Safety and Immunogenicity of Intranasally Administered Inactivated Trivalent Virosome-Formulated Influenza Vaccine Containing *Escherichia coli* Heat-Labile Toxin as a Mucosal Adjuvant


A trivalent influenza virosome vaccine containing hemagglutinin and *Escherichia coli* heat-labile toxin (HLT) was administered intranasally to young adults and elderly subjects. Symptoms that followed immunization were mild and transient. A significant increase in serum hemagglutination inhibition (HI) antibody was noted for the 3 vaccine strains. There was no significant difference in postimmunization geometric mean titers or seroconversion rates between age groups. The percentage of subjects attaining protective HI titers (≥40%) was comparable in both groups for the A/Bayern (P = .5) and B/Beijing (P = .3) strains but was higher among young adults (92.2%) versus elderly subjects (76.5%; P = .057) for the A/Wuhan strain. The proportion of subjects with nonprotective baseline titers who attained protective levels after immunization was similar in both age groups for the A/Bayern and B/Beijing components. For the A/Wuhan component, significantly (P = .017) more young adults achieved protective titers versus elderly subjects (85.7% and 53.8%, respectively). Vaccination evoked a significant (P < .005) increase in anti-HLT antibody titers.

Administration of a wide variety of vaccine antigens by the intranasal route evokes a vigorous local and systemic immune response in animals [1, 2]. Furthermore, the magnitude of this response can in many cases be augmented by the coadministration of bacterial toxins expressing adenosine diphosphate ribosylating activity, including the heat-labile toxin (HLT) of *Escherichia coli*, which appear to act as mucosal surface adjuvants [3–5]. Although this concept holds much promise, few data are available concerning the safety and immunogenicity of vaccines with or without mucosal adjuvants administered intranasally to humans. Inactivated influenza vaccine combined with the B subunit of HLT (LTB) is more immunogenic when administered intranasally than vaccine alone [6]. However, the B subunit has trace quantities of HLT, which may contribute to the effect noted.

We have shown that the immunogenicity of influenza HA can be augmented by intercalating HA into unilamellar liposomes, thereby yielding virosomes ~150 nm in diameter [7, 8]. Such virosome-formulated influenza vaccines administered by the parenteral route are safe and significantly more immunogenic in elderly persons than classical inactivated vaccines [7, 8]. In preliminary studies, such vaccines induced a significant humoral hemagglutination inhibition (HI) antibody response when given intranasally to young healthy adults [9]. Furthermore, the immune response could be augmented by the inclusion of E. coli HLT. In the present study, we expanded on these findings by investigating the safety and immunogenicity of inactivated trivalent virosome-formulated influenza vaccine containing HLT as a mucosal adjuvant in both young adults and elderly subjects.

**Methods**

**Vaccines.** Trivalent influenza virosome vaccines were produced as follows. The following strains of influenza were cultivated in embryonated hens’ eggs: A/Bayern (H1N1) and B/Beijing and A/ Wuhan (H3N2). Influenza virus was purified from the chorioallantoic fluid by classical separation techniques, including clarification by low-speed centrifugation, filtration, precipitation, ultrafiltration, and zonal ultracentrifugation on a sucrose gradient. The purified virions were inactivated by treatment with β-propiolactone. The inactivated virions were suspended in PBS, pH 7.4, containing 0.1 M octaethyleneglycol mono (n-dodecyl) ether (Nikko Chemicals, Tokyo). The mixture was incubated for 20 min at 21°C, to allow for extraction of the HA. The suspension was then centrifuged at 100,000 g for 30 min, and the HA rich supernatant was collected. HA purified in this manner contained ~5% neuraminidase and trace quantities of viral phospholipids. Phosphatidylcho-
line (PC; Lipoid, Ludwigshafen, Germany) was added to yield a PC:HA ratio of 2.6:1. The detergent was removed by batch chromatography over polystyrol biobeads. The spontaneously formed virosomes elute in the void volume, which was pooled and stored at 4°C. The HA content of this concentrate was then determined. HLT (purity 95%) was manufactured as described elsewhere [10] and was prepared as a lyophilized bulk. The final trivalent vaccine was formulated by adding appropriate amounts of the virosomes concentrate diluted in PBS and reconstituted HLT. A dose of vaccine contained 7.5 µg of each HA antigen per nostril per immunization (200 µL), equal to 22.5 µg of total HA per dose and 1 µg of HLT per dose (2 µg total). The intranasal preparation was packaged into an applicator (Dispray; Piffer, Eigeltingen, Germany) capable of delivering a 100-µL aerosolized dose. The vaccines were tested for sterility, general safety, and pyrogenicity.

