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Chimeras of Labile Toxin One and Cholera Toxin Retain Mucosal Adjuvanticity and Direct Th Cell Subsets Via Their B Subunit

Prosper N. Boyaka,* Mari Ohmura,‡ Kohtaro Fujihashi,* Toshiya Koga,* Masafumi Yamamoto,* Mi-Na Kwoun,* Yoshifumi Takeda,* Raymond J. Jackson,* Hiroshi Kiyono,* Yoshikazu Yuki,* and Jerry R. McGhee*

Native cholera toxin (nCT) and the heat-labile toxin 1 (nLT) of enterotoxigenic Escherichia coli share 83% amino acid sequence homology and both consist of an enzymatically active A subunit that catalyzes the ADP-ribosylation of the Gs protein. The B subunits of CT (CT-B) and LT (LT-B) bind to GM1 gangliosides (1, 2), while LT-B also binds asialo GM1, lactosylceramide, and galactoproteins (3–6). The binding of nCT or nLT to intestinal epithelial cells induces a cascade of events that ultimately results in secretion of chloride and water with subsequent diarrhea (7). Both nCT and nLT are mucosal adjuvants and induce mucosal and systemic immunity when given with protein vaccines by either oral or nasal routes (8–10). Furthermore, the A subunit mutants of both CT (11–13) and LT (13–17) have been shown to be devoid of diarrheagenic activity, but to retain full mucosal adjuvanticity, when given by the nasal route.

It has been shown that the adjuvant activity of both nCT and nLT as well as nontoxic mutants of CT involves up-regulation of costimulatory molecule expression by APCs (18–21). On the other hand, CT-B and LT-B fail to stimulate APC costimulatory molecule expression (19, 21). In addition, studies have now shown that neither CT-B nor LT-B enhances immune responses to mucosally coadministered protein Ags (2, 14, 21, 22). However, some reports have suggested that CT-B and LT-B display mucosal adjuvant activity when large doses are given with proteins by the nasal route (2, 13, 23, 24) or when enterotoxin B subunits are directly conjugated to the Ag itself (2, 23). Previous studies have shown that nCT as adjuvant elicits potent mucosal and systemic CD4+ Th2-type immune responses (25–28). Proof that IL-4 is involved in nCT-induced mucosal secretory IgA (S-IgA) Ab responses to coadministered protein Ags was provided by the finding that nCT fails to induce mucosal S-IgA Ab responses in IL-4 gene knockout (IL-4−/−) mice (25, 27, 28). This ability of nCT to promote polarized Th2-type responses is in part explained by recent findings that nCT inhibits IL-12 production by monocytes and dendritic cells (19, 29) and abrogates IL-12R expression by T cells (29). It has also been shown that nLT as mucosal adjuvant supports CD4+ Th1-type responses and IFN-γ production in the presence of IL-4-independent Th2-type responses (20, 30). It is clear that an in vivo network of Th1- or Th2-type cytokine responses can influence the overall nature of the immune response seen (31, 32). Thus, Th1-type cytokines support cell-mediated immunity and the production of complement-fixing IgG subclass Abs. On the other hand, Th2-type cytokines provide help for B cells and promote the production of IgE and noncomplement fixing. IgG1 and IgG2b
subclass Abs. Furthermore, CTL responses were induced after nasal immunization with either nCT or nLT as adjuvant (33, 34); however, higher CTL responses were noted after immunization with nLT or its nontoxic derivatives compared with nCT (35, 36). This suggests that a potent Th1 cell component characterizes the immune response induced when nLT is used as mucosal adjuvant.

Two recent studies suggest that slight alterations in the A subunits explain the differences in nCT and nLT adjuvanticity (37, 38). However, a major difference between nCT and nLT involves their B subunit ganglioside receptors, with nLT being more promiscuous. We reasoned that this could also explain the differences in Th1- or Th2-type responses induced by nLT or nCT, respectively. To address the relative contributions of the A and B subunits in Th1- vs Th2-type responses, we constructed the appropriate chimeras, i.e., CT-A/LT-B and LT-A/CT-B. These chimeras were assessed for mucosal adjuvant activity after nasal codelivery with protein Ags, and the results show that both A and B subunits contribute to adjuvanticity, with the B subunit associated with Th cell subset demarcation.

