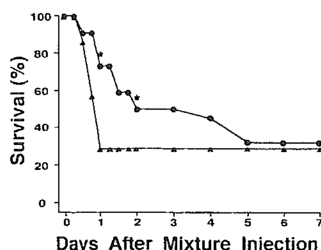

FIGURE 6. BoNT/A-specific S-IgA Abs in fecal extracts of mice given nasal vaccine neutralize BoNT/A toxicity. Fecal extracts (200 μl) from mice given nasal BoNToxoid/A (●) or OVA (▲) plus mCT E112K were incubated with 1 × i.p. LD_{50} of BoNT/A in 0.2 ml of PBS including 0.2% gelatin for 30 min at room temperature. Each mixture was then injected into mice via the i.p. route. Each group consisted of nine mice, and the data are representative of two separate experiments. The asterisks indicate a significant difference between the groups; *, $p < 0.01$.

can prevent mucosal BoNT intoxication as well as provide systemic immune protection comparable to that offered by the two current vaccines.

Our study provides the first evidence that nasal administration of BoNToxoid/A plus mCT E112K effectively induces BoNT/A-specific S-IgA Ab responses in addition to plasma IgG Abs. Thus, Ag-specific Ab responses were seen in plasma and mucosal secretions, while Ag-specific AFCs were detected in the NPs, the iLP, and the spleen, clearly showing that both mucosal and systemic immunity were induced in mice given the nasal BoNT vaccine. In this study, we have developed a unique oral BoNT/A challenge model that allows for investigation of protective mucosal S-IgA Ab responses. With this novel method, the levels of BoNT required for intoxication can be quantitated.

In showing that mice given a nasal BoNToxoid with mCT as mucosal adjuvant were protected from an oral challenge dose of 2 × LD_{50} BoNT/A, this study provided the first evidence that BoNT/A-specific S-IgA Abs play an important role in protecting against mucosally delivered BoNT/A. Furthermore, our study has also shown that BoNT/A-specific IgG Ab responses in plasma of mice given nasal vaccine also provide effective protection against i.p. BoNT/A intoxication. These results clearly demonstrate that this nasal BoNT/A vaccine establishes two layers of immune protection, one at the mucosal surfaces themselves and the second in the blood circulation via protective IgG Abs.

To assess the potential of mucosal vaccines, studies have relied on the oral delivery of mutant BoNT/B and on the nasal administration of the whole H chain component of BoNT/A. Although these reports showed successful induction of BoNT-specific Ab responses, they limited themselves to an examination of the IgG Ab isotype and did not investigate a role for S-IgA Ab responses in BoNT intoxication (39, 40). In contrast, our study has shown that BoNT/A-specific S-IgA Ab responses were induced in various mucosal secretions when mice were given a nasal BoNT/A vaccine. Indeed, like Ag-specific neutralizing plasma IgG Abs, BoNT/A-specific S-IgA Abs in fecal extract samples neutralized biologic activity and prevented BoNT/A intoxication. Furthermore, mice possessing high levels of BoNT/A-specific S-IgA Abs were completely protected against oral challenge with BoNT/A. These results indicate that intestinal mucosal BoNT/A-specific Abs, which are predominantly of the S-IgA isotype, play an important role in the prevention of GI tract botulism. To directly confirm the role of BoNT-specific S-IgA Abs in BoNT/A intoxication, we are currently testing the efficacy of nasal BoNT/A vaccine in both IgA-deficient and polymeric Ig receptor-deficient mice.

It is not enough, however, to consider the effects of BoNT on the GI tract. Because bioterrorists are more likely to disseminate BoNT by air than by food or water, it is of paramount importance to protect again nasal BoNT intoxication. For an example of terrorists' predilection for an airborne method of delivery, one need go no farther than the failed attempt by the Japanese cult Aum Shinrikyo to disseminate BoNT in downtown Tokyo (37).

The immunopathologic threat posed by nasal BoNT intoxication may be of a different order than those posed by orally ingested BoNT, because the nasal cavity is directly connected with the CNS through the olfactory nerves and epithelium as well as the olfactory bulbs. These neuronal tissues express an abundance of different types of gangliosides, including GD1a and GT1b, which are potent binding receptors for the BoNT/B serotype (41). The other type of ganglioside-binding toxins, such as CT and heat LT of *Escherichia coli*, has been shown to cause significant neuronal tissue damage when administered via the nasal route (42). Such a prospect of neuronal tissue damage underlines once again how imperative it is to devise a novel mucosal vaccine for the prevention of nasal BoNT intoxication. Our current study has shown that nasal BoNToxoid/A plus mCT E112K induces significant levels of BoNT/A-specific S-IgA and IgG Ab responses in nasal washes, indicating that this nasal vaccine protocol provides potent protection against nasal BoNT intoxication as well as neuronal tissue damage. We are currently developing a nasal BoNT challenge system to examine the efficacy of this mucosal BoNT vaccine and to assess any potential BoNT toxicity for the olfactory tissues and the CNS.

