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Enterotoxin-Based Mucosal Adjuvants Alter Antigen Trafficking and Induce Inflammatory Responses in the Nasal Tract

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The safety of nasal vaccines containing enterotoxin-based mucosal adjuvants has not been studied in detail. Previous studies have indicated that native cholera toxin (nCT) can alter antigen trafficking when applied nasally. In this study, we determined the enterotoxin-based variables that alter antigen trafficking. To measure the influence of enterotoxin-based mucosal adjuvants on antigen trafficking in the nasal tract, native and mutant enterotoxins were coadministered with radiolabeled tetanus toxoid (TT). The nCT and heat-labile enterotoxin type 1 (LTh-1) redirected TT into the olfactory neuroepithelium (ON/E). Antigen redirection occurred mainly across the nasal epithelium without subsequent transport along olfactory neurons into the olfactory bulbs (OB). Thus, no significant accumulation of the vaccine antigen TT was observed in the OB when coadministered with nCT. In contrast, neither mutant CT nor mutant LTh-1, which lack ADP-ribosyltransferase activity, redirected TT antigen into the ON/E. Thus, ADP-ribosyltransferase activity was essential for antigen trafficking across the olfactory epithelium. Accumulation of TT in the ON/E was also due to B-subunit binding to GM1 gangliosides, as was demonstrated (i) by redirection of TT by LTh-1 in a dose-dependent manner, (ii) by ganglioside inhibition of the antigen redirection by LTh-1 and nCT, and (iii) by the use of LT-IIb, a toxin that binds to gangliosides other than GM1. Redirection of TT into the ON/E coincided with elevated production of interleukin 6 (IL-6) but not IL-1β or tumor necrosis factor alpha in the nasal mucosa. Thus, redirection of TT is dependent on ADP-ribosyltransferase activity and GM1 binding and is associated with production of the inflammatory cytokine IL-6.

Enterotoxins are powerful mucosal adjuvants; however, the mechanisms for their adjuvanticity are still being defined. Native cholera toxin (nCT) and the *Escherichia coli*-derived heat-labile toxin (human type 1) (nLTh-1) are both potent mucosal adjuvants for coadministered protein antigens when given by the oral, nasal, or parenteral route (4, 9–11, 33, 42, 50). Despite extensive research on these enterotoxins, mucosal adjuvants for human use remain in experimental phases, and recent studies have focused on generating nontoxic mutants of CT (mCT) and LTh-1 (mLTh-1). Detoxification of these enterotoxins was accomplished by site-directed mutagenesis of the ADP-ribosylation site located in the A subunit of these AB_5 enterotoxins (3, 8, 51, 52, 53, 54). These mutants are effective mucosal adjuvants in mice and induce long-term memory for coadministered proteins given either by the nasal or parenteral route (3, 51, 52). In this regard, the nasal route is perhaps superior to oral delivery, since it requires much lower doses of both adjuvant and coadministered proteins/vaccines.

Both nCT and nLTh-1 are part of serogroup I of the heat-labile enterotoxins (38) and display somewhat different ganglioside binding specificities (12). For example, nCT binds predominantly to GM1, while nLTh-1 preferentially binds to GM1 and to a lesser extent to GD1b and binds weakly to GM2 and asialo-GM1 (12). Native LTh-I not only targets gangliosides, but also binds to other glycoproteins in the intestinal tract and is associated with a much larger repertoire of target molecules than has been reported for CT (18, 25). The heat-labile enterotoxins from serogroup II, such as LT-IIb, display different ganglioside binding specificities. LT-IIb binds to GD1a and to a lesser extent to GT1b and showed no affinity for GM1 (12). LT-IIb functioned as a mucosal adjuvant when given nasally and induced a mucosal immune response consistent with a mixed $CD4^+$ Th1/Th2 cell response (34), as was previously reported for nLTh-1 (42). A lack of ganglioside binding, which was accomplished by site-directed mutagenesis of amino acid 33, the G33D mutation, rendered both nCT and nLTh-1 deficient in GM1 binding and in the ability to function as mucosal adjuvants following oral (21) or nasal (7) application. Enterotoxin binding to gangliosides is functionally important for both mucosal adjuvanticity and enterotoxicity. Both nCT and nLTh-1 bind to GM1 on epithelial cells and are endocytosed and transported. Blocking GM1 sites is not sufficient to ameliorate the enterotoxicity of nLTh-1, since the molecule also binds to other intestinal epithelial glycoproteins (26, 55).

ADP-ribosyltransferase activity in nCT may potentially cause damage due to toxicity and inflammation of the nasal epithelium, and in so doing may allow passive entry of codelivered vaccine proteins into the olfactory nerve/epithelium (ON/E) (14). Increased permeability of the gut epithelium for

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low-molecular-weight dextran was seen when nCT was orally administered. This study suggested that increased permeability may be an intricate part of the ability of nCT to function as a mucosal adjuvant (32). This conclusion was supported by the fact that CT-B, which is a poor oral adjuvant, did not cause permeability changes in the gut epithelium (32).

The hypothesis proposed in this study was that part of the adjuvanticity of enterotoxin-based mucosal adjuvants may reflect their ability to alter antigen trafficking in the nasal tract and that this antigen redirection could contribute to enhanced inflammatory reactions, which may differentially boost mucosal immune responses.

In this study, we show that enterotoxin-based mucosal adjuvants, i.e., nCT and nLTh-1, alter codelivered protein vaccine trafficking into the ON/E when given nasally. This process of antigen redirection requires ADP-ribosyltransferase activity of the enterotoxin-based adjuvant, as well as binding to GM1 gangliosides, and coincided with the production of the inflammatory cytokine interleukin 6 (IL-6). On the other hand, mutants of CT and LTh-1 lacking ADP-ribosyltransferase activity did not redirect antigen into the ON/E, nor did the native toxin LT-IIb, which is not able to bind GM1. In conclusion, both ADP-ribosyltransferase activity and GM1 binding are required in order for enterotoxin to redirect antigen into the ON/E.

MATERIALS AND METHODS

Mice. Mice of the C57BL/6 strain 6 to 7 weeks of age were obtained from Charles River Laboratories (Wilmington, MA). The mice were maintained in horizontal laminar flow cabinets and were pathogen free as determined by plasma antibody screening and tissue histopathology performed on sentinel mice. All mice received sterile food and water ad libitum and were between 8 and 12 weeks of age when used for these experiments. All mouse studies were done in accordance with guidelines of both the NIH and the Animal Institutional Care and Use Committee of the University of Alabama at Birmingham to avoid pain and distress.

Enterotoxin production and purification. The enterotoxins were produced in our laboratory, with the exception of nCT, which was purchased (List Biological Laboratories, Inc., Campbell, CA). The mCT (E112K) was generated by site-directed mutagenesis of single-stranded DNA of *Escherichia coli* CJ236 transfected with M13 mp19, which included the CT gene, using the Mutant K system (Takara Biomedicals, Kyoto, Japan) as described previously (29, 53). The glutamate-to-lysine mutation of amino acid 112 was generated using the 5′-GATG AACAAAAAGTTTCTGCT-3′ oligonucleotide (53). The pUC119 plasmid carrying the mutated CT gene was transformed into *E. coli* DH5α. The *E. coli* strains containing the mCT gene were grown in LB broth (10 g NaCl, 10 g tryptone, and 5 g yeast extract/liter) with 100 μg/ml of ampicillin. The resulting mCT, derived from a sonicated cell suspension, was purified by binding to and elution from a D-galactose-immobilized column (Pierce Chemical Co., Rockford, IL).