Materials and Methods

Construction of CT-A/LT-B and LT-A/CT-B chimeras

Enterotxin chimeras were generated by spontaneous association of the A subunit of nCT or nLT with the B subunit of the opposite enterotoxin. The CT-A subunit was purchased from Sigma-Aldrich (St. Louis, MO) and applied to an immobilized α-galactoside column (Pierce, Rockford, IL) to remove any CT-B contamination. Eluted CT-A contained no B subunit, as shown by silver staining of SDS-PAGE gels. Recombinant CT-B was produced using a Brevibacillus choshinensis-expressing plasmid pNU 212, CTB (39) and was provided by JCR Biopharmaceuticals (San Diego, CA). LT-B was derived from an E. coli K12 strain DH5α transformant containing the plasmid (pLT10) that encodes the LT-B gene (40) and was purified by use of an immobilized α-galactoside gel column. The nCT was obtained from Lab Biological Laboratories (Campbell, CA), and nLT and nLT-B were purchased from Sigma-Aldrich. The CT-A/LT-B and LT-A/CT-B chimeras were generated by spontaneous association of A and B subunits in propionic acid, as previously described (41). Briefly, CT-A and LT-B were separately dialyzed in 0.1 M propionic acid (pH 4.0). A 2-fold molar excess of CT-B was then mixed with LT-A, and the two were allowed to spontaneously associate during a 24-h dialysis in 0.1 M propionic acid (pH 4.0). The associated CT-A/LT-B chimera was purified by gel filtration. The LT-A/CT-B chimera was generated by the same procedure used to construct the opposite chimera. Briefly, nLT was first denatured by dialysis in 0.1 M propionic acid (pH 4.0), followed by a second dialysis step in 0.1 M propionic acid/6 M urea, to separate the A and B subunits. The A subunit was then purified by HPLC using a TSK gel 2000 W (Tosoh Biosep, Montgomeryville, PA) and 0.1 M propionic acid/6 M urea plus 0.2 M NaCl as eluent. The urea was removed by dialysis in 0.1 M propionic acid before LT-A and CT-B were mixed at a 1:2 molar ratio and allowed to spontaneously associate. The resulting LT-A/CT-B chimera was purified by gel filtration as previously reported (42).

Mice and nasal immunization

C57BL/6 mice were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). Mice were maintained in horizontal laminar flow cabinets and were free of microbial pathogens as determined by plasma Ab screening and tissue histopathology performed on sentinel mice. All mice received sterile food and water ad libitum and were between 8 and 12 wk of age when used for these experiments.

Mice were nasally immunized at weekly intervals for 3 consecutive wk with 100 μg of OVA (Sigma-Aldrich) either alone or together with 0.5 μg of nCT, nLT, or CT-A/LT-B or LT-A/CT-B chimeras (12, 43). The nasal immunization was performed on lightly anesthetized mice with OVA and adjuvant in a total volume of 10 μl, with 5 μl placed into each nare. Blood samples were collected weekly (days 7, 14, and 21) just before reimmunization to monitor the development of plasma anti-OVA Ab responses as previously described (12, 43). Mucosal secretions (e.g., fecal extracts, nasal washes, and saliva) were collected on day 21 as previously described (12, 43) for assessment of mucosal S-IgA Ab responses.

Evaluation of OVA-specific Ab isotypes and IgG subclass responses

OVA-specific Ab titers in plasma and mucosal secretions were determined by ELISA as previously described (12, 25, 44). Briefly, serial 2-fold dilutions of plasma or mucosal secretions were added to plates coated with OVA (1 mg/ml). Anti-OVA Ab isotypes were detected with peroxidase-labeled goat anti-mouse μ, γ, or α chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL). For IgG subclass analysis, biotinylated rat anti-mouse γ1 (clone G1-7.3), γ2a (clone R19-15), γ2b (clone R12-3), or γ3 (clone R40-82) H-chain-specific mAbs (BD PharMingen, San Diego, CA) and streptavidin-conjugated peroxidase were employed. The colorimetric reaction was developed by the addition of ABTS substrate (Sigma-Aldrich). End-point titers were expressed as the reciprocal log2 dilution giving an OD415 of ≤0.1 above those obtained with control, nonimmunized mice.

Total plasma IgE levels were determined by ELISA as previously described (25, 26, 45) using a rat anti-mouse IgE mAb (BD PharMingen; clone R35-72) and a second biotinylated rat anti-mouse IgE mAb (BD PharMingen; clone R35-92) for capture and detection, respectively. The OVA-specific plasma IgE Ab responses were detected using a streptavidin-poly-HRP amplification system (Research Diagnostics, Flanders, NJ) and the ABTS substrate (25). A modified IgE capture luminometry assay (Sigma-Aldrich). End-point titers were expressed as the reciprocal dilution exhibiting relative light units which were 2-fold higher than background.

