Jpn. J. Infect. Dis., 53, 98-106, 2000

Review

A Proposal for Safety Standards for Human Use of Cholera Toxin (or *Escherichia coli* Heat-Labile Enterotoxin) Derivatives as an Adjuvant of Nasal Inactivated Influenza Vaccine

Shin-ichi Tamura* and Takeshi Kurata

Department of Pathology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan

(Received June 15, 2000. Accepted July 28, 2000)

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SUMMARY: Cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT) are not only the causative agents of diarrhea but are also strong mucosal adjuvants which enhance immune responses to mucosally coadministered bystander antigens. One of the most promising applications of these toxins would be as mucosal adjuvant of nasal influenza vaccine. In comparison to current inactivated vaccines, the nasal vaccine provides superior cross-protection by inducing production of cross-reacting anti-viral IgA antibodies in the respiratory tract even when the vaccine strain is different from the epidemic strain. On the use of the toxins as mucosal adjuvants in humans, toxicity and allergenicity of the toxins are problems which impinge on safety. To resolve these problems, various approaches have been attempted to produce less toxic and less allergenic CT (or LT) derivatives. We now propose the following standards for human use of safer CT (or LT) derivatives as an adjuvant of a nasal influenza vaccine. Thus, CT (or LT) derivatives can be administered intranasally together with a current inactivated influenza vaccine, provided they meet the following criteria. 1) A single dose of the derivatives, administered intranasally by spraying, should be around 100 μg/adult in a volume of less than 0.5 ml. 2) CT (or LT) derivatives should retain the properties of the native CT (or LT), i.e., the ability to augment secretory IgA and serum IgG Ab responses to viral surface glycoproteins, when administered intranasally together with an inactivated influenza vaccine. 3) CT (or LT) derivatives should not induce IgE Ab responses to the vaccine, as well as to the CT (or LT) itself. 4) The CT (or LT) should be nontoxic; the toxicity of the derivatives, as determined by the Y-1 adrenal cell assay, should not exceed 1/100 EC_{50} of the native CT (or 1/1000 ECi of the native CT). 5) CT (or LT) derivatives should not cause serious disease in guinea pigs when administered intranasally or intraperitoneally at the dose used in humans (around 100 μg).

1. Introduction

Influenza is a highly contagious acute respiratory disease, caused by influenza viruses that are implicated in both worldwide pandemics and local outbreaks by altering their surface hemagglutinin (HA) and neuraminidase either slightly or radically (1). Due to the short incubation period, the immunity raised within a few days after infection cannot prevent the onset of the respiratory symptoms. Therefore, an effective immunity must be induced in advance, by vaccination, in order to prevent the disease. Current trivalent inactivated vaccines, which contain A and B types of viruses, are administered subcutaneously. These vaccines are shown to induce production of high levels of serum antiviral IgG antibodies (Abs), which have a protective effect against homologous viral infection. However, the vaccination is less effective against heterologous viral infection within the same subtype, which explains the ineffectiveness of the current influenza vaccine when the vaccine strain is different from the epidemic strain. On the other hand, natural influenza viral infection has been shown to be superior to current inactivated vaccines for inducing cross-protection against variant viral infections (2). The cross-protection induced by natural infection seems to be largely due to the production of cross-reacting IgA Abs to the viral HA molecules in the respiratory tract (3). The cross-reactivity is derived from the polymeric nature of S-IgA (secretory IgA), which generates a greater reactivity for the

*Corresponding author: Tel: +81-3-5285-1111, Fax: +81-3-5285-1189, E-mail: stamura@nih.go.jp

influenza virus than serum IgG. This fact suggests that the development of an immunization procedure to stimulate mucosal IgA production would improve the protective efficacy of current inactivated vaccines. In this regard, intranasal immunization with a vaccine has been advocated as a means of inducing IgA and systemic IgG against influenza virus (4). However, a vaccine alone cannot easily generate S-IgA Abs. Therefore, we attempted to administer a vaccine together with cholera toxin (CT) as a mucosal adjuvant (5).

CT, which is produced by *Vibrio cholerae*, is the causative agent of cholera, while *Escherichia coli* heat-labile enterotoxin (LT), whose amino acid sequence shows 80% homology to that of CT, is one of the causative agents of traveler's diarrhea (6-8). At the same time, both toxins are strong mucosal adjuvants which enhance immune responses to mucosally co-administered bystander antigens (9-12). CT, as well as LT, consists of a binding region of five B subunits, into which the active toxic component, the A subunit, is inserted. CT (or LT) binds to a specific receptor, i.e., GM1 ganglioside, via the B subunits on the cell membrane. This enables translocation of the A subunit across the membrane into the cell, where it activates adenylate cyclase. The activation of adenylate cyclase results in an increase in the level of intracellular cyclic adenosine monophosphate, which induces diarrhea and other biological activities (13); the intracellular mechanisms by which CT (or LT) augments immune responses remain to be clarified.