The enterotoxin gene containing plasmid pMY1900 from *E. coli* strain 1032 was subcloned by PCR into the expression vector pTrc 99A (Amersham Pharmacia Biotech, Piscataway, NJ). The LTh-1 mutant E112K was constructed by site-directed mutagenesis with specific primers as described previously (43, 44). The mLT (E112K) and LTh-1 were purified from sonicated cell suspensions and resuspended in 0.9% NaCl in 10 mM Tris-HCl buffer, pH 8.6. After centrifugation, the supernatant was subjected to a 65% ammonium sulfate precipitation, resuspended in 0.2 M Tris (pH 8.0)–1 M sucrose–10 mM EDTA (TEAN) buffer, and purified on an immobilized D-galactose column (Pierce Chemical Co.) as reported previously (45).

Heat-labile enterotoxin IIb (LT-IIb) was produced with plasmid pTDC101-transformed *E. coli* XL-1 Blue (Stratagene, La Jolla, CA) (5). The recombinant *E. coli* was grown at 37°C with vigorous shaking (225 rpm) in Luria broth (Difco Laboratories, Detroit, MI) supplemented with ampicillin (150 μg/ml; Sigma-Aldrich, St. Louis, MO) in the presence of kanamycin (50 μg/ml; Sigma-Aldrich). The expression of LT-IIb was induced during mid-log phase by the addition of 1 mM isopropyl-β-D-thiogalactoside (Sigma-Aldrich). After 4 h of growth, the bacteria were harvested by centrifugation at 8,000 × *g* for 15 min and resuspended in ice-cold 100 mM Tris-HCl (pH 8.0) containing 20% sucrose, 5 mM EDTA, polymyxin B (100 μg/ml; Sigma-Aldrich), and lysozyme (0.5 mg/ml; Sigma-Aldrich) to release the periplasm content. The supernatants were harvested after 30 min of incubation at 4°C and precipitated by 60% ammonium sulfate saturation. The precipitate was dissolved in 10 mM Tris-HCl (pH 8.0) containing 0.3 M NaCl and filter sterilized prior to gel filtration on a Sephacryl-100 column (Amersham Pharmacia Biotech), followed by chromatography with an anion-exchange Mono Q column (Amersham Pharmacia Biotech). The LT-IIb preparations were analyzed for endotoxin content with the *Limulus* amoebocyte lysate assay kit (BioWhittaker, Inc., Walkersville, MD) using an *E. coli* K235 lipopolysaccharide standard.

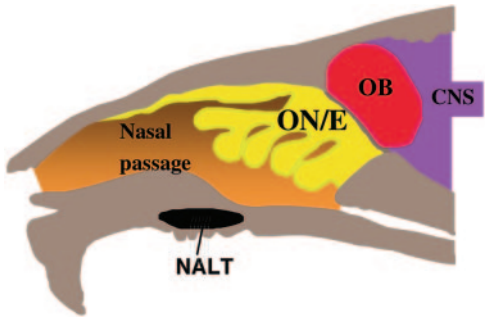

FIG. 1. Anatomy of the murine nasal tract and CNS. Indicated are the locations of the the ON/E, the NALT, and the OB in the nasal tract and adjacent CNS.

Radioiodination of proteins. Tetanus toxoid (TT) (kindly supplied by the Biken Institute, Osaka, Japan) was radiolabeled with ^{125}I. The radioiodination was performed with iodobeads (Pierce Chemicals) for 10 to 12 min at room temperature as described previously (46). Free, unincorporated ^{125}I was removed by dialysis using a Slide Dialyzer (Pierce Chemicals). The trichloroacetic acid-precipitable fraction of ^{125}I-labeled TT was used for all experiments described here. The specific activities of the radiolabeled proteins were 24.5 to 65 cpm/ng. A bicinchoninic acid protein assay (Pierce Chemicals) was used to determine the concentrations of radiolabeled proteins.

Nasal immunization. To assess the ability of ^{125}I-TT to target the ON/E following nasal application, a total of 20 μg of TT (~0.5×10^6 to 1.3×10^6 cpm) was administered in a 10-μl volume, i.e., 5 μl per nare, to naive mice. A total amount of 20 μg of ^{125}I-TT was given either alone or with the indicated enterotoxin delivered in the same volume as antigen alone. For the enterotoxins, we used 1 μg nCT, 10 μg mCT, and 10 μg mLTh-1, and for LTh-1, various amounts of protein were used, i.e., between 1 and 10 μg. For nasal application of the LTII-b enterotoxin, we used 5 μg of protein with ^{125}I-TT.

Trafficking of radiolabeled TT. We used radiolabeled TT protein to track its presence in both lymphoid and central nervous system (CNS) tissues. In these studies, ^{125}I-labeled-TT was given nasally. At 3, 6, 12, 24, and 48 h and 6 days, the ^{125}I-TT levels present in various lymphoid and CNS tissues were determined. For lymphoid tissues, the nasopharyngeal-associated lymphoreticular tissues (NALT), the cervical lymph nodes (CLNs), the mesenteric lymph nodes, the spleen, and blood (50 μl) were assessed. The isolation of NALT was performed as previously reported (49). For the CNS, we examined the ON/E, the olfactory bulbs (OB), and the remainder of the brain. These tissues were isolated as previously described (46). The radiolabeled TT in each tissue was quantitated by use of a gamma counter. The different nasal tract tissues isolated in this study are illustrated in Fig. 1.

In order to assess the influence of blocking the GM1 binding site of LTh-1 or nCT with subsequent tissue distribution of coadministered ^{125}I-TT after nasal application, the LTh-I and nCT were preincubated with a 15-fold molar excess of GM1 (Sigma-Aldrich) for 30 min at 25°C prior to nasal application. The cpm associated with different tissues 12 h after application were analyzed and compared with application without preincubation with GM1. A total of 20 μg of ^{125}I-TT and 5 μg of LTh-1 or 1 μg nCT either with or without preincubation with free GM1 was nasally administered to individual mice.

