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Phase I Evaluation of Intranasal Trivalent Inactivated Influenza Vaccine with Nontoxigenic *Escherichia coli* Enterotoxin and Novel Biovector as Mucosal Adjuvants, Using Adult Volunteers

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Trivalent influenza virus A/Duck/Singapore (H5N3), A/Panama (H3N2), and B/Guandong vaccine preparations were used in a randomized, controlled, dose-ranging phase I study. The vaccines were prepared from highly purified hemagglutinin and neuraminidase from influenza viruses propagated in embryonated chicken eggs and inactivated with formaldehyde. We assigned 100 participants to six vaccine groups, as follows. Three intranasally vaccinated groups received 7.5-μg doses of hemagglutinin from each virus strain with either 3, 10, or 30 μg of heat-labile *Escherichia coli* enterotoxin (LTK63) and 990 μg of a supramolecular biovector; one intranasally vaccinated group was given 7.5-μg doses of hemagglutinin with 30 μg of LTK63 without the biovector; and another intranasally vaccinated group received saline solution as a placebo. The final group received an intramuscular vaccine containing 15 μg hemagglutinin from each strain with MF59 adjuvant. The immunogenicity of two intranasal doses, delivered by syringe as drops into both nostrils with an interval of 1 week between, was compared with that of two inoculations by intramuscular delivery 3 weeks apart. The intramuscular and intranasal vaccine formulations were both immunogenic but stimulated different limbs of the immune system. The largest increase in circulating antibodies occurred in response to intramuscular vaccination; the largest mucosal immunoglobulin A (IgA) response occurred in response to mucosal vaccination. Current licensing criteria for influenza vaccines in the European Union were satisfied by serum hemagglutination inhibition responses to A/Panama and B/Guandong hemagglutinins given with MF59 adjuvant by injection and to B/Guandong hemagglutinin given intranasally with the highest dose of LTK63 and the biovector. Geometric mean serum antibody titers by hemagglutination inhibition and microneutralization were significantly higher for each virus strain at 3 and 6 weeks in recipients of the intramuscular vaccine than in recipients of the intranasal vaccine. The immunogenicity of the intranasally delivered experimental vaccine varied by influenza virus strain. Mucosal IgA responses to A/Duck/Singapore (H5N3), A/Panama (H3N2), and B/Guandong were highest in participants given 30 μg LTK63 with the biovector, occurring in 7/15 (47%; $P = 0.0103$), 8/15 (53%; $P = 0.0362$), and 14/15 (93%; $P = 0.0033$) participants, respectively, compared to the placebo group. The addition of the biovector to the vaccine given with 30 μg LTK63 enhanced mucosal IgA responses to A/Duck/Singapore (H5N3) ($P = 0.0491$) and B/Guandong ($P = 0.0028$) but not to A/Panama (H3N2). All vaccines were well tolerated.

Annual outbreaks of influenza A and B and pandemics of influenza A are responsible for substantial mortality and morbidity, particularly in high-risk groups, including the elderly and those with chronic underlying medical conditions. Our ability to reduce the impact of influenza depends mainly on immunization. However, despite the availability of effective parenteral vaccines, influenza still incurs considerable medical and socioeconomic costs. Barriers limiting vaccine uptake include the intramuscular route of injection and the perception of vaccine ineffectiveness (11, 31). Targeting influenza vaccines to the respiratory tract, the site of virus entry and principal location of replication, offers potential advantages over parenteral vaccination. While current intramuscular influenza vaccines are effective at inducing immunoglobulin G (IgG) for serum hemagglutination inhibition (HAI), they are poor at stimulating mucosal secretory IgA (8, 22, 32). Mucosal IgA exhibits both heterosubtypic cross-reactivity to influenza virus strains and potent immunological memory (10, 37), properties that offer potentially wider protection against variants of influenza virus that have drifted antigenically from the vaccine strain (33, 34). Thus, stimulation of both local and systemic immune responses following influenza vaccination may enhance vaccine efficacy, particularly among the elderly, who exhibit age-related reductions in immunity to vaccination.

The simple intranasal route of administration offers the possibility for self-administration and could reduce the costs of delivery and increase vaccine uptake. The potential value of a mucosal delivery method was recognized at a meeting convened recently by the WHO (6). Although a number of hurdles were recognized, it was suggested that the study of this approach for vaccine delivery should continue. The intranasal,

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live-attenuated, cold-adapted (*ca*) influenza vaccine is well tolerated and highly effective in the prevention of influenza in young children (3) but induces modest humoral and secretory immune responses in the elderly (29, 35) and provides no added efficacy over the inactivated vaccine in older adults with chronic obstructive pulmonary disease (17). Inactivated influenza virus antigens are not reliably immunogenic in people when delivered mucosally (10) unless they are administered with a potent mucosal adjuvant such as *Escherichia coli* heat-labile enterotoxin (16, 19). Heat-labile enterotoxin (LT) from *Escherichia coli* is composed of five B subunits (responsible for binding to the cells) linked to an A subunit which, after binding to the cells, dissociates into A1 and A2 polypeptide chains. The A1 portion is responsible for ADP-ribosylating activity, which results in increased intracellular levels of cyclic AMP, causing profuse diarrhea. To avoid the toxicity associated with the use of the native holotoxin, several genetically modified derivatives with single point mutations that inhibit or completely prevent the ADP-ribosylating enzyme activity have been constructed by site-directed mutagenesis. One of these mutants, LTK63, has a serine-to-lysine substitution at position 63 of the A1 fragment, which is located on the "floor" of the NAD-binding cavity. This mutant appears to be devoid of toxic activity in vivo and in vitro, while retaining the adjuvant effect (27, 30). Intranasal delivery of LTK63 with a bioadhesive delivery system (biovector), which acts as a carrier for antigens and adjuvants, facilitates the delivery of antigens to antigen-presenting cells and, after nasal delivery, helps mice to develop humoral responses comparable to those to conventional parenteral vaccination (2, 26). Multiple intranasal administrations of LTK63 in animals are safe and produce no histological inflammatory changes in the respiratory tract or olfactory or meningeal tissues (26).

We undertook a partially observer blind, phase I, single-center study of a trivalent intranasal vaccine containing antigens from two human strains, influenza virus A/Panama/2007/99 (H3N2) and B/Guandong/2000, and an avian strain, A/Duck/Singapore/97 (H5N3), which we evaluated previously as a vaccine candidate against human H5N1 influenza (25), with LTK63 used as an adjuvant. We compared the safety and immunogenicity of an intranasal influenza vaccine containing 3, 10, or 30 μg LTK63 and the biovector with those of an intranasal vaccine containing 30 μg LTK63 but lacking the biovector, an intramuscular vaccine formulation with adjuvant based on a licensed formulation containing the same antigens (which is licensed in certain countries in Europe but not in the United States), and an intranasal placebo consisting of phosphate-buffered saline. We used antigens from two circulating influenza virus strains (A/H3N2 and a B strain) and a novel avian apathogenic H5N3 virus strain so we could examine the immune responses of immunologically naïve and primed subjects. The comparator parenteral vaccine contained MF59 adjuvant, which significantly increases the antibody responses of adult volunteers to H5N3 virus compared to those to the conventional subunit vaccine (25). We delivered intranasal vaccines by drops rather than spray, as delivery by drops is as immunogenic or more so than spray (15, 20) and because drops are better distributed in the nasal cavity (1, 18).

