A Nontoxic Chimeric Enterotoxin Adjuvant Induces Protective Immunity in Both Mucosal and Systemic Compartments with Reduced IgE Antibodies

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A novel nontoxic form of chimeric mucosal adjuvant that combines the A subunit of mutant cholera toxin E112K with the pentameric B subunit of heat-labile enterotoxin from enterotoxigenic Escherichia coli was constructed by use of the Brevibacillus choshinensis expression system (mCTA/LTB). Nasal immunization of mice with tetanus toxoid (TT) plus mCTA/LTB elicited significant TT-specific immunoglobulin A responses in mucosal compartments and induced high serum immunoglobulin G and immunoglobulin A anti-TT antibody responses. Although TT plus native CT induced high total and TT-specific immunoglobulin E responses, use of the chimera molecule as mucosal adjuvant did not. Furthermore, all mice immunized with TT plus mCTA/LTB were protected from lethal systemic challenge with tetanus toxin. Importantly, the mice were completely protected from influenza virus infection after nasal immunization with inactivated influenza vaccine together with mCTA/LTB. These results show that B. choshinensis–derived mCTA/LTB is an effective and safe mucosal adjuvant for the induction of protective immunity against potent bacterial exotoxin and influenza virus infection.

An important feature of immune responses at mucosal surfaces is the production of secretory IgA antibodies and their transport across the epithelium. This immune response represents the first line of defense against invasion by viral and bacterial pathogens [1]. Therefore, recent efforts have been focused on the development of vaccines capable of inducing effective immune responses in mucosal tissues; however, most protein antigens are rather weak immunogens when given via a mucosal route. Thus, the development of effective and reliable mucosal adjuvants that can be safely coadministered with vaccine antigen is of central importance for new-generation vaccines.

Cholera toxin (CT) produced by Vibrio cholerae is structurally similar to the heat-labile enterotoxin (LT) of enterotoxigenic Escherichia coli, and both toxins act as adjuvants for enhancement of mucosal and serum antibody responses to coadministered protein antigens given by either oral or nasal routes [2]. Despite the potent mucosal adjuvant activity of native (n) CT and nLT, both enterotoxins cause severe diarrhea and thus are unsuitable for use in humans [3]. Therefore, a number of nontoxic mutant (m) derivatives of CT or LT have been constructed [4–10]. We also have generated mCT by substituting a single amino acid in the ADP-riboosyltransferase active center of the A subunit and have created 2 mutants of CT (S61F and E112K) [11]. These newly created forms of mCT did not induce ADP-ribosylation and cAMP formation but still have the protective center of the A subunit and have created 2 mutants of CT (S61F and E112K) [11]. These newly created forms of mCT did not induce ADP-ribosylation and cAMP formation but still served as a mucosal adjuvant by inducing CD4+ Th2-type cells. Those cells, in turn, provided effective help for antigen-specific mucosal secretory IgA, as well as serum IgG and IgA antibody responses [12–14]. Furthermore, antigen-specific mucosal IgA and serum IgG antibody responses induced by mCT were protective against challenge with bacterial or viral pathogens [12, 14, 15].

In this study, we have constructed a novel chimeric adjuvant that combines the A subunit of mCT E112K and the B subunit of LT (mCTA/LTB) in a Brevibacillus choshinensis host–vector system. We also further assessed mucosal adjuvant properties, as well as the usefulness of B. choshinensis–generated mCTA/LTB for the induction of protective immunity.
Materials and Methods

