Adjuvant Activity of the Heat-Labile Enterotoxin from Enterotoxigenic *Escherichia coli* for Oral Administration of Inactivated Influenza Virus Vaccine

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Alternative strategies for vaccination against influenza that elicit both systemic antibody and mucosal IgA responses are needed to improve the efficacy in protection against infection. This study demonstrated that oral delivery of inactivated influenza vaccine with the heat-labile enterotoxin (LT) from enterotoxigenic *Escherichia coli* elicited the spectrum of humoral and cell-mediated responses in BALB/c mice critical for the protection and recovery from influenza virus infection. Coadministration of LT with oral influenza vaccine increased antiviral serum IgG and mucosal IgA responses compared with administration of oral influenza vaccine alone. Serum hemagglutination-inhibition and neutralizing antibodies were also augmented by LT. The adjuvant potentiated protection from infection with influenza A H3N2 viruses in mouse lower and upper respiratory tracts, enabling the use of lower doses of oral vaccine. Coadministration of LT with oral inactivated influenza vaccine induced influenza virus–specific proliferative T cells, interleukin-2 production, and major histocompatibility complex class I–restricted cytotoxic T cells.

Current efforts to control the morbidity and mortality associated with yearly epidemics of influenza are based on the use of intramuscularly administered inactivated influenza vaccines [1]. The efficacy of such vaccines in preventing respiratory disease ranges from ~90% in younger healthy adults to <50% in the elderly [2, 3]. Variation in vaccine efficacy can be attributed to several factors, including the closeness of the antigenic match between the vaccine strain and the epidemic virus and the reduced immunogenicity of the vaccine in elderly, unprimed, and very young persons [4, 5]. Protection from infection with influenza viruses has been correlated with both serum IgG and mucosal IgA responses directed against viral hemagglutinin (HA) [6, 7], whereas cytotoxic T lymphocytes (CTL) are thought to play a role in clearance of the virus and recovery from infection [8]. Although the parenterally administered inactivated vaccine can elicit substantial hemagglutination-inhibition (HAI) antibody responses in a majority of younger adults, mucosal antibody is induced in only 25%–50% of previously primed vaccinees and in even fewer unprimed persons [6, 9]. Therefore, new vaccination strategies are sought that can elicit both systemic immune responses and improved mucosal immunity to influenza at the site of infection in the respiratory tract.

Effective vaccination that elicits both mucosal and systemic immunity has been difficult to achieve. The use of experimental intranasally (inl) administered live attenuated influenza vaccines is one approach for providing improved mucosal immunity in humans and shows much promise as an alternative to inactivated influenza vaccines, particularly for children. However, serum antibody responses induced by mucosal vaccination, such as inl delivery of live attenuated vaccine [6, 10] or oral vaccination with inactivated vaccine [11], are generally lower than responses achieved with intramuscular vaccination with inactivated virus. Therefore, vaccine strategies that target the common mucosal immune system through the use of novel adjuvants and delivery through mucosal routes are being explored [12, 13].

Delivery of vaccine antigen to the gastrointestinal tract leads to uptake of the antigen by the Peyer's patches and the preferential stimulation of secretory IgA synthesis. B and T cells primed in this mucosal inductive site can seed other mucosal tissues and provide mucosal immunity at distant effector sites (e.g., the respiratory tract). Oral delivery of influenza vaccines has been investigated in both mice [14–16] and humans [11, 17]. In mice, repeated oral administration of high doses of inactivated virus results in serum IgG and mucosal IgA responses that are protective against subsequent inl infection with influenza virus. To improve the efficiency of oral delivery of a killed or subunit influenza vaccine, a delivery system is needed that protects the antigen from the gastric environment or that has immunopotentiating properties. Strategies that have fo-

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cused on the improved delivery of oral influenza vaccines include the use of poly-(DL-lactide-coglycolide) microsphere encapsulation of inactivated vaccines [18] and the preparation of particulate virus vaccine [19] to protect antigen from degradation or to provide antigen of a size optimal for uptake by the Peyer's patches.

Adjuvants, such as the protein enterotoxins from *Vibrio cholera* (CT) or enterotoxigenic *Escherichia coli* (LT), that enhance the production of essential immune responses at mucosal surfaces have been used to facilitate oral immunization against a number of antigens [20–23]. The adjuvant activity of these related bacterial toxins is thought to be due to several properties, including the ability to enhance uptake of antigen through greater gut permeability [24], presentation of antigen by presenting cells [25], and induction of cytokines that promote immunoglobulin class switching [26]. Although CT and LT share many common features, there are significant differences between these molecules [23]. In particular, LT is at least 10-fold less toxic than CT in animals and, therefore, may prove to be a safer adjuvant for mucosal vaccination. Clements et al. [20] showed that LT can prevent the induction of tolerance to orally administered ovalbumin and enhance both mucosal IgA and systemic IgG antibody responses. Recently, LT was used to potentiate an immune response to a killed *Campylobacter* vaccine that promoted the rapid clearance of the enteric pathogen after challenge [22]. We report the use of LT as a mucosal adjuvant for oral administration with inactivated whole influenza virus vaccine.

Materials and Methods

Viruses. The high-growing reassortant influenza A virus, X-31, which possesses the surface glycoproteins of A/Aichi/2/68 (H3N2) and the internal proteins of A/Puerto Rico/8/34, was used to vaccinate mice. Additional influenza A and B viruses used as antigens for HAI assays or as infectious or inactivated antigens in T cell assays were A/Memphis/102/72 (H3N2), A/Victoria/3/75 (H3N2), A/Texas/1/77 (H3N2), A/Bangkok/1/79 (H3N2), A/Philippines/2/82 (H3N2), A/Puerto Rico/8/34 (H1N1), and B/Hong Kong/8/73 or B/Panama/45/90. Viruses were grown for 2–3 days at 35°C in the allantoic cavity of 10- to 11-day-old embryonated hens' eggs. Clarified allantoic fluid was stored in aliquots at −70°C until use.

Vaccine and adjuvant preparations. Virus to be used as vaccine was concentrated from allantoic fluid and purified by equilibrium density centrifugation through a 30%–60% linear sucrose gradient as described [27]. Inactivated whole virus vaccine was prepared by treating purified virus at a concentration of 1 mg/mL with 0.025% formalin at 4°C for 3 days. This treatment resulted in the complete loss of infectivity of virus, as determined by titration of the vaccine preparation in eggs. The vaccine doses given throughout are expressed as amounts of total viral protein. The HA protein has been estimated to make up ~30% of the total viral protein of purified X-31 virus [28]. Some of the recombinant LT used in this study (gift of J. Clements, Dept. of Microbiology and Immunology, Tulane University, New Orleans) was derived from a clone of *E. coli* carrying the LT gene from a human enterotoxigenic *E. coli* isolate H10407 [29]. Alternatively, recombinant LT prepared in an identical manner was purchased (Berna Products, Coral Gables, FL).