Sample collection. Blood was collected into heparinized collection tubes by retro-orbital bleeding of anesthetized mice. The plasma was separated from the cells by a 10-min centrifugation step at 10,000 × *g*. Nasal washes were collected by intubation of the trachea to access the nasopharyngeal cavity. This approach was used to avoid any blood contamination of the nasal washes. A total of 200 μl of phosphate-buffered saline (PBS) was inserted into the nasal cavity, and the

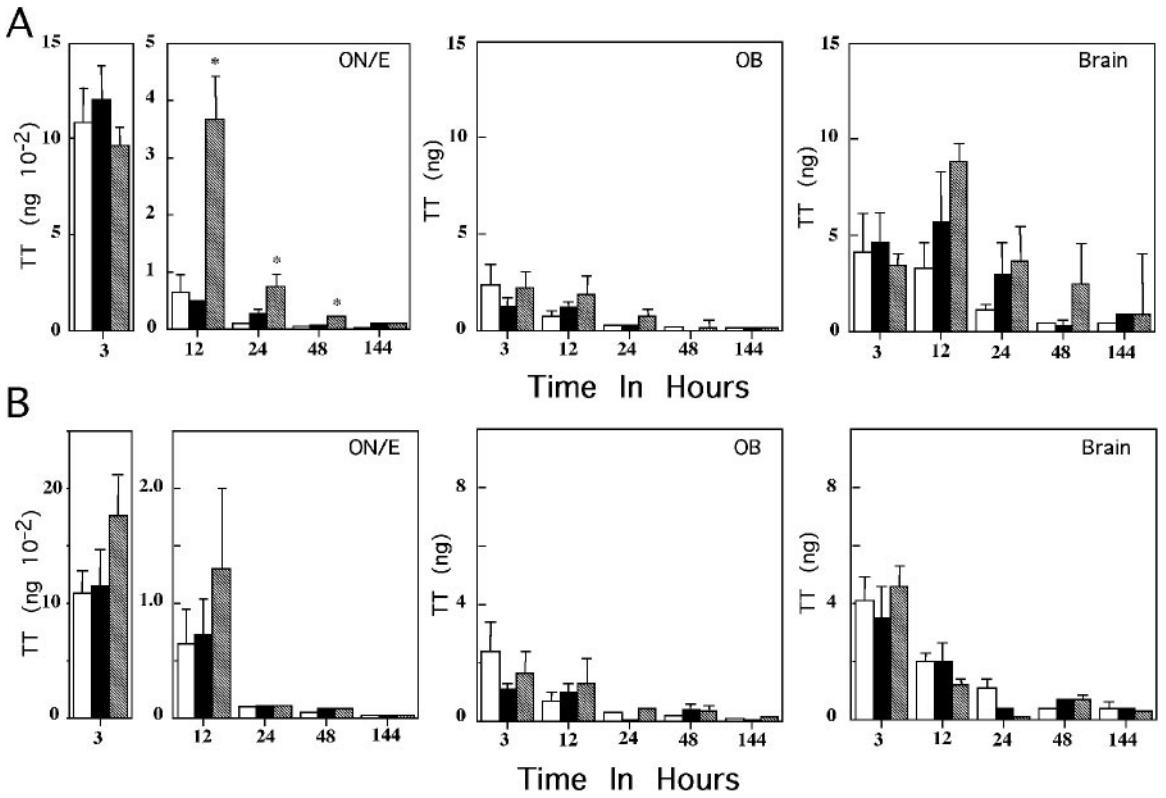

FIG. 2. Comparison of nCT- and mCT- (A) with nLTh-1- and mLTh-1-induced uptake (B) of ^{125}I-TT into olfactory and neuronal tissues. (A) Distribution of ^{125}I-TT in the ON/E, OB, and brain after nasal application of ^{125}I-TT alone (open bars) or in combination with 10 μg of mCT (solid bars) or 1.0 μg nCT (hatched bars) expressed as ^{125}I-TT protein (ng) accumulation. (B) Nasal application of ^{125}I-TT alone (open bars) or in combination with 10 μg of mLTh-1 (solid bars) or 1.0 μg of nLTh-1 (hatched bars) expressed as ^{125}I-TT protein (ng) accumulation. A total of 20 μg of ^{125}I-TT in 12 μl was given nasally either with or without enterotoxin (6 μl/nare). Significant differences between the ^{125}I-TT-only group and ^{125}I-TT-plus-enterotoxin group are indicated by an asterisk and mark *P* values smaller than 0.05. The averages of 4 to 10 mice plus 1 standard error of the mean are depicted.

exudate from the nares was collected in microcentrifuge tubes. Cells and debris were removed by a 10-min 10,000 × *g* centrifugation step. All samples were frozen at −80°C until they were analyzed by enzyme-linked immunosorbent assay (ELISA). Lymphoid and neuronal tissues were isolated as described previously (46).

Cytokine ELISA. The detection of the cytokines IL-6, IL-1β, and tumor necrosis factor alpha (TNF-α) in plasma and nasal washes was performed on Maxisorp 96-well immunoplates (Nunc A/S, Roskilde, Denmark). The plates were coated overnight at 4°C with the following capture monoclonal antibodies: rat anti-mouse IL-6 (clone MP5-20F3; BD PharMingen, San Diego, CA), rat anti-mouse IL-1β (clone 30311.1; R&D systems, Minneapolis, MN), and hamster anti-mouse TNF-α (clone TN3-19.12; BD PharMingen) at 2 μg/ml. The plates were washed with PBS-Tween 20 (0.05%) and blocked with 1% bovine serum albumin in PBS-Tween 20 (0.05%) for 1 hour at room temperature. Subsequently, the samples were added to 96-well plates and incubated overnight at 4°C. The plates were washed with PBS-Tween 20, and the biotinylated capture monoclonal antibodies rat anti-mouse IL-6 (clone MP5-32C11; BD PharMingen), goat anti-mouse IL-1β (R&D Systems), and rabbit anti-mouse TNF-α (BD PharMingen) at concentrations of 0.5 μg/ml, 300 ng/ml, and 0.5 μg/ml, respectively. For detection of IL-1β and TNF-α, streptavidin-conjugated to horseradish peroxidase (Life Technologies Inc., Rockville, MD) was used at a 1:2,000 dilution and anti-biotin-horseradish peroxidase at a 1:2,000 dilution (Vector Laboratories, Burlingame, CA) was used for IL-6. The ELISA plates were washed, followed by a 15-min incubation with 2,2′-azino-bis-(3)-ethylbenzylthiazoline-6-sulfonic acid substrate (Sigma Chemical Co., St. Louis, MO). The absorption at 415 nm was measured at various sample dilutions, and the cytokine levels were determined using standard curves. The detection limits of the ELISA for IL-6, IL-1β, and TNF-α were 10, 1, and 12 pg/ml, respectively.

Statistics. The data are expressed as the mean plus 1 standard error of the mean, and the results were compared by the two-tailed, unpaired Mann-Whitney or Student *t* test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Berkeley, CA) adapted for MacIntosh computers.