**Plasmid construction.** A plasmid containing both mCTA E112K and LTB genes was constructed as follows. First, the mCTA gene was amplified by use of polymerase chain reaction (PCR), using pUC119-E112K as a template and the following primer set: mCTA-M (5'-AACCATGGGCTTGCATGATGATAAGTATAT-3') and mCTA-R (5'-TTAAGCTTCAATTTCTCATCTCTTGAT-3') [11]. The PCR product was digested with NcoI and HindIII and then was inserted to pNCMO2 digested with the same enzymes to give pNCMO2-mCTA. Second, the LTB gene also was amplified by use of PCR with the following primer set (LTB-M, 5'-AACCATGGGCTTGCATGATGATAAGTATAT-3' and LTB-R, 5'-AACGATCTCAGAGGTGTTTCTATCAGAATT-3') from the genome of enterotoxigenic E. coli (K-12) [12]. The PCR product was cloned into the Neol/BamHI site of pNCMO2 after digestion with NcoI and BamHI to give pNCMO2-LTB. Third, the mCTA gene together with the preceding ribosome binding site (SD2) of B. choshinensis cell wall protein gene was amplified with BAMS primer (5'-AACGATGCTTAGGAGGAAGACACAGAGG-3') and mCTA-R primer from pNCMO2-mCTA [17]. The amplified SD2-mCTA gene was digested with BamHI and HindIII and then was cloned into the HindIII site of pNCMO2-LTB, resulting in pNCMO2-LTB-mCTA (figure 1A). The expression plasmid, pNCMO2-LTB-mCTA, was introduced into B. choshinensis by use of electroporation, as described elsewhere [18].

**Production and purification of mCTA/LTB chimera.** The E. coli JM109 strain (TaKaRa Shuzo) was used as cloning host, and B. choshinensis HPD31 was used as host for production of the recombinant protein [19]. The E. coli-B. choshinensis expression-secretion shuttle vector pNCMO2 was constructed by inserting the pUC119-derived ColE1 replication origin and ampicillin resistance gene (TaKaRa Shuzo) into pNH301 [20]. Additionally, the lac operator derived from pUC119 was inserted in front of a promoter 2 region, as described elsewhere [17]. For cultivation, 2SLN medium and LB broth were used for the B. choshinensis-harboring plasmid and E. coli, respectively. The transformation containing pNCMO2-LTB-mCTA was cultured in 2SLN medium for 3 days at 30°C. After cultivation, the mCTA/LTB chimera protein in the culture supernatant was purified by fractionation on an immobilized d-galactose column (Pierce Chemicals), according to the method described elsewhere [21]. The culture supernatant was applied to the column and then was eluted with 0.2 M galactose in 50 mM phosphate buffer (pH 8.0). The filtrate was concentrated and dialyzed against pyrogen-free 20 mM phosphate buffer (pH 8.0) by use of a 5000-molecular-weight ultrafiltration membrane (Biomax-5; Millipore) to remove endotoxin. The filtrate was concentrated and dialyzed against pyrogen-free 20 mM phosphate buffer (pH 8.0) by use of 5000-molecular-weight ultrafiltration membrane (Biomax-5; Millipore). The purity of the molecules was examined by SDS-PAGE. The protein concentration was determined by use of MicroBCA protein assay reagent (Pierce).

**Bioassay and toxicity of mCTA/LTB.** The ability of mCTA/LTB and nCT (List Biological Laboratories) to induce toxic effects on cultured Chinese hamster ovary (CHO) cells was investigated, as described elsewhere [11]. The toxicity of each adjuvant was determined as the induction of spindle formation in >20% of cultured CHO cells. For the cAMP assay, 10^5 CHO cells in MEM alpha medium containing 1% fetal calf serum were cultured with 1 ng/mL of nCT or mCTA/LTB at 37°C in a 5% CO_2 incubator for 24 h. Intracellular cAMP was measured by use of an EIA kit (Amersham). The endotoxin levels were determined by use of the Limulus J test (Wako), and in vitro toxicity was examined by use of a mouse ileal loop test [22]. In brief, mice were anesthetized, and 100 μL of PBS containing different doses of mCTA/LTB or nCT was injected into 2-cm sutured ileal loops. The mice were killed 18 h later, and the ratio of fluid to length was defined as being positive when the ratio was >40 μL/cm.