Since 1972, the mucosal adjuvants, CT and LT have been widely investigated (9-14). We have also been engaged in the development of an adjuvant-combined nasal influenza vaccine, which has become one of the most successful applications of the toxin in a human mucosal vaccine (5,15-17). Although both toxins function as mucosal adjuvants, the use of toxins as adjuvants of mucosal vaccines for humans is not feasible owing to their toxicity. In addition, these toxins have another potential drawback of possibly inducing IgE Ab responses to bystander antigens, as well as to the toxins themselves (18,19). Thus, two problems which impinge on the safety of these toxins, toxicity and allergenicity, should be examined carefully when these toxins are used as adjuvants of mucosal vaccines for humans. One approach being explored to resolve the problem is to use the nontoxic B subunits (CTB or LTB) alone (20), or B subunits coupled chemically or by gene fusion to vaccines (21-24). Another approach is to use B subunits containing a trace amount of the toxin (0.1-0.5%) (CTB* or LTB*), because the B subunit and a trace amount of the holotoxin act synergistically as an adjuvant, inducing mucosal immune responses to the vaccine (25-27). A third approach is to use a chemically modified (28) or mutated toxin without toxicity but retaining its adjuvant action (29-33). At present, there are no safety standards on the use of CT (or LT) derivatives as a mucosal adjuvant for humans, although there is much data on these derivatives. The objectives of this review are to propose safety standards for human use of CT (or LT) derivatives as an adjuvant of nasal influenza vaccine and to present the experimental system and data on which this proposal is based.

2. Advantages of using CT- (or LT-) adjuvant-combined nasal influenza vaccine

(1) Preclinical studies

We compared the protective efficacy of nasal immunization with the CTB* (or LTB*)- combined vaccine with that of subcutaneous immunization in BALB/c mice (34). As shown in Fig. 1, intranasal vaccination with A/Guizhou-X vaccine 4 weeks prior to a homologous viral challenge–which was administered in a small volume in an attempt to induce upper respiratory infection–provided almost complete protection against infection. In our experimental system, virus titers of less than $10^{0.5}$ EID_{50} were regarded as complete protection. In parallel with the protection, production of both HA-reactive IgA and IgG Abs were induced in the upper respiratory tract. Each of two vaccines derived from a variant within the same subtype, the A/Fukuoka (H3N2) and A/Sichuan (H3N2) vaccines, also provided almost complete cross-protection, accompanied by the production of a low level of cross-reacting IgA to A/Guizhou-X HA molecules in the upper respiratory tract. A different subtype of viral vaccine, A/PR8 (H1N1), gave slight cross-protection, whereas a different type of viral vaccine, B/Ibaraki, failed to give protection. Subcutaneous vaccination with A/Guizhou-X vaccine 4 weeks prior to a homologous viral challenge provided almost complete protection, accompanied by the production of anti-HA IgG Abs in the upper respiratory tract. However, the A/Fukuoka and A/Sichuan vaccines, as well as the PR8 and B/Ibaraki vaccines, failed to provide cross-protection and did not induce antibody production.

We drew the following conclusions from these results. Intranasal immunization with the current inactivated vaccine together with CTB*(or LTB*) provides effective cross-protection against variants within a subtype of influenza A viruses or within type B viruses in the upper respiratory tract. The cross-protection in the upper respiratory tract is provided mainly by cross-reacting anti-HA IgA Abs, whereas the cross-protection in the lower respiratory tract is provided by the IgG Abs (34). We further examined the vaccination conditions to apply the adjuvant-combined vaccine to humans. In terms of providing cross-protection against lethal infection, single immunization has been shown to be less effective than two doses of vaccine, i.e., primary immunization with a low dose of vaccine and CTB* and second immunization with another vaccine alone (35, 36). In addition, two doses of trivalent vaccines conferred cross-protection against various strains of viruses. Based on these studies, we theorized that application of an adjuvant-combined vaccine to humans could be performed effectively and safely via two successive intranasal immunizations at 4-week intervals, the first immunization with a CTB* (or LTB*)-combined trivalent vaccine (prepared from H1N1, H3N2 and B-type viral strains, recently circulating in humans) mixed with CTB* (or LTB*) and the second immunization with the trivalent vaccine (35,36).