RESULTS

Redirection of TT into the ON/E. In order to define the parameters involved in redirection of vaccine proteins into olfactory tissues by nCT and nLTh-1, the role of ADP-ribosyltransferase activity in antigen redistribution was first analyzed by comparison with nonenterotoxigenic mutants of CT (E112K) and LTh-1 (E112K). The presence of ^{125}I-TT in the ON/E, OB, and brain was assessed after nasal application of ^{125}I-TT only or in combination with nCT or mCT (E112K) (Fig. 2A) or with nLTh-1 or mLTh-1 (E112K) (Fig. 2B). The enterotoxin dose was based on the amount required to induce strong mucosal immune responses after nasal application. No significant differences were observed between the distribution of TT protein when given alone or with nCT (1 μg), mCT (10 μg), nLTh-1 (1 μg), or mLTh-1 (10 μg) in all tissues tested, with the exception of the ON/E. Strikingly elevated levels of TT protein were present at 12, 24, and 48 h in the ON/E (P = 0.004, P = 0.028, and P = 0.043, respectively) when given

nasally with nCT. However, minimal uptake was seen at these time points when TT was given alone or with mCT, nLTh-1 (1 μg), or mLTh-1 (Fig. 2A and B). Although the elevated redistribution of ^{125}I-TT into the ON/E induced by nCT was reproducible, it is unlikely that the 48-h time point was of biological importance, since the differences between the groups were relatively small. The nCT-versus-mCT results clearly show that ADP-ribosyltransferase activity is required for redirection of TT into the ON/E. However, it was noteworthy that nLTh-1 (1.0 μg) did not induce significant increases of TT protein in the ON/E at 12 h following nasal application. This TT accumulation was considerably lower than that seen with 1.0 μg of nCT and indicated that factors in addition to ADP-ribosyltransferase activity also play roles in protein redistribution. It should be noted that no preferential accumulation of TT was observed in the OB versus the brain as previously reported for CT-B and CT (46). Thus, unlike CT or CT-B, limited or no axonal transport of TT along olfactory neurons takes place after nasal application, and the distribution of TT in the OB and brain can be explained by the sizes of the organs and the blood associated with them.

Since nCT and nLTh-1 display different ganglioside binding specificities, where nLTh-1 binds in addition to GM1, as reported for nCT and also GD1b, GM2, asialo-GM1, and other intestinal glycoproteins (12, 18, 25), it remained possible that GM1 binding by the enterotoxin was required for protein redirection into the ON/E. Thus, decreased binding by nLTh-1 to GM1 may be due to competition by other nLTh-1 receptors in the nasal tract, which would inhibit protein redirection. In addition, it should be noted that the uptake of TT into various tissues, when 1 μg nLTh-1 was used, was about half of the total uptake observed when 1 μg of nCT was given nasally. To test the GM1 dependence of TT redirection, a dose-response experiment with nLTh-1 was performed (Fig. 3A). Increasing levels of nLTh-1 induced enhanced TT redirection, and this required between a two- and fivefold-higher dose to induce levels not significantly different from those seen with 1 μg of nCT. This observation is consistent with a requirement to target GM1 in order to redirect protein into the ON/E. A comparison between the abilities of nCT, mCT, nLTh-1, and mLTh-1 to redirect protein into ON/E was made (Fig. 3B). In conclusion, nCT was more potent than nLTh-1 in redirecting codelivered protein into the ON/E, while the nontoxic mCT or mLTh-1 was only marginally able to do so at the levels tested.

Distribution of TT in lymphoid tissues. After nasal application of ^{125}I-TT, the NALT, blood, spleen, and CLNs were isolated and analyzed for the distribution of protein when given alone or in combination with nCT, nLTh-1, mCT (E112K), and mLTh-1 (E112K). No significant differences were seen in these lymphoid tissues with the exception of NALT. A significantly decreased accumulation of TT was seen in NALT of mice given TT nasally with nCT (Fig. 4). A small decrease in TT protein accumulation in NALT was observed with mCT, and no significant differences were seen between nLTh-1 and mLTh-1 compared with TT given alone. The data from these observations are summarized in Table 1 and were compared with the observed immune responses induced by these enterotoxin-based mucosal adjuvants. It was interesting that only decreased antigen accumulation in NALT was observed when a strong Th2 response was induced.

The role of gangliosides in protein redirection. In order to assess the role of GM1 ganglioside binding by nLTh-1 and nCT for ^{125}I-TT redirection into the ON/E, the ganglioside binding site was blocked by prior incubation with a 15-fold molar excess of GM1. Blocking the ganglioside binding site of nLTh-1 and nCT with GM1 significantly inhibited redirection of ^{125}I-TT into the ON/E for both 5 μg of nLTh-1 ($P = 0.04$) and 1.0 μg of nCT ($P = 0.02$) 12 h after application (Fig. 5). Blocking the ganglioside binding site also elevated TT levels in the blood, spleen, and CLNs. A significant increase in TT accumulation was observed in the CLNs (which drain the nasal tract) 12 h after application with nLTh-1 (data not shown). Whether this increase of protein in the CLNs was due to a lack of ON/E targeting and resulted in subsequent drainage into the CLNs or was due to enhanced circulation in the blood, or a combination of the two, cannot be distinguished.

In order to determine whether binding to gangliosides other than GM1 would prevent antigen redirection into the ON/E, the heat-labile enterotoxin from serogroup two, LT-IIb, was used. This enterotoxin displays high-affinity binding to GD1a and GT1b and weak affinity for GM3 and does not bind at all to GM1 (12). Furthermore, LT-IIb is more toxic to Y1 adrenal cells than nCT based upon morphological changes and adenylate cyclase activation (24). When the ability of the LT-IIb enterotoxin was compared with that of nLTh-I for redirection of TT into the ON/E, it was very apparent that 5 μg of LT-IIb, unlike nLTh-I, was unable to redirect ^{125}I-TT into the ON/E and did not affect the TT distribution pattern observed in other tissues (Fig. 6). Thus, GM1 targeting appears to be an essential step in directing the ADP-ribosyltranferase to cause codelivered antigen redirection. As pointed out in Fig. 2, the TT associated with the OB was considerably lower than that observed in the brain and reflected the smaller size and lower amount of blood associated with these organs and argues against considerable axonal transport of TT from the nasal tract into the OB as observed with labeled CT and CT-B (46).

Differential production of inflammatory cytokines in the nasal tract. To determine if nasal application of mucosal adjuvants induces inflammatory cytokines, nasal washes and plasma were collected at various time points after nasal delivery. The nasal washes and plasma were analyzed for IL-1β, IL-6, and TNF-α. Differential expression of IL-6 was seen in the nasal washes (Fig. 7). Both nCT- and mCT-treated mice displayed IL-6 levels significantly elevated over those seen when TT was given alone (Fig. 7). Although the levels of IL-6 at 6 h were twofold higher in the nCT- than in the mCT-treated mice, no significant differences were seen between these groups until 12 h after application ($P = 0.026$). Markedly lower levels of IL-6 and IL-1β were seen in the plasma of the mice. The time frame between 3 and 12 h following administration of nCT and TT, when redirection of TT into the ON/E was observed (Fig. 2A), also represented the time when maximal IL-6 secretion was noted in nasal washes. Thus, local inflammatory responses were induced by nCT, and to a much lesser extent by mCT, during this time period. No detectable levels of TNF-α were observed in either plasma or nasal washes (data not shown), and IL-1β levels did not differ significantly among the three groups. These differences in production of IL-6 were not due to the differences in lipopolysaccharide, since the nCT contained ≤0.048 ng/μg and the mCT contained ≤1.0 ng/10