**Uptake and distribution of radiolabeled protein vaccines.** Tetanus toxoid (TT) and mCTA/LTB were radiolabeled with ^125^I, as described elsewhere [23]. To assess the redirection ability of vaccine antigen by mCTA/LTB, mice were given ^125^I-conjugated TT together with nCT or mCTA/LTB via the nasal route. Uptake of ^125^I-labeled TT into neuronal and lymphoid tissues was assessed over a 6-day period. In addition, ^125^I-conjugated mCTA/LTB was given nasally to address their presence in central nervous system tissues at 6, 12, 24, 72, and 144 h. The counts per minute present in the different tissues were determined by use of a gamma counter. The microBCA protein assay was used to determine the protein concentrations of radiolabeled proteins. This allowed for the calculation of the specific activity of ^125^I-labeled TT (87 cpm/ng) and ^125^I-conjugated mCTA/LTB (1605 cpm/ng), for the determination of the amount of protein associated with the different organs. A total of 6.8 μg of ^125^I-labeled TT was applied nasally with the mucosal adjuvants nCT (1.0 μg) or mCTA/LTB (2.5 μg) for antigen redirection studies, and a total of 2.0 μg of ^125^I-conjugated mCTA/LTB was used for the neuronal targeting studies. All nasal applications were given in a final volume of 10–12 μL, which is 5–6 μL per nares, to naive mice. About 43% of the ^125^I-conjugated mCTA/LTB bound to ganglioside GM1.

**Mice and immunization protocols.** Female C57BL/6 mice aged 5–6 weeks were purchased from Japan Clea and were housed in the experimental animal facility at the Research Institute for Microbial Diseases (Osaka University). Mice were immunized nasally on days 0, 7, and 14 with a 20-μL aliquot (10 μL/nostril) of PBS containing 5 μg of TT (provided by Dr. Yasushi Higashi, Osaka University, Biken Foundation, Osaka, Japan) alone or combined with 0.5 μg of nCT or 10 μg of mCTA/LTB. In the influenza virus studies, female BALB/c mice (SLC) were used in all experiments. Hemagglutinin (HA) vaccines (split-product virus vaccines) were prepared from influenza virus A/PR/8/34 (PR8, H1N1) by the method of Davenport et al. [24]. Mouse-adapted PR8 virus was passaged 148 times in the ferret, 596 times in the mouse, and 73 times in 10-day fertile chicken eggs. Mice were anesthetized and then were immunized nasally by dropping PBS containing mCTA/LTB chimera (5 μg/2 μL) plus HA vaccine (5 μg/2 μL) into each nostril [25]. Four weeks later, these mice were boosted with an identical immunization regimen.

**Sample collection.** Saliva, nasal wash, and serum samples were collected to examine TT- or HA-specific antibody responses. Saliva samples were obtained from mice after intraperitoneal injection with 100 μg of sterile pilocarpine [14]. Nasal wash specimens were collected by gently flushing the nasal passages with 100 μL of sterile PBS [14]. Alternatively, a hypodermic needle was inserted into the posterior opening of the nasopharynx, and 1 mL of PBS containing 0.1% bovine serum albumin was injected 3 times [26]. To obtain lung washes, the trachea and lungs were taken out and the washing...
Figure 1. Development of chimeric mucosal adjuvant that combines A subunit of mutant cholera toxin E112K with pentameric B subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* (mCTA/LTB). A, Structure of mCTA/LTB chimera expression-secretion shuttle vector pNCMO2-LTB-mCTA. Sequences encoding mature mCTA and LTB genes are directly fused to signal sequence. B, SDS-PAGE analysis of purified mCTA/LTB chimera. Proteins were stained with Coomassie brilliant blue. Samples were loaded onto 15% SDS-polyacrylamide gels under reducing (lane 1) or nonreducing (lane 2) conditions. Molecular mass markers (M) are at left. A, mCTA subunit; *Ampr*, ampicillin resistance gene; B, LTB subunit; SD1, SD2, ribosome-binding sites; SP, signal peptide-encoding sequence; P2, promoter 2 region of cell wall protein gene of *Brevibacillus choshinensis*; pUB110rep, pUB110 origin of replication.

**Antigen-specific antibody titers by ELISA.** Antigen-specific antibody titers in serum and mucosal secretions were determined by ELISA, as described elsewhere [25, 27]. To quantify the level of PR8 HA-specific antibodies, polyclonal PR8 HA-specific IgA and IgG antibodies were affinity-purified from the lung washes of mice immunized nasally with adjuvant (e.g., nCT)-combined PR8 HA molecules [25]. Affinity-purified IgG and IgA antibodies (100 ng/mL) were routinely used as a standard [25]. The antibody concentration of an unknown specimen was determined from the standard regression curve constructed for each assay.