Although the nasal vaccine regimen helped induce two layers of Ag-specific responses in mucosal and systemic lymphoid tissues, it succeeded in doing so only with the aid of a mucosal adjuvant. Concern has been expressed that the potent toxicity of toxin-based mucosal adjuvants such as CT and LT may induce CNS damage (42). To circumvent this problem, both current and our previous studies have examined whether nontoxic mCTs as well as cytokines and chemokines provide mucosal adjuvanticity. These studies have clearly shown that nasal application of cytokines or chemokines as mucosal adjuvants together with selected Ags induces Ag-specific Ab responses in both systemic and mucosal compartments (5, 10–12, 43–47). Although the toxicity of these cytokines and chemokines needs to be elucidated, our most recent studies show that nasal mCT E112K does not elicit nerve growth factor-β1 induction indicative of neuronal tissue damage in the CNS of nonhuman primates (48). Thus, we feel confident that our current approach may yield a nasal vaccine able to generate effective immunity against botulism.

Ag-specific plasma IgG subclass Ab responses are excellent indicators of Th1- or Th2-type cytokine responses by $CD4^+$ T cells. Our results showed significantly increased levels of IgG1, IgG2a, and IgG2b Ab responses in plasma of mice given nasal BoNToxoid/A plus mCT E112K when compared with those mice given nasal toxoid only. Among these elevated IgG subclass Abs, levels of anti-BoNT/A IgG1 and IgG2b Abs were higher than the IgG2a subclass Ab response. A similar pattern of Ag-specific IgG subclass responses has been seen in mice given native CT or mCT as nasal adjuvants, which are known to induce Th2-type cytokine responses (3, 4). Thus, we anticipate that BoNT/A-specific Ab responses induced by nasal BoNToxoid plus mCT E112K are mediated through Th2-type cytokine responses. To our knowledge, no studies have reported potential roles of Th1- and Th2-type responses by BoNT-specific $CD4^+$ T cells. To this end, our studies are the first to suggest that BoNT/A-specific and dominant Th2-type responses are associated with protective immunity against botulism. More precise and direct studies of BoNT vaccine-induced Th1 and Th2 cytokine responses are currently underway in our laboratory.

In summary, our studies have now provided the first evidence that nasal immunization with BoNToxoid/A plus mCT E112K induces BoNT/A-specific S-IgA Ab responses in mucosal secretions as well as plasma IgG Ab responses. Furthermore, mice given nasal BoNT/A vaccine were protected from both parenteral and oral challenge with BoNT/A. These results directly demonstrate that mucosal immunization is able to provide two layers of immunity, a first line of defense at mucosal surfaces and a second line via systemic blood circulation. The precise roles of BoNT-specific S-IgA Abs will require further investigation; however, our findings in this study show that this BoNT-based mucosal vaccine is an effective and perhaps essential strategy for the protection of the population against botulism used as a weapon of bioterrorists.

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Nontoxic Shiga Toxin Derivatives from *Escherichia coli* Possess Adjuvant Activity for the Augmentation of Antigen-Specific Immune Responses via Dendritic Cell Activation

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Shiga toxin (Stx) derivatives, such as the Stx1 B subunit (StxB1), which mediates toxin binding to the membrane, and mutant Stx1 (mStx1), which is a nontoxic doubly mutated Stx1 harboring amino acid substitutions in the A subunit, possess adjuvant activity via the activation of dendritic cells (DCs). Our results showed that StxB1 and mStx1, but not native Stx1 (nStx1), resulted in enhanced expression of CD86, CD40, and major histocompatibility complex (MHC) class II molecules and, to some extent, also enhanced the expression of CD80 on bone marrow-derived DCs. StxB1-treated DCs exhibited an increase in tumor necrosis factor alpha and interleukin-12 (IL-12) production, a stimulation of DO11.10 T-cell proliferation, and the production of both Th1 and Th2 cytokines, including gamma interferon (IFN-γ), IL-4, IL-5, IL-6, and IL-10. When mice were given StxB1 subcutaneously, the levels of CD80, CD86, and CD40, as well as MHC class II expression by splenic DCs, were enhanced. The subcutaneous immunization of mice with ovalbumin (OVA) plus mStx1 or StxB1 induced high titers of OVA-specific immunoglobulin M (IgM), IgG1, and IgG2a in serum. OVA-specific CD4$^+$ T cells isolated from mice immunized with OVA plus mStx1 or StxB1 produced IFN-γ, IL-4, IL-5, IL-6, and IL-10, indicating that mStx1 and StxB1 elicit both Th1- and Th2-type responses. Importantly, mice immunized subcutaneously with tetanus toxoid plus mStx1 or StxB1 were protected from a lethal challenge with tetanus toxin. These results suggest that nontoxic Stx derivatives, including both StxB1 and mStx1, could be effective adjuvants for the induction of mixed Th-type CD4$^+$ T-cell-mediated antigen-specific antibody responses via the activation of DCs.

For the design of effective vaccines in the areas of immunology and infectious diseases, a primary focus of research is the development of effective and safe adjuvants, which instruct and control the selective induction of the appropriate type of antigen-specific immune response. Thus far, several bacterial enterotoxins, including the cholera toxin (CT) of *Vibrio cholerae* and the heat-labile enterotoxin (LT) of enterotoxigenic *Escherichia coli*, are known to be potentially strong adjuvants when given by the oral, nasal, or parenteral route (7, 9, 18, 52). As early as 1972, it was reported that CT acts as an adjuvant for antibody responses following intravenous administration (32). The mucosal administration of CT was shown to elicit antigen (Ag)-specific Th2-type CD4$^+$ T-cell responses via high levels of interleukin-4 (IL-4) and IL-5 production, which in turn enhanced Ag-specific systemic immunoglobulin G1 (IgG1) and IgE and mucosal secretory IgA responses (28). In contrast, LT was shown to induce mixed CD4$^+$ Th1- and Th2-type cells producing gamma interferon (IFN-γ), IL-4, IL-5, IL-6, and IL-10, with subsequent serum IgG1 and IgG2a and mucosal secretory IgA responses (47). Other bacterial toxins, such as pertussis toxin and a genetically detoxified derivative of pertussis toxin, PT-9K/129G, have also been shown to possess mucosal adjuvant activities (3, 11, 36). Pertussis toxin potentiates Th1 and Th2 responses to a coadministered antigen (37). The administration of a chimeric molecule composed of the gp120 V3 loop region of the MN strain of human immunodeficiency virus type 1 (HIV-1) and a nontoxic form of *Pseudomonas* exotoxin resulted in strong antigen-specific immune responses to an integrated HIV Ag (30).