ELISPOT assay for detection of Ab-forming cells (AFC)

OVA-specific AFC in mucosal and systemic tissues were evaluated as previously described (12, 45, 47). Dispersed cells were resuspended in RPMI 1640 medium (Cellgro; Mediatech, Virginia). RPMI 1640 medium containing 10% FCS, 15 mM HEPES, 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (complete medium). Different dilutions of cells were then added to 96-well nitrocellulose-based plates (Millipore, Bed ford, MA) coated with 100 μl of a 1 mg/ml solution of OVA and incubated for 6 h at 37°C in a 5% CO2 atmosphere. Ag-specific AFC were detected with peroxidase-labeled anti-mouse μ, γ, or α chain Abs (Southern Biotechnology Associates). Spots were visualized by adding the chromogenic substrate, 3-amino-9-ethylcarbazole (Moss, Pasadena, MA) and were counted with the aid of a dissecting microscope (SZH Zoom Stereo Microscope System; Olympus, Lake Success, NY).

Ag-specific CD4+ T cell and cytokine responses

Single-cell suspensions from the spleen and from cervical lymph nodes (CLN) were obtained as previously described (12, 44). The cells were first added to a nylon wool column (Polysciences, Warrington, PA) and incubated for 1 h at 37°C to obtain an enriched T cell fraction. The nonadherent, T cell-enriched population was stained with biotinylated anti-CD4 mAb (clone GK1.5), followed by streptavidin-coupled microbeads (MACS; Miltenyi Biotec, Auburn, CA). The CD4+ T cells were then obtained by positive sorting using a MACS system (Miltenyi Biotec). In some experiments CD4+ T cells were enriched (>98% purity) using Mouse CD4 Collect Plus columns (Biotec, Edmonton, Canada). Purified CLN and splenic CD4+ T cells were cultured at a density of 4 × 10^6 cells/ml and stimulated with OVA (1 mg/ml) in the presence of T cell-depleted, irradiated (3000 rad) splenic feeder cells (8 × 10^6 cells/ml) and IL-2 (10 U/ml; BD PharMingen) in complete medium. To measure cell proliferation, 0.5 μCi of tritiated thymidine ([3]H[3]TdR; DuPont/NEN, Boston, MA) was added to individual culture wells 4 days later. Eighteen hours after the addition of [3]H[3]TdR, the cells were harvested onto glass microfiber filter paper (Whatman, Clifton, NJ), and [3]H[3]TdR incorporation was determined by liquid scintillation counting.

Analysis of OVA-specific CD4+ T cell cytokine responses

Supernatants from OVA-stimulated CD4+ T cell cultures were collected after 5 days of incubation, and cytokine levels were determined by ELISA as previously described (12, 26, 44, 45, 48). Briefly, Nunc MaxiSorp Immunoplates (Nunc, Naperville, IL) were coated with anti-mouse IFN-γ (clone R4-6A2), IL-2 (clone JE56-1A12), IL-4 (clone BV4D1-1D1), IL-5 (clone MQ5-21E3), IL-6 (clone MP5-20F3), or IL-10 (clone JES5-2A5) mAbs (BD PharMingen) in 0.1 M sodium bicarbonate buffer (pH 9.2). After blocking, cytokine standards and serial dilutions of culture supernatants were added in duplicate. The plates were washed and incubated with secondary biotinylated mouse anti-IFN-γ (clone XMG-1.2), IL-2 (clone JE56-7H4), IL-4 (clone BVD6-24G2), IL-5 (clone TRFK4), IL-6 (clone MP5-32C11), or IL-10 (clone JES5-16E3) mAbs (BD PharMingen), followed by peroxidase-labeled goat anti-biotin Ab (Vector Laboratories, Burlingame,
Flow cytometric analysis of IL-12Rβ1 expression

Splenic T cells were isolated from naïve mice and seeded into culture plates at a density of 5 × 10⁶ cells/ml. To investigate the effects of nCT, nLT, and the two chimeras on IL-12Rβ1 expression, T cells were incubated for 1 h with 100 ng/ml of nCT, nLT, LT-A/CT-B, or CT-A/LT-B. Anti-mouse CD3e chain (1 μg/ml; clone 145-2C11; BD Pharmingen) was then added. After 3 days of incubation the T cells were incubated for 30 min at 4°C with PE-labeled anti-mouse IL-12Rβ1 (clone 114) and FITC-labeled anti-mouse CD3 (clone 145-2C11) or anti-CD4 (clone GK1.5). Cells were then washed and fixed in 1% paraformaldehyde in PBS and analyzed by flow cytometry using a FACSCalibur equipped with the CellQuest software (BD Biosciences, Mountain View, CA).

Statistics

The results shown are reported as the mean ± 1 SE. Statistical significance (p < 0.05) was determined by Student’s t test and the Mann-Whitney U test for unpaired samples. The results were analyzed using the StatView II statistical program (Abacus Concepts, Berkeley, CA) for Apple computers (Cupertino, CA).