Subjects and trial design. Adult subjects of both genders (≥18 years old) were enrolled after medical screening to ensure they were in good health. Exclusion criteria included contraindication against influenza immunization, immunization against influenza during the 1997/1998 season, acute disease state present, severe atopy, immunosuppression due to either underlying disease or therapy, pregnancy or lactation, receipt of an investigational substance, receipt of immune globulin or blood transfusion within the past 3 months, participation in another clinical trial, and a history of nosebleeds. Subjects were assigned to 1 of 2 groups on the basis of age at the time of enrollment, 18–60 years and >60 years.

One dose of vaccine was administered in each nostril on days 1 and 8. Safety assessments were made by the clinical investigators immediately after administration of the vaccine and at days 8 and 29. The study subjects were told to record all systemic and local reactions for 4 days after immunization. Serum samples obtained at the time of immunization and 28 days after the first dose were coded and stored frozen. Adverse events were classified as mild (presence of symptoms), moderate (symptoms impacted normal activities), or severe (symptoms curtailed normal activities).

Serology methods. Serum HI antibody titers were determined by an HI assay as described elsewhere [8]. A reciprocal titer ≥40 was considered to be protective. Seroconversion was defined as a ≥4-fold increase in titer over baseline and attaining a titer ≥40.

Serum anti-HLT. Serum anti-HLT antibody titers were measured by ELISA. Microplates (Nunc Maxisorp U96; Life Technologies, Basel, Switzerland) were coated with E. coli HLT (1 µg/mL) overnight at 4°C. Serum samples were serially diluted and the optical density determined. Absorbance values within the linear portion of the curve were used to calculate antibody titers. Seroconversion is defined as an increase in antibody titers over baseline values of ≥4-fold.

Statistical analysis. Significance between seroconversion rates and the percentage of subjects who achieved protective antibody titers subsequent to immunization was determined by χ² analysis. Difference in baseline and postimmunization geometric mean antibody titers (GMTs) were determined by Student’s t test.

Results

In total, 106 subjects received 2 doses of vaccine. The mean ages of the study populations were 32.7 and 62.8 years for the groups designated as 18–60 (n = 52) and >60 years of age (n = 54), respectively. The vaccine was well tolerated by subjects in both age groups. About 50% of subjects experienced some type of local or systemic adverse reaction (rhinorrhea, stuffiness, sneezing, and headache were the most common symptoms noted) subsequent to vaccination. No febrile events were associated with immunization. One episode of transient diarrhea was reported. The vast majority of symptoms were classified as mild and most resolved within 24–48 h of onset. The type, frequency, and severity of both systemic and local adverse events were comparable after either the first or second dose and among both age groups. For example, 44.2% of participants in the 18–60 years group reported a local reaction after the first dose versus 53.8% after the second (P > .05), whereas 48.1% and 46.0% experienced a systemic reaction after the first and second doses, respectively (P > .05). Two local reactions (rhinorrhea) were classified as severe subsequent to the initial immunization and 5 (rhinorrhea, stuffiness, and discomfort) after the second dose.

The serum HI antibody response following immunization is shown in table 1. There was no statistically significant difference (P ≥ .3) in the seroconversion rate between the 2 groups for any of the 3 vaccine strains. Vaccination engendered a significant increase (P < .005) in the GMT over baseline for all 3 vaccine components in both age groups. The absolute magnitude of the increase varied among the vaccine strains, ranging from a 7.7-fold increase (A/Bayern, >60 year age group) to 2.51-fold (B/Beijing, >60 year age group). There was no significant difference in the magnitude of the immune response between