It is interesting that in the case of Shiga toxin (Stx), oral administration confers immunogenicity but not adjuvanticity (43). Stx is produced by Stx-producing *E. coli* and is one of the major virulence factors for infectious diseases by Stx-producing *E. coli*. Stx is a holotoxin composed of an approximately 32-kDa A subunit in noncovalent association with a pentameric ring of identical nontoxic B subunits, each of which has a

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molecular mass of 7.7 kDa. The A subunit is the enzymatic component of the toxin and acts as a highly specific *N*-glycosidase enzyme, hydrolyzing the bond between ribose and a single adenine residue found on a prominent loop structure in the 28S rRNA component of eukaryotic ribosomes (10, 39). The B subunits mediate toxin binding to the membrane-neutral glycolipids glogotriaosylceramid and globotetraosylceramid (38). Stx toxins are classified into the following two groups: Stx1, which is identical to Shiga toxin at the amino acid sequence level, and Stx2, which is immunologically different from Stx1 (42).

In previous studies, we generated E167Q/R170L (mStx1), a double mutant of Stx1 which harbors amino acid substitutions in the RNA *N*-glycosidase active center which were derived by site-directed mutagenesis. mStx1 lacks RNA *N*-glycosidase activity, cytotoxicity, and mouse lethality (33). For the present study, we assessed the capability of mStx1 and StxB1 to provide activation signals to dendritic cells (DCs) and T cells and then addressed the issue of whether this capability of mStx1 and StxB1 is connected to in vivo adjuvanticity when these molecules are subcutaneously coadministered with a protein antigen. The results obtained in this study suggest that both mStx1 and StxB1 act as effective adjuvants for the induction of Ag-specific antibody (Ab) responses via DC activation.

MATERIALS AND METHODS

Mice. C57BL/6 and BALB/c mice were purchased from SLC (Shizuoka, Japan) and were maintained and bred in the experimental animal facilities of Niigata University Graduate School of Medical and Dental Science, the Research Institute for Microbial Diseases, Osaka University, and the Institute of Medical Science, University of Tokyo, under pathogen-free conditions using microisolator cages. DO11.10 T-cell receptor (TCR)-transgenic mice, which recognize the OVA peptide 323-329 in association with I-A^d (31), were kindly provided by Kazuhiko Yamamoto (University of Tokyo, Tokyo, Japan). All mice were provided sterile food and water ad libitum and were maintained in our experimental animal facility. C57BL/6 and BALB/c mice of 8 to 12 weeks of age and DO11.10 Tg mice of 5 to 12 weeks of age were used for this study.

Bacterial toxins. A mutant of Stx1 (mStx1) and native Stx1 (nStx1) were purified from *E. coli* MC 1061 strains M 23 and 87-27, respectively, according to a previously described method (14, 33). Purification steps included ion-exchange chromatography, chromatofocusing, and high-performance liquid chromatography as described previously (14). The B subunit of Stx1 (StxB1) was derived from *Bacillus brevis* pNU212-VT1B and was purified by the use of ion-exchange chromatography and gel filtration (5).

The amounts of endotoxin in the toxin preparations were measured with an Endospec-SP test (Seikagaku Co., Tokyo, Japan). The nStx1, mStx1, and StxB1 preparations contained 7.03 pg, 34.0 pg, and 3.05 pg of lipopolysaccharide (LPS) per 10 μg of protein, respectively. These ranges of LPS contamination have been shown to be ineffective for the stimulation of lymphoid cells (22, 50).

Culture conditions, treatment of BMDCs in vitro, and treatment of BMDCs with Stx1 derivatives. For the generation of bone marrow-derived DCs (BMDCs), male C57BL/6 or BALB/c mice were sacrificed, and their bone marrow was isolated and then flushed from the femur and tibia (12). Erythrocytes were depleted with ammonium chloride. DCs were generated from bone marrow precursors as described previously (12). Following 6 days of incubation in the presence of an optimal dose of granulocyte-macrophage colony-stimulating factor (10 ng/ml), nonadherent cells were collected and used as a source of BMDCs.

BMDCs were cultured at 5 × 10^5 cells/ml in 24-well plates (Corning, Inc., Corning, N.Y.) in culture medium containing granulocyte-macrophage colony-stimulating factor (10 ng/ml) (12) in the presence or absence of an optimal dose of a Stx1 derivative (1 μg/ml) for 48 h at 37°C. Culture supernatants were collected and frozen at −70°C until assayed for the synthesis of cytokines, including tumor necrosis factor alpha (TNF-α) and IL-12 p70, by enzyme-linked immunosorbent assays (ELISAs) (AN'LYZA immunoassay kit; R&D Systems, Minneapolis, Minn.).

