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Development of two novel nontoxic mutants of *Escherichia coli* heat-labile enterotoxin

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Abbreviations: ETEC, enterotoxigenic *Escherichia coli*; LT, *Escherichia coli* heat-labile enterotoxin; CT, cholera toxin; G_{M1}, monosialoganglioside; ELISA, enzyme-linked immunosorbent assay

Abstract

***Escherichia coli* heat-labile enterotoxin (LT) is composed of catalytic A and non-catalytic homo-pentameric B subunits and causes diarrheal disease in human and animals. In order to produce a nontoxic LT for vaccine and adjuvant development, two novel derivatives of LT were constructed by a site-directed mutagenesis of A subunit; Ser^{63} to Tyr^{63} in LTS63Y and Glu^{110}, Glu^{112} were deleted in LTΔ110/112. The purified mutant LTs (mLTs) showed a similar molecular structural complex as AB_5 to that of wild LT. In contrast to wild-type LT, mLTs failed to induce either elongation activity, ADP-ribosyltransferase activity, cAMP synthesis in CHO cells or fluid accumulation in mouse small intestine *in vivo*. Mice immunized with mLTs either intragastrically or intranasally elicited high titers of LT-specific serum and mucosal antibodies comparable to those induced by wild-type LT. These results indicate that substitution of Ser^{63} to Tyr^{63} or deletion of Glu^{110} and Glu^{112} eliminate the toxicity of LT without a change of AB_5 conformation, and both mutants are immunogenic to LT itself. Therefore, both mLTs may be used to develop novel anti-diarrheal vaccines against enterotoxigenic *E. coli*.**

Keywords: mutant LT (mLT), ADP-ribosyltransferase activity, cAMP, immunogenicity, secretory IgA (sIgA)

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strain causes diarrheal disease in human and animals due to production of toxins such as heat-labile enterotoxin (LT) (Spangler, 1992). LT is a multimeric protein composed of two functionally distinct domains; the enzymatically active A subunit (LTA; Mr, ~30,000 daltons) with ADP-ribosylating activity, and the pentameric B subunits (LTB; Mr, ~11,600 daltons) that contain G_{M1} (monosialoganglioside) receptor-binding site (Bäckström *et al*., 1997). Upon thiol reduction, the A subunit dissociates into two polypeptide chains, A1 (Mr, 23,000 daltons) and A2 (Mr, 6,000 daltons) (Tsuji *et al*., 1985; Grant *et al*., 1994). The A1 subunit, in particular, intoxicates eucaryotic cells by catalyzing ADP-ribosylation of the protein Gs, a GTP-binding protein that regulates the levels of the second messenger cAMP (Guerrant *et al*., 1974; Field *et al*., 1989). The resulting increase in cAMP levels causes secretion of water and electrolytes into the small intestine through interaction with two cAMP-sensitive ion transport mechanisms including (i) NaCl co-transport across the brush border of villous epithelial cells and (ii) electrogenic Na^+-dependent Cl^- secretion by crypt cells (Guidry *et al*., 1997).

Both the cholera toxin (CT) from *Vibrio cholerae* and heat-labile enterotoxin (LT) from ETEC belong to the most potent mucosal adjuvants and immunogens known to date by oral and other mucosal routes, where most of antigens are unable to induce immune responses (Jackson *et al*., 1993; Takahashi *et al*., 1996). However, their toxi-cities have precluded usages in human (Douce *et al*., 1995). One approach to overcome the problem of toxicity is the generation of genetically detoxified derivatives of LT (Lobet *et al*., 1991; Dickinson and Clements, 1995) and CT (Fontana *et al*., 1995; Yamamoto *et al*., 1997b) by a site-directed mutagenesis of amino acids which are located on the β-strand that constitutes the 'floor' of NAD-binding cavity.

The most important factor for immunogenicity is shown to be the ability to bind to the receptor on eucaryotic cell (Nashar *et al*., 1996). In fact, a nonbinding mutant of the B subunit of LT was found to be nonimmunogenic (Guidry *et al*., 1997). Also, another group found that the ADP-ribosylating activity was unnecessary for immunogenicity because nontoxic derivatives of LT obtained by a site-directed mutagenesis of the A subunit retained the immunological properties of the wild-type LT (Pizza *et al*., 1994).