Immunization of mice. Female 6- to 10-week-old BALB/c mice (Charles River Laboratories, Wilmington, MA, or Harlan Sprague Dawley, Indianapolis) were used in all experiments. They were provided food and water ad libitum. Mice were lightly anesthetized with CO_2 and vaccinated orally (intragastrically) with various doses of inactivated purified X-31 virus suspended in 0.4 *M* $NaHCO_3$ in the presence or absence of LT (25 μg, unless stated otherwise) using a 20-gauge (3.8-cm) feeding needle (Popper and Sons, New Hyde Park, NY) attached to a 1-mL syringe. Mice received three doses of vaccine at weekly intervals. In some experiments, mice were vaccinated with a single dose of inactivated X-31 vaccine without LT by the intraperitoneal (ip) route or received a final ip boost without LT 1 week after the final oral vaccine booster dose.

Antibody sample collection. Three weeks after the final vaccine boost, 5 mice from each vaccine and control group were anesthetized (ip administration of Avertin: 2,2,2-tribromoethanol; 0.15 mL/10 g of body weight); blood samples from the orbital plexus provided immune sera. Mice were then exsanguinated from the axilla. To obtain a bronchoalveolar (lung) wash sample, the trachea was exposed, and an 18-gauge plastic cannula (Baxter, Deerfield, IL) with a 1-mL syringe attached was inserted through an incision in the trachea into the lungs. Lungs were washed with 1 mL of PBS containing 1% bovine serum albumin (BSA). The 1-mL volume was infused and withdrawn from the lungs five times. Nasal wash samples were recovered by flushing 1 mL of fresh PBS-BSA through the tracheal incision and forward into the nasal passage 3 times. The fluid expelled through the nares was collected in 35-mm petri dishes. The degree of blood contamination, which was minimal, was estimated by counting erythrocytes in the lung and nasal wash samples. Lung and nasal wash samples were clarified by centrifugation and were stored at −20°C.

Antibody assays. All sera and lung and nasal wash samples were treated with receptor-destroying enzyme (RDE) from *V. cholera* (WHO Center for Surveillance, Epidemiology, and Control of Influenza, CDC, or Denka Seiken, Tokyo). Briefly, RDE was rehydrated in physiologic saline according to the manufacturer's instructions. Three volumes of RDE were mixed with 1 vol of biologic sample and incubated overnight at 37°C. Six volumes of saline were then added so that the RDE-treated material was at a 1/10 dilution of the original sample. RDE-treated samples were tested for the presence of antiviral IgG and IgA by ELISA. Immulon II plates (Dynatech Laboratories, Chantilly, VA) were coated with 50 hemagglutinating units (HAU) of purified X-31 virus in PBS and incubated at room temperature overnight. The plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked by the addition of PBS containing 4% fetal bovine serum (FBS) for 1 h at 37°C. Plates were washed, and serial dilutions of samples in PBST containing 1% FBS were added and incubated for 2 h at 37°C. Plates were washed, and bound antibody was detected by the addition of goat antimouse IgG or IgA conjugated to horseradish peroxidase (Kirkegaard & Perry, Gaithersburg, MD). Plates were washed, and a substrate consisting of 10 mg of *o*-phenylenediamine dihydrochloride and 0.015% H_2O_2 in citrate buffer, pH 5.0, was added. After 10 min at room temperature, the reaction was stopped

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with 2 *N* H_2SO_4, and the optical density at 490 nm was determined. Positive control samples were also tested (serum, lung wash, and nasal wash samples harvested and pooled from 5 mice infected inl with 100 HAU of live X-31 virus 3 weeks previously).

Amounts of total IgG and IgA in antibody samples were detected by a similar procedure, except that a sheep anti-mouse immunoglobulin antibody (Boehringer-Mannheim, Indianapolis) was used to coat plates at a concentration of 4 μg/mL in PBS. Standard curves of purified IgG and IgA (Southern Biotechnology Associates, Birmingham, AL) and positive and negative antiviral control samples were run in each experiment. Antibody titers are expressed as the reciprocal of the end-point dilution of sample, with an A_{490} reading three times the mean A_{490} of wells that lacked antibody.

Sera were tested for the presence of HAI antibody by standard methods, using 4 HAU of virus and 0.5% chicken red blood cells [30]. Neutralizing antibody titers, determined as described previously [31], are expressed as the reciprocal of the highest dilution of serum that neutralized 200 $TCID_{50}$ of virus in 50% of infected MDCK cell cultures.

Viral challenge. Three weeks or 3 months after vaccination, mice were challenged inl with 50 mouse infectious doses $(MID)_{50}$ of X-31 virus (equivalent to 250 EID_{50}) in a volume of 50 μL. Four days later, at peak viral replication in unvaccinated animals, mice were euthanized and nasal and lung tissues (or both) were collected, rinsed in PBS, and stored at -70°C until virus titration. Thawed tissues were homogenized in 1 mL of PBS and titrated for virus infectivity in 10- to 11-day-old embryonated eggs. Virus end-point titers are expressed as $\log_{10}$ EID_{50}/mL.

Proliferative and IL-2 responses. Single-cell suspensions were prepared from spleen, mediastinal, or mesenteric lymph nodes collected from mice vaccinated $\geq$4 weeks previously. Cells (4×10^5/well) were cultured with Dulbecco's MEM (DMEM) in 96-well plates with various doses (10–0.01 HAU) of UV-inactivated influenza virus for 4 days at 37°C in 5% CO_2. The DMEM was supplemented with 2 m*M* glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 1 m*M* sodium pyruvate, 10 m*M* HEPES buffer (all from GIBCO BRL, Grand Island, NY), 5×10^{-5} 2-mercaptoethanol, and 10% heat-inactivated FBS. To determine the level of stimulation of cells, 0.5 μCi of [^{3}H]thymidine was added to each well in the final 18 h of culture. Cellular DNA was harvested onto glass-fiber filters for liquid scintillation counting.

Similar cell cultures were set up to detect interleukin (IL)-2 produced by stimulated T cells and secreted into the culture medium. Culture supernatants were harvested after 72 h of culture and stored at -20°C until they were tested for their ability to support the IL-2–dependent growth of the CTLL-2 cell line (TIB 214; American Type Culture Collection, Rockville, MD). CTLL-2 cells were washed free of residual IL-2 and cultured at 5000 cells/well in a total volume of 0.1 mL in which the culture supernatants from virus-stimulated spleen cell cultures had been serially diluted 2-fold. After 24 h of culture, the degree of proliferation was detected as described above. IL-2 activity is expressed as mean $\pm$ SE of triplicate CTLL-2 cell cultures.

CTL activity. Spleen cells (5×10^6/mL) from mice vaccinated 6 months previously were restimulated by coculture with γ-irradiated (25 Gy), X-31 virus–infected BALB/c spleen cells (10^6/mL) for 5 days in the culture medium defined above. The virus-specific CTL activity of this secondary T cell population was determined in a ^{51}Cr release assay. Major histocompatibility complex (MHC) class I antigen (H-2^d)–bearing P815 cells or MHC-mismatched EL-4 cells (H-2^b) were labeled with ^{51}Cr (100 μCi/10^6 cells) for 30 min at 37°C before the addition of 100 μL of allantoic fluid containing influenza virus. Cells were incubated for 1 h, supplemented with 10 mL of culture medium, and incubated another 3 h. Target cells were washed and incubated (5×10^3 cells/well) with restimulated effector cells at various effector-to-target ratios. The amount of ^{51}Cr released into 100 μL of culture supernatant was determined after 6 h of incubation. The percent specific activity was calculated as [(test counts per minute [cpm] $-$ spontaneous release cpm)/(total cpm $-$ spontaneous release cpm] $\times$ 100.