Fluorescence-activated cell sorting analysis. BMDCs were analyzed 48 h after treatment with a variety of toxin derivatives since a preliminary time kinetics study showed that maximum levels of surface antigen expression were achieved and maintained between 24 and 48 h. Cells were analyzed by use of a FACScan cytometer (Becton Dickinson) using the following antibodies from BD Pharmingen and Beckman Coulter, Inc. (Fullerton, Calif.): fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD11c (clone HL3), biotin-conjugated anti-mouse CD80 (clone 16-10A1), biotin-conjugated anti-mouse CD86 (clone GL1), biotin-conjugated anti-mouse I-A^b (clone AF6-120.1), biotin-conjugated anti-mouse CD40 (clone 3/23), and phycoerythrin (PE)-conjugated streptavidin. BMDCs and splenic DCs were characterized with FITC-conjugated anti-mouse CD11b (Mac-1; M1/70), PE-conjugated anti-mouse CD11c (HL3), Cy-chrome-conjugated anti-mouse CD8α (53-6.7), allophycocyanin-conjugated anti-mouse CD4 (RM-4-5), FITC-conjugated hamster anti-mouse CD11c (HL3), and PE-conjugated anti-mouse CD45R/B220 (RA3-6B2).

Purification of TCR-transgenic T cells. T cells were purified from the spleens of naive BALB/c mice expressing a transgenic α/β-TCR specific for peptide 323-329 of ovalbumin (OVA) (31) by magnetic bead-activated cell sorting (MACS) using a CD4$^+$ T-cell purification system with CD4$^+$-specific MACS beads (Miltenyi Biotech, Sunnyvale, Calif.). More than 90% of the resulting T-cell population was CD4$^+$ and expressed the OVA-specific TCR transgene. These purified CD4$^+$ T cells (2 × 10^6 cells/well) were then cultured in RPMI 1640 plus 10% fetal calf serum (FCS) with toxin derivative-treated DCs (5 × 10^5 cells/well) and 0.3 μM OVA peptide (ISQAVHAAHAEINEAGR-COOH; Peptide Institute, Inc., Minoh, Osaka, Japan) for 3 days at 37°C. In a preliminary experiment, three different amounts of toxin derivative-treated DCs were tested, and 5 × 10^5 cells/well consistently provided the most reproducible data. CD4$^+$ T cells were then stimulated with 50 ng/ml phorbol myristate acetate (PMA; Sigma, St. Louis, Mo.) and 500 ng/ml ionomycin (Sigma) overnight. Culture supernatants from the different wells were tested for the synthesis of the cytokines IFN-γ, IL-4, IL-5, IL-6, and IL-10 by ELISAs (AN'LYZA immunoassay kit; R&D Systems).

Induction of T-cell proliferation. BMDCs (1.7 × 10^4 cells/well) were incubated in round-bottomed 96-well plates (Corning) in the presence of 1 μg of Stx1 derivative for 48 h at 37°C and then were irradiated with 30 Gy of radiation. The plates were extensively washed with RPMI 1640 followed by complete RPMI 1640 containing 10% FCS, HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). OVA-specific transgenic T cells (5 × 10^4 cells/well) were added to the DC-coated wells. The plates were then incubated in the presence of 0.3 μM OVA peptide for an additional 3 days at 37°C. [^{3}H]thymidine (0.5 μCi; Amersham Pharmacia Biotech, Buckinghamshire, England) was added to each well 18 h before harvesting, and incorporated radioactivity was then measured with an LS1701 scintillation counter (Beckman Coulter Inc., Hialeah, Fla.). The results are expressed as stimulation indexes (E/C), defined as the ratios between the amounts of [^{3}H]thymidine incorporated into T cells incubated with toxin derivative-treated DCs and the amount of [^{3}H]thymidine incorporated into T cells incubated with untreated DCs.

Isolation of splenic DCs. Spleens were isolated from mice receiving subcutaneous administrations of Stx1 derivatives and were then suspended in RPMI 1640 medium containing 2% FCS, HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). The spleens were digested with collagenase D (400 Mandl units/ml; Roche, Indianapolis, Ind.) as previously described (45), and then the red blood cells were lysed with ammonium chloride-potassium lysing buffer. Briefly, the spleens were incubated with collagenase D (400 Mandl units/ml) and DNase I (15 μg/ml; Roche) for 35 min at 37°C in RPMI 1640 medium, and EDTA was added to a final concentration of 5 mM during the last 5 min of incubation. For DC enrichment, released cells were layered over a metrizamide gradient column (Accurate, Westbury, N.Y.) (14.5 g of metrizamide added to 100 ml of complete medium) and centrifuged, and the low-density fraction was collected as DCs (26). The enriched DCs were counted and then stained with appropriate monoclonal antibodies as described above for fluorescence-activated cell sorting analysis.

Immunization protocol. A standard subcutaneous immunization protocol was used for this study (55). Mice were subcutaneously immunized on days 0 and 14 with a 100-μl aliquot containing 100 μg of ovalbumin (OVA; Sigma) alone or combined with an optimal dose of mStx1 (10 μg), StxB1 (10 μg), or nStx1 (50 ng) as an adjuvant. This dose of OVA has been shown to be optimal and is routinely used in our laboratory (55). The optimal doses of the Stx1 derivatives were determined in preliminary experiments. In the case of the native form, the dose was selected as the concentration which did not show in vivo toxicity. For an assay of protection against tetanus toxin, mice were subcutaneously immunized on days 0 and 14 with a 100-μl aliquot of tetanus toxoid (TT; 307 μg/ml, 900 limit of flocculation (Lf)/ml, 2,932 Lf/mg PN; provided by Y. Higashi, Osaka University, Biken Foundation, Osaka, Japan) alone or combined with mStx1 (1, 10, or 25 μg) or StxB1 (1, 10, or 25 μg) as an adjuvant.