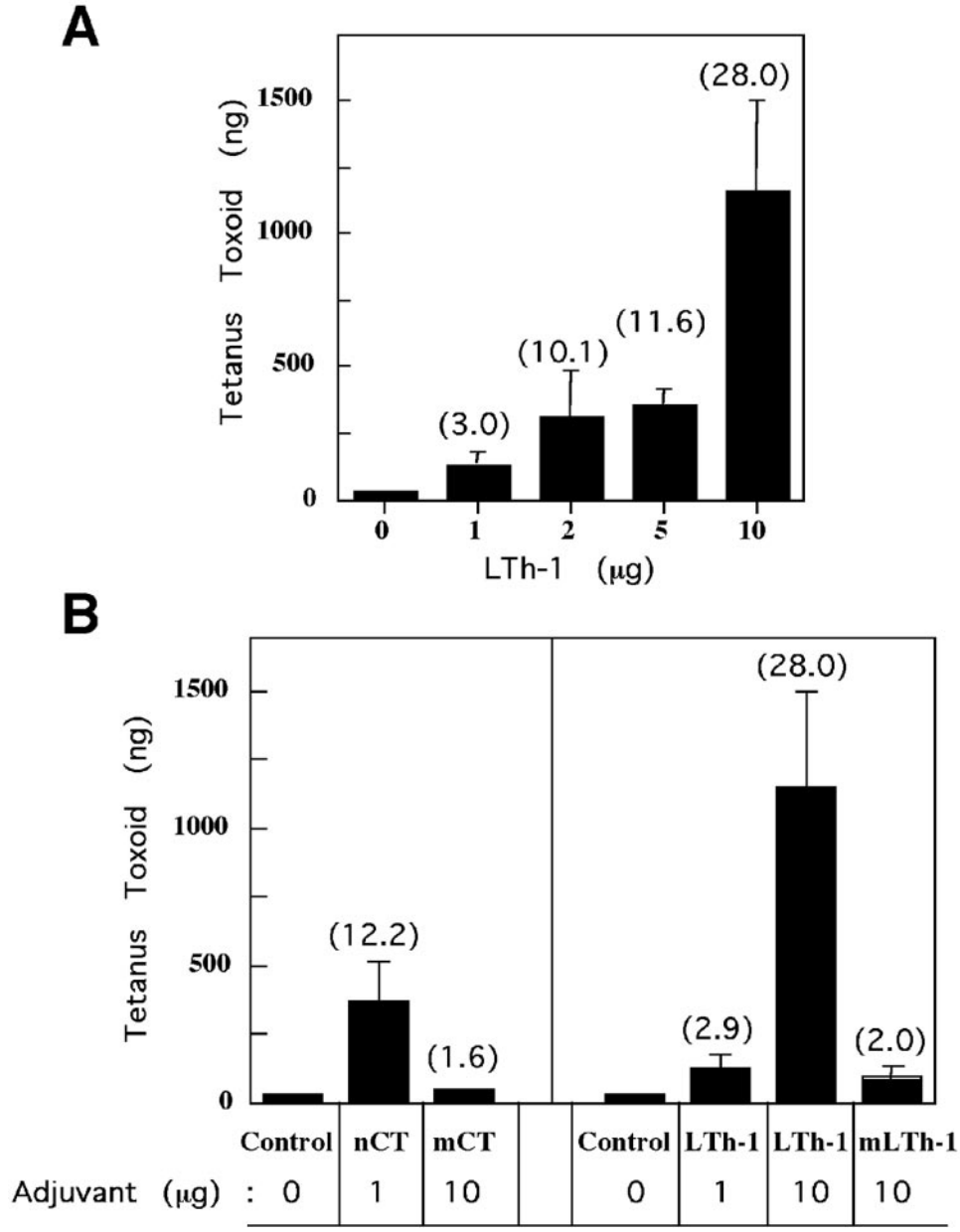

FIG. 3. Enterotoxin-dependent redirection of ^{125}I-TT into the ON/E. Various doses of nLTh-1, i.e., 0, 1, 2, 5, and 10 μg, were combined with 20 μg ^{125}I-TT and applied nasally. The ON/E were collected 12 h after application to assess redirection of the protein. (A) Indicated is the increase (*n*-fold) over control values, i.e., ^{125}I-TT alone, when administered with nLTh-I. (B) Comparison of the degrees of redirection of ^{125}I-TT into ON/E observed with nCT, mCT (E112K), nLTh-1, and mLTh-1 (E112K). Indicated are the means plus standard errors of the mean.

μg. The observations for IL-6 were confirmed by real-time reverse transcription-PCR on RNA derived from the ON/E (data not shown).

To determine whether LTh-1 had similar effects on IL-1β and IL-6 production, nasal washes and plasma were collected from mice treated 12 h prior with TT alone or TT with 1.0 or 5.0 μg of LTh-1. A significant increase in IL-6 was seen in nasal washes when 5 μg of LTh-1 was given with TT, while no significant increase was observed in plasma (Fig. 8). Elevated secretion of IL-6 was also seen in nasal washes with the 1.0-μg LTh-1 dose; however, this increase in IL-6 was not significant compared with TT alone.

DISCUSSION

The redirection of TT protein by native enterotoxin-based mucosal adjuvants raises questions regarding both safety and the molecular mechanisms involved. In this study, we addressed the parameters influencing redirection of the vaccine protein TT into the olfactory nerve/epithelium, NALT, and related lymphoid tissues, as well as the associated production of inflammatory cytokines in the nasal tract. To redirect nasally coadministered ^{125}I-TT into the ON/E by enterotoxin-based mucosal adjuvants, ADP-ribosyltransferase activity is clearly required. This is based upon the finding that both nCT and

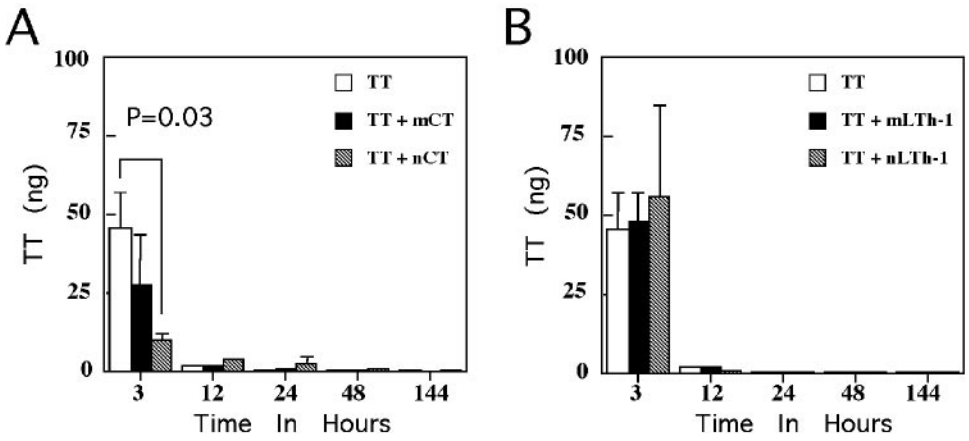

FIG. 4. Comparison of nCT- and nLTh-1- with mCT (E112K)- and mLTh-1 (E112K)-induced uptake of ^{125}I-TT into NALT. Distribution of ^{125}I-TT in NALT is expressed as TT (ng) accumulation per organ. A total of 20 μg of ^{125}I-TT alone or in combination with 10 μg of mCT or 1.0 μg of nCT (A) or ^{125}I-TT alone or in combination with 10 μg of mLTh-1 or 1.0 μg nLTh-1 (B) was given nasally (6 μl/nare). Accumulation of ^{125}I-TT was analyzed at various time points after application. The average of 5 to 10 mice plus standard error of the mean per data point are depicted.