The attempt to define the role of ADP-ribosylating activity in LT adjuvanticity has generated conflicting results. For example, it was reported that a nontoxic derivative of LT (LTE112K) when co-administered with keyhole limpet hemocyanin (KLH) by an oral route in mice, lacked the adjuvant properties, thus suggesting that the adjuvanticity of LT is linked to its ADP-ribosylating activity (Lycke *et al*., 1992). However, more recently, the adjuvant

activity of the LTE112K was found to be identical to that of the LT holotoxin when delivered with influenza virus surface antigen by an intranasal route (Verweij *et al*., 1998). On the other hand, other investigators showed that another LT derivative, LTK63, lacking enzymatic activity and toxicity was still able to elicit antibody responses against the co-administered antigen in mice immunized orally, intranasally, or intravaginally (Di Tommaso *et al*., 1996; Giuliani *et al*., 1998; Marchetti *et al*., 1998).

In an effort to develop anti-diarrheal vaccine or mucosal adjuvant, we constructed nontoxic mLTs, LTS63Y and LTΔ110/112 by site-directed mutagenesis, each of which contains a single amino acid substitution and deletion of two amino acids, respectively, in the ADP-ribosyltransferase active center. We have demonstrated that in contrast to wild-type LT, both the LTS63Y and LTΔ110/112 did not induce any toxic activities. Both the mutants elicited high and comparable levels of anti-LT antibodies when delivered either intragastrically or intranasally, inducing systemic and local responses in serum and fecal extracts. Thus, they might be useful for the development of a novel diarrheal vaccine in human and animals.

Materials and Methods

Plasmid construction and mutagenesis

A 1.5-kb *Bam*HI DNA fragment including LT gene from porcine-origin, entero-toxigenic *E. coli* K88ac strain was cloned into pBluescript KS$^-$ vector (pBlueKS$^-$/rLT). Single-stranded DNA was prepared from the culture supernatant of *E. coli* CJ 236 transformed with pBlueKS$^-$/rLT and then subjected to a site-directed mutagenesis using Mutan K (Takara Biomedicals, Kyoto, Japan). The sequences of oligonucleotides used for the substitution (S63Y) and deletion (Δ110/112) were 5'-ATATGATGACGGATATGTTTC CACTTACCTTAGTTTGAGAAGTGCTCACTTG–3' and 5'-AG GCGTATACAGCCCTCACCCATATCAGGTTTCTGCGTTAGGT GGAATACCAT–3', respectively. Serine at position 63 was substituted for tyrosine at LTS63Y and glutamic acids in position 110 and 112 were deleted in LTΔ110/112. These residues are in proposed ADP-ribosyltransferase active center of LT and their substitutions or deletions have been shown to inactivate ADP-ribosyltransferase activity and enterotoxicity. We confirmed the changes of DNA sequences using Sequenase Version 2.0 sequencing kit (Amersham Life Science, USA).

Purification of recombinant mLTs

pBluescript KS$^-$ vectors containing mutant LT genes were transformed into *E. coli* Top10F' (Invitrogen, USA). The mutant LTs were purified from cultures grown overnight. The cells were harvested by centrifugation, resuspended in TEAN buffer (0.2 M NaCl, 50 mM Tris, 1 mM EDTA and 3 mM NaN_3 [pH 7.5]), and lysed with microfluidizer (Microfluidics Corporation, USA). The lysates were clarified by centrifugation and then filtered using 0.45-μm membrane (Micro Filtration Systems, Japan) prior to chromatography on immobilized D-galactose column (Pierce, USA) (Uesaka *et al*., 1994). The mLTs were eluted with 0.3 M galactose in TEAN buffer. Holotoxin (AB_5) fraction was separated from the free B-subunit pentamers by size exclusion chromatography using FPLC Superdex 200 column (Pharmacia, Sweden).

Cell elongation assay

The ability of mLTs to induce morphological changes in cultured Chinese hamster ovary-K1 (CHO-K1) (ATCC, USA) cells was tested as previously described (Guerrant *et al*., 1974). CHO-K1 cells were incubated for 24 h as monolayer cultures in minimal essential medium alpha (MEM-α) (GibcoBRL, USA) supplemented with 10% fetal bovine serum (FBS) in a humidified, 5% CO_2 atmosphere at 37˚C. The cells were washed once with Hanks's balanced salt solution (HBSS) and then removed from the flask by incubation of the cells with 0.1% trypsin for 5 min. After centrifugation, they were washed once, and then resuspended in the growth medium. To each well of a 48-well tissue culture plate, the same numbers of CHO-K1 cells (10^4 in a 200-μl volume per well) were added. The cells were allowed to adhere for 4 h prior to the addition of the toxin dilutions and then incubated for 24 h in a humidified, 5% CO_2 atmosphere at 37˚C. Cells were then washed with phosphate-buffered saline (PBS), fixed with methanol, and stained with 0.04% Trypan Blue Stain (GibcoBRL). After staining, the cells were washed, air dried, and analyzed for morphological changes by light microscopy.