**ELISA for total and TT-specific IgE antibodies in serum.** For detection of total and TT-specific serum IgE antibody levels, 96-well plates (Nunc) were coated with rat anti-mouse IgE monoclonal antibody (R35-72; BD PharMingen) [28]. After blocking, serial dilutions of serum samples were made, and standard mouse IgE (27-74L; BD PharMingen) was added. After incubation, biotinylated rat anti-mouse IgE monoclonal antibody (R35-118; BD PharMingen) for determining total IgE levels or biotinylated TT for determining TT-specific IgE antibodies was added, followed by horseradish peroxidase–labeled anti-biotin monoclonal antibody (Vector Laboratories). Endpoint titers of TT-specific IgE were expressed as reciprocal log2 titers.

**Tetanus toxin challenge.** Tetanus toxin for the challenge experiments was provided by Dr. Yasushi Higashi.
diluted in 0.5% gelatin/PBS, and the appropriate minimum lethal dose (130 LD₅₀) was given subcutaneously to each mouse group. The mice were monitored daily for paralysis and death [27].

Influenza virus infection. Two weeks after the last nasal immunization, the mice were infected nasally by dropping 20 μL of PBS containing a PR8 virus suspension with 10⁴ 100% EID₀ per mouse [29]. This procedure induced a total respiratory tract infection that caused virus shedding from nose and lung and led to viral pneumonia and death ~7 days after infection. Lung wash on day 3 after infection was collected, and 200-μL aliquots of each serial 10-fold dilution were injected into Madin-Darby canine kidney cells in a 6-well plate. After 1 h of absorption, each well in the plate was overlaid with 2 mL of agar medium, according to methods described elsewhere [30, 31]. The plaques were developed for 2 day during incubation in a CO₂ incubator and then were counted. The virus titer was expressed as plaque-forming units per milliliter.

Data and statistical analysis. Data were expressed as mean ± SE and were evaluated by use of the Mann-Whitney U test for unpaired samples, using a Statview II statistical program designed for the Macintosh computer. P < .05 was considered to be statistically significant.

Results

Properties of mCTA/LTB chimera constructed with B. choshinensis HPD31. A plasmid consisting of LTB and mCTA E112K was constructed by using the pNCMO2 shuttle vector system (figure 1A). A large amount of mCTA/LTB chimera protein (2 mg/L) was secreted by B. choshinensis harboring pNCMO2-LTB-mCTA, and SDS-PAGE revealed it to have 2 bands, corresponding to mCTA and LTB (figure 1B). The ratio of the amounts of these 2 components was 1:5, which suggests that each component combined to form 1 molecule of mCTA/LTB with the pentameric LT complex. This assumption was supported further by SDS-PAGE analysis of the chimeric protein together with nLT carried out under nonreducing and denaturing conditions. Both proteins migrated to identical positions in the gel (data not shown).

The mCTA/LTB chimera is nontoxic and enzymatically inactive. The biologic properties and toxicity of mCTA/LTB were examined and were compared with those of nCT (table 1). Although 1 ng of nCT induced extensive spindle formation in CHO cells, mCTA/LTB chimera, at doses as high as 1 μg, did not. Furthermore, CHO cells treated with mCTA/LTB did not produce cAMP. We confirmed that the mCTA/LTB chimera was nontoxic by use of mouse ileal loop test. Although 100 ng of nCT induced significant fluid accumulation, a 1000-fold-higher level of mCTA/LTB did not induce any detectable fluid accumulation (table 1).