nLTh-1 redirected protein into the ON/E while mCT (E112K) and mLTh-1 (E112K), which both lack ADP-ribosyltransferase activity (28, 54), did not. Thus, ADP-ribosyltransferase activity of the A subunit is an essential, although not sufficient, element for redirection of protein into the ON/E by AB_5 enterotoxins. Binding to GM1 by the B subunit, in addition to ADP-ribosyltransferase activity of the A subunit, also appears to be a prerequisite for redirection of protein into the ON/E, since incubation of nCT or nLTh-1 with excess GM1 prior to administration prevented accumulation of ^{125}I-TT in the ON/E.

The finding that LT-IIb does not redirect TT into the ON/E while it retains full ADP-ribosyltransferase activity and functions as a mucosal adjuvant when delivered nasally (34) may be explained by a requirement for GM1 binding by the enterotoxin to induce protein redirection, since LT-IIb, unlike nCT or nLTh-1, does not bind to GM1 gangliosides (12, 38). Using a human intestinal epithelial cell line (T84), others found that both nCT and LT-IIb bound with high affinity (2 to 5 μM) to the apical membranes of T84 cells (48). However, only nCT was able to elicit a cyclic-AMP-dependent secretory response. Moreover, while nCT-GM1 fractionated with a caveola-like, detergent-insoluble membrane fraction, the LT-IIb-GD1a complex was solubilized by 1% Triton X-100. The authors suggested that signal transduction may require the formation of caveola-like structures and demonstrated that the chimera composed of the LT-IIb A subunit and CT-B was capable of inducing a secretory response. Native CT binding to polarized epithelial cells takes place on the apical membrane surface but targets a basolaterally located effector molecule, i.e., adenylate cyclase (31). It could be hypothesized that the requirement to bind GM1 is associated with the endocytotic pathway taken following GM1 endocytosis. The LT-IIb binds to GD1a, a ganglioside that is not located in the caveola-like membrane domains as reported for GM1 (48), and may thus follow a different intracellular path in epithelial cells. As a consequence of this, the A1 subunits or the ADP-ribose-Gsα may not reach the adenylate cyclase located in the basolateral domain of polarized epithelial cells. This could be an important step for enhanced permeability of the epithelium and the ability of antigen to cross the nasal epithelial barrier.

The nasal tract is covered by a pseudostratified epithelium. Underneath this epithelium, a dense network of fenestrated capillaries provides a readily available blood supply (14). The nasal administration of enterotoxin-based adjuvants would target this epithelium through GM1 ganglioside binding. Our observations and those of others reporting the use of rabbit mucosa in vitro (14) clearly raise questions regarding the safety of nasal use of these adjuvants in humans. Human studies performed previously using nasal nLTh-1 and CT-B suggest that precautions need to be taken when applying these mole-

TABLE 1. Antigen-specific immune response and antigen distribution in NALT following nasal immunization

Adjuvant	Antigen	Plasma IgG[a]	S-IgA[b]	T helper activity	TT in NALT[c]	Reference(s)
	TT	+[d]	+	Th1/Th2	++++	30, 47, 51
nCT	TT	++++	+++	Th2	+	47, 50, 51
mCT(E112K)	TT	++++	+++	Th2	++	50, 51
nLTh-1	TT	++++	+++	Th1/Th2	++++	2, 7, 42
mLTh-1(E112K)	TT	++++	+++	Th1/Th2	++++	2, 7
LTIIb	AgI/II[e]	++++	+++	Th1/Th2	++++	34

[a] IgG, immunoglobulin G.
[b] S-IgA, mucosally-derived secretory IgA.
[c] Data were obtained in this study and indicate the relative TT antigen accumulation in NALT.
[d] +, low-, ++, medium-; +++, high-; ++++, very high.
[e] AgI/II, Antigen I/II from *Streptococcus mutants*.

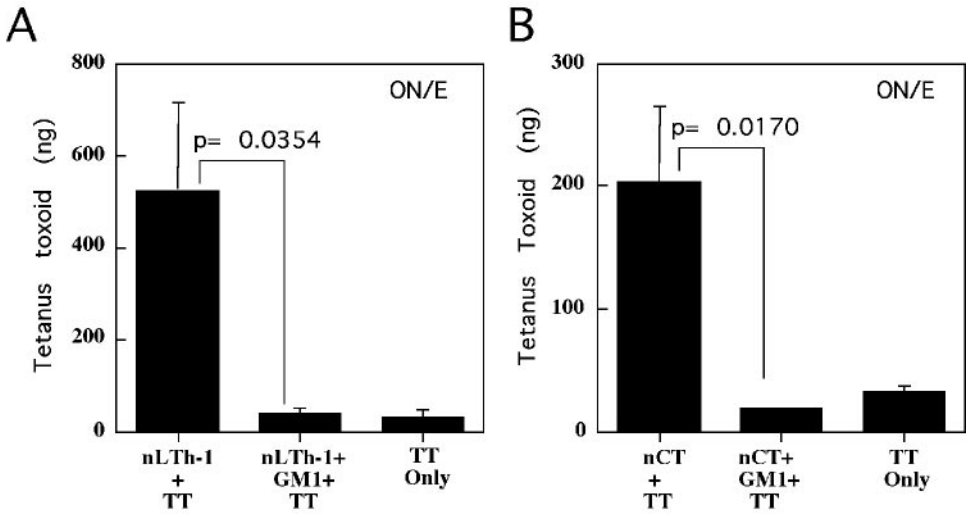

FIG. 5. Influence of blocking the GM1 binding site on nLTh-1 and nCT on tissue distribution of coadministered ^{125}I-TT in the ON/E after nasal application. nLTh-I (A) and nCT (B) were preincubated with a 15-fold molar excess of GM1 for 30 min at room temperature prior to nasal application together with ^{125}I-TT. The cpm associated with the ON/E 12 h after application were analyzed and compared with application without preincubation with GM1 and with administration of ^{125}I-TT antigen alone. A total of 20 μg ^{125}I-TT with 5 μg of nLTh-1 or 1.0 μg of nCT was nasally delivered to individual mice. The results are from five mice per group. Indicated are the means plus standard errors of the mean.

cules to the human nasal tract. Human studies involving nasal application of CT-B reported mild adverse effects, which resolved within 24 h (1, 39–41). The tolerated and effective dose was between 100 and 500 μg for nasally applied CT-B in a nasal spray/aerosol. At the lower end of an effective immunization range, i.e., 100 μg of CT-B, 7 out of 20 patients and all in the high range (the 1,000-μg group), experienced adverse effects. The symptoms consisted of profuse nasal secretions, itching, and violent sneezing, which resolved within 1 day (1). Although the use of a high-dose CT-B is different from our toxin-mediated antigen redirection, it could be argued that high doses of CT-B will accumulate in the olfactory neuroepithelium, as has been reported for mice (46), and consequently would lead to induction of proinflammatory cytokines throughout the nasal tract.