ADP-ribosyltransferase activity test

For the preparation of crude membranes, CHO-K1 cells were maintained in monolayer culture by serial passages in MEM-α medium supplemented with 10% FBS (Locht *et al*., 1987). The cells were detached from the flask, resuspended in PBS (pH 7.2) and then sedimented by centrifugation at 1,000 *g* for 10 min. The cells were resus-pended in ice-cold 25 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, allowed to equilibrate for 15 min on ice and then homogenized. The homogenate was centrifuged at 600 *g* for 10 min at 4˚C to remove nuclei and intact cells. The postnuclear supernatant fractions were centrifuged at 18,000 *g* for 7 min to yield a microsomal or membrane pellet. The pelleted material was suspended in 50 mM Tris-HCl (pH 8.0) and centrifuged at 18,000 *g* for 7 min. The final washed membrane pellet was resuspended in 50 mM Tris-HCl (pH 8.0) at a concentration of 1 mg of protein per ml and stored at -70˚C until used.

ADP-ribosyltransferase activity was determined as the ability to catalyze the transfer of labeled ADP-ribose from [*adenylate*-^{32}P]NAD to the 41 kDa G protein in CHO-K1 membranes (Locht *et al*., 1987). Reaction mixtures (100 μl) containing 32 μM [*adenylate*-^{32}P]NAD (2 μCi) (NEN, USA), 10 mM thymidine, 100 μM ATP, 20 mM DTT, 100 μM GTP, 50 μg of CHO-K1 membrane proteins, 50 mM Tris-HCl (pH 8.0) and 10 μg of wild-type LT or mLT were incubated at 37˚C for 30 min. The reactions were terminated by the addition of 1 ml of ice-cold 50 mM Tris-HCl (pH 8.0), and the membranes were sedimented by centrifugation at 15,000 *g* for 7 min at 4˚C. The membrane pellet was resuspended in ice-cold Tris-HCl (pH 8.0) and sedimented once more by centrifugation before solubilization in 50 μl of electrophoresis sample buffer containing 5% β-mercaptoethanol. The samples were heated to 95˚C for 5 min and then analyzed by SDS-PAGE and autoradiography.

Measurement of intracellular cAMP accumulation

CHO cells (ATCC) were maintained in MEM-α medium supplemented with 10% FBS in 24-well plate at a concentration of 5×10^4 cells per well, grown to near confluency, and incubated in MEM-α containing 1% FBS and 1 mM IBMX for 30 min prior to addition of toxins (Grant *et al*., 1994). Either CT, CTB, trypsin-activated wild-type LT, LTS63Y, or LTΔ110/112 was added to each well and incubated for 18 h. The cells were then washed three times with PBS. Intracellular cAMP was extracted by adding 200 μl of 50 mM HCl to each well and incubating the plates in -70˚C deep freezer for 20 min. cAMP was measured with a Biotrak cAMP enzyme-immunoassay (EIA) system (Amersham Life Science) as described by manufacturer's instructions.

Assessment of toxicity using mouse ileal loops

The enterotoxicity of mLTs was examined using a mouse ileal loop test (Yamamoto *et al*., 1997b). Groups of mice were anesthetized, and 100 μl of PBS containing different doses of toxins were injected into ileal loops (LT, 100 ng or 1 μg per mouse; mLT, 10 μg or 100 μg per mouse). The mice were killed 18 h after the injection, and the ratio of fluid to length was determined and defined as positive when the ratio was more than 40 μl/cm.

Mice and their immunization

Female Balb/c mice aged 6 weeks old were purchased from Charles River (Japan). For intragastric immunization, antigens were resuspended in PBS (pH 7.2) buffer containing 0.35 M $NaHCO_3$ and delivered in a volume of 0.5 ml per mouse. Mice were immunized intragastrically with 25 μg of each toxin on days 0, 7, 14, and 21 (Takahashi *et al*., 1996). For intranasal immunization, mice were delivered with a 20-μl aliquot (10 μl per nostril) containing 2 μg of each toxin on days 0, 7, and 14 (Yamamoto *et al*., 1997a).