MATERIALS AND METHODS

Vaccine formulations. The A/Duck/Singapore/97 (H5N3), A/Panama/2007/99-like (H3N2), and B/Guandong/120/2000 strains of influenza virus were cultivated in embryonated hen eggs with biosafety level 2 containment, using conventional procedures. The intranasal vaccine formulations contained 7.5 μg hemagglutinin from each virus per 0.3-ml dose, with either 3, 10, or 30 μg of LTK63 and 990 μg the bioadhesive delivery system (biovector) or 30 μg LTK63 without the biovector. The biovector is a supramolecular, nanoparticulate drug delivery system with a positively charged polysaccharide core enclosed by a phospholipid-cholesterol double layer (2). LTK63, a genetically mutated *Escherichia coli* heat-labile enterotoxin, was made by substituting lysine for serine at amino acid position 63 of the A subunit. The surface antigen vaccine with MF59 adjuvant for parenteral administration contained 15 μg hemagglutinin from each strain per 0.5-ml dose, with 9.75 mg squalene, 1.175 mg polysorbate 80, 1.175 mg sorbitan trioleate, sodium citrate dihydrate, and citric acid monohydrate. The neuraminidase contents of vaccines are not standardized and are not known for any formulation. The dilutant for intranasal and intramuscular vaccines was phosphate-buffered saline. A second control group received an intranasal placebo of phosphate-buffered saline.

Participants. We assessed healthy volunteers of 18 to 40 years of age during May to August 2002 at the Leicester Royal Infirmary, Leicester, United Kingdom. We excluded people if they had serious underlying chronic illness (mild and stable asthma was allowed), allergic rhinitis, or immunosuppression due to illness or treatment; were pregnant or were women who refused to use a reliable method of contraception during the study; had received blood products during the preceding 3 months; had been vaccinated or taken experimental drugs during the previous 4 weeks; had experienced anaphylaxis; had an allergy to eggs, vaccine, antibiotics, or mercury-containing products; had laboratory-confirmed influenza or had been vaccinated against influenza in the previous 6 months; had been vaccinated against H5N3 influenza; had acute respiratory illness needing antibiotics or antivirals in the previous 7 days; had a temperature of higher than 38°C in the preceding 3 days; or could not give informed consent. The Medicines Control Agency gave regulatory approval, the Leicester ethics committee approved the study, and volunteers gave signed informed consent.

Clinical protocol. The study was a single-center, partially observer blind, randomized, controlled, dose-ranging phase I study with six groups (Table 1). Volunteers received two identical 0.3-ml doses of trial intranasal vaccines, separated by 7 days, by instillation of 0.15 ml into each nostril on each occasion by syringe. Two intramuscular injections of comparator vaccine with MF59 adjuvant were given 3 weeks apart in the deltoid muscle of the nondominant arm. The second dose was given to boost the immune response to the H5 hemagglutinin (25). A dose-escalating approach was used to assign intranasal vaccines containing increasing amounts of LTK63. The first vaccinees were randomized to receive intranasal placebo, intranasal vaccine containing 3 μg LTK63 with biovector, or intramuscular vaccine. Two weeks later, provided that no serious vaccine-related event had occurred during the first 2 weeks, a second cohort was randomly allocated placebo or the vaccine containing 10 μg LTK63 with biovector. Two weeks later, a third cohort was randomized to receive intranasal placebo or the intranasal vaccine containing 30 μg LTK63 with or without biovector, provided that no serious vaccine-related event had occurred during the previous 4 weeks. The volunteers receiving intranasal vaccines and the investigators, with the exception of one nurse who gave the vaccines, were unaware of the vaccine type given. The nurse had no further contact with the volunteers after the vaccine had been given. Vaccines were given in coded prefilled syringes. We used a computer-generated randomization code, which was held by the manufacturers until data entry was completed, adverse experiences were graded, and serological results were collated.

Serum samples for antibody titrations were obtained immediately before each vaccine administration and then 3 weeks and 6 weeks later, i.e., on days 0, 28, and 49 for intranasal groups and days 0, 21, and 63 for the intramuscular group. We collected nasal secretions before and 6 weeks after vaccination, i.e., on days 0 and 49 for the intranasal group and days 0 and 63 for the intramuscular group. We measured blood pressure, pulse, and temperature before and 30 min after each vaccination. Participants were assessed before and 30 min after vaccination for local and systemic reactions. They used a diary card to record local and systemic symptoms, temperature, and analgesic drugs taken for up to 7 days after each dose. Participants recorded symptoms as none, mild (defined as the occurrence of a symptom, but not often enough to cause inconvenience), moderate (interference with daily activities but not needing medical intervention), or severe (interference with daily activities and needing medical intervention). We made a follow-up telephone call 2 and 7 days after each dose to check that no serious reactions (life-threatening or disabling symptoms needing admission) had oc-

TABLE 1. Baseline characteristics by study group

Vaccine group[a]	No. of subjects in group		Age (yrs) (median [range])	Ethnicity (no. of subjects)		
	Male	Female		White	Asian	Black
Intranasal placebo	1	12	29 (21–40)	13	0	0
Intranasal vaccine (7.5 μg HA per virus strain on days 0 and 7) plus biovector and mucosal adjuvant LTK63						
3 μg LTK63	8	9	25 (18–35)	12	2	3
10 μg LTK63	3	13	25.5 (20–39)	13	2	1
30 μg LTK63	2	16	26 (20–40)	15	3	0
Intranasal vaccine (7.5 μg HA per virus strain on days 0 and 7) plus 30 μg LTK63 and no biovector	3	16	30.0 (21–38)	14	2	3
Intramuscular vaccine with MF59 adjuvant (15 μg HA per virus strain on days 0 and 21)	4	13	26.0 (19–36)	14	0	3

[a] HA, hemagglutinin.

curred so that the next highest dose of LTK63 could be given to participants. Adverse events were noted throughout the study.

Evaluation of the immune response. Blood and nasal wash samples were coded for analysis. We did hemagglutination inhibition tests in duplicate, using turkey erythrocytes, by standard methods (14). Serum sample dilutions ranged from 1 in 8 to 1 in 2,048 in serial twofold dilutions. The titer is expressed as the reciprocal of the highest dilution of serum that completely inhibited hemagglutination. Hemagglutination inhibition tests are insensitive for the detection of antibody to H5 hemagglutinin, so we also measured neutralizing antibodies by a microneutralization (MN) assay based on described methodology (25). Serum sample dilutions ranged from 1 in 10 to 1 in 1,280 in serial twofold dilutions. The end-point MN antibody titer was measured at the 50% neutralization point. We did three assays with at least six replicates and used the geometric mean (GM) in the final analysis. Test viruses (influenza viruses A/Panama/2007/99 [H3N2], A/Duck/Singapore/97 [H5N3], and B/Guandong/2000) were grown in 10-day-old embryonated hen eggs. We used polyclonal sheep antiserum to A/Chick/Scotland/97 (H5N1) and homologous postinfection ferret antiserum samples as positive controls, with pooled human sera as the negative control. Pre- and postvaccination samples were tested together at the same time.