The results with nasally applied CT-B in humans are consistent with our observations that high levels of IL-6, and to a lesser extent IL-1β, are present in the nasal tract during the first 24 h after application. Interleukin 6 was expressed during the peak of TT protein redirection into the ON/E, and at 12 h was significantly higher in nCT-treated mice than in mice treated with mCT E112K. Furthermore, both enterotoxins induced significantly higher IL-6 levels in nasal washes than were seen in mice given TT only. IL-6 is a multifunctional cytokine that influences both innate immune reactions, such as inflammation (36) and acute-phase responses, and specific immunity, such as B-cell differentiation. IL-6 is produced by a variety of cells, including epithelial cells, macrophages, fibroblasts, and T cells. Native CT rapidly induces IL-6 secretion by the rat intestinal epithelial cell line IEC-6 (35). Whether IL-6 plays a

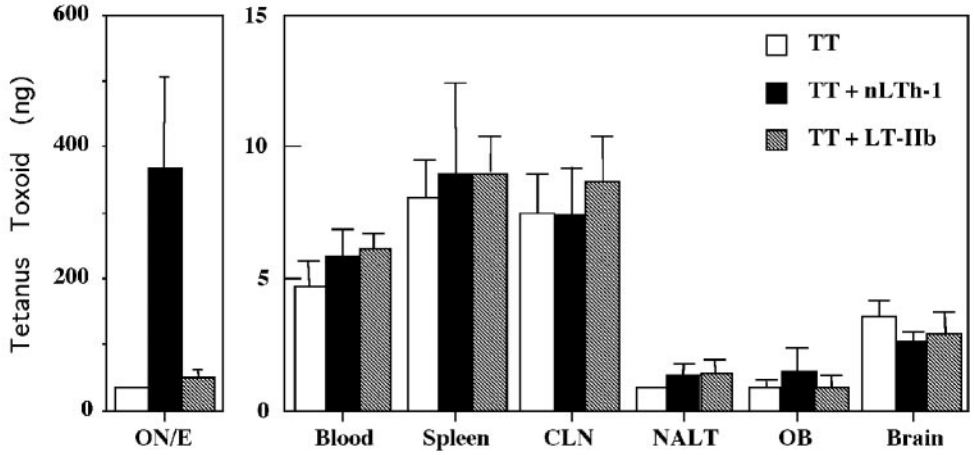

FIG. 6. Trafficking of ^{125}I-TT (20 μg) given nasally without or with nLTh-1 (5 μg) or LT-IIb (5 μg) as mucosal adjuvant. The uptake of ^{125}I-TT into the ON/E, OB, brain, NALT, CLNs, blood, and spleen is shown 12 h after nasal application. The results depicted are from five mice/group and are representative of three separate experiments. Indicated are the means plus standard errors of the mean.

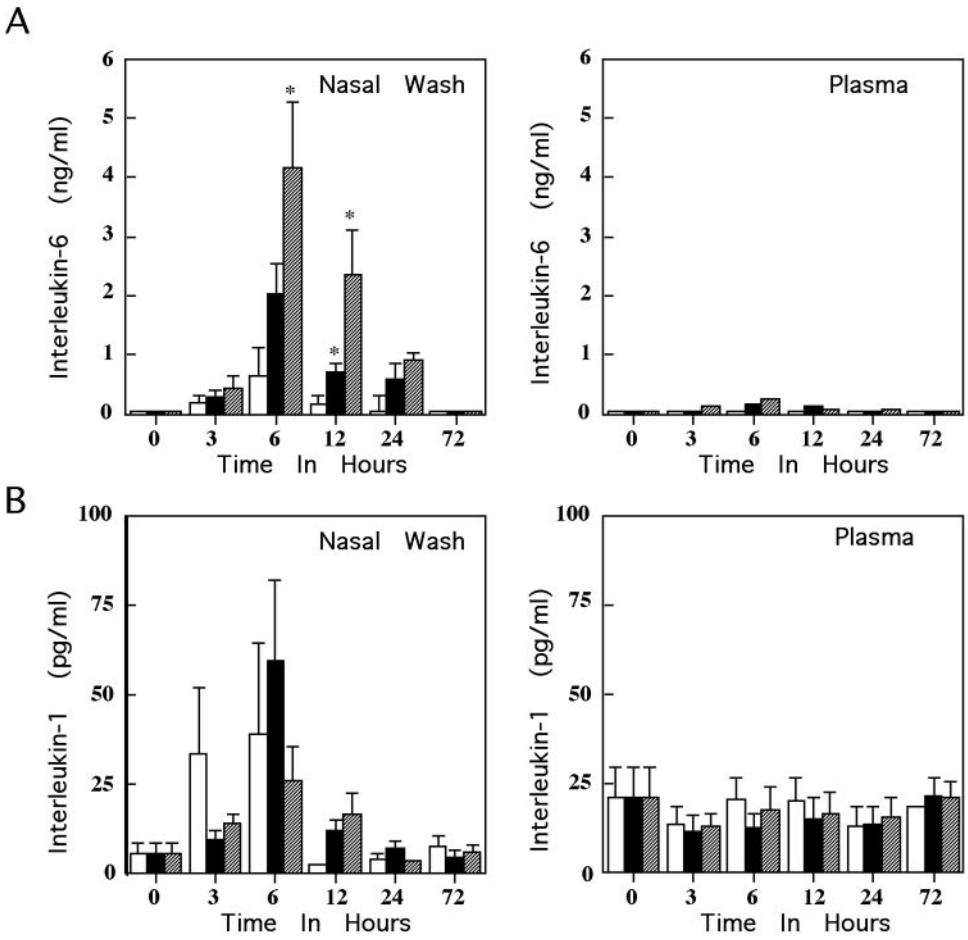

FIG. 7. Inflammatory cytokine expression in the nasal tract after nasal application of TT with or without mCT or nCT. Nasal washes were collected at 0, 3, 6, 12, and 24 h after nasal application of TT alone (open bars), TT and mCT (solid bars), or TT and nCT (hatched bars). The inflammatory cytokine levels for IL-6 (A), IL-1β (B), and TNF-α were determined by ELISA. No TNF-α was detected in the nasal washes. Indicated are the means plus standard errors of the mean for IL-6 and IL-1β. The asterisks indicate significantly elevated cytokine levels ($P < 0.05$) when nCT or mCT with TT was compared with TT alone. The results are representative of two separate experiments.

role in antigen redirection remains to be determined; however, it is more likely that multiple factors contribute to antigen redirection into the ON/E. Specifically, neuropeptides could be major players in this process. For example, vasoactive intestinal peptide (VIP) plays an important role in fluid accumulation in the rat jejunum following stimulation with nCT or nLTh-1 (27). Furthermore, CT-B seems to specifically target VIP-containing neurons in the intestinal tract (15). These observations indicate that VIP could also be important for nasal reactogenicity and antigen redirection.