G_{M1}-ELISA

LT-specific antibodies were measured with a G_{M1} capture enzyme-linked immunosorbent assay (G_{M1}-ELISA) (Spiegel, 1990; Douce *et al*., 1997). Plates were coated with 150 ng of G_{M1} (Sigma, USA) suspended in PBS per well of 96-well EIA/RIA plate (Costar, USA) and then incubated at 37˚C for 1 h. Plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and blocked for 1 h at 37˚C with 2.5% skim milk (Difco, USA) in PBST. After washing with PBST three times, 100 ng of wild-type LT was added into wells and plates were incubated for 1 h at 37˚C and washed three times with PBST. Sera or fecal samples obtained (Jackson *et al*., 1993; Yamamoto *et al*., 1997a) from each mouse were tested by using two-fold serial dilutions and incubated for 2 h at 37˚C. After washing with PBST six times, the plates were incubated for 1 h at 37˚C with an appropriate anti-mouse immunoglobulin G (IgG) or IgA antibody (KPL, USA) conjugated with horseradish peroxidase (HRP) and washed as described above. Bound antibodies were visualized by adding 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Absorbancies were read at 450 nm and ELISA titers were arbitrarily determined as the dilution of serum which gave an optical density value above the level measured in preimmune samples.

Results and Discussion

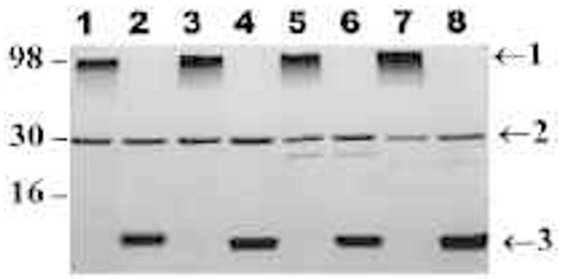

Figure 1. Analytical SDS-PAGE of wild-type LT and mLTs. *E. coli* lysates containing LT, LTS63Y, and LTΔ110/112 were chromatographed on an immobilized D-galactose column and then eluted with 0.3 M galactose in TEAN buffer as described in 'Materials and Methods'. Lane 1 and 2, LT purchased from Sigma; 3 and 4, recombinant LT; 5 and 6, LTS63Y; 7 and 8, LTΔ110/112. Each well contained 10 μg and samples in lanes 2, 4, 6, and 8 were heated to 95˚C for 5 min in the presence of β-mercaptoethanol. Arrow 1, LT holotoxins and LTB pentamers; arrow 2, LTA subunits; arrow 3, LTB monomers.

Expression and purification of mLTs

Ser^{63} was substituted to Tyr^{63} in LTS63Y and Glu^{110} and Glu^{112} were deleted in LTΔ110/112. These residues located in or near the NAD-binding site of LT have been shown to be essential for the ADP-ribosyltransferase activity of LT (Domenighini *et al*., 1994). We expressed the mLTs, LTS63Y and LTΔ110/112, in the plasmid vector (pBlueKS$^-$/rLTS63Y or pBlueKS$^-$/rLTΔ110/112), containing the coding (1.2 kb) and regulatory (160 bp of 5'- and 197 bp of 3'-noncoding genes) regions. The recombinant proteins were purified by immobilized D-galactose column chromatography. The homogeneity of LTS63Y and LTΔ110/112 was confirmed by SDS-PAGE, as shown in Figure 1. When the purified mLTs were analyzed without boiling, two protein bands were appeared; one band with the size of 70-100 kDa corresponding to the holotoxin and LTB pentamers, and the other band with the size of about 30 kDa corresponding to the LTA subunit. When the purified mLTs were boiled for 5 min, the holotoxins were dissociated into two bands of about 30 and 11 kDa, corresponding to the A and B subunits of LT, respectively. Since the mobilities of mLTs were identical to those of the wild-type LT (Figure 1), the molecular weights of the mLT subunits were presumed to be identical to those of wild-type LT. These results suggested that the innate structure of the A subunit associated with pentameric B subunits of LT was not affected by substitution of tyrosine for Ser^{63} or deletion of Glu^{110} and Glu^{112} residues on NAD-binding pocket. Moreover, it was demonstrated that the binding ability of the B subunit of mLTs to G_{M1} ganglioside was similar to that of the normal B subunit using a G_{M1}-ELISA, and mLTs were reacted with anti-LT antibody in Western blot analysis (data not shown). These results imply that mLTs retain the AB_5 conformation similar to wild-type LT.