We measured IgA and IgG responses to influenza viruses A/Panama/2007/99 (H3N2), A/Duck/Singapore/97 (H5N3), and B/Guandong/2000 in nasal wash samples by enzyme-linked immunosorbent assay (ELISA) methods. Plates were coated with hemagglutinin and neuraminidase antigens from each virus at concentrations of 1.5, 1.5, and 0.75 μg/ml, respectively. Test and control sera were added in duplicate and diluted from 1 in 3 to 1 in 6,561 in serial threefold dilutions. Influenza virus-specific IgA or IgG was detected using goat anti-human IgA or IgG conjugated with horseradish peroxidase, orthophenylenediamine, and H_2O_2 as the enzyme substrate. The end-point titer was determined as the reciprocal dilution giving an absorbance at 450 nm of 0.4 above the background. Total IgA and IgG antibody concentrations in nasal secretions were determined by ELISA as described previously (4). The ELISA for influenza virus-specific or total IgG was repeated if the end-point titers determined in duplicate differed more than twofold. Samples with titers below the detection limit were assigned a value of one-half the lowest dilution. The influenza virus-specific IgA and IgG concentrations were adjusted to the total IgA and IgG concentrations in nasal secretions to account for variations in the immunoglobulin contents of samples collected on different occasions. Mucoconversion was defined as a 2.5-fold rise in influenza virus-specific immunoglobulin. This level was based on initial parallel testing of coded pre- and postimmunization samples from eight people, with each sample tested in duplicate for both specific and total IgA and IgG and with analysis of the variation for each assay. Analysis showed that a ≥2.5-fold rise in titer exceeded the maximal assay variation, indicating a statistically significant difference with a P value of <0.01.

Statistical analysis. Our intended group size of 15 to 20 subjects for each group is usual for phase I trials and was not based on sample size, power calculations, or licensing criteria. The measures of immunogenicity were increases in systemic geometric mean HAI and MN titers and mean influenza virus-specific mucosal IgG and IgA concentrations in nasal secretions after vaccination. We assessed the HAI serological responses to vaccination against the Committee for Human Medicinal Products (CHMP) criteria for interpandemic vaccines. Fulfillment of these criteria is a requirement for annual registration of interpandemic vaccines in the European Union (9). These vaccine registration criteria for adults (18 to 60 years old) are seroconversion in >40% of participants, a mean geometric ratio of postvaccination to prevaccination HAI titers of >2.5, and seroprotection (postvaccination HAI titers of ≥1:40) in over 70% of those vaccinated. Data were analyzed with SAS, version 8.2. We compared the numbers of participants who recorded reactions after immunization between vaccine types by the χ^2 test. A generalized linear model was used to compare $\log_{10}$-transformed serum antibody titers. No data transformation was done for comparisons of influenza virus-specific IgG and IgA concentrations in nasal secretions. The percentage of subjects achieving seroprotection (reciprocal HAI titer of ≥40) or seroconversion (i.e., those who developed fourfold or higher rises in HAI or MN serum antibody titers), ≥2.5-fold increases in IgA or IgG concentration in nasal secretions, and associated 95% confidence intervals (CIs) were computed for each vaccine group and compared by the χ^2 test or Fisher's exact test when appropriate. Analysis was performed for each protocol.

RESULTS

Characteristics of volunteers and adverse reactions. One hundred persons (Table 1), mostly nurses, were recruited to the study. One, two, and ten participants were withdrawn from the intramuscular, placebo, and intranasal groups, respectively, because they did not attend for the second vaccine dose. Two individuals were withdrawn from the study because they did not attend for final blood samples and review 6 weeks after vaccination. All four intranasal vaccine formulations, as well as the parenteral vaccine, were well tolerated (Table 2). There were no significant differences between recipients of the intranasal vaccine and placebo in the occurrence of solicited local and systemic reactions of any severity. There was no linear trend for increasing frequencies of nasal symptoms with increasing doses of LTK63. Occasionally, some local and systemic reactions were reported to interfere with daily activities, but these events were no more frequent in recipients of the intranasal vaccine than in those of the placebo. The intranasal vaccine was better tolerated than the intramuscular vaccine, with fewer recipients reporting pain at the site of vaccine delivery (17/70 versus 14/17; $P < 0.0001$) or the use of analgesics or antipyretics (16/70 versus 10/17; $P = 0.0125$). Most symp-

TABLE 2. Adverse reactions to both doses of trivalent influenza vaccine

Adverse reaction	No. of vaccinees with adverse reaction						*P* value	
	Intranasal vaccine[a]					Intramuscular vaccine with MF59 and no BV ($n = 17$)[a]	All intranasal groups versus placebo group	All intranasal groups versus intramuscular group
	Placebo, no BV ($n = 13$)	LTK63 (3 μg) + BV ($n = 17$)	LTK63 (10 μg) + BV ($n = 16$)	LTK63 (30 μg) + BV ($n = 18$)	LTK63 (30 μg), no BV ($n = 19$)			
Local reactions								
Nasal discomfort	6	6	3	2	5		0.1598	
Sneezing	6	7	8	10	11		0.9623	
Stuffy nose	8	8	5	9	11		0.5148	
Runny nose	8	5	9	10	13		0.7842	
Loss of smell	4	2	3	1	2		0.1640	
Red eyes	3	2	0	1	7		0.6999	
Lacrimation	2	1	2	0	3		0.8005	
Facial swelling	1	2	0	0	0		0.9611	
Nasal pain	4	4	3	5	5		0.8835	
Pain at injection site						14		
Erythema of >10 mm						2		
Induration of >10 mm						1		
Systemic reactions								
Fever of >38°C	0	0	0	1	0	1	0.3491	0.8438
Chills	4	2	1	3	2	3	0.1640	0.7755
Fatigue	3	5	5	6	3	3	0.9704	0.9266
Cough	5	1	2	1	4	1	0.0406	0.0801
Myalgia	3	3	2	1	3	3	0.5941	0.9266
Headache	7	9	9	10	8	7	0.8871	0.7489
Nausea	4	7	3	2	5	3	0.8835	0.6844
Arthralgia	1	0	1	0	1	1	0.9611	0.5881
Diarrhea	1	1	0	0	1	2	0.9611	0.8060
Analgesic/antipyretic use	1	1	4	6	5	10	0.3843	0.0125

[a] BV, biovector.

toms resolved within 72 h, and no absenteeism was recorded. There was no significant difference between recipients of the intranasal vaccine and placebo in the occurrence of unsolicited adverse events (21% to 47% versus 31%). There was no trend of increasing reactions with increasing doses of adjuvant. Pharyngitis was the most common unsolicited adverse event (16% overall), with the most cases occurring with the placebo (23%).