Analysis of Ag-specific Ab isotype and IgG subclass responses. Ag-specific Ab titers in serum were determined by ELISAs as described previously (28, 51). Briefly, plates were coated with OVA (1 mg/ml) or TT (5 μg/ml) and blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS). After the plates were washed, serial dilutions of serum were added in duplicate. Following incubation, the plates were washed and a peroxidase-labeled goat anti-mouse μ, γ, or α heavy chain-specific Ab (Southern Biotechnology Associates, Birmingham, Ala.) was added to appropriate wells. Finally, 3,3′,5,5′-tetramethylbenzidine (TMB) with H_2O_2 was added for color development. For IgG subclass analysis, biotinylated rat monoclonal anti-mouse γ1 (G1-7.3), γ2a (R19-15), γ2b (R12-3), and γ3 (R40-82) heavy chain-specific Abs (Pharmingen) and streptavidin-conjugated peroxidase (Vector Laboratories, Inc., Burlingame, Calif.) were employed. End-point titers were expressed as reciprocal $\log_2$ values of the last dilutions giving optical densities at 450 nm of ≥0.1 above the negative control.

Detection of Ag-specific AFCs. For the elucidation of Ag-specific Ab-forming cells (AFCs), an enzyme-linked immunospot (ELISPOT) assay was employed as previously described in detail (51, 52). Splenic mononuclear cells were resuspended in complete medium. Ninety-six-well nitrocellulose-based plates were coated with 1 mg/ml of OVA diluted in PBS for the enumeration of Ag-specific AFCs. The wells were blocked with complete medium. Cells at various dilutions were added and incubated for 6 h at 37°C in 5% CO_2 in moist air. Antigen-specific AFCs were detected with a peroxidase-labeled anti-mouse μ, γ, or α heavy chain Ab (Southern Biotechnology Associates) and then visualized by adding the chromogenic substrate 3-amino-9-ethylcarbazole (Moss Inc., Pasadena, Md.). Spots were counted with the aid of a dissecting microscope (SZH Zoom stereo microscope system; Olympus, Lake Success, N.Y.).

Analysis of OVA-specific $CD4^+$ T-cell responses. $CD4^+$ T cells were purified from splenic cell suspensions by use of a magnetic bead-activated cell sorter system (Miltenyi Biotech) (51). Splenic mononuclear cells were initially applied to a nylon wool column (Polysciences, Warrington, Pa.) and incubated at 37°C for 1 h to remove adherent cells. Purified $CD4^+$ T cells were then obtained by positive sorting using a magnetic bead separation system consisting of anti-CD4 monoclonal Ab (clone GK1.5)-conjugated microbeads (Miltenyi Biotech). Purified splenic $CD4^+$ T cells (>98% pure) were cultured at a density of 4×10^6 cells/ml with OVA (1 mg/ml), T-cell-depleted, irradiated (30 Gy) splenic feeder cells (8×10^6 cells/ml), and recombinant IL-2 (rIL-2; 10 units/ml) (Pharmingen) in complete medium (51). These $CD4^+$ T-cell cultures were incubated for 3 days at 37°C in 5% CO_2 in air. For measurements of the levels of Ag-specific T-cell proliferation, 0.5 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech) was added to individual cultures 18 h before termination, and the uptake of [^{3}H]thymidine was determined in counts per minute (cpm) by use of a scintillation counter (55).

Tetanus toxin challenge. Tetanus toxin was diluted in 0.5% gelatin–PBS, and an appropriate lethal dose (130 50% lethal doses [LD_{50}s]) was given subcutaneously to each group of mice as described previously (15, 20). Mice were then monitored daily for paralysis and death.

Statistical analysis. The results are presented as means ± 1 standard error (SE). Statistical significance ($P < 0.05$) was determined by Student's *t* test and by the Mann-Whitney U test of unpaired samples.

RESULTS

mStx1 and StxB1 up-regulate cell surface expression of costimulatory molecules and MHC class II molecules on BMDCs. On day 6 of bone marrow-derived DC (BMDC) cultures, >90% of the cells were determined to be $CD11c^+$ (data not shown). The CD11b, CD8α, CD4, and B220 cells identified among the BMDCs were characterized as having the $CD11b^+$, $CD8\alpha^-$, $CD4^-$, and $B220^-$ phenotypes, respectively (data not shown). These BMDCs were incubated with or without Stx1 derivatives for 48 h, and the expression of cell surface molecules was analyzed by flow cytometry. Even nonactivated BMDCs showed moderate expression of the costimulatory molecules CD80 (B7-1) and CD86 (B7-2) and of major histocompatibility complex (MHC) class II molecules. The addition of SxtB1 resulted in a moderate up-regulation of CD86, MHC class II, and CD40 expression on BMDCs. Furthermore, StxB1 enhanced the expression of CD80; however, the CD80 level was lower than that of CD86 (Fig. 1).