Results

Adjuvant activity of nasal CT-A/LT-B and LT-A/CT-B chimeras for serum Ab responses

We initially determined whether chimeric molecules generated by spontaneous association of the A subunit of CT or LT and the B subunit of the reciprocal enterotoxin would display adjuvanticity when nasally coadministered with a weak protein Ag, i.e., OVA. For this purpose, mice were nasally immunized with OVA and CT-A/LT-B, LT-A/CT-B, nCT, or nLT as mucosal adjuvants. Both CT-A/LT-B and LT-A/CT-B enhanced plasma anti-OVA Ab isotype responses, demonstrating that both chimeras were effective adjuvants for nasally coadministered OVA (Fig. 1A). No significant differences were noted in the levels of plasma IgM, IgG, or IgA anti-OVA Ab responses induced by the two chimeras compared with the two native enterotoxins (Fig. 1A). Since nCT and nLT promote distinct patterns of IgG Ab subclass responses to mucosally coadministered protein Ags (20, 25, 26, 28, 30, 45), we next investigated the patterns of plasma anti-OVA IgG subclass Ab responses resulting from the adjuvant activity of the two chimeras compared with the native enterotoxins. The plasma IgG subclass responses in mice that received OVA and nLT or the CT-A/LT-B chimera were characterized by high anti-OVA IgG2a, with IgG1 and IgG2b Ab responses (Fig. 1B). On the other hand, mice that received the nasal vaccine consisting of OVA and nCT or LT-A/CT-B showed high levels of IgG1, followed by IgG2b, and only low levels of IgG2a anti-OVA Abs (Fig. 1B). These results clearly show that both CT-A/LT-B and LT-A/CT-B chimeras are mucosal adjuvants, and their activities mimic the native enterotoxin with which they share a common B subunit.

To further elucidate differences between CT-A/LT-B and LT-A/CT-B chimeras, we next analyzed their potential to promote plasma IgE Ab responses. Previous studies in mice orally or nasally immunized three times at weekly intervals with protein vaccines and nCT as mucosal adjuvant have shown that a transient IgE Ab response occurs by 7 days following the second immunization (day 14) (12, 20, 25, 26, 45). It has also been shown that nLT induces lower IgE Ab responses compared with nCT (20, 30). In this study we noted that both CT-A/LT-B and LT-A/CT-B chimeras induced plasma IgE Ab responses (Table I). These IgE responses were higher in mice given either LT-A/CT-B or nCT compared with mice that received CT-A/LT-B or nLT as nasal adjuvants (Table I). Interestingly, no significant differences were noted between Ag-specific IgE Ab responses induced by nCT or LT-A/CT-B. On the other hand, the anti-OVA IgE Ab responses elicited by CT-A/LT-B were slightly higher than those induced by nLT; however, the differences were not statistically significant (Table I).

Both CT-A/LT-B and LT-A/CT-B chimeras support mucosal S-IgA Ab responses

Mucosal secretions from mice nasally immunized with OVA in the presence of CT-A/LT-B or LT-A/CT-B were analyzed to determine whether these chimeric enterotoxins also induced mucosal S-IgA Ab responses. No significant anti-OVA S-IgA Ab responses were seen in the saliva of mice given OVA alone. In contrast, higher levels of salivary IgA anti-OVA Abs were noted in mice that received OVA with either CT-A/LT-B or LT-A/CT-B as mucosal adjuvant (Fig. 2A). Similar levels of IgA anti-OVA Abs were
induced in this secretion by both nCT and nLT (Fig. 2A). We also compared mucosal S-IgA Ab responses induced by chimeras and nCT or nLT in nasal washes. Both CT-A/LT-B and LT-A/CT-B induced similar levels of S-IgA anti-OVA Abs, which were not significantly different from those induced by nCT or nLT (Fig. 2B).

To establish the mucosal origin of anti-OVA IgA Abs seen in these external secretions, we next evaluated numbers of IgA AFCs in the CLN of nasally immunized mice. High numbers of OVA-specific IgA AFCs were detected in CLNs of mice nasally immunized with OVA and nCT, nLT, or chimeras as mucosal adjuvants (Fig. 3A). Analysis of splenic OVA-specific AFCs of mice nasally immunized with OVA and either CT-A/LT-B or LT-A/CT-B as mucosal adjuvants showed higher numbers of OVA-specific IgG compared with IgA AFCs, which is consistent with higher levels of serum OVA-specific IgG than IgA Abs (Fig. 3B). The frequency of IgA AFCs in mucosal tissues of mice nasally immunized with the chimeras was of the same magnitude as that in mice induced by nCT and nLT. Further, higher numbers of IgA AFCs were noted in the CLNs compared with spleen (Fig. 3).