<table>
<thead>
<tr>
<th>Age group</th>
<th>Geometric mean titer (GMT) (range)</th>
<th>Seroconversion (%)</th>
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<tbody>
<tr>
<td></td>
<td>A/Bayern Before</td>
<td>After</td>
</tr>
<tr>
<td>18–60 years</td>
<td>51.7 (5–5120)</td>
<td>371 (5–10,480)</td>
</tr>
<tr>
<td>&gt;60 years</td>
<td>38.9 (5–5120)</td>
<td>299 (5–5120)</td>
</tr>
<tr>
<td></td>
<td>7.17</td>
<td>3.42</td>
</tr>
<tr>
<td></td>
<td>7.70</td>
<td>3.15</td>
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the 2 age groups for any of the 3 vaccine components, as determined by comparing the fold increase in GMTs over baseline. Furthermore, there was no significant difference ($P \geq .2$) between the 2 groups for either baseline GMTs ($P \geq .2$) or those attained after immunization ($P \geq .1$) for any of the 3 vaccine strains. The seroconversion rate was highest against the A/Bayern vaccine component (62.7%–64.7%) and lowest for the B/Beijing component (29.4%–37.3%) among both groups.

Immunogenicity was also assessed by determining the percentage of subjects who achieved protective HI antibody titers ($\geq 40$) after immunization (table 2). At baseline, $\sim 50$%–60% of subjects in both age groups possessed a protective titer to the A/Bayern and A/Wuhan strains but only 15%–20% did so for the B/Beijing strain. After immunization, $\geq 90$% of vaccinees in both groups possessed protective antibody levels to the A/Bayern strain, as did 76.5%–92.2% for the A/Wuhan component. The response to the B/Beijing strain was significantly ($P < .05$) lower, compared with the other 2 strains, with only 49.0%–58.8% of subjects attaining protective titers after immunization. There was no significant difference between the 2 groups as to the percentage of subjects who attained protective titers subsequent to immunization. However, the response rate was generally lower among the older age group and approached significance for the A/Wuhan strain ($P = .057$).

We also evaluated the ability of intranasal immunization to increase HI antibody titers to $\geq 40$ among persons with non-protective titers at baseline. The response to the A/Bayern strain was comparable in both age groups with 78.9%–84.0% of volunteers attaining protective levels. For the A/Wuhan component, the response rate ranged from 53.8% ($\geq 60$ year olds) to 85.7% (18–60 year olds), which was statistically significant ($P = .017$). A comparable lower percentage (39.5%–47.5%; $P > .05$) of subjects in both age groups achieved protective titers to the B/Beijing strain. However, the overall response rate was substantially lower than with the other 2 vaccine strains. For example, among subjects 18–60 years old, the percentage who achieved protective titers was significantly higher for the A/Bayern and A/Wuhan versus the B/Beijing strain ($P = .04$ and .008, respectively). For subjects $\geq 60$ years of age, significantly more achieved titers $\geq 40$ versus for the A/Bayern versus B/Beijing component ($P = .009$). There was no significant difference between the A/Bayern and B/Beijing components ($P > .3$).

Intranasal immunization also engendered a vigorous serum anti-LT antibody response. The mean fold increases in GMTs were 13.98 and 11.13 for the 18–60 and $\geq 60$ year old age groups, respectively. Even though the baseline GMT for subjects $\geq 60$ years old was significantly ($P = .017$) higher than among 18–60 year olds, there was no significant difference ($P > .05$) between the postimmunization GMTs. The vast majority of those vaccinated showed a $\geq 4$-fold increase in serum anti-LT antibody titer (92% and 86% for those 18–60 and $\geq 60$ years, respectively; $P > .5$).

Discussion

Live attenuated influenza vaccine given intranasally confers a high degree of protection in children and demonstrates the utility of this approach [11]. However, inactivated antigens administered either orally or intranasally do not necessarily engender a vigorous immune response [12, 13]. This has led to the search for adjuvants capable of enhancing the immune response of antigens applied to mucosal surfaces. Native and mutant HLT, along with cholera toxin have been effective mucosal adjuvants in laboratory animals when utilized with a variety of antigens [3–5]. Experience to date in humans has been limited. Intranasally administered inactivated influenza vaccine delivered with LT containing 0.5% native HLT was found to engender a superior immune response in humans, compared with vaccine alone [6]. The relative contribution of the trace quantities of HLT versus LTB regarding the adjuvant effect noted could not be ascertained. In preliminary studies involving small numbers of young adults, we found that the addition of HLT enhanced the local and systemic immune response to intranasally administered inactivated influenza vaccine. The present study compared the safety and immunogenicity of this vaccine in healthy young and elderly subjects, who are at increased risk of serious illness.