The expression of CD86, but not that of CD80, was up-regulated when BMDCs were exposed to mStx1. In contrast, nStx1 failed to enhance the expression of these activation molecules on BMDCs. The expression of CD40 on BMDCs was also up-regulated by treatment with mStx1 or StxB1 (Fig. 1). An increase in the expression of these activation molecules also occurred after the treatment of cells with an optimal concentration (1 μg/ml) of LPS (data not shown), a known activator of DCs (17). To exclude any effects of contaminating endotoxins, we incubated BMDCs, with or without Stx1 derivatives, after the pretreatment of Stx1 derivatives with 5 μg/ml polymyxin B. Polymyxin B did not affect Stx1 derivative-induced surface marker expression (data not shown).

Induction of cytokine synthesis by StxB1-treated BMDCs. To analyze whether the observed phenotypic maturation (e.g., the expression of CD80, CD86, and MHC class II) was associated with cytokine production, we tested StxB1- and mStx1-treated BMDCs for an enhancement of TNF-α and IL-12 p70 synthesis. BMDCs incubated with StxB1 for 48 h produced modest amounts of TNF-α and IL-12 (Table 1), which was consistent with the observation of the expression of functional molecules of StxB1-treated BMDCs. In contrast, the incubation of BMDCs with nStx1 and mStx1 failed to invoke any increases in cytokine production. Thus, BMDCs activated by treatment with StxB1 exhibited the most enhanced capacity to secrete cytokines such as TNF-α and IL-12.

Enhanced stimulation of T cells by Stx1 derivative-activated BMDCs. In the next experiment, we tested whether the activation of DCs by mStx1 or StxB1 translated to an increased functional ability of DCs to stimulate T-cell proliferation and subsequent Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-6, and IL-10) cytokine production. In this assay, Stx1 derivative-stimulated DCs were cocultured with an OVA-specific peptide and splenic T cells isolated from OVA Tg mice. Stx1 derivative-treated BMDCs promoted higher levels of OVA-specific $CD4^+$ T-cell proliferation than did untreated DCs (Fig. 2A), with StxB1-treated BMDCs inducing the highest levels and mStx1-treated BMDCs inducing the next highest levels. In contrast, nStx1-treated BMDCs only weakly enhanced T-cell responses. Similarly enhanced T-cell proliferative responses were also noted when alloreactive responder T cells were cocultured with Stx1 derivative-treated BMDCs (data not shown).

To determine whether the observed increase in OVA-specific $CD4^+$ T-cell proliferation induced by StxB1- or mStx1-treated BMDCs was associated with Th1 and Th2 cytokine production, we harvested culture supernatants and subjected them to IFN-γ-, IL-4-, IL-5-, IL-6-, and IL-10-specific ELISAs. StxB1-treated DCs promoted an increased synthesis of cytokines such as IFN-γ, IL-4, IL-5, IL-6, and IL-10 (Fig. 2B) most effectively, followed by those treated with mStx1 and nStx1 (Fig. 2B). However, it should be pointed out that Stx1 derivatives significantly enhanced only IL-6 synthesis, prompting little or no release of the other cytokines. Among the Stx1 derivatives, StxB1 possessed the most potent immunoenhancing activity for an increase of T-cell proliferation and subsequent Th1 and Th2 cytokine production through the activation of BMDCs.

In vivo effects of mStx1 and StxB1 on the up-regulation of costimulatory molecules and MHC class II on splenic DCs. It