Statistical analysis. Statistical significance of the data was determined using Fisher's exact or Student's *t* tests.

Results

Serum and mucosal antibody responses after oral vaccination with inactivated influenza vaccine. The work of Clements et al. [20] suggested that administration of multiple doses of oral vaccine was necessary to elicit both systemic and mucosal antibody responses in mice. In a preliminary study, we confirmed that antiviral mucosal IgA responses were detected only after a third oral dose of influenza virus vaccine, whereas low serum IgG responses were elicited after a single oral dose of vaccine and were boosted with subsequent doses (unpublished data). Therefore, an initial experiment examined the antibody responses elicited in BALB/c mice given a high (150 μg) dose of formalin-inactivated purified whole X-31 virus vaccine with or without the mucosal adjuvant LT (25 μg) three times at weekly intervals. Antiviral IgG, IgA, and IgM responses in sera and lung wash samples collected 3 weeks after oral vaccination were compared with those elicited by a single parenteral ip vaccination with 30 μg of X-31 vaccine or an ip boost (30 μg of X-31 vaccine) 1 week after oral vaccination. As shown in figure 1A, oral vaccination with X-31 vaccine alone resulted in serum antiviral IgG and IgA antibody responses. However, coadministration of LT enhanced the IgG response to oral X-31 vaccination 5-fold and serum antiviral IgA and IgM responses ~3-fold. Thus, the levels of these serum antibody responses were similar to those observed in mice receiving parenteral vaccination.

The administration of high doses of protein by the oral route in many studies has resulted in the induction of tolerance to subsequent parenteral vaccination with the same antigen [20]. However, in our experiment, an ip booster dose after oral vaccination enhanced the serum antiviral IgG and IgA responses above those obtained by oral vaccination alone. The low levels of antiviral IgM induced by oral vaccination were enhanced $\geq$10-fold after the parenteral boost, resulting in IgM titers $\geq$10-fold higher than those induced by a single-dose parenteral vaccination. This result was obtained regardless of whether LT had been coadministered with the oral vaccine. Therefore, no evidence for the induction of B cell tolerance was observed using this immunization regimen with influenza vaccine. Figure

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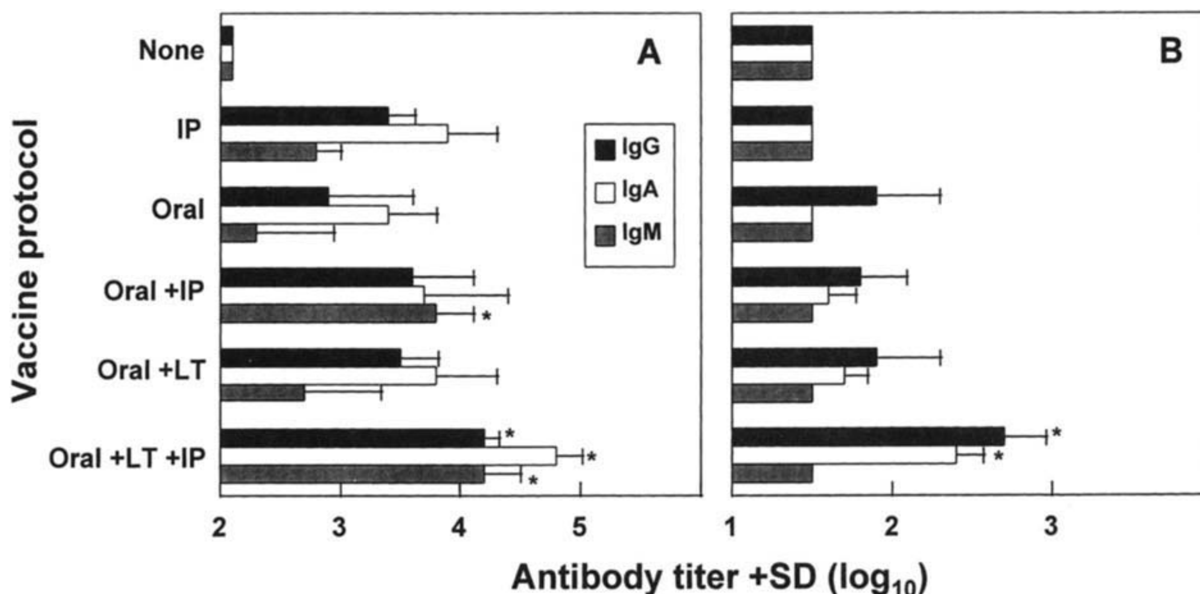

Figure 1. Antiviral IgG, IgA, and IgM antibody responses in sera and lung wash samples after oral vaccination of mice with high-dose inactivated influenza vaccine and effect of parenteral boosting. 5 mice/group were left unvaccinated (None), vaccinated with single 30-μg intraperitoneal inoculation of inactivated X-31 virus (IP) or with 3 150-μg intragastric doses of inactivated X-31 virus with (Oral+LT) or without (Oral) LT (heat-labile enterotoxin) adjuvant or with IP boosting with 30 μg of X-31 vaccine after oral vaccination (Oral+IP; Oral+LT+IP). Anti–X-31 virus antibody responses in serum (**A**) and lung lavage fluid (**B**) were determined by ELISA and expressed as reciprocal end-point titers. Anti–X-31 antibody titers of positive control samples from X-31 virus–infected mice were as follows: serum IgG, IgA, and IgM, 4.0, 3.8, and 2.8, respectively; lung IgG, IgA, and IgM, 3.0, 2.6, and <1.5., respectively. * $P < .005$ vs. Oral, Oral+LT, or IP vaccine groups.

1B shows the levels of antiviral antibody in lung lavage fluids. Oral X-31 vaccination in the absence of LT induced an antiviral IgG but not an IgA response in the lungs. Coadministration of LT with the oral vaccine resulted in both IgG and IgA responses in the lungs that were further enhanced by an ip vaccine boost. In contrast, parenteral vaccination alone failed to induce any detectable antiviral IgG or IgA antibody in the lungs. No virus-specific IgM antibody was detected in lung lavage samples from any vaccination group.

In a second experiment, the effect of LT on oral vaccination with a low dose (20 μg) of vaccine was investigated. Responses induced by oral vaccination were again compared with those induced by a single ip inoculation of inactivated X-31 vaccine alone. The virus-specific IgG and IgA serum antibody responses were examined 3 weeks after vaccination (figure 2). Oral immunization with 20 μg of inactivated vaccine alone resulted in production of antiviral serum IgG antibody and low levels of serum IgA (figure 2A). Again, the addition of LT to the low-dose oral influenza vaccine enhanced the serum anti–X-31 IgG and IgA responses 6- to 8-fold and resulted in serum IgG responses comparable with those elicited by parenteral vaccination. Oral immunization without LT induced a virus-specific IgG response in the lungs, which was enhanced 8-fold when LT was coadministered with the oral influenza vaccine (figure 2B). Only oral vaccination in the presence of LT led to substantial levels of antiviral IgA in the lungs of mice. In contrast, parenteral vaccination again failed to induce detectable antiviral IgA antibody in the lungs.