### Distinct CD4+ Th cell subsets are induced by CT-A/LT-B and LT-A/CT-B chimeras

The pattern of IgG subclass responses elicited by the two chimeras suggests that separate mechanisms account for adjuvanticity. Further, the results show that LT-like immunity was induced by CT-A/LT-B, while LT-A/CT-B supported CT-like immune responses. To clarify the precise Th cell subset pathways associated with the Ab responses elicited by CT-A/LT-B or LT-A/CT-B as adjuvant, we next analyzed the profile of CD4+ Th1- and Th2-cell cytokine responses supported by these two chimeras. We noted that OVA-specific CD4+ T cells from CLNs and spleen of mice nasally immunized with OVA and either nLT or CT-A/LT-B secreted high levels of IFN-γ after in vitro restimulation with OVA (Fig. 4). On the other hand, only minimal levels of IFN-γ were seen in culture supernatants of OVA-stimulated CLN and splenic CD4+ T cells isolated from mice immunized with OVA and LT-A/CT-B or nCT as mucosal adjuvants (Fig. 4).

Previous studies have shown that nCT induces significant IL-4 responses compared with nLT (20, 30). Further, the mucosal adjuvant activity of nCT, but not nLT, requires IL-4, and no adjuvant effect was seen in IL-4 knockout mice (25, 27, 28). Therefore, we compared the ability of our chimeric enterotoxins to support IL-4 and CD4+ Th2-type responses. Analysis of culture supernatants of OVA-stimulated CD4+ T cells from CLNs and spleen of mice nasally immunized with OVA and native enterotoxins or with the chimeras showed no significant differences in levels of IL-5, IL-6, and IL-10 (Fig. 5). Conversely, the adjuvant activity of CT-A/LT-B and LT-A/CT-B differentially affected IL-4 synthesis by OVA-specific CD4+ T cells. Thus, the cultures of CD4+ T cells from mice nasally immunized with OVA and LT-A/CT-B as adjuvant produced high levels of IL-4, which were essentially identical with those seen in the cultures of CD4+ T cells from mice immunized with OVA and nCT (Fig. 5). On the other hand, significantly lower and similar levels of IL-4 production were seen in culture supernatants of CD4+ T cells from mice that received CT-A/LT-B or nLT as nasal adjuvants (Fig. 5). Taken together, these results show that the induction of CD4+ Th2-type responses by LT-A/CT-B closely mimics that induced by nCT, while Th1- and select Th2-type responses were promoted by both CT-A/LT-B and nLT.

### Both LT-A/CT-B and nCT inhibit T cell IL-12Rβ1 expression

We next addressed the potential mechanism involved in the induction of biased Th cell cytokine responses by nCT, nLT, and the chimeras LT-A/CT-B and CT-A/LT-B by analyzing the effects of these enterotoxins on IL-12Rβ1 expression and IFN-γ secretion by activated T cells. Addition of anti-CD3 mAb to splenic T cell cultures enhanced IL-12Rβ1 expression (Fig. 6). Pretreatment of splenic T cells with nCT, but not nLT, abolished the anti-CD3 mAb-induced IL-12Rβ1 expression (Fig. 6). When cells were pretreated with the LT-A/CT-B chimera, we observed an inhibition of IL-12Rβ1 expression similar to that induced when cells were pretreated with nCT (Fig. 6). Conversely, no inhibition of IL-12Rβ1 expression was seen in spleen cell cultures pretreated with the CT-A/LT-B chimera (Fig. 6), again suggesting that chimeric enterotoxins reproduce the effect of the native enterotoxin with which they share the same B subunit. Neither CT-B nor LT-B alone affected IL-12Rβ1 expression by anti-CD3 mAb-stimulated T cells.

### Table 1. Total and OVA-specific IgE Ab responses in mice nasally immunized with OVA and chimeric or native enterotoxins

<table>
<thead>
<tr>
<th>Nasal OVA Plus Adjuvant</th>
<th>Total IgE (µg/ml)</th>
<th>OVA-Specific IgE (reciprocal log₂ titers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Below detection&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Below detection&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CT-A/LT-B</td>
<td>2.3 ± 0.4</td>
<td>7.5 ± 1.2</td>
</tr>
<tr>
<td>nLT</td>
<td>1.9 ± 0.1</td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td>LT-A/CT-B</td>
<td>6.5 ± 0.3</td>
<td>11.1 ± 0.7</td>
</tr>
<tr>
<td>nCT</td>
<td>8.6 ± 0.6</td>
<td>13.6 ± 0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Groups of five mice were nasally immunized with 100 µg of OVA and 0.5 µg of chimeras or native enterotoxin three times at weekly intervals (days 0, 7, and 14). Total and OVA-specific plasma IgE levels were determined on day 14 as described in Materials and Methods. Results are from one experiment and are representative of three separate experiments.

<sup>b</sup>Below the limits of detection. Total IgE, <150 ng/ml; OVA-specific IgE, <4 reciprocal log₂ titer.