Influence of nasal mCTA/LTB on trafficking of coadministered vaccine. To determine whether mCTA/LTB redirects vaccine protein, 125I-labeled TT distribution in various tissues was analyzed after nasal administration with mCTA/LTB plus 125I-labeled TT and was compared with groups inoculated with 125I-labeled TT given alone or together with nCT (figure 2A). Interestingly, lower levels of 125I-labeled TT accumulation were observed in the olfactory nerves and epithelium isolated from the group given 125I-labeled TT plus mCTA/LTB nasally than from the group given 125I-labeled TT plus nCT. In contrast, no significant difference was seen in several lymphoid tissues, the olfactory bulbs, or brain of mouse groups given nasal 125I-labeled TT plus mCTA/LTB or nCT or 125I-labeled TT alone. In the next study, 125I-conjugated mCTA/LTB alone was given nasally, and neuronal tissues (olfactory bulbs, epithelium, and brain) were analyzed for the presence of 125I-conjugated mCTA/LTB (figure 2B). In contrast to the 125I-labeled TT redirection experiments, the 125I-conjugated mCTA/LTB itself was present in the olfactory nerves and epithelium and peaked at 6 h, plateaued, and remained detectable for 6 days. In addition, 125I-conjugated mCTA/LTB in the olfactory bulbs peaked at 12 h and remained relatively constant over 6 days. We have reported that an accumulation of 1–2 ng of nCT was seen routinely in the olfactory bulbs during the 6 days when these tissues were analyzed after 10 μg of nCT was given nasally [23]. There was an ~2-fold–higher accumulation in the olfactory bulbs (3–4 ng) with the chimeric mCTA/LTB compound when 2.0 μg was given nasally (figure 2B). On the other hand, the targeting levels of mCTA/LTB were similar or even lower in the olfactory nerves and epithelium and in brain after 2.0 μg of 125I-conjugated mCTA/LTB was given, compared with that of 125I-labeled nCT administered nasally [23] (figure 2B). Overall, although nasal administration of mCTA/LTB targeted neuronal tissues, it did not affect trafficking of coadministered vaccine antigens into the neuronal tissues.

The mCTA/LTB chimera adjuvant supports TT-specific mucosal secretory IgA and serum IgG antibody responses. To assess the mucosal adjuvant properties of mCTA/LTB, mice were immunized nasally with TT plus mCTA/LTB. Nasal immunization with TT plus mCTA/LTB resulted in significant TT-

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**Table 1.** Biologic characteristics and potential toxicity of chimeric mucosal adjuvant that combines A subunit of mutant cholera toxin E112K with pentameric B subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* (mCTA/LTB).

<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>CHO assay, ng of protein</th>
<th>cAMP induction, pmol</th>
<th>Ileal loop test, ng of protein</th>
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<td>nCT</td>
<td>1.0</td>
<td>3.92 ± 0.01</td>
<td>100</td>
</tr>
<tr>
<td>mCTA/LTB</td>
<td>&gt;10⁴</td>
<td>0</td>
<td>&gt;10⁴</td>
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*NOTE.* CHO, Chinese hamster ovary; nCT, native cholera toxin.

* a CHO cells were cultured in MEM alpha medium containing 1% FBS with log₁₀ dilutions of each adjuvant for 24 h and the toxic effect were determined as spindled formation in >20% of cultured cells.

* b CHO cells (1 × 10⁴ cells/well) were cultured in medium containing 1% FBS with 1 ng/mL of each adjuvant for 24 h, and the cAMP induction were assessed using ELISA.

* c Enterotoxicity of each adjuvant were measured by use of ileal loop test, where 100 μL of PBS containing different levels of each adjuvant were injected into 2 cm ileal loop of anesthetized mice. The amount of fluid to length were measured 18 h later and were defined as positive when the ratio was >40 μL/cm².
specific serum IgG and IgA antibody responses, compared with those induced by TT together with nCT (figure 3A). In particular, the profiles of serum IgG1 and IgG2a antibody responses of mice given TT plus mCTA/LTB nasally were similar to those of mice given TT together with nCT (figure 3A). On the other hand, nasal administration of nCT or mCTA/LTB alone failed to elicit TT-specific antibody responses in the starting dilution (log\(^2\) of 6) used in these experiments (data not shown). In addition, nasal administration of TT plus mCTA/LTB chimera induced high levels of TT-specific IgA antibody responses in saliva, as well as nasal wash samples, compared with those induced by TT plus nCT (figure 3A).