Taken together, these results demonstrated that coadministration of LT with oral inactivated X-31 vaccine augmented systemic virus-specific IgG and IgA responses. More importantly, the addition of LT to the vaccine formulation resulted in an antiviral IgA response in the lungs of mice that was not achieved by oral immunization with either the low or high dose of X-31 vaccine alone or parenteral vaccination. The ability of LT to enhance lung IgA responses was dependent on the mucosal route of delivery, since parenteral administration of vaccine in the presence of LT also failed to elicit IgA at this site (data not shown).

Serum HAI and neutralizing antibody responses after oral vaccination. We next compared the ability of oral vaccination with and without LT to elicit X-31 strain–specific and cross-reactive anti-HA antibodies capable of neutralizing virus infectivity in vitro. Table 1 shows the titers of HAI and neutralizing antibody in sera from vaccinated mice against 3 related H3N2 viruses and viruses of the H1N1 and H2N2 subtypes. Titers of serum HAI and neutralizing antibodies reacting with X-31 and A/Memphis/72 (H3N2) were 4-fold higher in mice given X-31 vaccine with LT compared with titers in mice given oral X-31 vaccine alone. The former titers were comparable to those in mice vaccinated parenterally with inactivated X-31 virus. Serum from all groups of mice did not have detectable HAI

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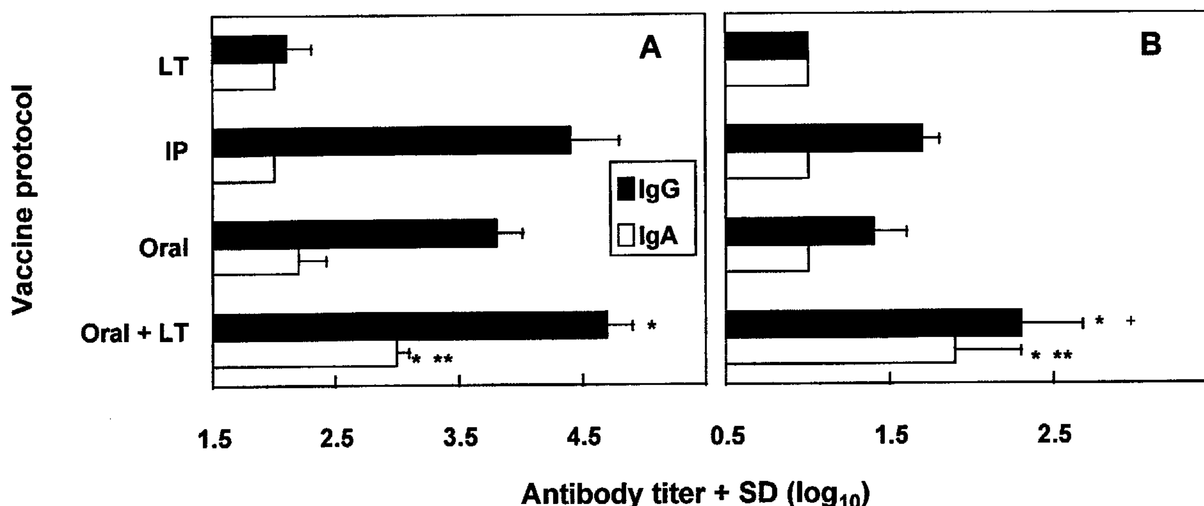

Figure 2. Antiviral IgG and IgA antibody responses in sera and lung wash samples after oral or parenteral vaccination with low dose of inactivated influenza vaccine in presence and absence of LT (heat-labile enterotoxin). 5 mice/group received 1 intraperitoneal inoculation of inactivated X-31 virus (20 μg) in PBS (IP) or 3 intragastric doses of 25 μg of LT alone (LT), 20 μg of X-31 vaccine (Oral), or X-31 vaccine with LT (Oral+LT). Anti–X-31 virus antibody responses in serum (**A**) and lung lavage fluid (**B**) were determined by ELISA. Responses are expressed as reciprocal end-point titers. Anti–X-31 antibody titers of positive control samples from X-31 virus–infected mice were as follows: serum IgG, 5.0, and IgA, 3.0; lung IgG, 3.0, and IgA, 2.6. * $P < .005$ vs. Oral. ** $P < .001$ and $^{+}$ $P < .05$ vs. IP group.

or neutralizing antibody titers against A/England/75 (H3N2) virus or other influenza A virus subtypes. A/England/75 differs from A/Memphis/72 by 15 aa in HA1, the region of the HA molecule that contains the major antibody-binding sites, and by 29 aa from the HA1 region of A/Aichi/2/68, from which X-31 derives its HA protein [32, 33]. HAI antibody titers comparable to those presented in table 1, for mice vaccinated with 20 μg of X-31 vaccine, were also obtained when higher doses (150 μg) were used (data not shown). Therefore, the HAI antibody titers paralleled the virus-specific IgG titers obtained by ELISA in that the 20- and 150-μg vaccine doses elicited similar titers.

Neutralizing antibody specific for X-31 virus was also detectable (titer = 40) in lung wash samples from mice administered oral X-31 vaccine with LT, whereas samples from mice vaccinated with oral X-31 alone had no detectable neutralizing activity (titer, <20). These results indicate that LT enhanced not only serum antiviral IgG antibody induced by oral vaccination but specifically the anti-H3 HA responses capable of neutralizing infectivity of the homologous virus and an antigenically related H3N2 virus.

The protective efficacy of oral vaccination is augmented by LT adjuvant. A series of challenge experiments was done to determine the dose-dependence and the degree and longevity of protection against influenza virus challenge afforded by oral vaccination with influenza in the presence or absence of LT. Vaccination with a high dose (150 μg) of vaccine resulted in complete protection of animals challenged inl with 50 MID_{50}

Table 1. Serum hemagglutination-inhibition (HAI) and neutralizing antibody response of vaccinated mice.

	HAI and neutralizing antibody titer against*				
Vaccine protocol	A/X-31 (H3N2)	A/Mem/72 (H3N2)	A/Eng/75 (H3N2)	A/AA/6/60 (H2N2)	A/PR/8/34 (H1N1)
Oral X-31	160 (80)	20 (<40)	<20 (<40)	<20 (<40)	<20 (<40)
Oral X-31 + LT	320 (320)	80 (80)	<20 (<40)	<20 (<40)	<20 (<40)
IP X-31	320 (ND)	80 (ND)	<20 (ND)	<20 (ND)	<20 (ND)
Oral LT	20 (<40)	<20 (<40)	<20 (<40)	<20 (<40)	<20 (<40)

NOTE. Mice were vaccinated with 20 μg of formalin-inactivated whole X-31 virus with or without 25 μg of heat-labile enterotoxin (LT). Sera from 5 mice/group were collected 3 weeks after 3rd oral vaccination or single intraperitoneal (IP) immunization and pooled for assay. ND, not determined.

* HAI titers represent reciprocal of highest dilution of sera inhibiting agglutination of 0.5% chicken erythrocytes by 4 hemagglutination units of virus. Neutralization titers (in parentheses) are expressed as reciprocal of highest dilution of serum that neutralized 200 $TCID_{50}$ virus in 50% of infected MDCK cell cultures.