(2) Clinical studies

We conducted the first field trial to evaluate the efficacy of the LTB*-combined nasal influenza vaccine during the winter of 1993-1994 (16). A current inactivated trivalent vaccine composed of A/Yamagata (H1N1), A/Kitakyushu (H3N2) and B/Bangkok influenza virus strains, was administered intranasally into volunteers by spraying with or without recombinant LTB*. Vaccination was performed twice, 4 weeks apart. Titers of salivary S-IgA and serum hemagglutination-inhibiting (HI) Abs were measured before and 8 weeks after the primary vaccination. The volunteers were divided into two groups: 73 volunteers were administered the LTB*-combined vaccine, and 49 volunteers were administered the vaccine alone. For the IgA Ab response, a 1.4-fold or greater increase in units of specific IgA per microgram of total IgA after vaccination was regarded as a positive response. This was because only less than 10% of the vaccinees, who received a subcutaneous

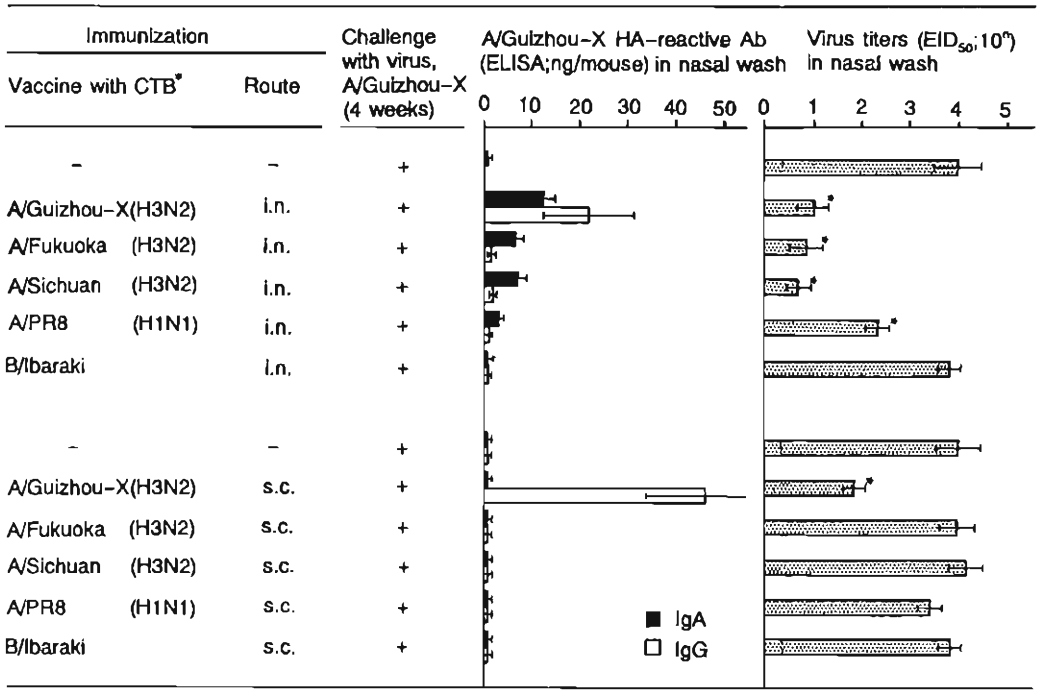


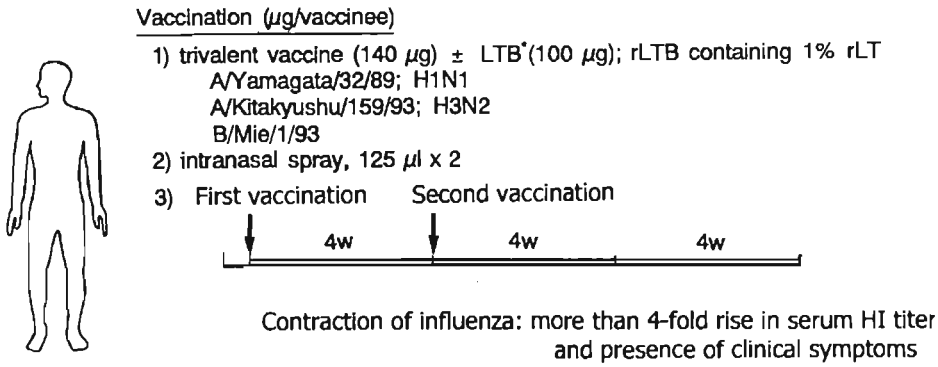
Fig. 1. Protection against influenza A virus infection and production of cross-reactive anti-HA IgA and IgG Abs in the upper respiratory tract in mice immunized intranasally or subcutaneously with different adjuvant-combined vaccines. The mice were immunized intranasally or subcutaneously with 0.5 μg of a vaccine combined with 1 μg of CTB* (CTB containing about 0.1% of CT) 4 weeks previously. The immunized mice were infected intranasally with a small volume (1 μl × 2) of a mouse-adapted A/Guizhou-X virus (H3N2) suspension. Three days later, their nasal virus titers were assayed. Each column represents the mean ± SD of nasal virus titers (EID_{50}) of all mice in each group of six. An asterisk (*) represents a statistically significant difference from a non-immunized control at $P < 0.05$.