To determine whether IgA antibody responses in external secretions were mucosa-associated or, alternatively, were exudates from serum, the numbers of antigen-specific IgA antibody–forming cells were measured. High numbers of TT-specific IgA antibody–forming cells were found in the submandibular gland and nasal passages of mice given TT plus mCTA/LTB or nCT, whereas low numbers of antibody-forming cells were detected in the submandibular gland and nasal passages of mice given TT alone (data not shown). These findings demonstrate that a new chimeric molecule of mCTA/LTB is a potent mucosal adjuvant for the induction of vaccine antigen–specific mucosal IgA and systemic IgG antibody responses.

**Induction of neutralizing antibody responses to tetanus toxin by nasal immunization.** Because nasal TT plus mCTA/LTB elicited high levels of antigen-specific IgG and IgA antibody responses, we determined if these antibodies also were protective. Mice given TT plus mCTA/LTB chimera or nCT as mucosal adjuvant, or TT alone, were challenged with a lethal dose (130 LD\(_{50}\)) of tetanus toxin and were monitored for paralysis and death. As expected, nasal immunization with TT plus nCT provided complete protection (figure 3B). Of importance, equal protection was provided when TT was given with mCTA/LTB and when nCT was used as a mucosal adjuvant. In contrast, TT, mCTA/LTB, or nCT alone provided no protection for mice against the paralysis and death that normally occurs within 1 day after administration of tetanus toxin (figure 3B). These findings clearly demonstrate the effectiveness of mucosally induced serum TT-specific IgG antibodies by coadministered mCTA/LTB chimera as mucosal adjuvant.

**Lack of induction of IgE antibody responses by mCTA/LTB chimera.** To determine whether the newly constructed *B. choshinensis*–derived chimeric mCTA/LTB enhanced IgE antibody responses, we measured both total and TT-specific IgE antibody titers in serum samples of mice immunized nasally with TT plus mCTA/LTB or nCT. As might be expected, high levels of total and TT-specific IgE antibodies were induced in serum of mice given TT plus nCT (figure 4). Interestingly, however, significantly lower levels of total and TT-specific IgE antibodies were noted in mice given nasal TT plus mCTA/LTB than in mice immunized with TT plus nCT (figure 4). It is possible that mCTA/LTB or mCT E112K may lead to different kinetics for TT-specific IgE antibody responses. There-
Figure 3. Mucosal adjuvant activity of chimeric adjuvant that combines A subunit of mutant cholera toxin E112K with pentameric B subunit of heat-labile enterotoxin from enterotoxigenic Escherichia coli (mCTA/LTB). A, Nasally administered mCTA/LTB-supported antigen-specific systemic IgG (including IgG subclass) and mucosal IgA antibody responses. Groups of mice were immunized nasally with 5 μg of tetanus toxoid (TT) alone (open bars), with 0.5 μg of native cholera toxin (nCT; hatched bars), or with 10 μg of mCTA/LTB (solid bars) on days 0, 7, and 14. Samples were collected 1 week after last immunization. Bars represent mean antibody titers ± SE in each group. B, Nasal immunization with TT and mCTA/LTB-induced protective immunity against challenge with tetanus toxin. One week after the last immunization, all groups were challenged on day 21 by subcutaneous injection of 130 minimum lethal doses of tetanus toxin in 0.5 mL of PBS including 0.2% gelatin. Each group consisted of 5 mice; data are representative of 2 separate experiments.

Therefore, we assessed total and TT-specific IgE antibodies at weekly intervals. Both total and TT-specific IgE antibody responses reached maximum levels by day 21 (data not shown). These findings suggest that the levels of IgE induced by the chimera were much lower than those evoked by the native form of CT.