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Table 2. Protective efficacy of oral vaccination is augmented by heat-labile enterotoxin (LT) adjuvant.

Experiment no., dose of X-31 vaccine (μg)	Time after vaccination	Vaccination protocol	Degree of protection: No. protected*/total mice (P)[†]	Degree of protection: Mean lung virus titer ± SD, $\log_{10}$ EID_{50}/mL (P)[‡]
1, 150	3 weeks	Oral X-31	4/5 (<.05)	1.7 ± 1.5 (<.005)
		Oral X-31 + LT	5/5 (<.005)	≤1.0 ± 0 (<.001)
		IP X-31	5/5 (<.005)	≤1.0 ± 0 (<.001)
		None	0/5	7.2 ± 0.6
2, 100	6 weeks	Oral X-31	1/5 (NS)	4.0 ± 1.9 (NS)
		Oral X-31 + LT	4/4 (<.01)	≤1.5 ± 0 (<.01)
		Oral LT	0/5	4.9 ± 1.2
3, 75	3 months	Oral X-31	3/5 (NS)	1.8 ± 1.1 (.01)
		Oral X-31 + LT	5/5 (<.005)	≤1.0 ± 0 (<.001)
		IP X-31	2/4 (NS)	1.7 ± 0.7 (<.01)
		None	0/5	5.7 ± 0.4
4, 20	3 months	Oral X-31	0/9 (NS)	5.1 ± 1.2 (NS)
		Oral X-31 + LT	9/9 (<.005)	≤1.5 ± 0 (<.005)
		Oral LT	0/4	6.4 ± 0.9

NOTE. Mice were challenged intranasally with 50 MID_{50} of X-31 virus and euthanized 4 days later. Lungs were homogenized in 1 mL of PBS and titrated for virus infectivity in eggs. IP, intraperitoneal; NS, not significant.

* Mice were considered protected if no virus was detectable in 0.1 mL of undiluted lung homogenate (virus titer ≤1.0, experiments 1, 3) or in 1:10 dilution of lung homogenate (virus titer ≤1.5, experiments 2, 4). These titers represent limits of sensitivity of assay.

† Significance of results (Fisher's exact test) from vaccinated mice vs. those from unvaccinated controls (experiments 1, 3) or control mice receiving oral LT only (experiments 2, 4).

‡ Significance of results (Student's t test) relative to unvaccinated controls (experiments 1, 3) or control mice receiving oral LT only (experiments 2, 4).

X-31 virus 3 weeks later, regardless of vaccination route or whether LT was coadministered with oral vaccination (table 2; experiment 1). In experiment 3, we investigated the protective efficacy induced using a lower dose (75 μg) of viral vaccine with 3 months between vaccination and challenge. In this case, protection against infection was incomplete in groups of mice given either parenteral or oral viral vaccine alone. In contrast, all mice given oral vaccine with LT were completely protected from infection with live X-31 virus, and this result was statistically significant when compared with the unvaccinated control group ($P < .005$, Fisher's exact test). Nevertheless, all groups of vaccinated mice showed significant reduction in lung virus titers compared with unvaccinated mice.

When the vaccine dose was lowered to 20 μg (experiment 4) or oral vaccine doses were given at 2-day rather than 7-day intervals (experiment 2), the efficacy of oral vaccination with inactivated influenza virus alone was reduced further. In contrast, coadministration of influenza vaccine with LT resulted in complete protection from infection in all cases. Therefore, coadministration of oral influenza vaccine with the adjuvant LT enabled the use of a lower dose of vaccine and improved the durability of the protective effect of oral vaccination.

Specificity of the protective effect of oral vaccination in the upper and lower respiratory tracts of mice. Since mucosal IgA responses are thought to be particularly important for protection in the upper respiratory tract, we investigated the protective efficacy of oral influenza vaccination in nasal tissues and in the lower respiratory tract (lungs). In addition, the specificity of the protective effect was evaluated. Table 3, experiment 1, shows that although oral X-31 vaccination in the absence of LT failed to protect mice from infection in the lower respiratory tract, a 20-fold reduction in virus titer was seen in the upper respiratory tract compared with control mice given LT alone ($P < .05$). However, oral influenza vaccination with LT completely protected mice from lower respiratory tract infection, and virus titers in nasal tissues were 300-fold lower than those of control animals. Therefore, the reduction in virus shedding in the upper respiratory tract was significantly greater in mice receiving LT with oral X-31 vaccine compared with those given oral vaccine alone ($P < .05$).

To investigate the cross-protective efficacy of oral vaccination, another group of animals was challenged with the related influenza A H3N2 virus A/Memphis/72. Mice administered oral vaccine without LT were not completely protected from infection of the lower respiratory tract but had reduced lung virus titers compared with animals administered LT alone. These same animals showed no reduction in nasal virus titers. In contrast, mice administered LT with the oral influenza vaccine were completely protected from viral replication in the lungs and had 500-fold lower titers of virus in the upper respiratory tract compared with control mice given oral LT ($P < .005$). Therefore, the cross-protective efficacy against a heterologous

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Table 3. Heat-labile enterotoxin (LT) augments the protective efficacy of oral influenza vaccination in the lower and upper respiratory tract of mice challenged with influenza A (H3N2) viruses.

Experiment no., vaccination protocol	Challenge virus	Virus titer ($\log_{10}$ EID_{50}/mL) in	
		Lung (*P*)*	Nose (*P*)†
1, Oral X-31	X-31	5.2 ± 0.6 (NS)	3.5 ± 0.2 (<.05)
Oral X-31 + LT		≤1.5 ± 0 (<.005)	2.2 ± 0.8 (<.05)
Oral LT		6.4 ± 0.9	4.8 ± 0.3 (.05)
Oral X-31	A/Mem/102/72	3.0 ± 0.8 (<.05)	4.3 ± 0.8 (NS)
Oral X-31 + LT	(H3N2)	≤1.5 ± 0 (<.005)	2.2 ± 0.9 (<.005)
Oral LT		4.3 ± 0.6 (<.05)	4.9 ± 0.6
2, Oral X-31	A/PR/8/34	5.1 ± 0.7 (NS)	ND
Oral X-31 + LT	(H1N1)	4.0 ± 2.2 (NS)	ND
Oral LT		4.9 ± 1.8	ND

NOTE. Mice were vaccinated with 20 μg of formalin-inactivated X-31 virus ± 25 μg LT or with 25 μg of LT alone. 3 months after vaccination, 5 mice/group were challenged with 50 MID_{50} of influenza A virus. 4 days after challenge, mice were exsanguinated and lung and nasal tissues were collected, homogenized, and titrated for virus infectivity in eggs. NS, not significant; ND, not determined; infection procedure used results in poor replication of A/PR/8/34 virus in upper respiratory tract.

* Determined using Student's *t* test and represents significance for vaccinated mice vs. control mice receiving oral LT alone.

H3N2 virus was significantly greater in mice coadministered LT adjuvant with X-31 vaccine than in mice given X-31 vaccine alone.