injection of the same trivalent vaccine, showed a 1.4-fold increase in their salivary IgA titer. For the HI Ab response, a 4-fold or greater increase in HI Ab titer after vaccination was regarded as a positive response. The results show that in the group administered the nasal LTB*-combined vaccine, 37 (50.7%) and 36 (49.3%) of 73 vaccinees showed positive IgA and HI Ab responses to one or more of the vaccine strains, respectively. On the other hand, in the group given the vaccine alone, 16 (32.8%) and 15 (30.6%) of 49 vaccinees showed positive IgA and HI Ab responses. There was a statistically significant difference between these two groups. Thus, the nasal LTB*-combined vaccine could induce both nasal IgA and serum IgG Ab responses at levels higher than the nasal vaccine alone. Whether the increase in the salivary IgA Ab titer was effective in preventing influenza could not be determined in the present study because there was no epidemic of influenza during the test period.

In the winter of 1994-1995, we conducted a second field trial to evaluate the efficacy of the nasal LTB*-combined influenza vaccine (Fig. 2) (17). The conditions of vaccination in the second field trial were the same as those in the first, except that B/Mie/1/93 was used instead of B/Bangkok/163/90. Contraction of influenza was monitored up to 8 weeks after the second vaccination. Outbreaks of H3N2-subtype virus and B-type virus occurred over the trial period. The results showed that 3 of 18 vaccinees (16.7%) who received the nasal LTB*-combined vaccine contracted influenza, while 6 of 14 nonvaccinees (42.7%) contracted influenza. There was no statistically significant difference between the two groups because the number of subjects was small. Thus, we can only speculate on the effectiveness of the nasal LTB*-combined influenza vaccine (estimated effectiveness, 61%). In addition, because we did not compare the efficacy of the nasal vaccine with that of subcutaneous injection of the current inactivated vaccine in the second field trial, we could not assess the superiority of the nasal vaccine to the current subcutaneous vaccine. Further field trials are required to assess the effectiveness of the nasal LTB*-combined influenza vaccine.

(3) Other advantages of CT (or LT) derivatives as an adjuvant

CT and LT act similarly as mucosal adjuvants of the nasal influenza vaccine, although more LT is required for positive mucosal adjuvant activities than CT (26, 27). Both toxins appear to have the following advantages as adjuvants of the nasal vaccine. (i) In mice, the ability of CTB* (or LTB*) to augment mucosal immune responses to the vaccine is generated only when the derivatives were administered simultaneously with the vaccine via the same route (37). (ii) The mechanisms by which the CTB*-combined vaccine augments mucosal Ab responses in mice can be explained by the stimulation of innate immunity by CT (or LT) derivatives (38). (iii) CTB*(or LTB*) (around 1 μg) does not cause any damage to the nasal mucosa of mice. In addition, 0.1 μg of CTB* does not cause any local brain damage, i.e., so-called encephalopathy, even when injected intracerebrally into mice (39,40). (iv) CTB* (or LTB*) could be repeatedly administered to mice as an adjuvant for nasal vaccination, without inhibition of preexisting immunity to the CTB (or LTB) (41,42). (v) The adjuvant effect of CTB* is not genetically restricted by MHC genes in mice (43); this finding would favor the combined application of current vaccines with CTB* to genetically heterogeneous humans. (vi) CT induces almost no IgE Ab responses to bystander antigens, as well as to CT



Group	Number of volunteers (female)	Age distribution (means ± SD)	Morbidity of influenza
None	14 (4)	44.1 ± 11.3	6/14 (42.9%)
LTB*-combined vaccine	18 (5)	37.7 ± 11.3	3/18 (16.7%)

* Effectiveness = (42.9 - 16.7 / 42.9) x 100 = 61.0 (%)

Fig. 2. Preliminary field trial using nasal LTB*-combined influenza vaccine (1994-1995). A trivalent inactivated vaccine, composed of A/Yamagata/32/89 (H1N1), A/Kitakyushu/159/93 (H3N2) and B/Mie influenza virus strains, as well as the adjuvant, recombinant LTB supplemented with 1% recombinant LT (LTB*), were administered intranasally into 18 volunteers (mean age, 37.7 ± 11.3). The control group was composed of 14 non-vaccinated volunteers (mean age, 44.1 ±11.3). Vaccination was performed twice 4 weeks apart.

itself, when used as an adjuvant for nasal influenza vaccine in mice, as described in the following section (44).