Mucosal protection against influenza virus infection by mCTA/LTB chimera. To determine the ability of mCTA/LTB chimera to support the generation of protective immunity in mucosal compartments, BALB/c mice were immunized nasally with inactivated influenza HA vaccine together with mCTA/LTB chimera and then infected nasally with a lethal dose of PR8 viruses. Interestingly, high levels of anti-PR8 HA IgA antibodies in nasal wash and anti-PR8 HA IgA and IgG antibodies in lung wash were detected after nasal immunization (figure 5A). In addition, high levels of anti-PR8 HA IgG antibody responses were detected in serum samples (figure 5A). Just as with the antibody responses, virus titers in the lung wash showed that the 2-dose regimen conferred complete protection against infection (figure 5B). These results clearly indicate that nasally administered mCTA/LTB is a useful adjuvant against mucosal diseases such as influenza infection.

Discussion

In the present study, we have developed a novel chimera molecule consisting of the A subunit of mCT E112K and the B subunit of LT produced by the B. choshinensis host-vector system as a new generation of safe and effective mucosal adjuvants. It is well known that CT and LT are effective adjuvants and that they are capable of enhancing both mucosal IgA and systemic IgG antibody responses to coadministered protein antigens; however, both enterotoxins cause severe diarrhea and thus are unsuitable for use in humans [32–34]. Therefore, several groups, including ours, have developed nontoxic derivatives of CT or LT that may be suitable for use in humans [4–11, 35]. Newly created B. choshinensis–derived mCTA/LTB chimera molecule did not induce any increases in intracellular cAMP and failed to elicit fluid accumulation in ligated ileal loops. Furthermore, nasal administration of TT together with mCTA/LTB as adjuvant could elicit TT-specific serum IgG and IgA antibody responses with minimal induction of IgE antibody responses. These mucosally induced serum IgG antibody responses provided complete protection against systemic challenge with tetanus toxin. Even more importantly, influenza vaccine given with this nontoxic chimera adjuvant elicited antigen-specific IgA and IgG antibodies in the lungs and provided complete protection against mucosal infection with influenza virus. These results indicate that this novel chimeric mCTA/LTB molecule is a potent nontoxic mucosal adjuvant for the induction of protective immunity in both mucosal and systemic compartments.
Figure 4. Nasally administered tetanus toxoid (TT) with chimeric mucosal adjuvant that combines A subunit of mutant cholera toxin E112K with pentamer B subunit of heat-labile enterotoxin from enterotoxigenic Escherichia coli (mCTA/LTB) did not induce total and TT-specific IgE antibodies in serum samples. Groups of mice were immunized nasally with 5 mg of TT alone (open bars), with 0.5 mg of native cholera toxin (CT; hatched bars), with 10 mg of mCTA/LTB (solid bars), or with 10 mg of mCT E112K (dotted bars) on days 0, 7, and 14. Serum samples were collected 1 week after last immunization. Bars represent mean antibody titers ± SE in each group. *P < .05, compared with that of mice immunized with TT alone.

Figure 5. Nasal immunization with inactivated influenza vaccine together with chimeric mucosal adjuvant that combines A subunit of mutant cholera toxin E112K with pentamer B subunit of heat-labile enterotoxin from enterotoxigenic Escherichia coli (mCTA/LTB) protected mice from influenza virus infection. BALB/c mice were inoculated nasally without (open bars) or with 5 mg of PR8 vaccine together with 5 mg of mCTA/LTB (hatched bars), and second administration was done 4 weeks later (solid bars). Two weeks after second immunization, groups of mice were infected with lethal dose of PR8 influenza virus. Levels of anti-PR8 hemagglutinin (HA) antibodies in nasal wash, lung wash, and serum samples (A) and virus titer in lung wash samples (B) were assessed 3 days after infection. *P < .05, compared with that of nonimmunized mice.

It has been reported that a number of children are prone to anaphylaxis when given certain live vaccines, such as measles, mumps, rubella, and varicella vaccines [36]. These vaccine preparations include high amounts of gelatin, and the anaphylactic reaction is caused by anti-gelatin IgE antibodies [37, 38]. Therefore, an important criterion for the development of mucosal adjuvants should be to reduce or diminish the potential for IgE antibody production without losing the ability to generate antigen-specific mucosal IgA and serum IgG antibody responses. In a previous study, we demonstrated that among different mutant forms of CT tested, mCT E112K was selected as a safe and effective adjuvant because it supported antigen-specific IgA responses with lower levels of total and anti-CTB IgE antibodies than those observed with different toxin-derived adjuvants after nasal immunization [15]. Interestingly, the present results showed that nasal administration of mCTA/LTB did not enhance IgE antibody responses. Collectively, point mutations in the A subunit of CT may be a key element in the regulation of IgE antibody responses. Current studies are focused on elucidating the molecular basis for the regulation of IgE responses by A subunit that emerged from the present and previous studies. The mCTA/LTB chimera has unique and potentially beneficial features of both enterotoxins and should be considered as a new candidate mucosal adjuvant for future application in humans, which will avoid the danger of anaphylactic shock and/or allergic reactions provoked by IgE antibodies.