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*FIGURE 2. Anti-OVA S-IgA Ab responses in mucosal secretions following nasal immunization with CT-A/LT-B or LT-A/CT-B chimeras. Saliva (A) and nasal washes (B) were collected on day 21 from mice nasally immunized with OVA only (●), OVA with CT-A/LT-B (□), nLT (○), LT-A/CT-B (●), or nCT (■). OVA-specific IgA Ab responses were evaluated by ELISA. The results are expressed as individual end-point (log₂) Ab titers ± 1 SE from four experiments each with five mice per group per experiment.*
To further establish the functional relevance of the adjuvant effects of nCT, nLT, and the chimeric enterotoxins on IL-12Rβ2 expression, we investigated how pretreatment with enterotoxin would affect IFN-γ secretion by anti-CD3 mAb-stimulated T cells. The ability of anti-CD3 mAb-stimulated T cells to produce IFN-γ was essentially abolished by pretreatment of T cells with nCT (Fig. 7). In contrast, pretreatment with nLT only marginally affected IFN-γ production by anti-CD3 mAb-stimulated T cells (Fig. 7). Interestingly, pretreatment of cells with LT-A/CT-B altered IFN-γ production, while CT-A/LT-B had no significant effect on this response (Fig. 7).

Discussion

Both nCT and nLT have been extensively used as mucosal adjuvants in various animal models; however, little is known about the precise mechanisms that govern the nature of CD4+ Th cell responses induced by these related molecules for support of mucosal and plasma Ab responses. We have investigated the involvement of the A and B subunits of these two enterotoxins in CD4+ Th cell subset responses by analyzing the patterns of immune responses promoted by the reciprocal CT-A/LT-B or LT-A/CT-B chimeras compared with the parent nCT or nLT. Both chimeras were effective adjuvants for a nasally coadministered protein Ag, OVA, which is poorly immunogenic when given alone. Both chimeras elicited mucosal and plasma Ab responses similar in magnitude to those induced when nCT or nLT was used as mucosal adjuvant. Further, the adjuvant activity of CT-A/LT-B resulted in OVA-specific IgG subclass and CD4+ Th2-type responses was further supported by the finding that LT-A/CT-B, like nCT, inhibited IL-12Rβ1 expression and IFN-γ production by T cells, while these effects were not seen after treatment with nLT or the chimera CT-A/LT-B.

The first important finding of this study was that both the A and B subunits of CT and LT could be freely substituted without altering their ability to serve as mucosal adjuvants. Chimeras generated by genetic expression of the A subunit of one enterotoxin and the B subunit of the other were reported to stimulate plasma IgG Ab responses at levels comparable to those achieved by native enterotoxins (37). In addition, our recent and separate study showed that a nontoxic CT-A-E112K/LT-B chimera produced with a *Brevibacillus choshinensis* expression system was an effective adjuvant for mucosal and systemic immunity (42). Here we provide evidence that CT-A/LT-B and LT-A/CT-B chimeras, which were generated by spontaneous reassociation of A and B subunits, also retain their mucosal adjuvant activity. Despite the close similarity between nCT and nLT (1), there are subtle functional differences between these two molecules. The stability and conformation of these enterotoxins are important factors in their adjuvant activity, and thus one could not exclude the possibility that altered conformation or stability would affect the adjuvanticity of the chimeras. In this regard, CT-A2 was reported to more efficiently stabilize interactions between the A and B subunits than did LT-A2 (49). Further, the same mutation in CT or LT differentially affected their adjuvant activity. For example, a nontoxic LT mutant with a serine to lysine substitution at position 63 in the A subunit (LT-K63) was shown to be an excellent mucosal adjuvant (13, 16, 50, 51); however, the CT mutant bearing the same single amino acid substitution (CT-K63) did not display adjuvant activity (14). It has also been shown that receptor binding mutants of LT (LT-B G33D) fail to evoke systemic and mucosal immune responses (52).
Further, higher doses of nontoxic mutants of LT or CT were required to induce similar response levels as native enterotoxins (2, 12, 13, 16, 43, 51). Our findings that both CT-A/LT-B and LT-A/CT-B possess adjuvant activity indicate that the A and B subunits of CT and LT can be interchanged, and the resulting chimeric enterotoxins retain both ganglioside binding and ADP ribosyltransferase activities. In contrast to studies suggesting an essential role of A subunits in Th cell subset responses, our findings support a major role for the B subunits in directing the nature of Th cell cytokine responses to coadministered protein Ags. This possibility was initially suggested by separate studies in which the nontoxic CT-A-E112K/LT-B chimera exhibited an LT-like adjuvant activity (42). However, the single amino acid substitution in the A subunit of this chimera could also have influenced the profile of the Th cell cytokine responses induced.