Adverse events temporally associated with vaccination were predominantly mild and transient in both age groups and usually resolved within 24–48 h. Local reactions manifested (e.g., sneezing, stuffiness, and rhinorrhea) have been previously reported in association with the intranasal administration of LTB combined with influenza vaccine [6]. Many events may have been triggered in response to the physical sensation associated with vaccine application. Systemic reactions were composed primarily of nonspecific complaints such as fatigue and headache. No case of fever ($\geq 38.5^\circ$C) or flulike illness was reported.

**Table 2.** Protective hemagglutination inhibition (HI) antibody levels before and after immunization.

<table>
<thead>
<tr>
<th>Age group</th>
<th>A/Bayern Before</th>
<th>A/Bayern After</th>
<th>A/Wuhan Before</th>
<th>A/Wuhan After</th>
<th>B/Beijing Before</th>
<th>B/Beijing After</th>
</tr>
</thead>
<tbody>
<tr>
<td>18–60 years</td>
<td>32/51 (62.7)</td>
<td>47/51 (92.2)</td>
<td>30/51 (58.8)</td>
<td>47/51 (92.2)</td>
<td>11/51 (21.6)</td>
<td>30/51 (58.8)</td>
</tr>
<tr>
<td>$\geq 60$ years</td>
<td>26/51 (51)</td>
<td>46/51 (90.2)</td>
<td>25/51 (49)</td>
<td>39/51 (76.5)</td>
<td>8/51 (15.7)</td>
<td>24/51 (49)</td>
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NOTE. Protective HI antibody titer, $\geq 40$.
Thus, it can be concluded that the presence of native HLT did not compromise vaccine safety.

The intranasal application of trivalent influenza vaccine engendered an excellent humoral HI and anti-HLT antibody response. The magnitude of the HI antibody response as judged by the percentage of subjects who attained protective levels of antibody after immunization was comparable to that seen after a single parenteral immunization with virosome-formulated, classical whole virion or subunit vaccines [7, 8]. Perhaps of greater importance was the fact that influenza vaccine administered intranasally was equally effective at eliciting protective levels of HI antibody titers in subjects with nonprotective baseline levels. The serum HI antibody response elicited in the present study appeared to be superior to that previously reported for intranasally administered influenza vaccine containing LTB as an adjuvant [6]. There may be several reasons for this observation. First, native HLT may be a far more potent adjuvant than LTB alone [14]. Second, virosome-formulated influenza vaccine, such as that used here, is more immunogenic than conventional inactivated vaccines [7, 8]. Finally, there may have been innate differences in immunogenicity between the influenza strains comprising the 2 vaccines.

The elderly are at increased risk of acquiring a life-threatening infection with influenza [15]. Unfortunately, this population has a diminished capacity to mount a protective HI antibody response after immunization with classical parenteral influenza vaccines [16]. Therefore, in the present study, we compared the performance of intranasal influenza vaccine in the elderly (mean age, 62.8 years) and young adults (mean age, 32.7 years). The overall HI serum antibody response was comparable among the 2 age groups with 1 exception, the percentage of subjects with nonprotective baseline levels to the A/Wuhan strain who achieved protective levels after immunization (85.7% and 53.8%, for young adults and elderly, respectively; \( P = .017 \)).

Of interest, even the relatively small quantity (2 \( \mu g/dose \)) of HLT administered was able to elicit a significant serum anti-HLT immune response. The majority of subjects (86%–92%) in both age groups mounted a 4-fold increase in titer accompanied by a >10-fold increase in GMT over baseline. These findings also support the belief that small amounts of other protein antigens may prove to be highly immunogenic after intranasal administration.

The present findings demonstrate the potential utility of the intranasal route of immunization in humans. The results also indicate that small quantities of native HLT can be safely administered. Although preliminary, these novel results should serve as an impetus for additional clinical studies aimed at delivering vaccine antigens intranasally. Further studies are underway to compare the local immune response elicited by intranasally and parenterally administered influenza vaccines.

References