3. Assessment of CT (or LT) derivatives as nasal influenza vaccine adjuvants for human use

The human field trials showed that the adjuvant-combined vaccine induced production of a significantly high level of anti-HA secretory IgA and systemic IgG, compared with the vaccine alone, suggesting that this is effective in preventing influenza (16,17). Although no serious adverse effects associated with the vaccine were observed in the preliminary human trials, further trials on the reduction of the toxicity and allergenicity of these toxins will be required to avoid any untoward effects of adjuvant-combined vaccines. We prepared four CT mutants, namely, CT7K (Arg to Lys substitution at amino acid position 7 from the N-terminus of the A1 fragment of the A subunit) (45), CT61F (Arg to Phe substitution at amino acid position 61 from the N-terminus) (31), CT112K (Glu to Lys substitution at amino acid position 112 from the N-terminus) (32) and 118E (Glu to Gly substitution at amino acid position 112 from the N-terminus), as useful adjuvant models since all of these were less-toxic than LTB*. Then we examined experimental systems for assessing the effectiveness and safety of the toxin derivatives as adjuvants for nasal influenza vaccines (44).

(1) Ability of various CT (or LT) mutants to enhance protective Ab responses

BALB/c mice were used to assess the efficacy of mucosal adjuvant activities of CT mutants in mice, because they showed intermediate levels of response to either CT derivatives or influenza HA molecules (43,46). Mice were immunized intranasally (1 μl into each nostril) with PR8 vaccine (0.1 μg) together with CT (or LT) derivatives (0.1 μg) and boosted in the same manner 4 weeks later. Two weeks after the second vaccination, the immunized mice were challenged with either a small volume or a lethal dose of PR8 virus. Three days after infection, nasal wash, lung wash and serum specimens were collected to measure either virus titers based on 50% egg-infecting dose (EID_{50}) or anti-HA IgA and IgG Abs using ELISA. The dose of adjuvant or vaccine (0.1 μg per 20-g weight of mouse) and the volume of the vaccine solution (1 μl per each nostril) used in this experiment correspond to 100 μg per 20 kg body weight and 1 ml per nostril in humans, respectively. The method used in mice was similar to that used in preliminary human trials, i.e., 100 μg of recombinant LTB containing 0.5-1% LT, together with 140 μg of trivalent influenza vaccine per 250 μl, was sprayed intranasally in a two-dose regimen (twice at 4-week intervals) (16,17). Thus, the data obtained in this screening system using BALB/c mice should provide clues for estimating the efficacy of adjuvant-combined vaccines for humans. Figure 3 shows that relatively high anti-HA IgA and IgG Ab responses were induced in mice immunized with the CT112K-combined vaccine, although the Ab responses were approximately one-third lower than those with the native CT. Table 1 shows that the anti-HA IgA and IgG Ab levels, induced in mice immunized with the CT112K-combined vaccine, were sufficient to provide almost complete protection against the virus infection. In addition, the Ab titers and the degree of protection in mice immunized with the CT112K-combined vaccine roughly corresponded to those in mice immunized with the LTB-1% LT (LTB*)-combined vaccine (Fig. 3 and Table 1). These results suggest that CT112K, as well as LTB-1% LT, could be used effectively as safer adjuvants for nasal influenza vaccines in humans.

To assess the efficacy of mucosal adjuvant activities of CT mutants, the ability of the mutants to induce delayed-type hypersensitivity (DTH) reaction is also available. Mice are immunized intranasally (1 μl into each nostril) with influenza vaccine (0.1 μg) together with CT (or LT) derivatives (0.1 μg). Seven days after the vaccination, the peak DTH response, which is elicited by injecting the vaccine into the footpad of the immunized mouse, is induced (47). Twenty-four hours after the elicitation, footpad swelling is measured as an index

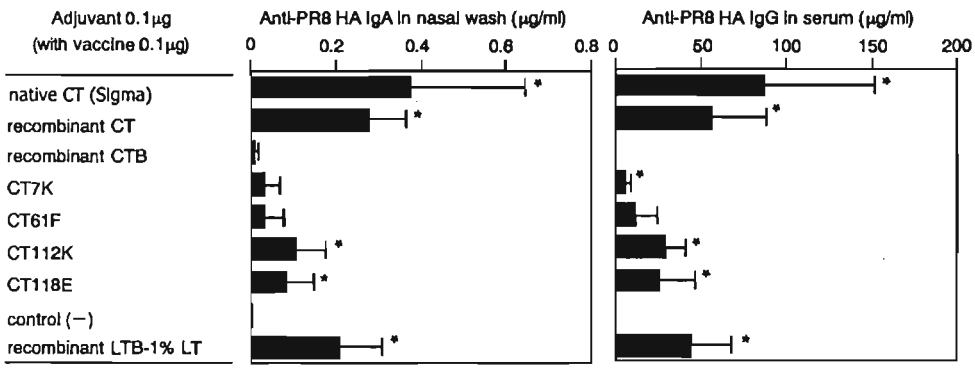


Fig. 3. Secondary anti-PR8 HA IgA and IgG Ab responses in mice immunized twice intranasally with 0.1 μg of various CT derivatives and 0.1 μg of PR8 viral vaccine, at 4-week interval. Nasal wash and serum samples were collected from the immunized mice 2 weeks after the second immunization. Each column represents the mean Ab titer ± SD of five mice in each group measured by ELISA. An asterisk (*) represents a statistically significant difference from a control group immunized with vaccine alone at $P < 0.05$.