Humoral immune response. Figure 1 shows the GM serological immune responses. Significant increases in GM HAI antibody titers to B/Guandong/2000 were measured at 3 ($P = 0.0004$) and 6 weeks ($P = 0.0002$) in volunteers who received 30 μg LTK63 with biovector compared with those receiving the placebo. GM serum antibody titers by HAI and MN were significantly higher for each virus strain at 3 and 6 weeks in recipients of the intramuscular vaccine than in those receiving the intranasal vaccine (Fig. 1). The increases in GM HAI titer (i.e., the GM ratio of postimmunization to preimmunization titers) were significantly higher at 6 weeks for the A/Panama (H3N2) virus ($P = 0.04$) and at 3 and 6 weeks for influenza virus B/Guandong ($P = 0.002$ for each occasion) when all combined intranasal vaccine groups were compared to the placebo group (Table 3). Increases in GM HAI titer were significantly higher for the intramuscular vaccine group than for all combined intranasal vaccine groups—at 3 weeks, the maximum *x*-fold increases in GM HAI titer with intranasal vaccine were 1.9 (95% CI, 1.4 to 2.7) for the A/Panama (H3N2) vaccine and 4.8 (95% CI, 2.4 to 9.6) for the B/Guandong vaccine; the values for the intramuscular vaccine were 5.3 (95% CI, 2.6 to 11) and 27 (95% CI, 12 to 59), respectively (Table 3).

We used the A/Panama/2007/99 (H3N2) and B/Guandong/2000 viruses as test antigens to assess HAI serological responses to vaccination against the CHMP criteria for interpandemic vaccines (Table 3). One dose of intramuscular vaccine passed two of the three criteria (at least one of three criteria must be satisfied in at least 50 people) for its A/Panama/2007/99 (H3N2) component and all three criteria for its B/Guandong/2000 component. The immunogenicity of the intranasally delivered experimental vaccine varied by influenza virus strain. The intranasal vaccine containing 30 μg LTK63 with biovector passed two of three criteria for its B/Guandong component 3 weeks after vaccination and all three criteria at 6 weeks. No intranasal formulation passed any criteria when assessed against A/Panama/2007/99 (H3N2). There are no comparable criteria for pandemic vaccines and none involving the MN test. The A/Duck/Singapore/97 (H5N3) MN results showed significant rises in geometric mean titers (mean rises of 5.92-fold versus 1.92-fold; $P < 0.0001$) and seroconversion rates (87% versus 33%; $P = 0.0013$) (data not shown) at week 6 compared to week 3 after a second dose of intramuscular vaccine, but the serum antibody responses to the intranasal formulations were not greater than those to the placebo on either occasion (Fig. 1). On day 42, the GM MN A/Duck/Singapore (H5N3) antibody titer after the second 15-μg intramuscular dose of A/Duck/Singapore (H5N3) vaccine was almost 1 in 30.

Specific IgA response in nasal secretions. The mucosal antibody responses measured in nasal secretions 6 weeks after vaccination with the intranasal (15 μg hemagglutinin per

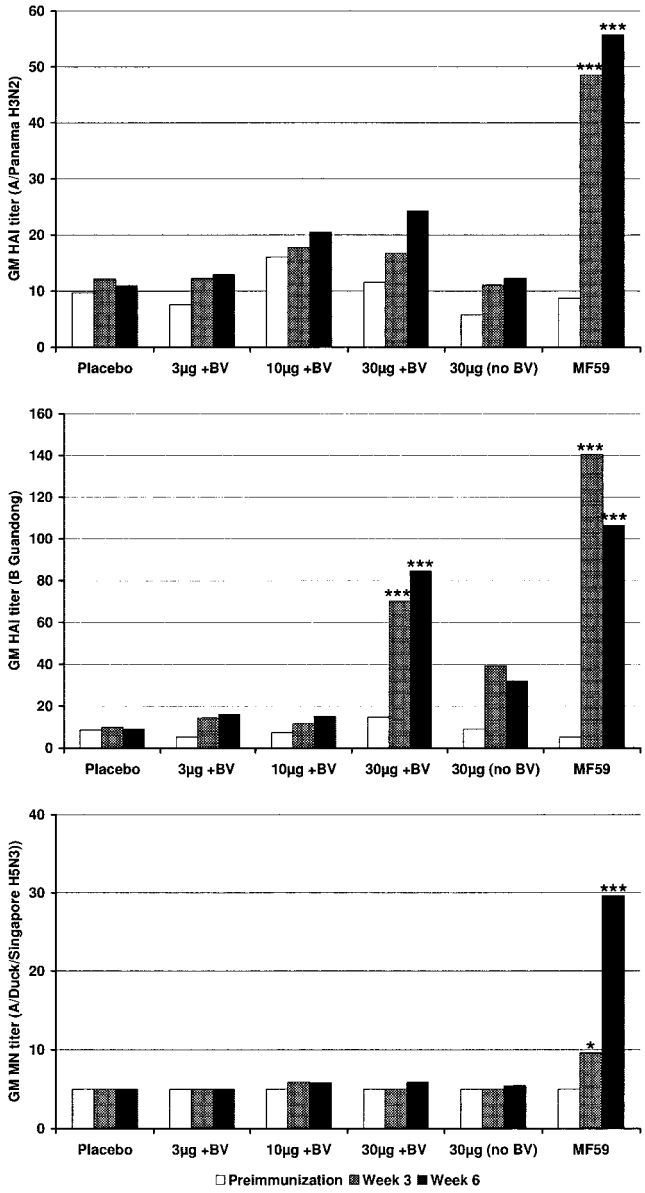

FIG. 1. GM HAI or MN antibody titers pre- and postimmunization with placebo and intranasal and intramuscular vaccines containing influenza virus A/Panama (H3N2), B/Guandong, and A/Duck/Singapore (H5N3) antigens. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ (for comparison of the intramuscular vaccine with all intranasal vaccine groups or for comparison of any intranasal vaccine group with the placebo). BV, biovector.

strain) and intramuscular (30 μg hemagglutinin per strain) vaccines are shown in Fig. 2 (*x*-fold increases) and 3 (mucoconversion rates). The largest increase in virus-specific IgA was measured in subjects given 30 μg LTK63 and biovector, where results were significantly better for all strains, including A/Duck/Singapore/97 (H5N3), than those for the placebo or intramuscular vaccine. In subjects given the intranasal vaccine containing 30 μg LTK63 with biovector, the mean virus-specific mucosal IgA concentrations increased 2.8- to 6.3-fold (Fig. 2). IgA mucoconversion for A/Duck/Singapore (H5N3), A/Panama (H3N2), and B/Guandong was significantly higher than that for the placebo, occurring in 7/15 (47%) ($P = 0.0103$), 8/15 (53%) ($P = 0.0362$), and 14/15 (93%) ($P = 0.0033$) volunteers, respectively (Fig. 3) ($P = 0.0154$ for comparison of mucoconversion rates across groups). The mucoconversion rates to B/Guandong and A/Duck/Singapore antibody in subjects given 30 μg LTK63 and biovector were significantly greater than that for recipients of 30 μg LTK63 without biovector ($P = 0.0028$ and $P = 0.0491$, respectively).