Our previous studies have shown that CT accumulates in the olfactory nerves and epithelium regions when given nasally [23]. This uptake of CT has been shown to be ganglioside GM1-dependent. Furthermore, CT as mucosal adjuvant redirects co-administered protein antigen into these neuronal tissues [23]. The finding raised some concerns about a potential role for ganglioside GM1-binding molecules that target neural tissues, including the central nerve system, in nasal immunization. However, a recent study provided new evidence that deposition of CT via the olfactory tissues did not lead to obvious pathologic changes in brain tissue after nasal administration [39]. Although we still do not know the exact biologic and pathologic significance of enterotoxin deposition in the central nervous system mediated by ganglioside GM1–binding of olfactory tissues, we have investigated the distribution of mCTA/LTB and
TT in olfactory nerves and epithelium and in olfactory bulbs after nasal immunization. Interestingly, unlike the native form of CT, mCTA/LTB did not influence trafficking of TT into the olfactory nerves and epithelium or into olfactory bulbs, although mCTA/LTB itself accumulated in olfactory central nervous system regions. A separate study has indicated that mCT E112K, the parent molecule of the A subunit in our chimera, also fails to redirect TT into the central nervous system (authors’ unpublished data). Thus, our chimeric adjuvant inherits this unique feature of mCT E112K. These results suggest an interesting possibility that redirected trafficking of protein antigen by CT may be mediated by ADP-ribosyltransferase activity.

It has been reported that CTB selectively binds to ganglioside GM1, whereas LT uses several ligands, including ganglioside GM1, ganglioside GM2, and asialo ganglioside GM1, as target cell surface receptors [3]. Thus, an alternative possibility would be that the difference in these binding sites may influence the capacity for redirection antigen trafficking.

In the present study, the B. choshinensis host-vector system was chosen for production of chimeric mCTA/LTB molecule, because it has several beneficial properties. First, the B. choshinensis expression construct has been shown to be the most effective mass production system for recombinant protein [40]. For example, the quantity of CTB produced by B. choshinensis is 1000-fold higher than that of wild-type V. cholerae [41]. Second, B. choshinensis is a gram-positive bacterium that is known to be nonpathogenic; thus, the possibility of contamination by endotoxin and other virulence factors was excluded. Indeed, our results showed that endotoxin levels of purified mCTA/LTB were at the lower limit of detection (16 EU/mg). Furthermore, mice given mCTA/LTB did not possess any clinical or pathologic symptoms caused by endotoxin (data not shown). These results clearly indicate that the B. choshinensis host-vector system has significant advantages in the preparation of mucosal adjuvants, such as the mCTA/LTB chimera, for use in humans.

In summary, a novel chimeric mucosal adjuvant possessing beneficial features of mCTA and LTB was constructed and produced by the B. choshinensis host-vector system. Our results have provided evidence that nasal vaccination with TT plus mCTA/LTB elicited antigen-specific mucosal IgA and serum IgG antibodies without IgE responses. Furthermore, TT-specific antibody responses induced by TT plus mCTA/LTB provided protective immunity against challenge with tetanus toxin. It is also important to emphasize that nasal immunization with influenza HA vaccine together with chimeric mCTA/LTB resulted in effective production against viral challenge in the respiratory tract. This newly developed chimeric molecule that combines the A subunit of mCT E112K and the B subunit of nLT should be considered as a novel candidate mucosal adjuvant.

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References

coli heat-labile enterotoxin and cholera toxin using immobilized galactose. 