Table 1. Protection against PR8 virus infection in mice immunized intranasally with CT mutant-combined influenza vaccine

Nasal immunization[a] vaccine (0.1 μg)	CT (0.1 μg)	Virus infection PR8	Nasal virus titer[b] (EID_{50}:10^n)	Lung virus titer[b] (EID_{50}: 10^n)
+	native CT (Sigma)	+	<0.5*	<0.5*
+	recombinant CT	+	<0.5*	<0.5*
+	CT7K	+	0.5±0.2*	N.D.
+	CT61F	+	<0.5*	N.D.
+	CT112K	+	<0.5*	0.7±0.3*
+	CT118E	+	0.6±0.3*	0.9±0.9*
+	–	+	3.0±0.3	6.5±0.7
+	recombinant LTB-1% LT	+	<0.5*	<0.5*

[a] Mice were immunized by intranasal administration of a CT mutant (0.1 μg) together with influenza PR8 virus vaccine (0.1 μg), twice at 4-week interval. Two weeks after the second immunization, the mice were challenged by intranasal administration of either a small or a large volume of PR8 virus suspension. Three days after the challenge, nasal or lung washes from the immunized mice were assayed for virus titer.

[b] Values represent mean ± SD of each group of five mice.

* Statistically significant difference from control mice immunized with vaccine alone at $P < 0.05$.

N.D.:not done.

of DTH reaction. This assay can detect the ability of CT (or LT) derivatives to enhance immune responses within 8 days. Figure 4 shows that in mice immunized with the CT112K-combined vaccine, a relatively high DTH response, which is slightly lower than that in mice immunized with the native CT-combined vaccine, is induced. The DTH response detected by this assay seems to be mediated by $CD4^+$ T helper cells that are capable of producing IFN-γ (47).

Strictly speaking, further studies in humans will be needed to confirm that the dose/body weight for adjuvant activity is sufficient. In one previous study, 100 μg of recombinant CTB, which is estimated to correspond to 0.1 μg of CTB in BALB/c mice, was used as a strong mucosal immunogen in humans (20), although less than 1 μg of CTB had no adjuvant activity in BALB/c mice (26). This finding suggests that humans are more sensitive to CT derivatives than BALB/c mice. If this is the case, the efficacy of CT derivatives as mucosal adjuvants, as detected in this experiment using BALB/c mice, will be much higher in humans. In any case, the experimental system used for assessing the ability of CT (or LT) derivatives to enhance immune responses will be useful to select ideal candidates for effective adjuvants for nasal influenza vaccines. However, whether this system is useful for assessing the ability of CT (or LT) derivatives to enhance immune responses in the case of antigens other than the current influenza vaccine remains to be examined.

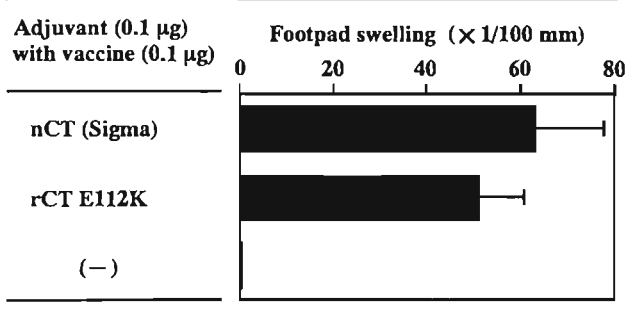


Fig. 4. DAH reaction in mice immunized intranasally with both 0.1 μg of CT derivatives and 0.1 μg of PR8 virus vaccine. Six days after immunization, DAH reaction was elicited by injecting PR8 virus vaccine into the footpad. Twenty-four hours after elicitation, the footpad thickness (swelling) was measured as an index of DTH reaction. Each bar represents the mean footpad swelling (± SE) of five mice.