Specific IgG response in nasal secretions. The mucosal immune responses in nasal secretions are shown in Fig. 2 and 3. The largest increases in virus-specific IgG were measured in subjects given the intramuscular vaccine, where GM rises and IgG mucoconversion rates were significantly better for all strains. The mean mucosal IgG concentrations in subjects given the intramuscular vaccine increased 3.5- to 15.5-fold (Fig. 2), and the mucoconversion rate was 60% to 93% (Fig. 3). There was no significant increase in virus-specific IgG in any intranasal group.

DISCUSSION

Our study is the first conducted in humans of an intranasal vaccine with LTK63 as an adjuvant. We showed that intranasal influenza vaccines with LTK63 as an adjuvant and an intramuscular vaccine with MF59 as an adjuvant stimulate different antibody responses, which is consistent with comparisons of live *ca* influenza vaccine and plain, inactivated vaccine (5). The strongest virus-specific mucosal IgA responses in our study occurred with the intranasal vaccine containing 30 μg LTK63 and biovector, which significantly increased IgA levels to all three virus strains. In contrast, two doses of intramuscular vaccine with MF59 as an adjuvant failed to elicit significant rises in mucosal IgA to any virus strain. Virus-specific IgA mucoconversion rates (i.e., ≥2.5-fold increases in IgA antibody concentration) for A/Panama/2007/99 (H3N2), A/Duck/Singapore/97 (H5N3), and B/Guandong/2000 ranged from 47% to 93% in those given the vaccine containing 30 μg LTK63 and biovector, and the mean virus-specific IgA levels increased 2.8- to 6.3-fold. The addition of the biovector enhanced the mucosal IgA responses to two of the three vaccine strains. The IgA response to the intranasal vaccine containing 30 μg LTK63 and biovector is comparable with that of an inactivated intranasal virosomal vaccine, which was licensed in Switzerland for the 2000–2001 influenza season and contained wild-type *E. coli* heat-labile holotoxin as a mucosal adjuvant—the corresponding IgA mucoconversion rates for the A-H1N1, A-H3N2, and influenza B virus antigens in the Swiss vaccine were 50 to 57%, and geometric mean titers increased 2.5- to 2.8-fold compared to the baseline (13). Similarly, the IgA responses in our study elicited to the vaccine formulation containing 30 μg LTK63 and biovector are comparable with those elicited by the live *ca* influenza vaccine that is licensed in the United States, as roughly one-half of recipients given live vaccine respond with local IgA production to influenza A H3N2 and A H1N1 viruses, while the response to influenza B virus is evidently lower (5). For reasons that are unclear, we found that the serum hemagglutination inhibition responses and mucosal IgA responses to currently circulating virus strains in intranasally delivered vaccine formulations differed by the strain of influenza virus.

TABLE 3. HAI results in relation to the CHMP criteria for the assessment of interpandemic influenza vaccines[a]

Virus and parameter	Value for indicated vaccine[b]						*P* value[c]	
	Intranasal vaccines					Intramuscular vaccine with MF59 and no BV ($n = 15$)	Intranasal vaccines versus placebo	Intramuscular vaccine versus intranasal vaccines
	Placebo, no BV ($n = 11$)	LTK63 (3 μg) + BV ($n = 13$)	LTK63 (10 μg) + BV ($n = 14$)	LTK63 (30 μg) + BV ($n = 15$)	LTK63 (30 μg), no BV ($n = 17$)			
A/Panama (H3N2)								
Geometric mean fold increase								
Wk 3 to wk 0	1.2	1.6	1.3	1.5	1.9	5.3[b]	NS	<0.01
Wk 6 to wk 0	1.1	1.7	1.3	2.1	2.2	6.4[b]	<0.05	<0.001
% of subjects with HAI titers of ≥1:40 (seroprotection)								
Wk 3	9	8	14	13	0	67	NS	<0.01
Wk 6	18	8	21	27	0	73[b]	<0.05	<0.05
% of subjects with seroconversion								
Wk 3	0	0	0	13	18	53[b]	NS	<0.01
Wk 6	0	0	0	27	24	67[b]	NS	<0.001
B/Guandong								
Geometric mean fold increase								
Wk 3 to wk 0	1.1	2.8	1.8	4.8[b]	4.3[b]	27[b]	<0.01	<0.001
Wk 6 to wk 0	1.1	3.1	2.0	5.8[b]	3.5[b]	20[b]	<0.01	<0.001
% of subjects with HAI titers of ≥1:40 (seroprotection)								
Wk 3	18	11	21	67	47	87[b]	NS	<0.01
Wk 6	18	23	29	73[b]	35	87[b]	NS	<0.01
% of subjects with seroconversion								
Wk 3	0	31	14	47[b]	35	93[b]	NS	<0.001
Wk 6	0	46	21	67[b]	53	80[b]	<0.01	<0.001

[a] The CHMP criteria are: a GM titer increase of >2.5 (18 to 60 years); proportion of individuals with postvaccination HAI titers of ≥1 in 40 (seroprotection) of >70%; and seroconversions (≥4-fold increase) in >40% of participants.

[b] Fulfillment of CHMP criteria. Weeks 3 and 6 are days 21 and 42 after the first dose of intramuscular vaccine and days 28 and 49 after the first dose of intranasal vaccine.

[c] All *P* values were generated by general linear models. NS, not significant.

The percentages of subjects with a fourfold rise in HAI titer (seroconversion) after two doses of vaccine with 30 μg LTK63 and biovector were 27% for A/Panama (H3N2) and 67% for B/Guandong virus. As expected, the HAI results showed that participants were more likely to seroconvert if they received the parenteral vaccine than if they received the intranasal vaccine, which is consistent with such comparisons with live intranasal vaccine or inactivated intranasal vaccine containing *E. coli* heat-labile holotoxin (5, 13). This effect is more pronounced in populations with low or absent prevaccination antibody than in those with various degrees of seropositivity prior to vaccination (5) and was evident in our study, where the neutralizing antibody response to A/Duck/Singapore/97 (H5N3) by recipients of the intranasal vaccine was no better than that to the placebo. Nonetheless, although the number of participants was small, we showed that the intranasal formulation containing 30 μg LTK63 and biovector fulfilled all three CHMP criteria for the assessment of interpandemic vaccines, when assessed at six weeks, for the B/Guandong component. The measurement of serum HAI titers may not be the most appropriate method for assessing the immune response to intranasal vaccines, which is an issue that needs to be urgently addressed in Europe because of the likely application for licensure for a live *ca* intranasal vaccine. The live *ca* intranasal vaccine is as efficacious as the parenteral vaccine in preventing culture-positive influenza illness, but variable proportions of recipients (ranging from 10 to 32% for influenza B virus, 39 to 92% for influenza A H1N1 virus, and 28 to 86% for influenza A H3N2 virus) achieve reciprocal HAI titers of ≥32 (≥40 is one of the CHMP criteria for vaccine assessment) (5). Thus, while many studies indicate that following immunization with inactivated virus vaccines, HAI antibody titers of approximately 1:30 to 1:40 represent the 50% protective level of antibody (28), serum HAI responses to intranasally delivered vaccine correlate less well with protection.