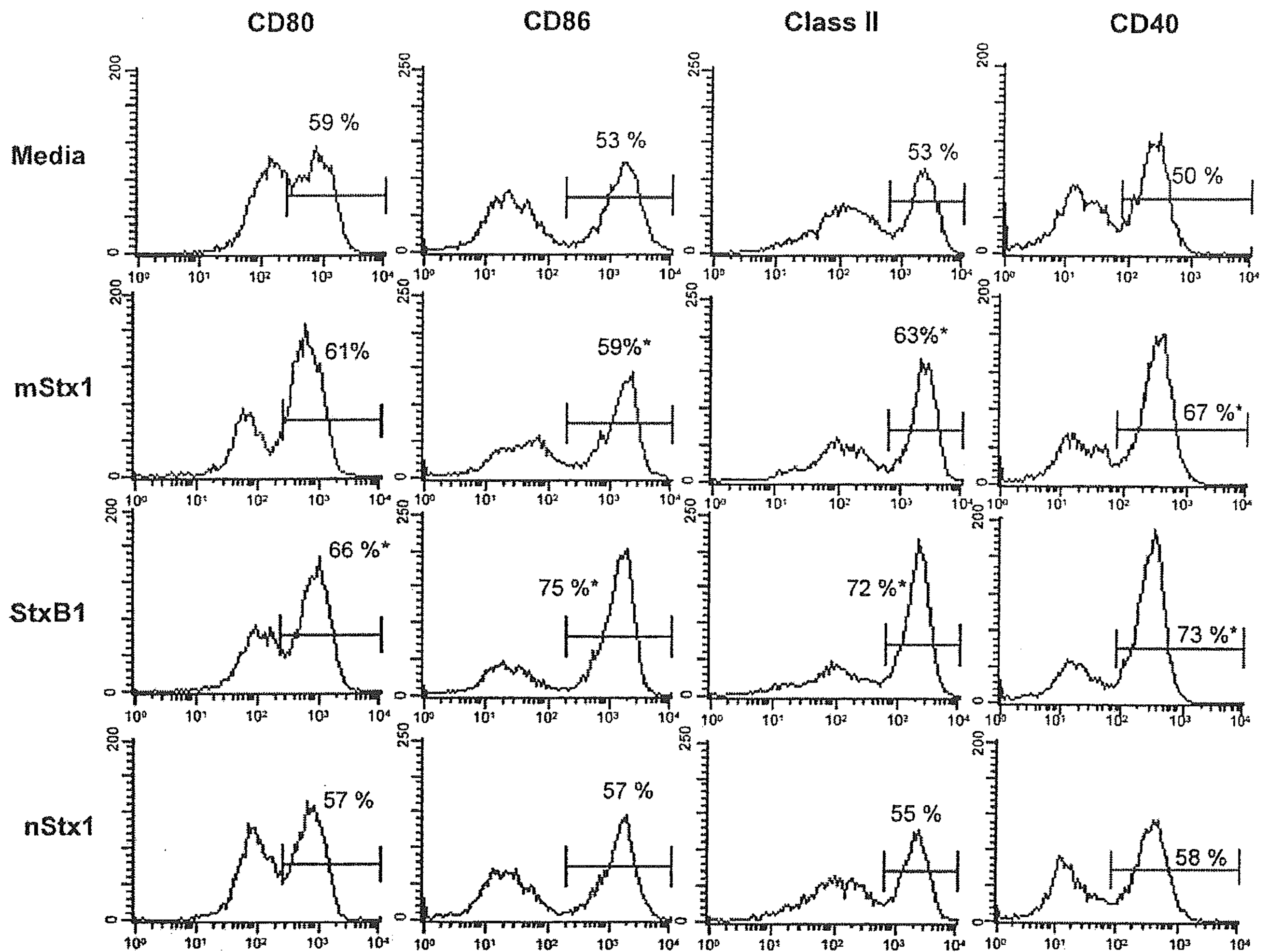

FIG. 1. Effects of StxB1, mStx1, and nStx1 on the expression of CD80, CD86, CD40, and MHC class II by bone marrow-derived DCs (BMDCs). BMDCs were cultured with Stx1 derivatives (StxB1, 1 μg/ml; mStx1, 1 μg/ml; nStx1, 1 μg/ml) for 48 h since a preliminary study showed that the maximum levels of surface antigen expression occurred between 24 and 48 h. Cell surface Ag expression was analyzed by flow cytometry as described in Materials and Methods. The data are presented as histograms and are expressed as means of three independent experiments. The percentage within each panel indicates the number of cells staining strongly for the indicated marker. *, $P < 0.05$ compared with the control medium-treated culture. Data were obtained by using the $CD11c^+$ gated cell fraction.

was important to examine whether in vivo administration of the Stx1 derivatives could modulate DC function. Thus, the expression of costimulatory molecules and MHC class II on splenic DCs was analyzed by flow cytometry 12 h and 48 h after Stx1 derivatives were subcutaneously administered to healthy mice (Table 2).

TABLE 1. TNF-α and IL-12 p70 synthesis by StxB1-, mStx1-, or Stx1-treated murine BMDCs[a]

Stimulator	TNF-α concn (pg/ml)	IL-12 concn (pg/ml)
mStx1	260 ± 81	90 ± 18
StxB1	470 ± 92*	260 ± 34*
nStx1	240 ± 42	76 ± 13
media	280 ± 92	82 ± 14

[a] Culture supernatants were harvested and then analyzed for the production of secreted cytokines by the use of appropriate cytokine-specific ELISAs. The results are expressed as means ± SEM and were taken from a total of three separate experiments. *, $P < 0.05$ compared with a culture to which no stimulator was added.

As shown above for nonactivated BMDCs, the splenic DCs isolated from healthy mice also expressed $CD11b^+$ (data not shown). Nonactivated splenic DCs were also found to naturally express moderate levels of the costimulatory molecules CD80 and CD86, MHC class II, and CD40 (Table 2), whose levels were up-regulated after the administration of StxB1. Interestingly, the up-regulation of CD80 expression was observed as early as 12 h after the administration of StxB1, with the expression of CD86, CD40, and MHC class II appearing 48 h after administration. After a subcutaneous injection of mStx1 into mice, the expression of CD40 and MHC class II was up-regulated. In contrast, as seen with BMDCs, nStx1 failed to enhance the expression of any of these costimulatory molecules, except for MHC class II, on splenic DCs.

Enhancement of Ag-specific Ab responses by subcutaneous immunization of mice with OVA and mStx1 or StxB1. To examine in vivo the immunoenhancing activities of Stx1 derivatives, we subcutaneously immunized mice with an optimal