(2) Assay for allergenicity of various CT (or LT) mutants

A possible drawback of CT as a mucosal adjuvant is its potential to induce IgE Ab responses to bystander antigens, as well as to CT itself (18,19). Under the experimental condi-

tions described above, sera can be obtained for assessing the ability of CT (or LT) derivatives to induce IgE Ab responses using ELISA or passive cutaneous anaphylaxis (PCA) (48,49). Table 2 shows both total IgE and anti-CTB IgE Ab responses in mice immunized intranasally with 0.1 μg of a CT mutant and vaccine in a two-dose regimen (44). One microgram of native CT-, recombinant CT- or CT7K-combined vaccine induced only a low level of total IgE Ab responses. When given in doses of 1 μg or 0.1 μg, neither the native CT-combined vaccine nor the other CT derivative-combined vaccine induced IgE Ab responses to CTB. On the other hand, frequent intraperitoneal injections of 2 μg of native CT (positive control) could induce both total IgE and anti-CTB IgE Ab responses (PCA titer less than 2^5). These findings suggest that 0.1 μg of native CT produces only a low level of total IgE Ab responses even after several administrations. Moreover, Yamamoto et al. have already shown that the ability of native CT (0.5 μg) to induce IgE Ab responses is significantly higher than that of CT61F (5 μg) in C57BL/6 mice (31). Thus, when the CT mutants, which produce IgE Abs at a lower level than the native CT, are used at a low dose (0.1 μg), they cannot induce IgE Ab responses even after several vaccinations.

(3) Assay system for toxicity of various CT (or LT) mutants

There are several assay systems for assessing the toxicity of CT (or LT) derivatives (50-56): the vascular permeability test (50), the rabbit ileal loop test (51,52), the Y-1 adrenal cell assay (53), the Chinese hamster ovary cell assay (54), the cAMP-stimulation test (55) and the ADP-ribosyltransferase activity assay (56). Of these, the Y-1 adrenal cell assay is the simplest and most sensitive (53). Thus, we used this assay to assess the toxicity of the CT mutants. Briefly, mouse Y-1 adrenal tumor cells were cultured in 96-well flat bottom plates at a concentration of 10^4 cells per well, to which the CT (or LT) derivatives were added. After overnight incubation, the cells were examined by light microscopy to determine the minimum concentration of the toxin required for greater than 50% cell rounding (EC_{50}) and the toxin concentration required to initiate cell rounding (ECi). Table 3 shows the data for toxicity of the CT mutants on Y-1 cells. All four CT mutants examined were less toxic than LTB*.

(4) Another assessment of toxicity of CT (or LT) mutants

To ensure the safety of CT (or LT) derivatives for human use, we must consider another toxicity assessment factor. CT (or LT) derivatives should not cause serious disease in guinea pigs when administered intranasally or intraperitoneally at the dose used in humans (around 100 μg) (57).

4. A proposal for safety standards for human use of CT (or LT) derivatives

From the results described above, it was shown that the effectiveness and safety of CT (or LT) derivatives as a nasal influenza vaccine adjuvant for human use can be assessed by the following assays. (i) The ability of CT (or LT) derivatives

Table 2. IgE responses induced by CT mutants

CT derivatives	Total IgE (ng/ml)[a]		Anti-CTB IgE (PCA:2^n)	
	1 μg	0.1 μg	1 μg	0.1 μg
native CT (Sigma)	178±85	<160	<1	<1
recombinant CT	259±74	<160	<1	<1
CT7K	236±12	<160	<1	<1
CT61F	<160	<160	<1	<1
CT112K	<160	<160	<1	<1
CT118E	<160	<160	<1	<1
vaccine alone	<160	<160	<1	<1
positive control[b]	536±235		4	

[a] Mice were immunized by intranasal administration of a CT mutant (1 μg or 0.1 μg) together with inactivated influenza vaccine (1 μg or 0.1 μg), twice at a 4-week interval. Two weeks after the second immunization, sera from the immunized mice were assayed for total IgE and anti-CTB IgE Ab responses.

[b] Positive control sera were obtained 2 weeks after the last immunization from mice immunized by intraperitoneal injection of native CT (2 μg, Sigma) 3 times at a 2-week interval.

Table 3. Toxicity of CT mutants on Y-1 cells

	EC_{50}[a] (pg/well)		ECi[b] (pg/well)	
native CT (Sigma)	26	(1)[c]	0.2	(1)[c]
recombinant CT	240	(1/9)	5	(1/25)
recombinant CTB	<1000000	(>1/38462)	<1000000	(>1/5000000)
CT7K (Arg→Lys)	11100	(1/427)	1200	(1/6000)
CT61F (Arg→Phe)	19300	(1/742)	3700	(1/18500)
CT112K (Glu→Lys)	19300	(1/742)	5100	(1/25500)
CT118E (Gly→Glu)	11100	(1/427)	3700	(1/18500)
recombinant LTB-1% LT	2140	(1/82)	46	(1/230)

[a] Concentration of CT (or LT) derivatives inducing rounding of 50% of cells.

[b] Concentration of CT (or LT) derivatives initiating rounding of Y-1 cells.