In another experiment, orally vaccinated mice were challenged with an influenza A virus of the H1N1 subtype. None of the mice vaccinated with X-31 (H3N2) virus were protected from infection of the lower respiratory tract with A/PR/8/34 (H1N1) virus. Therefore, the specificity of the protective efficacy induced by oral vaccination with LT in the lower respiratory tract correlated with the subtype-specific HAI and virus neutralization antibody responses observed in similarly vaccinated mice (table 1).

LT adjuvant activity dose response and correlation of protective efficacy with mucosal antibody response in the upper respiratory tract. A 25-μg dose of LT was used in initial experiments based on the adjuvant activity observed by others using other purified protein or vaccine systems [20]. To determine whether lower doses of LT had equivalent adjuvant activity for oral vaccination with influenza virus, groups of mice were orally vaccinated with inactivated X-31 vaccine and 0, 1, 5, or 25 μg of LT. To directly relate the protective efficacy of oral vaccination in the presence of different doses of LT, animals were challenged inl with X-31 virus 3 months after vaccination, and the IgG and IgA antibody responses in lung and nasal wash samples and virus titers in lung and nasal tissues were determined for the same groups of animals 4 days later.

Figure 3A shows mean levels (±SD) of anti–X-31 IgG and IgA in the lungs of mice vaccinated with X-31 virus and different doses of LT and the corresponding degree of protection determined as lung virus titers. Levels of antiviral IgG and IgA rose with increasing amounts of LT, and complete protection from viral replication was observed in all mice vaccinated with inactivated X-31 virus and 5 or 25 μg of LT ($P < .01$). In the upper respiratory tract, oral vaccination with or without LT did not stimulate a detectable antiviral IgG antibody response. In contrast, increasing doses of LT resulted in an increase in IgA and a concomitant protection from viral replication in the nose (no viral shedding detected in any mouse) at doses of 5 and 25 μg of LT ($P < .01$). The reduced amounts of antiviral IgA obtained with a dose of 25 μg of LT in this experiment most likely reflects a problem with sampling, since the IgA antibody response in similarly vaccinated mice 3 weeks after vaccination was greater than the IgA antibody response induced with X-31 vaccine and 5 μg of LT (data not shown).

Total IgA in lung and nasal wash samples was also estimated to ensure that the rises in X-31 virus–specific antibody induced by LT were not merely a consequence of increases in polyclonal IgA antibody. The average amounts of total IgA in lung washes were 0.5, 0.6, and 0.9 μg/mL; in nasal washes they were 0.4, 0.5, and 0.6 μg/mL for antibody samples from mice receiving oral X-31 vaccine with 0, 5, and 25 μg of LT, respectively. Therefore, the enhanced antiviral IgA antibody responses observed after oral vaccination with LT were presumably due to adjuvant activity specific for the vaccine antigen and not due to a nonspecific polyclonal activation of B cells.

LT promotes a proliferative response and IL-2 production in orally vaccinated mice. To determine whether LT could also augment influenza virus–specific T cell responses to oral influenza vaccination, spleen cells from groups of mice vaccinated 3 months earlier were tested for proliferative responses to a panel of type A (H3N2) viruses, the A(H1N1) parent virus A/PR/8/34 (from which X-31 derives all proteins except the HA and the neuraminidase), and an unrelated influenza B virus. Figure 4 shows the proliferative response of spleen cells to 5.0

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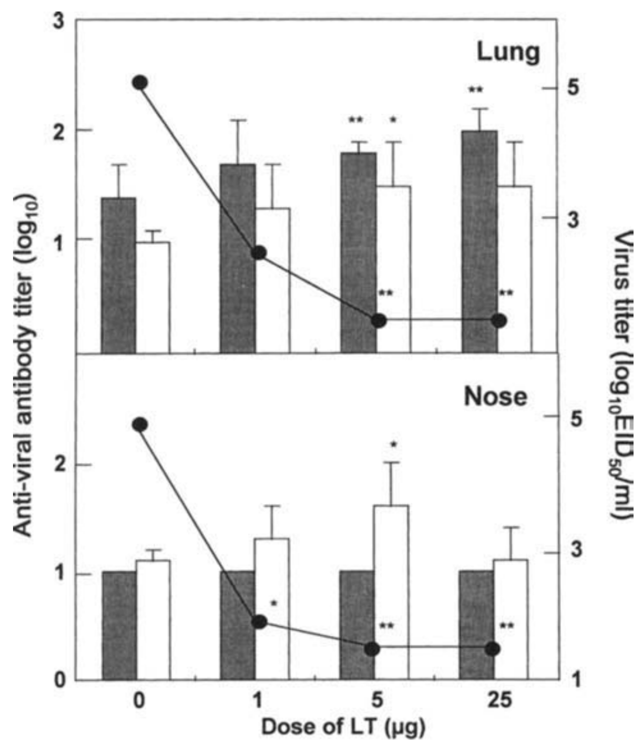

Figure 3. Dose dependence of LT (heat-labile enterotoxin) activity in induction of antibody responses in mucosal tissues and protection from infection in lower and upper respiratory tracts of mice. Mice (5/group) that received oral X-31 vaccine (20 μg) coadministered with different amounts of LT 3 months earlier were challenged with 50 MID_{50} of X-31 virus. Antiviral IgG (shaded bars) and IgA (open bars) antibody responses in lung and nose wash samples and virus titers (●) in lung and nose tissues were determined 4 days later. Antibody responses are expressed as reciprocal end-point titers. Anti–X-31 antibody titers of positive control samples from X-31 virus–infected mice were as follows: lung IgG, 3.0, and IgA, 2.6; nasal IgG, 2.7, and IgA, 2.3. Limit of virus detection was $10^{1.5}$ EID_{50}/mL. * $P < .05$ and ** $P < .01$ vs. vaccine group receiving no LT.

and 0.5 HAU/mL UV-inactivated whole virus. Only minimal proliferation to the H3N2 viruses X-31, Memphis/72, and Texas/77 was observed in spleen cells from mice vaccinated with oral X-31 vaccine without LT. In contrast, spleen cells from mice given X-31 vaccine with LT (25 μg) demonstrated substantial proliferation in response to all influenza A viruses tested, but not to the influenza B virus. In a further experiment, spleen cells from vaccinated mice were purified over nylon wool [34]. The proliferative response of the enriched T cell populations from mice vaccinated orally with or without adjuvant was 11,746 ± 664 and 1337 ± 370, respectively, in culture stimulated with 5 HAU/mL UV-inactivated X-31 virus and 505 ± 12 and 493 ± 58 in cultures lacking antigen. These results suggest that the proliferation observed in unseparated lymphocyte populations was due to T cells.

The IL-2 activity present in 72-h supernatants from spleen cell cultures stimulated with 5.0 HAU/mL X-31 or B/Hong Kong/73 virus was also determined using IL-2–dependent CTLL cells (table 4). As with the proliferative response, there was minimal IL-2 activity in spleen cells cultured from mice vaccinated orally with X-31 vaccine alone. However, spleen cells from mice given LT with the oral X-31 vaccine had substantial IL-2 activity after in vitro stimulation with X-31 but not with influenza B virus. Virus-stimulated spleen cells from mice administered only LT had no detectable IL-2 activity. In a second experiment, cultures of mesenteric and mediastinal lymph node cells from mice vaccinated with, but not without, LT adjuvant and X-31 virus produced substantial IL-2 activity upon stimulation with X-31 virus relative to stimulation with B/Hong Kong/73 virus. The production of IL-2 in response to virus-specific stimulation of lymphocytes most probably reflects the activity of CD4 T cells. Nevertheless, since unseparated cell populations were used, we cannot exclude the contribution of other lymphoid cells (e.g., CD8 T cells) to IL-2 production.