It is generally considered that IgA is the main effector antibody of the mucosal immune system, whereas the origin and role of the observed IgG antibody in the nasal cavity are less certain. One possibility is that this IgG reaches the mucosal lumen by transudation from the circulation (39), with studies of mice indicating that IgG antibodies are capable of providing protective mucosal immunity (12, 21). In this regard, it seems important to elicit a strong mucosal IgG response to vaccination in order to achieve optimal virus neutralization at the portal of virus entry. In our study, the parenterally administered vaccine elicited significantly better mucosal IgG antibody

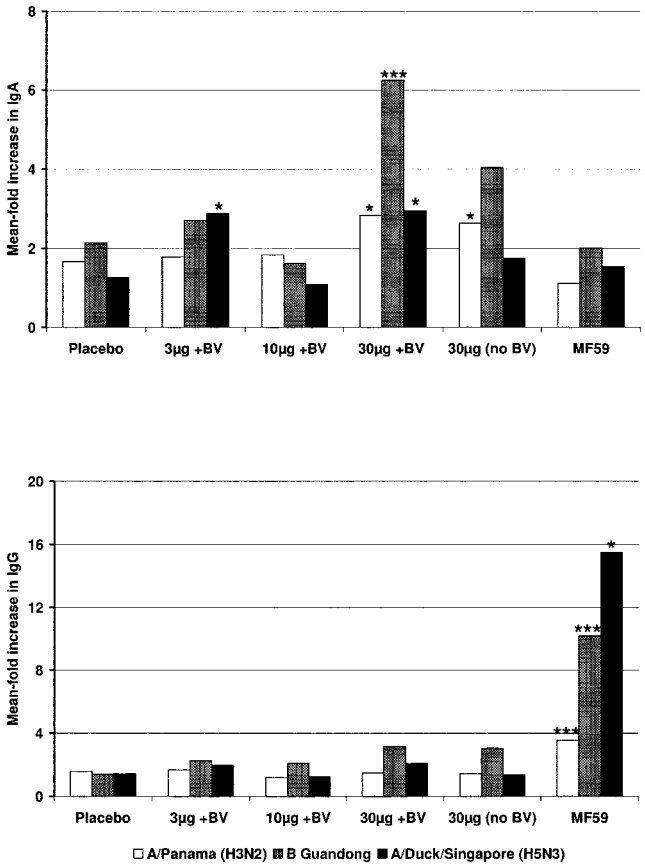

FIG. 2. Mean increases (n-fold) in IgA and IgG concentrations in nasal secretions 6 weeks after vaccination compared with baseline. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P < 0.001$ (for comparison of the intramuscular vaccine with all intranasal vaccine groups or for comparison of any intranasal vaccine group with the placebo). BV, biovector.

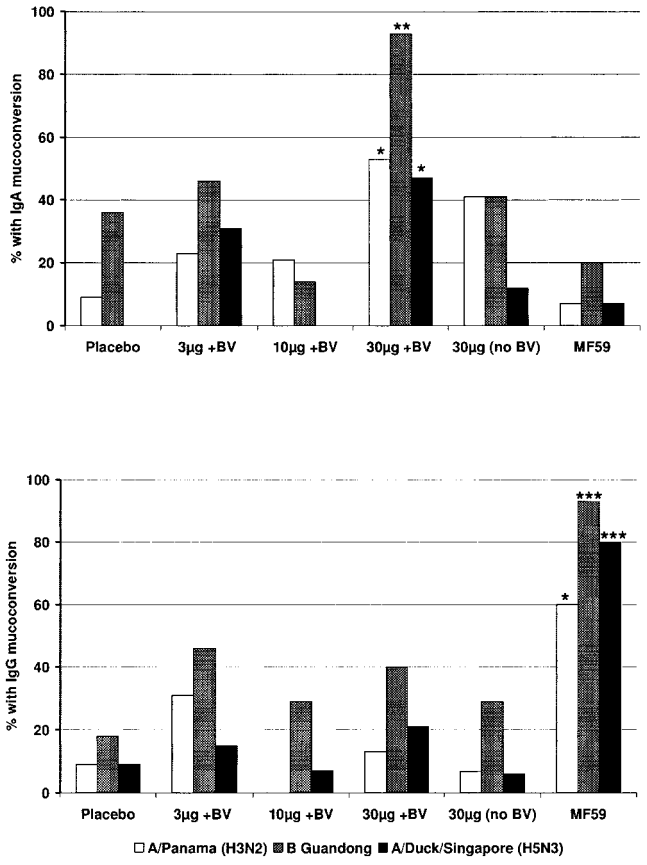

FIG. 3. Percentages of subjects with ≥2.5-fold increases (mucoconversions) in mucosal IgA and IgG 6 weeks after vaccination compared with baseline. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ (for comparison of the intramuscular vaccine with all intranasal vaccine groups or for comparison of any intranasal vaccine group with the placebo).

responses to each vaccine strain than the intranasal vaccines, which contrasts with its ability to elicit mucosal IgA responses. Some recipients of the intranasal vaccine containing 30 μg LTK63 and biovector developed mucosal IgG responses.

Immunity to influenza virus infection in humans is multifactorial, and the precise contributions of innate immunity, serum IgG to hemagglutinin and neuraminidase, local IgG and IgA, and Th1- and Th2-type immune responses have been difficult to ascertain. Observations indicate that live vaccine virus infection-induced and inactivated vaccine-induced immunity involve different arms of the immune system, with sufficient antibody in either serum or nasal secretions being capable of conferring resistance (7). We and others have shown that the current parenteral influenza vaccines elicit strain-specific HAI humoral antibodies in most healthy individuals but that only a minority develop nasal IgA responses (5, 8). Parenterally administered vaccines are expensive and inconvenient to deliver, and the need for injections affects vaccine uptake (31). In order to achieve better protection, new influenza vaccines should aim to induce both mucosal and systemic antibodies. The use of an intranasal vaccine containing LTK63 with biovector as a mucosal adjuvant and a special delivery system shows promise in this respect, but whether the immune response in humans is adequate to prevent or ameliorate influenza virus infection has not been demonstrated, and further studies are required to answer this question. Overall, both intranasal and intramuscular vaccines were easy to administer and well tolerated, although the incidence of transient local pain and the use of analgesics or antipyretics were greater after intramuscular vaccination than after intranasal vaccination, which is consistent with previous findings (13, 23). The safety of the intranasal vaccines containing the LTK63 enterotoxin mutant could not be evaluated effectively with the small numbers of subjects enrolled in this study. In 2002, the Swiss inactivated intranasal virosomal vaccine that contained *E. coli* heat-labile holotoxin as an adjuvant was withdrawn from the market when postmarketing surveillance suggested a strong association between vaccination and Bell's palsy (24). No serious adverse events were reported with the Swiss vaccine in prelicensure trials conducted among 1,218 volunteers during four winter seasons in 1996 to 1999. Subsequently, 107 case reports of Bell's palsy with vaccine exposure were identified in German-speaking parts of Switzerland, corresponding to 13 excess cases per 10,000 vaccinees (24). Herpes simplex virus has been implicated in the pathogenesis of Bell's palsy, but the involvement of herpes simplex virus in the Swiss cases and the possible role of residual enterotoxin activity or local inflammatory responses to the holotoxin-containing vaccine are unknown. Cholera