[c] The values in parenthesis represent the ratio of the toxicity of CT (or LT) derivatives to the toxicity of native CT (Sigma).

to enhance immune responses against vaccine and to provide protection against virus infection can be assayed in BALB/c mice immunized intranasally with 0.1 μg of both CT (or LT) derivatives and vaccine in a two-dose regimen at 4-week intervals, or in BALB/c mice which received the first intranasal immunization with 0.1 μg of both CT (or LT) derivatives and vaccine, and the second intranasal immunization with 0.1 μg of the vaccine alone at 4-week intervals (unpublished data). (ii) The ability of CT (or LT) derivatives to induce IgE Ab responses against CT (or LT) derivatives or vaccine (allergenicity) can also be assayed in BALB/c mice immunized intranasally with 0.1 μg of both CT (or LT) derivatives and vaccine in a two-dose regimen at 4-week intervals. (iii) The toxicity of CT (or LT) derivatives can be assayed using Y-1 adrenal cells. (iv) The toxicity of CT (or LT) derivatives can also be assayed based on their ability to cause disease in guinea pigs when administered intranasally or intraperitoneally at the dose used in humans (around 100 μg).

From the assessment of the effectiveness and safety of CT (or LT) derivatives as a nasal influenza vaccine adjuvant, we can propose the following "Safety Standards for Human Use of CT (or LT) Derivatives as an Adjuvant of Nasal Inactivated Influenza Vaccine". CT (or LT) derivatives can be administered intranasally together with a current inactivated influenza vaccine, provided they meet the following criteria. 1) CT (or LT) derivatives should retain the properties of the native CT (or LT), i.e., the ability to augment secretory IgA and serum IgG Ab responses to viral surface glycoproteins, when administered intranasally together with the influenza inactivated vaccine. 2) A single dose of the derivatives, administered intranasally by spraying, should be less than 300 μg in a volume of less than 0.5 ml. 3) CT (or LT) derivatives should not induce IgE Ab responses to the vaccine, as well as to CT (or LT) itself. 4) CT (or LT) should be nontoxic; the toxicity of the derivatives, as determined by Y-1 adrenal cell assay, should not exceed 1/100 EC_{50} of the native CT (or 1/1000 EC_{50} of the native CT). 5) CT (or LT) derivatives should not cause serious disease in guinea pigs when administered intranasally or intraperitoneally at the dose used in humans.

As shown in Fig. 3 and Tables 1-3, there was no statistically significant difference among the four CT mutants in regard to either adjuvant activities or toxicities. In addition, the A subunit of some mutants seems to be more structurally stable than that of other mutants (44). If this information is taken into consideration in the "Safety Standards for Human Use of CT (or LT) Derivatives as an Adjuvant of Nasal Inactivated Influenza Vaccine", mutant CT112K (32) is recommended as one of the safer, stabler and more effective adjuvants for nasal influenza vaccine. However, the yields of CT112K from bacterial culture are very low. Further studies are required to improve the yield of CT112K.

The preparation and conditions for effective and safe administration of CT (or LT) derivative-combined nasal influenza vaccine are as follows. (i) Immediately before vaccination, a fraction of a current inactivated influenza vaccine (about 100 μg for an adult) is mixed equally with the same amount of the adjuvant fraction to make 250 μg/ml. Vaccination is carried out by spraying the mixture in a volume of 200 μl to each nostril. (ii) The vaccine fraction is 3-fold higher concentration than the current trivalent inactivated vaccine, which contains H1N1, H3N2 and B-type viruses circulating in humans. (iii) The adjuvant should be a CT (or LT) derivative having an effective adjuvant activity equal to or greater than that of LTB* (LTB containing 1% LT) and having less toxicity than LTB*, as described in the safety standards of CT (or LT) derivatives. (iv) Vaccination is carried out before the onset of epidemic of influenza in two successive intranasal administrations at 4-week intervals, primary immunization with the adjuvant-combined vaccine and a secondary immunization with the vaccine alone. This two-dose regimen would be used for naive people who have not yet contracted influenza. The single-dose regimen using the vaccine alone would be recommended for those who have already contracted influenza.

Further detailed field trials are required to confirm the effectiveness of the adjuvant-combined inactivated influenza vaccine in preventing or in attenuating illness without negative effects. The adjuvant-combined nasal influenza inactivated vaccine will be useful in preventing influenza and its complications not only in children and adults, but also in the elderly at high risk (those with conditions such as diabetes, chronic obstructive pulmonary disease or congestive heart failure) who cannot receive an attenuated influenza virus vaccine. The "Safety Standards for Human Use of CT (or LT) Derivatives as an Adjuvant of Nasal Inactivated Influenza Vaccine" will be useful in the realization of such an effective nasal influenza vaccine in the near future.

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