Oral vaccination with inactivated influenza vaccine and LT elicits a secondary CTL response in mice. The induction of influenza virus–specific CTL activity is a desirable feature of influenza vaccines because CTL activity has been associated with enhanced clearance of virus and recovery from influenza infection. However, in general, inactivated influenza vaccines administered by parenteral routes are poor inducers of CTL activity [35]. We therefore investigated whether administration of inactivated influenza vaccine, with or without LT, could prime for a secondary CTL response in vitro. Spleen cells from orally vaccinated mice were restimulated in vitro with irradiated X-31 virus–infected BALB/c spleen cells for 5 days. As shown in table 5, spleen cells from mice orally vaccinated with inactivated X-31 virus in the presence of LT showed substantial cytotoxic activity that was specific for MHC class I–matched P815 target cells infected with A/X-31 virus but not with an influenza B virus. No cytotoxic activity was observed on MHC-mismatched EL-4 (H-2^b) target cells infected with X-31 virus. Spleen cells from mice given oral X-31 vaccine alone or LT alone had no virus-specific cytotoxic activity. Therefore, these data suggest that coadministration of LT with oral inactivated influenza vaccine can induce a class I–restricted memory CTL response.

Discussion

Our results demonstrate that LT is an effective adjuvant for the oral delivery of inactivated influenza vaccine in BALB/c mice. LT augmented antiviral IgA responses in mucosal tissues and systemic IgG responses to orally administered inactivated influenza vaccine and induced serum HAI and neutralizing antibody responses comparable to those induced by parenteral immunization. In addition, oral coadministration of LT with inactivated influenza vaccine induced IL-2–producing cells and class I–restricted CTL responses important for regulation

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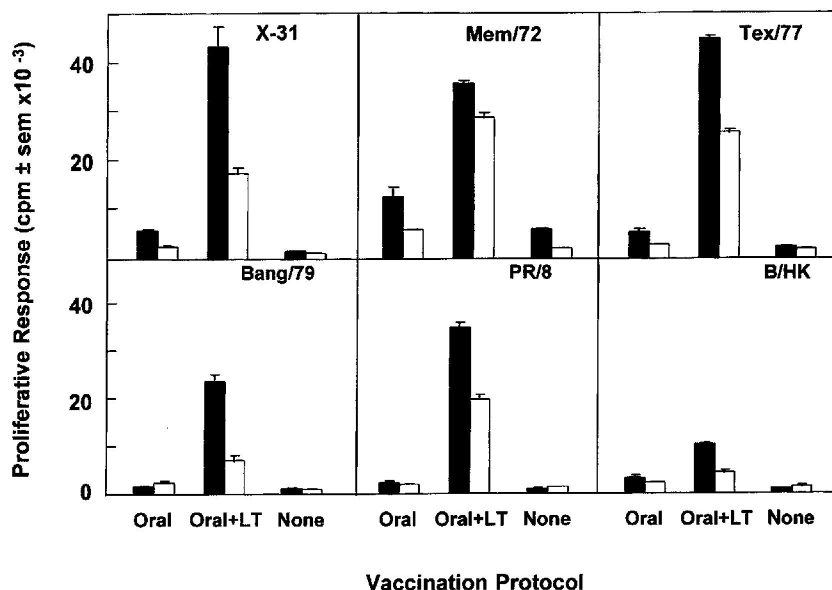

Figure 4. Effect of LT (heat-labile enterotoxin) on proliferative response of spleen cells from orally vaccinated mice. Groups of 3–5 mice were vaccinated orally 3 months earlier with 75 μg of inactivated X-31 vaccine with or without LT (Oral+LT and Oral, respectively) or were not vaccinated (None). Responses are of pooled spleen cells to 5 HAU/mL (solid bars) or 0.5 HAU/mL (open bars) of different type A influenza H3N2 viruses, H1N1 virus PR/8, or negative control type B influenza virus.

of the immune response and recovery from infection, respectively.

The induction of antiviral IgA in mucosal tissues was dependent on the coadministration of LT with either a high or low dose of inactivated vaccine and the oral route of delivery. No virus-specific IgA antibody was induced in respiratory tissues when LT was used as an adjuvant for multiple parenteral immunizations with inactivated vaccine (data not shown), suggesting that the mucosal route of delivery, rather than the larger total amount of vaccine administered in multiple doses, was critical for the detection of IgA antibody in mucosal tissues. The antiviral IgA antibody detected in the respiratory tract appeared to be derived locally and not from the serum, since both parenterally and orally vaccinated animals had comparable amounts of serum IgA, but only mice vaccinated orally with the LT had substantial respiratory antiviral IgA, suggesting that this antibody was not serum-derived. However, further characterization of the IgA in mucosal tissue washings or by isolation of IgA-secreting cells from respiratory tissues is required for confirmation. The total IgA response in the mucosal wash samples was not significantly greater in mice given X-31 vaccine with LT than in mice vaccinated with virus alone, indicating that the enhanced IgA response was specific for the immunizing viral antigen and not merely a polyclonal expansion of total IgA antibody.

Table 4. Virus-specific interleukin (IL)-2 activity in lymphocytes from orally vaccinated mice.

Experiment no., origin of lymphocytes	Vaccine protocol	IL-2 activity (mean cpm ± SE) after stimulation with*	
		A/X-31	B/Hong Kong
1, Spleen	Oral X-31	916 ± 179	167 ± 21
	Oral X-31 + LT	8033 ± 412	651 ± 36
	Oral LT	135 ± 10	127 ± 5
2, Mesenteric lymph nodes	Oral X-31	4713 ± 158	1309 ± 30
Mediastinal lymph nodes	Oral X-31 + LT	13,430 ± 142	1304 ± 23
	Oral X-31	878 ± 32	308 ± 5
	Oral X-31 + LT	12,604 ± 120	1397 ± 70

NOTE. Spleen or lymph node cells from 3–5 mice/vaccine group were cultured in triplicate with influenza A or B virus in volume of 0.2 mL for 72 h. Supernatants were collected and assayed for presence of IL-2 using IL-2–dependent CTLL-2 cell line (see Materials and Methods). LT, heat-labile enterotoxin.

* UV-inactivated A/X-31 or B/Hong Kong/8/73 viruses were used to stimulate cells at 5.0 HAU/mL.

Table 5. Secondary cytotoxic activity in spleen cells from orally vaccinated mice.

Vaccine protocol	E:T ratio	% specific ^{51}Cr release		
		P815 + A/X-31	P815 + B/Pan	EL-4 + A/X-31
Oral X-31	10:1	5.1	5.8	2.5
	5:1	0	3.1	0
Oral X-31 + LT	10:1	32.1	3.7	0
	5:1	19.1	4.6	0
Oral LT	10:1	9.4	10.4	3.5
	5:1	3.2	5.9	0

NOTE. Mice were vaccinated with 3 oral doses of 50 μg of X-31 vaccine and/or 25 μg of heat-labile enterotoxin (LT). Spleen cells from 3 mice/vaccine group were cultured with irradiated X-31–infected BALB/c spleen cells for 5 days at effector:stimulator cell ratio of 5:1. Effector cells were tested in ^{51}Cr release assay using target cells infected with A/X-31 or B/Panama/45/90 viruses. E:T, effector to target.