Earlier studies comparing the receptors for nCT and nLT indicated that while both molecules bound GM1 and, to a lesser extent, GD1b, nLT bound other gangliosides as well as glycoproteins (6, 53, 54). Studies performed in rabbit small intestine revealed that blocking of GM1 and GD1b with CT-B did not affect the binding of nLT to glycoproteins and subsequent intestinal fluid secretion (4, 53). In the human small intestine, extraction of lipids from intestinal epithelial cells resulted in removal of all nCT binding sites, but only 50% of the nLT binding sites (54). Moreover, nLT was found to bind polyglycosylceramides in rabbits, while nCT did not (5). Thus, this pleiotrophic binding to the above sites probably contributes to the Th1 component of the LT-induced immune response.

It is now well documented that nCT as a mucosal adjuvant promotes CD4\(^+\) Th2-type responses to coadministered protein Ags (12, 21, 25–28, 45, 55). The key role played by IL-4 was demonstrated by the loss of nCT-induced mucosal adjuvanticity in IL-4 knockout mice (27, 28). In vitro studies have also shown that nCT inhibits IL-12 production by human monocytes and DCs (29). Interestingly, these studies showed that higher doses of nLT were required to inhibit IL-12 production (29). These observations add support to in vivo studies that showed predominant Th2-type responses after mucosal immunization with nCT (12, 20, 25–28, 45, 55) and Th1- with low IL-4 Th2-type responses when nLT was used as the mucosal adjuvant (17, 20, 30). However, the precise role of the A vs B subunits in adjuvanticity had not been studied in detail. In this regard, CT-B, like nCT, was reported to stimulate the synthesis of arachidonic acid metabolites (56) including PGE\(_2\), which is known to promote the differentiation of Th2-type cells.

FIGURE 5. The production of Th2-type cytokines by OVA-specific CD4\(^+\) T cells from mice nasally immunized with chimeric CT-A/LT-B or LT-A/CT-B enterotoxins. The CLN (A) and splenic (B) CD4\(^+\) T cells were isolated 21 days after the initial immunization with OVA only (○), OVA with CT-A/LT-B (△), nLT (□), LT-A/CT-B (■), or nCT (●). After in vitro restimulation, Th2-type cytokine secretion in culture supernatants was evaluated by IL-4-, IL-5-, IL-6-, and IL-10-specific ELISA. The results are expressed as the mean ± 1 SE and are representative of five separate experiments, each with five mice per group per experiment.
FIGURE 6. Treatment with nCT and LT-A/CT-B inhibits IL-12Rβ1 expression by anti-CD3 mAb-stimulated T cells. Naïve splenic T cells were treated for 1 h with nCT, nLT, CT-ALT-B, or LT-A/CT-B (100 ng/ml) and then were cultured for 3 days in the presence of anti-CD3 mAb (1 μg/ml). Control T cells were either not treated with enterotoxin nor stimulated with anti-CD3 mAb (left panel) or were only stimulated with anti-CD3 mAb (right panel). Flow cytometry was performed after staining with PE-labeled anti-mouse IL-12Rβ1 and FITC-labeled antimouse CD3 or anti-CD4 mAbs. Data are representative of four separate experiments.

Chimera enterotoxins for Th1- or Th2-type mucosal immunity

(57–60). While this would indicate a predominant role of the binding B subunit for the induction Th2-type responses, other reports favor a role for the A subunit. For example, single amino acid substitutions in the A subunit of LT (LTK63 and LTR72 mutants) were shown to promote protein Ag-specific mixed Th1/Th2- or Th2-type responses, respectively (38). Another study showed that genetically produced CT-A/LT-B and LT-A/CT-B as nasal adjuvants induced IFN-γ and IL-5 secretion in vitro, and these authors suggested that the A subunits (i.e., CT-A and LT-A) of these adjuvants actually controlled the nature of CD4+ Th cell cytokine responses induced (37). Unfortunately, this study did not investigate the complete pattern of Th2-type cytokines, including IL-4, which is the major cytokine for Th2 cells differentially affected by nCT compared with nLT. It is important to note that immune responses induced by native (i.e., nCT and nLT) or chimeric (CT-A/LT-B or LT-A/CT-B) enterotoxins could be different if these enterotoxins were used as adjuvants for more complex Ags or Ags with intrinsic biological activity (i.e., endotoxin). In this regard, high IgG1 and IgG2b Ab responses to Hemophilus influenzae were seen in mice immunized nasally with a recombinant outer membrane protein P6 of nontypeable H. influenzae and nCT as adjuvant (61). In contrast, no IgG1 and high IgG2a Ab responses as well as and CD4+ T cell-derived IFN-γ responses were induced by mucosal (i.e., nasal, oral, and intratracheal) immunization with H. influenzae membranes and nCT as adjuvant (62).