toxin binds to GM1 gangliosides expressed on epithelial cells and is able to enter the olfactory bulb via the olfactory epithelium after intranasal delivery, causing inflammatory responses in the central nervous system (38). In contrast, the LTK63 mutant of the heat-labile enterotoxin from *E. coli* appears to be safe and noninflammatory in the olfactory bulb in animal models (26, 27). Nevertheless, despite evidence for its safety in animals, reassurance that LTK63 does not cause neurological problems in humans will require large clinical trials, which should proceed cautiously.

The reemergence of highly pathogenic avian influenza H5N1 viruses in humans in 2004–2005 in Vietnam, Thailand, Cambodia, Indonesia, China, and, most recently, Turkey highlights the continuing pandemic threat posed by these viruses. In this study, the recipients of two trivalent intramuscular doses of vaccine, with each dose containing 15 μg A/Duck/Singapore/97 (H5N3) hemagglutinin, responded with a GM MN antibody titer of almost 1 in 30, a level comparable to that observed in our study of the monovalent H5N3 vaccine (25). Our findings after one and two 15-μg doses of A/Duck/Singapore/97 (H5N3) vaccine suggest that the immune response to the H5N3 vaccine given in a multivalent preparation is no better or worse than that in a monovalent formulation, with the caveat that the serological tests were not concurrent and the studies were phase 1 evaluations with a small number of vaccinees. Recent WHO meetings have highlighted the need for improved influenza vaccines providing long-lasting, cross-subtype protection. The finding that mucosal delivery of an inactivated monovalent H3N2 vaccine that was administered with a mutant derivative of *E. coli* heat-labile enterotoxin, LT(R192G) (which possesses reduced toxicity), can completely protect mice against lethal challenge with a highly pathogenic avian H5N1 virus isolated from humans suggests that a strategy of mucosal vaccination might stimulate cross-protection against multiple influenza virus subtypes, including those with pandemic potential (36). We conclude that the vaccine containing the detoxified mutant LTK63 derivative of heat-labile enterotoxin from *E. coli* represents a promising novel vaccine candidate that warrants further study.

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REFERENCES

1. **Aoki, F. Y., and J. C. Crowley.** 1976. Distribution and removal of human serum albumin-technetium 99m instilled intranasally. Br. J. Clin. Pharmacol. **3:**869–878.
2. **Baudner, C., O. Balland, M. M. Giuliani, P. Von Hoegen, R. Rappuoli, D. Betbeder, and G. Del Giudice.** 2002. Enhancement of protective efficacy following intranasal immunization with vaccine plus a nontoxic LTK63 mutant delivered with nanoparticles. Infect. Immun. **70:**4785–4790.
3. **Belshe, R. B., P. M. Mendelman, J. Treanor, J. King, W. C. Gruber, P. Piedra, D. I. Bernstein, F. G. Hayden, K. Kotloff, K. Zangwill, D. Iacuzio, and M. Wolff.** 1998. The efficacy of live attenuated cold adapted trivalent intranasal influenza virus vaccine in children. N. Engl. J. Med. **338:**1405–1412.
4. **Bergquist, C., E.-L. Johansson, T. Lagergård, J. Holmgren, and A. Rudin.** 1997. Intranasal vaccination of humans with recombinant cholera toxin B subunit induces systemic and local antibody responses in the upper respiratory tract and vagina. Infect. Immun. **63:**2021–2025.
5. **Beyer, W. E. P., A. M. Palache, J. C. de Jong, and A. D. M. E. Osterhaus.** 2002. Cold-adapted live influenza vaccine versus inactivated vaccine: systemic vaccine reactions, local and systemic antibody responses and vaccine efficacy: a meta-analysis. Vaccine **20:**1340–1353.
6. **Cassetti, M. C., R. Couch, J. Wood, and Y. Pervikov.** 2005. Report of meeting on the development of influenza vaccines with broad spectrum and long-lasting immune responses, World Health Organization, Geneva, Switzerland, 26–27 February 2004. Vaccine **23:**1529–1533.
7. **Clements, M. L., R. F. Betts, E. L. Tierney, and B. R. Murphy.** 1986. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild-type virus. J. Clin. Microbiol. **24:**157–160.
8. **Clements, M. L., and B. R. Murphy.** 1986. Development and persistence of local and systemic antibody responses in adults given live attenuated or inactivated influenza A virus vaccine. J. Clin. Microbiol. **23:**66–72.
9. **Committee for Proprietary Medicinal Products.** 1996. Note for guidance on harmonisation of requirements for influenza vaccines, London, CPMP/BWP/214/96. Circulaire no. 96-0661:1-22. Committee for Proprietary Medicinal Products, London, United Kingdom.
10. **Corrigan, E. M., and R. L. Clancy.** 1999. Is there a role for mucosal influenza vaccine in the elderly? Drugs Ageing **15:**169–181.
11. **Cox, N. J., and K. Subbarao.** 1999. Influenza. Lancet **354:**1277–1282.
12. **de Haan, L., W. R. Verweij, M. Holtrop, R. Brands, G. J. van Scharrenburg, A. M. Palache, E. Agsteribbe, and J. Wilschut.** 2001. Nasal or intramuscular immunisation of mice with influenza subunit antigen and the B subunit of Escherichia coli heat-labile toxin induces IgA- and IgG-mediated protective mucosal immunity. Vaccine **19:**2898–2907.
13. **Durrer, P., U. Gluck, C. Spyr, A. B. Lang, R. Zurbriggen, C. Herzog, and R. Gluck.** 2003. Mucosal antibody response induced with a nasal virosome based influenza vaccine. Vaccine **21:**4328–4334.
14. **Ellis, J. S., and M. C. Zambon.** 1997. Molecular investigation of an outbreak of influenza in the United Kingdom. Eur. J. Epidemiol. **13:**369–372.
15. **Freestone, D. S., C. H. Bowker, E. Letley, R. D. Ferris, W. G. White, and G. M. Barnes.** 1976. A clinical trial of WRL 105 strain live attenuated influenza vaccine comparing four methods of intranasal vaccination. J. Hyg. (London) **76:**459–466.
16. **Glück, U., J. O. Gebbers, and R. Glück.** 1999. Phase I evaluation of intranasal virosomal influenza vaccine with and without *Escherichia coli* heat-labile enterotoxin in adult volunteers. J. Virol. **73:**7780–7786.
17. **Gorse, G. J., T. Z. O'Connor, S. L. Young, P. M. Mendelman, S. F. Bradley, K. L. Nichol, J. H. Strickland, D. M. Paulson, K. L. Rice, R. A. Foster, A. M. Fulambarker, J. W. Shigeoka, W. G. Kuschner, R. P. Goodman, K. M. Neuzil, J. Wittes, K. D. Boardman, and P. N. Peduzzi.** 2003. Efficacy trial of live, cold-adapted and inactivated influenza virus vaccines in older adults with chronic obstructive pulmonary disease: a VA cooperative study. Vaccine **21:**2133–2144.
18. **Hardy, J. G., S. W. Lee, and C. G. Wilson.** 1985. Intranasal drug delivery by spray and drops. J. Pharm. Pharmacol. **37:**294–297.
19. **Hashigucci, K., H. Ogawa, T. Ishidate, R. Yamashita, H. Kamiya, K. Watanabe, N. Hattori, T. Sato, Y. Suzuki, T. Nagamine, C. Aizawa, S. Tamura, T. Kurata, and A. Oya.** 1996. Antibody responses in volunteers induced by nasal influenza vaccine combined with *Escherichia coli* heat-labile enterotoxin B subunit containing a trace amount of the holotoxin. Vaccine **14:**113–119.
20. **King, J. C., R. Lagos, D. I. Bernstein, P. A. Piedra, K. Kotloff, M. Bryant, I. Cho, and R. B. Belshe.** 1998. Safety and immunogenicity of low and high doses of trivalent live cold-adapted influenza vaccine administered intranasally as drops or spray to healthy children. J. Infect. Dis. **177:**1394–1397.
21. **Mbawuike, I. N., S. Pacheco, C. L. Acuna, K. C. Switzer, Y. Zhang, and G. R. Harriman.** 1999. Mucosal immunity to influenza without IgG: an IgA knockout mouse model. J. Immunol. **162:**2350–2357.
22. **Muszkat, M., A. B. Yehuda, M. H. Schein, Y. Friedlander, P. Naveh, E. Greenbaum, M. Schlesinger, R. Levy, Z. Zakay-Rones, and G. Friedman.** 2000. Local and systemic immune response in community dwelling elderly after intranasal or intramuscular immunization with inactivated vaccine. J. Med. Virol. **61:**100–106.
23. **Muszkat, M., E. Greenbaum, A. Ben-Yehuda, M. Oster, E. Yeu'l, S. Heimann, R. Levy, G. Friedman, and Z. Zakay-Rones.** 2003. Local and systemic immune responses in nursing-home elderly following intranasal or intramuscular immunization with inactivated influenza vaccine. Vaccine **21:**1180–1186.
24. **Mutsch, M., W. Zhou, P. Rhodes, M. Bopp, R. T. Chen, T. Linder, C. Spyr, and R. Steffen.** 2004. Use of inactivated intranasal influenza vaccine and risk of Bell's palsy in Switzerland. N. Engl. J. Med. **350:**896–903.
25. **Nicholson, K. G., A. E. Colegate, A. Podda, I. Stephenson, J. Wood, E. Ypma, and M. C. Zambon.** 2001. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet **357:**1937–1942.
26. **Peppoloni, S., P. Ruggiero, M. Contorni, M. Morandi, M. Pizza, R. Rappuoli, A. Podda, and G. Del Giudice.** 2003. Mutants of the *Escherichia coli* heat-labile enterotoxin as safe strong adjuvants for intranasal delivery of vaccines. Expert Rev. Vaccines **2:**285–292.
27. **Pizza, M., M. M. Giuliani, M. R. Fontana, E. Monaci, G. Douce, G. Dougan,**