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The use of LT in the oral vaccine regimen reduced the amount of viral antigen required for the induction of protection in mice. Protection of the upper respiratory tract correlated with the induction of antiviral IgA responses, while protection in the lower respiratory tract was associated with elevated levels of antiviral IgG and IgA. There was complete protection from infection in the lower respiratory tract for 3 months after vaccination with influenza virus in the presence but not in the absence of LT. Protection was subtype-specific; animals vaccinated orally with X-31 (H3N2) were not protected from lower respiratory tract infection with the H1N1 virus A/PR/8/34. Therefore, the specificity of the protective effect in the lower respiratory tract correlated with that observed for HAI and virus-neutralizing antibody responses (table 1).

Since cell-mediated mechanisms of protection are generally directed against internal antigens, which are identical in the X-31 and A/PR/8/34 viruses, this result also suggested that the protective effect observed in the lower respiratory tract of mice was mediated by antibody. The subtype-specificity of the protective effect in the upper respiratory tract could not be addressed by infection of mice with A/PR/8/34, since the virus replicated poorly at this site. Furthermore, the single early sampling time point chosen (day 4 after challenge) was not sufficient to address the contribution of cell-mediated immunity, which typically enhances viral clearance and is often detected at later time points of infection. Therefore, although virus-specific IgA antibody was associated with protection in the upper respiratory tract, a role for cell-mediated immunity in protection at this site cannot be ruled out and requires further study.

Another important feature of the adjuvant action of LT was the ability to enhance influenza virus–specific T cell responses. Oral vaccination with inactivated influenza vaccine in the presence but not in the absence of LT induced a virus-specific proliferative T cell response and secretion of IL-2 after in vitro stimulation with type A but not type B influenza viruses. Oral administration of the mucosal adjuvant CT selectively induces Peyer's patches and splenic T helper cells, which produce Th2-type cytokines (e.g., IL-4 and IL-5), whereas LT can induce T cells with Th1- or Th2-type profiles [36]. These results suggest that compared with CT, LT adjuvant may stimulate a more balanced immune response.

A noteworthy finding was that oral vaccination with inactivated influenza virus coadministered with LT induced a type A influenza virus–specific class I–restricted CTL response. Inactivated and subunit influenza vaccines administered intramuscularly are traditionally poor immunogens for CTL in naive animals [35] and elicit only short-lived memory CTL responses in primed individuals [37]. The virus-specific memory CTL response to oral inactivated influenza vaccine and LT in mice was evident 6 months after vaccination and was, therefore, relatively long-lived.

Although a number of novel strategies for the induction of CTL with nonreplicating antigens have been reported, these approaches have primarily used systemic immunization to induce CTL activity. One exception is the study by Bowen et al. [38], which demonstrated that oral administration of large doses of ovalbumin in the presence of CT primed for ovalbumin-specific CTL. The results of the present study demonstrated that oral vaccination with a relatively small dose of killed viral antigen and LT adjuvant elicited CTL that recognized and killed virus-infected target cells. Whether these CTL enhanced viral clearance and recovery from infection was not addressed in this study. While the precise mechanism(s) for the adjuvant effect of LT and CT remains unresolved, the ability of LT to augment proliferative responses and CTL activity may, by analogy with CT, reside in the adjuvant's effects on antigen presentation and cytokine production [25].

Earlier investigations into oral vaccination with influenza established the validity of this route of immunization for the induction of local respiratory antibodies. In general, oral vaccination required substantially higher doses and multiple administrations of vaccine compared with parenteral vaccination. In the present study, the use of LT reduced the amount of inactivated viral vaccine required for the induction of protection in mice, although the delivery of multiple doses of vaccine was required for the induction of virus-specific IgA antibody in mucosal tissues. Administration inl appears to be a more sensitive route of mucosal vaccination, although inl delivery of inactivated influenza vaccine also results in mucosal and systemic antibody responses that are substantially augmented by the coadministration of LT adjuvant. However, inl administration of the LT holotoxin has been associated with lethal toxic effects in some animals, in contrast to the total lack of reactogenicity observed in mice administered multiple oral doses of LT (Katz JM, unpublished observation).

Hirabayashi et al. [39] have used the B subunit of CT as an adjuvant for a single-dose inl vaccination with HA subunit vaccine. When compared with inl delivery, oral vaccination required 100-fold higher doses of HA to elicit mucosal antibody and failed to induce any serum HAI antibody. More recently, Tamura et al. [40] reported the use of the B subunit of LT (2 μg) supplemented with 0.2–2 ng of the holotoxin as a potent and less toxic adjuvant for inl vaccination.

There are several considerations in the use of the LT holotoxin as a mucosal adjuvant for oral vaccination with influenza virus. First, in addition to its adjuvant activity, LT is also immunogenic. Oral administration of LT results in anti-LT serum IgG and mucosal IgA antibody responses, and therefore the efficacy of repeated oral vaccinations with influenza virus and LT may be reduced in the presence of circulating anti-LT antibodies. However, the work of Clements et al. [20] suggests that adjuvant activity may be reduced but not abrogated in the presence of an anti-LT antibody response. The effect of anti-LT antibodies on the adjuvant activity of LT for influenza virus–specific antibody and cellular immune responses is currently under investigation. Second, responsiveness to CT and LT as immunogens and adjuvants is genetically restricted with

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"high" and "low" responder phenotypes [41]. It remains to be determined whether LT has broad adjuvant activity in humans. Finally, any novel strategy for improved vaccination with influenza virus must elicit protective immunity in the face of a preexisting immune response. In preliminary studies, we observed that oral vaccination of animals with preexisting anti-H3 antibody resulted in rises in serum antibody titers to the antigenically distinct H3N2 vaccine virus strains when LT was coadministered with the vaccine. To what extent oral influenza vaccination with LT can also augment mucosal IgA responses in the face of preexisting antibody remains to be determined.

The advantages offered by oral influenza vaccination include ease and simplicity of administration and the acceptability of the noninvasive delivery route, particularly for children. It is also possible that an oral vaccine would have less stringent preparation and purification requirements compared with an injectable vaccine. Finally, a more immunogenic and effective vaccine formulation could encourage the broader use of the vaccine in the community. Phase I clinical trials recently completed by the Navy Medical Research Institute in association with the US Army indicated that at doses of up to 5 μg, LT was safe for oral administration to humans. Preliminary findings from this study also suggested that LT had adjuvant activity for mucosal and serum antibody responses in humans (Scott D, personal communication). However, a safer form of LT adjuvant may now be at hand. Dickinson and Clements [42] recently described a mutant LT protein that is insensitive to activation by cleavage with trypsin, has negligible enterotoxic activity, and retains adjuvant activity for mucosal IgA responses when coadministered orally with ovalbumin. Our preliminary findings suggest that the mutant LT protein also retains adjuvant activity for influenza virus–specific protective immune responses. The potential of the mutant LT protein for use in humans is being investigated.

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