Human studies involving nasal application of CT-B have focused on the induced immune responses to CT-B rather than on its properties as a mucosal adjuvant (1, 39–41). However, the adjuvant properties of nLTh-1 were assessed in humans given two nasal applications a week apart with an aerosolized virosome-formulated influenza vaccine containing 1.0 μg or 2.0 μg of nLTh-1 for induction of influenza virus-specific immune responses (16, 17). The nLTh-1 functioned as a mucosal adjuvant in humans and induced influenza virus-specific immune responses; however, about 50% of the subjects experienced some type of local or systemic adverse reaction. These reactions included rhinorrhea, stuffiness, sneezing, and headaches, but most of them were mild and resolved within 48 h (16). Similar reactions have also been reported with nasal influenza virus vaccine given with 99.5 μg LT-B and 0.5 μg nLTh-1 (23). The results are consistent with our observations with nCT and nLTh-1 in that redirection of coadministered antigen into the ON/E and production of inflammatory cytokines resolved within 48 h, indicating that similar events could have taken place in humans.

Despite the similarities between mice and humans, the relative surfaces of the nasal tract that constitute the olfactory epithelium are quite different. In mice, approximately 45% of the nasal tract surface constitutes olfactory epithelium (19), while in humans it is an estimated 2.5 cm^2 which would translate to ~2 to 3% of the nasal surface (13, 22). Thus, in mice, nasal application is probably more likely to target olfactory neurons than in humans. Furthermore, the nasopharynx has a 90° angle in humans while there is only a 15° angle in mice. Due to the much larger volume of the human nasopharynx (20 ml) than the mouse nasopharynx (30 μl) and the larger angle, it is likely much harder to consistently target the olfactory

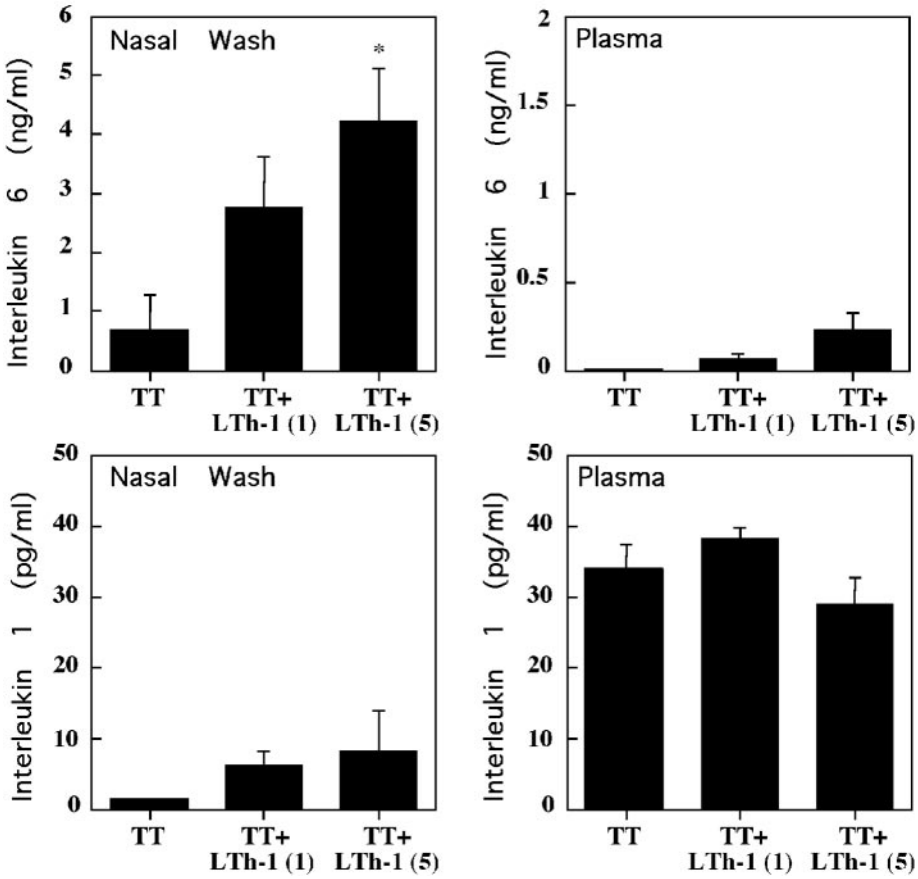

FIG. 8. Inflammatory cytokine expression in the nasal tract after nasal application of TT with or without LTh-1. Nasal washes and plasma were collected 12 h after nasal application of TT (10 μg) alone, TT and LTh-1 (1) (1.0 μg), or TT and LTh-1 (5) (5.0 μg). The levels of IL-6 and IL-1β in nasal washes and plasma were measured by ELISA. Indicated are the means plus standard errors of the mean for IL-6 and IL-1β of five mice per group. The asterisk indicates significantly elevated cytokine levels ($P < 0.05$) when LTh-1 given with TT was compared with TT given alone. The results are representative of three separate experiments.

epithelium in humans using nasal drops, while in mice this would be very reproducible (13). It could be argued that because of the above-outlined reasons nasal sprays would more consistently target the olfactory epithelium in humans than nasal drops.

The observation that nCT significantly reduces TT accumulation in NALT 3 h after nasal application compared to TT alone or TT plus nLTh-1 (Fig. 4) is interesting from the perspective that exposure to a low dose of soluble protein is associated with induction of a Th2-type T helper cell response (6, 20, 37, 47). The induction of potent Th2-type helper activity specific for antigens codelivered with nCT (33, 50) or mCT (30, 51, 53, 54) and the induction of a mixed Th1/Th2 response to antigen coadministered with nLTh-1 (2, 7, 42), mLTh-1 (2, 7), or LTIIb (34) coincide with decreased antigen accumulation in NALT with a strong Th2 response but not with the mixed Th1/Th2 response (Table 1). For example, antigen accumulation in NALT is approximately sixfold lower with nCT than with nLTh-1. It will be interesting to see in future studies whether this altered antigen level will translate into an altered cytokine environment in the NALT for induction of a TT-specific immune response.

In summary, the redirection of a vaccine protein into the olfactory tissues by enterotoxin-based mucosal adjuvants following nasal administration is associated with reactogenicity in the nasal mucosa. The differential accumulation of TT protein in NALT when administered with nCT or nLTh-1 may have consequences for the induced TT-specific T helper cell responses. The parameters controlling antigen redirection into the ON/E include ADP-ribosyltransferase activity of the A subunit and GM1 ganglioside binding by the B subunit. Thus, redirection of vaccine antigen into the ON/E by enterotoxin-based mucosal adjuvants, such as nCT and nLTh-1, clearly requires both ADP-ribosyltransferase activity and targeting of GM1 gangliosides.

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