Table 1. Comparison of biologic and enzymatic activity of wild-type LT, LTS63Y, and LTΔ110/112.

Toxin assessed	Cell elongation[a]	Ileal loop test[b]
PBS	10%<	Negative
Wild-type LT	90%> at 100 ng	Positive at 100 ng
S63Y	10%< at 10 μg	Negative at 100 μg
Δ110/112	10%< at 10 μg	Negative at 100 μg

[a] 10^4 cells of CHO-K1 cells were cultured with 100 ng of wild-type LT or 10 μg of each mLT for 24 h and a positive toxin effect on the CHO-K1 cells was defined as elongation of > 20% of the cells according to published criteria (Yamamoto *et al*., 1997b).

[b] The enterotoxicity of mLTs was examined using an ileal loop test, where mice were anesthetized, and 100 μl of PBS containing 100 ng of wt LT or 100 μg of each mLT were injected into an ileal loop. Loops were examined 18 h later and the ratio of fluid to length was defined as positive when the ratio was > 40 μl/cm (Yamamoto *et al*., 1997b).

Assays for biologic, enzymatic, and toxic activities of mLTs

Enzymatic and biologic characterizations of mLTs were carried out to compare their properties with wild-type LT, including cell elongation assay, ADP-ribosyltransferase activity test, cAMP assay and mouse ileal loop test. The morphological changes on the CHO-K1 cells (Grant *et al*., 1994) were used to detect the toxic activity of mLTs. As little as 100 ng/ml of LT induced longitudinal growth of approximately 90% of the CHO-K1 cells, a response previously shown to be dependent upon adenylate

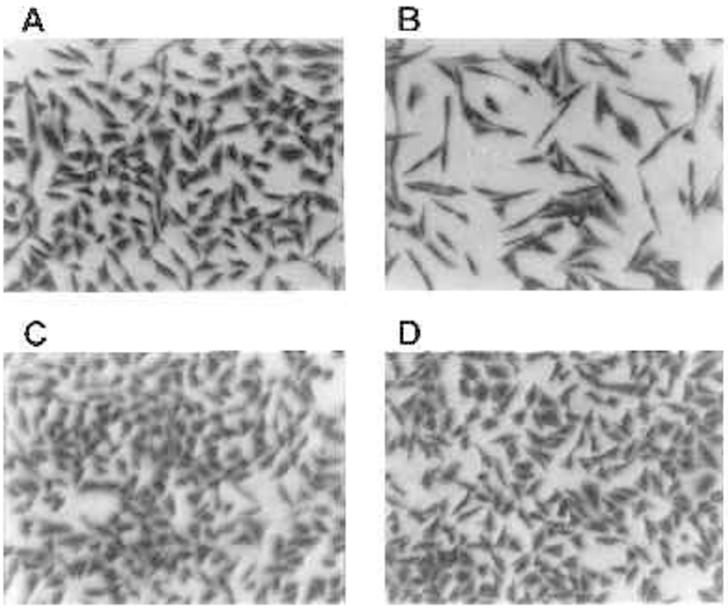

Figure 2. Cell morphologies changed by treatment with wild-type LT or mLTs. A, toxin-untreated; B, wild-type LT-treated (100 ng/ml); C, LTS63Y-treated (10 μg/ml); D, LTΔ110/112-treated (10 μg/ml) CHO-K1 cells (400 ×). The same numbers of CHO-K1 cells (10^4 in a 200-μl volume per well) were added to each well of a 48-well tissue culture plate.

Figure 3. ADP-ribosyltransferase activity of LT, LTS63Y and LTΔ110/112. The membrane proteins (50 μg) of CHO-K1 cells were incubated without toxins (lane 1), with 10 μg of wild-type LT (lane 2), 10 μg of LTS63Y (lane 3), or 10 μg of LTΔ110/112. The conditions are described in 'Materials and Methods'. Reaction mixtures were then analyzed by SDS-PAGE in a 12.5% gel followed by autoradiography. The arrow denotes the position of the Mr-41,000 band corresponding to the Gs protein.