We now report that the mucosal adjuvant activity of CT-A/ALT-B results in plasma Ab responses characterized by the presence of Ag-specific IgG2a with IgG1 and IgG2b Abs. This pattern of IgG subclass response is similar to that induced by nLT and strongly suggests a major involvement of the LT-B subunit in this response profile. Interestingly, a completely different pattern of IgG subclass responses, characterized by IgG1 and IgG2a Abs with IgE Ab responses, was seen after nasal immunization with the LT-A/CT-B chimera. This latter profile of Ab responses mimicked those induced by nCT. These results support a major role for the CT-B subunit in Th2-type adjuvanticity. Taken together, the present findings and our recent study with the nontoxic CT-A-E112K/LT-B chimera (42) clearly indicate that the major changes associated with substitution of the B subunit of one enterotoxin with the reciprocal molecule from the other involve Th cell cytokine responses and the associated pattern of IgG subclass and IgE Ab responses.

It is now well accepted that specific cytokines produced by Th cell subsets control the pattern of Ig isotype and IgG subclass Ab responses (31, 63). In this study we confirmed these biased patterns of plasma Ab responses induced by CT-A/ALT-B and LT-A/CT-B by analyzing cytokine production by Ag-specific CD4+ T cells. Consistent with the well-established role of IFN-γ for enhancing IgG2a responses in mice (31, 32, 63), in vitro restimulated, OVA-specific CD4+ T cells from mice nasally immunized with OVA and CT-A/ALT-B or nLT as adjuvants produced high levels of IFN-γ. On the other hand, elevated levels of IL-4, but not IFN-γ, were seen in culture supernatants from OVA-specific CD4+ T cells isolated from mice given OVA and LT-A/CT-B or nCT as mucosal adjuvants. Both IFN-γ and IL-4 are major cytokines that down-regulate the expression of the opposite Th cell phenotype (32, 64). In fact, IFN-γ production by Th1 cells down-regulates IL-4, a major cytokine produced by CD4+ Th2 cells, and conversely, IL-4 effectively diminishes IFN-γ production by CD4+ Th1 cells (32, 64). Thus, high levels of IL-4 produced by CD4+ T cells from mice that received LT-A/CT-B or nCT most likely resulted in inhibitory signals for CD4+ Th1 cells, while IgG1/IgG2a subclasses and IgE Ab responses would be enhanced. On the other hand, a potent IFN-γ environment elicited by CT-A/ALT-B or nLT would inhibit IL-4 production by Th2 cells and would support the development of plasma IgG2a responses. Taken together, our results clearly indicate that biased Th2- or Th1-type responses are induced by LT-A/CT-B or CT-A/ALT-B, respectively. We have also provided direct evidence that nCT and LT-A/CT-B down-regulate IL-12R expression on T cells, an effect consistent with a biased Th2 cell subset response.

Since mucosal Ag delivery is critical for the induction of mucosal and systemic immunity, considerable efforts have been dedicated to the development of safe adjuvants for mucosal vaccines that promote host immunity (2, 11, 12, 65). The newly developed nontoxic derivatives of bacterial enterotoxins represent a significant step toward the incorporation of these powerful adjuvants in
human vaccines. In this regard better protection against intracel-
lar pathogens or extracellular Ags and toxins would require the ability to target Th1- or Th2-type responses. The results reported here show that such targeted immune responses could be achieved by chimeric CT/LT molecules. The efficacy of chimeras as adju-
ants when administered by the oral route remains to be deter-
mined. Since CT-A2 appears to more efficiently stabilize interac-
tions between the A and B subunits than LT-A2 (49), one cannot exclude that the CT-ALT-B molecules would display adjuvant-
ity at lower concentrations than nLT when given by the oral route. This possibility is currently under investigation. The CT-B conju-
gated to an Ag was recently shown to more effectively enhance Ag-specific immunity than did LT-B-Ag conjugates after oral ad-
administration (23). The mechanism underlying this difference in adjuvant activity is still unknown. Our chimeric CT/LT molecules could represent unique probes to investigate the mechanisms un-
derlying the mucosal adjuvant activity of these enterotoxins and for the development of mucosal vaccines that induce either Th1- or Th2-type responses.

In summary, we have shown that chimeric molecules made by spontaneous association of the A subunit of CT or LT with the B subunit of the corresponding toxin are both effective mucosal ad-
juvants for protein vaccines that elicit a pattern of Th cell re-
sponses dictated by the origin of the B subunit. Our results clearly show that biased Th1- or Th2-type responses can be elicited by CT/LT chimeras and depend upon the presence of the LT-B or the CT-B subunit, respectively. Since the enterotoxinity of CT and LT can be eliminated by single amino acid substitutions in the A sub-
unit, the development of chimeras composed of the mutant A sub-
unit and either CT-B or LT-B may lead to safe adjuvants for Th1-
or Th2-type responses and may thus be suitable for use in human mucosal vaccines.

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