K. H. Mills, R. Rappuoli, and G. Del Giudice. 2001. Mucosal vaccines: nontoxic derivatives of LT and CT as mucosal adjuvants. Vaccine **19:**2534–2541.
28. **Potter, C. W., and J. S. Oxford.** 1979. Determinants of immunity to influenza in man. Br. Med. Bull. **35:**69–75.
29. **Powers, D. C., B. R. Murphy, L. F. Fries, W. H. Adler, and M. L. Clements.** 1992. Reduced infectivity of cold-adapted influenza A H1N1 viruses in the elderly: correlation with serum and local antibodies. J. Am. Geriatr. Soc. **40:**163–167.
30. **Rappuoli, R., M. Pizza, G. Douce, and G. Dougan.** 1999. Structure and mucosal adjuvanticity of cholera and *Escherichia coli* heat-labile enterotoxins. Immunol. Today **20:**493–500.
31. **Stephenson, I., J. P. Roper, and K. G. Nicholson.** 2002. Healthcare workers and their attitudes to influenza vaccination. Commun. Dis. Public Health **5:**247–252.
32. **Subbarao, K.** 1999. Influenza vaccines: present and future advances. Virus Res. **54:**349–373.
33. **Takada, A., S. Matsushita, A. Ninomiya, Y. Kawaoka, and H. Kida.** 2003. Intranasal immunization with formalin-inactivated virus vaccine induces a broad spectrum of heterosubtypic immunity against influenza A virus infection in mice. Vaccine **21:**3212–3218.
34. **Tamura, S., H. Asanuma, and Y. Ito.** 1994. Formulation of inactivated influenza vaccines for providing effective cross-protection by intranasal vaccination in mice. Vaccine **12:**310–316.
35. **Treanor, J., G. Dumyati, D. O'Brien, M. A. Riley, G. Riley, S. Erb, and R. Betts.** 1994. Evaluation of cold-adapted, reassortant influenza B virus vaccines in elderly and chronically ill adults. J. Infect. Dis. **169:**402–407.
36. **Tumpey, T. M., M. Renshaw, J. D. Clements, and J. M. Katz.** 2001. Mucosal delivery of inactivated influenza vaccine induces B-cell-dependent heterosubtypic cross-protection against lethal influenza A H5N1 virus infection. J. Virol. **75:**5141–5150.
37. **Van den Dobblesteen, P. J. M., and E. P. van Rees.** 1995. Mucosal immune responses to pneumococcal saccharides: implications for vaccination. Trends Microbiol. **3:**155–159.
38. **Van Ginkel, F. W., R. J. Jackson, Y. Yuki, and J. R. McGhee.** 2000. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. J. Immunol. **165:**4778–4782.
39. **Wagner, D. K., M. L. Clements, C. B. Reimer, M. Snyder, D. L. Nelson, and B. R. Murphy.** 1987. Analysis of immunoglobulin G antibody responses after administration of live and inactivated influenza A vaccine indicates that nasal wash immunoglobulin G is a transudate from serum. J. Clin. Microbiol. **25:**559–562.