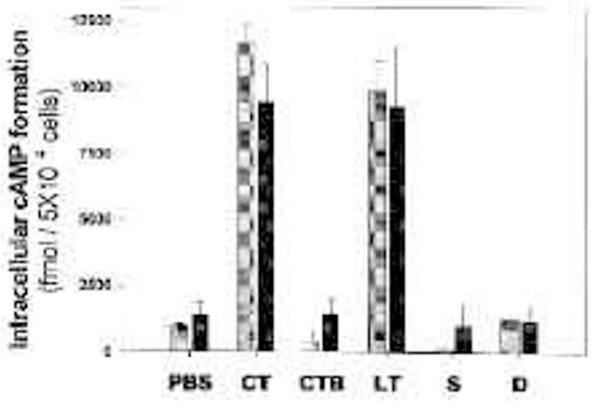

Figure 4. Assay for intracellular cAMP formation. CHO cells were cultured in 24-well plate at a concentration of 5×10^4 cells per well, grown to near confluency, and incubated with each toxin for 18 h. Intracellular cAMP was extracted and measured by an enzyme immunoassay system. CT, cholera toxin; CTB, cholera toxin B subunit; LT, *E. coli* heat-labile enterotoxin; S, LTS63Y; D, LTΔ110/112. Gray bars, 500 ng- (CT and LT) or 5 μg- (CTB, S and D); black bars, 50 ng- (CT and LT) or 500 ng-treated cells (CTB, S and D).

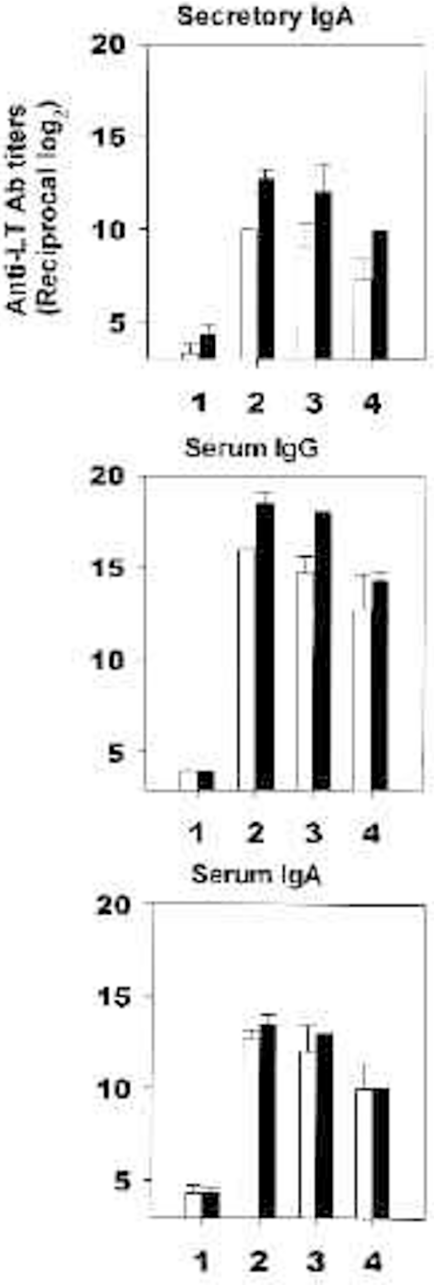

Figure 5. Anti-LT secretory IgA and serum IgG and IgA antibody responses on intragastric (white bars) or intranasal (black bars) immunization. IgA immune responses to LT in fecal extracts and IgG and IgA in sera of mice immunized four- (intragastric) or three- (intranasal) times. 1, PBS; 2, LT; 3, LTS63Y; 4, LTΔ110/112. Results are shown as mean titers and error bars indicate the standard deviation from the mean titer.

cyclase-induced increases in cAMP (Guerrant *et al*., 1974). However, the cells treated with mLT at the level of 10 μg/ml showed no morphological changes of the CHO-K1 cells (Figure 2 and Table 1).

