 2007. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. *Lancet* **370**:580–589.
 14. Lin, J., J. Zhang, X. Dong, H. Fang, J. Chen, N. Su, Q. Gao, Z. Zhang, Y. Liu, Z. Wang, M. Yang, R. Sun, C. Li, S. Lin, M. Ji, Y. Liu, X. Wang, J. Wood, Z. Feng, Y. Wang, and W. Yin. 2006. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. *Lancet* **368**:991–997.
 15. National Institute of Pharmacy. 1995. License number OGYI-T-8998/01. National Institute of Pharmacy, Budapest, Hungary.
 16. Nolan, T. M., P. C. Richmond, M. V. Skeljo, G. Pearce, G. Hartel, N. T. Formica, K. Höschler, J. Bennet, D. Ryan, K. Papanaoum, R. L. Bassier, and M. C. Zambon. 2008. Phase I and II randomised trials of the safety and immunogenicity of a prototype adjuvanted inactivated split-virus influenza A (H5N1) vaccine in healthy adults. *Vaccine* **26**:4160–4167.
 17. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **27**:493–497.
 18. Rowe, T., R. A. Abernathy, J. Hu-Primmer, W. W. Thompson, X. Lu, W. Lim, K. Fukuda, N. J. Cox, and J. M. Katz. 1999. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. *J. Clin. Microbiol.* **37**:937–943.
 19. Stephenson, I., K. G. Nicholson, J. M. Wood, M. C. Zambon, and J. M. Katz. 2004. Confronting the avian influenza threat: vaccine development for a potential pandemic. *Lancet Infect. Dis.* **4**:499–509.
 20. Takatsy, G. 1952. Purified precipitated virus obtained by a new simple method. *Acta Med. Acad. Sci. Hung.* **3**:185–191.
 21. Treanor, J. J., J. D. Campbell, K. M. Zangwill, T. Rowe, and M. Wolff. 2006. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N. Engl. J. Med.* **354**:1343–1351.
 22. Ungchusak, K., P. Auewarakul, S. F. Dowell, R. Kitphati, W. Auwanit, P. Puthavathana, M. Uiprasertkul, K. Boonnak, C. Pittayawonganon, N. J. Cox, S. R. Zaki, P. Thawatsupha, M. Chittaganpitch, R. Khontong, J. M. Simmerman, and S. Chunsutthiwat. 2005. Probable person-to-person transmission of avian influenza A (H5N1). *N. Engl. J. Med.* **352**:333–340.
 23. U.S. Food and Drug Administration. 2007. Draft guidance on clinical data needed to support the licensure of pandemic influenza vaccines. U.S. Food and Drug Administration, Washington, DC. <http://www.fda.gov/cber/gdlns/pandfluvac.htm>.
 24. Vajo, Z., L. Kosa, I. Visontay, M. Jankovics, and I. Jankovics. 2007. Inactivated whole virus influenza A (H5N1) vaccine. *Emerg. Infect. Dis.* **13**:807–808.
 25. Vajo, Z., L. Kosa, I. Szilvasy, Z. Pauliny, K. Bartha, I. Visontay, M. Jankovics, A. Kis, and I. Jankovics. 2008. Yearly licensing studies from 1997–2007 of the inactivated whole virus seasonal influenza vaccine Fluvax—a useful approach to pandemic vaccine development even in less well developed countries? *Influenza Other Respir. Viruses* **2**:211–218.
 26. Vajo, Z., L. Kosa, I. Szilvasy, Z. Pauliny, K. Bartha, I. Visontay, A. Kis, I. Tarjan, N. Rozsa, and I. Jankovics. 2008. Safety and immunogenicity of a pre-pandemic influenza A (H5N1) vaccine in children. *Pediatr. Infect. Dis. J.* **27**:1052–1056.
 27. Voordouw, A. C., M. C. Sturkenboom, J. P. Dieleman, T. Stijnen, D. J. Smith, J. van der Lei, and B. H. Stricker. 2004. Annual revaccination against influenza and mortality risk in community-dwelling elderly persons. *JAMA* **292**:2089–2095.
 28. Walls, H. H., M. W. Harmon, J. J. Slagle, C. Stocksdales, and A. P. Kendal. 1986. Characterization and evaluation of monoclonal antibodies developed for typing influenza A and influenza B viruses. *J. Clin. Microbiol.* **23**:240–245.
 29. Wood, J. M., G. C. Schild, R. W. Newman, and V. Seagroatt. 1977. An improved single-radial-immunodiffusion technique for the assay of influenza haemagglutinin antigen: application for potency determinations of inactivated whole virus and subunit vaccines. *J. Biol. Stand.* **5**:237–247.
 30. World Health Organization. 13 June 2009, accession date. Cumulative number of confirmed human cases of avian influenza A (H5N1) reported to WHO. World Health Organization, Geneva, Switzerland. http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_06_02/en/index.html.
 31. World Health Organization. 2009. Tables on the clinical trials of pandemic influenza prototype vaccines. World Health Organization, Geneva, Switzerland. http://www.who.int/vaccine_research/diseases/influenza/flu_trials_tables/en/index.html.
 32. World Health Organization. 13 June 2007, posting date. WHO and manufacturers move ahead with plans for H5N1 influenza global vaccine stockpile. World Health Organization, Geneva, Switzerland. www.who.int/mediacentre/news/statements/2007/s14/en/index.html.
 33. Yang, Y., M. E. Halloran, J. D. Sugimoto, and I. M. Longini, Jr. 2007. Detecting human-to-human transmission of avian influenza A (H5N1). *Emerg. Infect. Dis.* **13**:1348–1353.