In general, the A1 fragment of LT is capable of binding NAD and catalyzing the ADP-ribosylation of Gs, a GTP-binding regulatory protein associated with adenylate cyclase (Spangler, 1992). The consequence is a sharp increase in cAMP production resulting in excessive accumulation of salts and water in the intestinal lumen (Field *et al*., 1989). A subunits of LT is known to catalyze ADP-ribosylation of the membrane-bound substrate G-proteins. As shown in Figure 3, when the membrane proteins (50 μg) from CHO-K1 cells were incubated with wild-type LT in the presence of [*adenylate*-^{32}P]NAD, it

specifically ADP-ribosylated the Mr-41,000 proteins, which correspond to the α-subunits of the GTP binding Gs protein (lane 2 in Figure 3). In contrast, no ADP-ribosylation of this protein was detected in reaction mixtures incubated with the same amounts (10 μg) of LTS63Y (lane 3) or LTΔ110/112 (lane 4). This result was identical to that of the negative control treated without toxins (lane 1). Therefore, the substitution of Ser^{63} to Tyr^{63} or deletion of Glu^{110} and Glu^{112} in A1 subunit did cause changes in structural integrity of NAD binding crevis that may be important for enzymatic activity of LT. To investigate cAMP accumulation induced by mLT, the levels of cAMP were determined in CHO cells treated with CT, CTB, LT, LTS63Y, or LTΔ110/112. As shown in Figure 4, the addition of 50 ng/ml concentration of wild-type CT or LT caused about tenfold higher levels of cAMP production than those of untreated cultures. On the other hand, cAMP formation in cultures treated with CTB, LTS63Y, or LTΔ110/112 was undetectable even at a concentration as high as 5 μg/ml. These data showed that the presence of wild-type LTA subunit (accurately LTA1 subunit) is necessary for an increase in the intracellular cAMP concentration and the mutant derivatives, LTS63Y and LTΔ110/112, devoid of enzymatic activity, are unable to form cAMP.

The toxicity of LTS63Y or LTΔ110/112 was also assessed in a mouse ileal loop assay. One hundred nanogram of wild-type LT induced significant fluid accumulation in small intestine, however, no fluid accumulation was observed in the loop treated with thousand-fold higher levels (100 μg) of mLTs (Table 1). These data strongly indicate that the mLTs possess negligible enterotoxicity *in vivo*.

The derivatization of the wild-type toxin via substitution of a single amino acid or deletion of double amino acids known to be associated with the NAD-binding site on the A subunit resulted in complete loss of enzymatic, biologic, and toxic activities of LT. In particular, two novel mutants LTS63Y and LTΔ110/112 that are shown to be devoid of ADP-ribosyltransferase activity, were unable to induce longitudinal growth of CHO-K1 cells, increase intracellular cAMP, and elicit fluid accumulation in mouse-ligated ileal loops. These results have revealed that LTS63Y or LTΔ110/112 is qualitatively, physiologically distinct from wild-type LT.

Immunogenicity of LTS63Y and LTΔ110/112

The mucosal immunogenicities of LTS63Y and LTΔ110/112 were tested via two immunization routes. Groups of mice were immunized intragastrically four times at weekly intervals with 25 μg of LTS63Y or LTΔ110/112 and intranasally three times at the same intervals with 2 μg of the antigens. The control groups received PBS alone. The serum and fecal antibody titers to LT were determined using samples prepared on day 7 following the last immunization; the results are shown in Figure 5. The mice immunized with LTS63Y or LTΔ110/112 contained high and comparable levels of anti-LT antibodies in sera and fecal extracts compared with those immunized with wild-type LT. The LTS63Y was slightly more immunogenic than LTΔ110/112 on both intragastric and intranasal administration. On the other hand, titers of anti-LT in the serum or fecal extracts of mice intranasally immunized with wild-type LT or mLTs were slightly higher than those observed in mice intragastrically administered. Intranasal immunization offers several advantages compared with other immunization routes. Lower doses of proteins are required to induce antibody responses and would decrease the cost for vaccine (Yamamoto *et al*., 1997a). As shown in Figure 5, when administered intranasally, only 6% of the quantity of mLT used in intragastric immunization was required to elicit slightly higher levels of secretory IgA responses and this dose also effectively induced systemic IgG and IgA antibody responses. Thus, intranasal immunization using mLT could be an effective method for vaccination in human and animals.

In summary, this study has shown that both novel mLTs, namely LTS63Y and LTΔ110/112, lacked toxicity but elicited mucosal immunogenicity via two mucosal routes and could be useful for the development of anti-diarrheal vaccines. Particularly, both mLTs appeared to be more immunogenic on intranasal administration. We are currently assessing the mucosal adjuvanticity of these mLTs to *Helicobacter pylori* antigens. Further, the protection test using mLTs as adjuvants against *H. pylori* has been investigated in mice following intranasal immunization

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