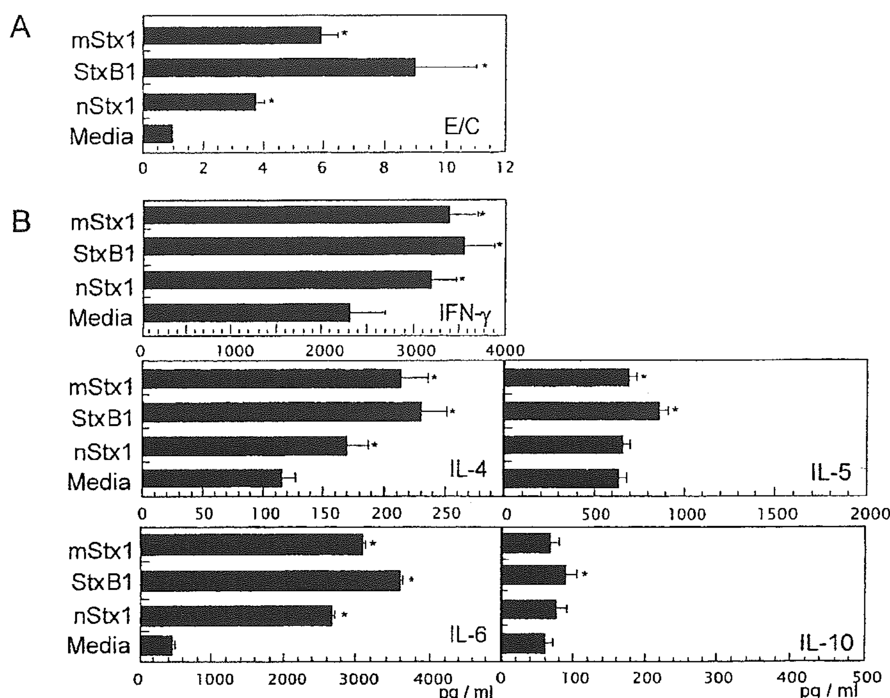

FIG. 2. Activation of OVA-specific $CD4^+$ T-cell responses by Stx1 derivative-treated BMDCs. T-cell proliferation (A) and Th1 and Th2 cytokine production (B) by $CD4^+$ T cells from DO10.11 Tg mice stimulated with Stx1 derivative-treated BMDCs were examined. BMDCs pretreated with 1 μg/ml Stx1 derivative were washed and then cocultured with purified $CD4^+$ T cells (10^6/ml) from DO11.10 Tg mice in the presence of 0.3 μM $OVA_{323-329}$ peptide for 3 days. An aliquot of cell culture was subjected to DNA synthesis by the addition of [^{3}H]thymidine during the last 18 h of incubation. For cytokine analysis, another aliquot of $CD4^+$ T cells was harvested and then treated with 50 nM PMA and 500 nM ionomycin overnight. No or little cytokine release was detected for $CD4^+$ T cells without PMA and ionomycin. The results are expressed as mean E/C ± standard errors of the means (SEM) for triplicate cultures. *, $P < 0.05$ compared with the control medium-treated culture. The count for the control culture was 6,880 ± 380 cpm. The results of the T-cell proliferation assay (A) are expressed as mean E/C (experimental, stimulated value/control, nonstimulated value) ± SEM of triplicate cultures.

dose of OVA in the presence or absence of the toxin derivatives. The coadministration of 10 μg of mStx1 or StxB1 resulted in high levels of OVA-specific IgG, IgM, and IgA (Fig. 3A). In contrast, 50 ng of nStx1 did not support the generation of any isotype of anti-OVA Ab. As one might expect, when mice were immunized with OVA alone, antigen-specific Ab responses were not induced (Fig. 3A). An analysis of antigen-specific IgG antibody-forming cells (AFCs) in the spleens of mice immunized with OVA plus Stx1 derivatives confirmed the results obtained for the characterization of OVA-specific Ab titers in sera. Thus, significant numbers of OVA-specific IgG AFCs were detected in the spleens of mice subcutaneously immunized with OVA plus mStx1 or StxB1 as an adjuvant (Fig. 3B). In contrast, obvious OVA-specific IgG AFCs were not seen in the spleens of mice given OVA alone or OVA plus nStx1 (Fig. 3B). A subsequent analysis of the OVA-specific IgG subclasses revealed that the major antigen-specific IgG subclass response was IgG1, followed by IgG2a, after the coadministration of mStx1 or StxB1 (Fig. 3C). These findings demonstrate that Stx1 derivatives, especially nontoxic forms of StxB1 and mStx1, are potent immunoenhancing molecules in vivo.

Induction of OVA-specific $CD4^+$ Th1- and Th2-cell responses after immunization with OVA and Stx1 derivatives.

TABLE 2. Characterization of CD80, CD86, CD40, and MHC class II expression by mStx1-, StxB1-, or nStx1-treated splenic DCs

Stimulator	% of the highest intensity of the expressed molecule							
	CD80		CD86		CD40		MHC class II	
	12 h	48 h	12 h	48 h	12 h	48 h	12 h	48 h
mStx1	43.0 ± 3.9	16.5 ± 9.3*	43.6 ± 2.9*	45.3 ± 2.5	58.3 ± 5.8*	37.6 ± 1.7	48.8 ± 3.1	65.9 ± 7.5*
StxB1	55.8 ± 2.6*	33.9 ± 1.6*	20.0 ± 4.3*	75.3 ± 14.3*	37.6 ± 1.4*	63.3 ± 4.4*	45.1 ± 2.2*	70.4 ± 6.3*
nStx1	33.2 ± 1.4*	15.7 ± 8.6*	17.4 ± 10.5*	30.2 ± 7.5*	27.4 ± 3.0*	9.7 ± 9.1*	42.5 ± 4.7*	57.3 ± 2.7*
PBS	42.4 ± 1.3	50.9 ± 6.6	49.6 ± 1.7	49.2 ± 6.6	34.7 ± 0.5	37.2 ± 7.5	51.3 ± 2.6	49.2 ± 6.2

Twelve or 48 h after the subcutaneous administration of StxB1 (10 μg/mouse), mStx1 (10 μg/mouse), or nStx1 (50 ng/mouse), mice were sacrificed for the preparation of splenocytes. The cells were then analyzed by flow cytometry. The data are means ± SEM are representative of three independent experiments. *, $P < 0.05$ compared with mice administered PBS.

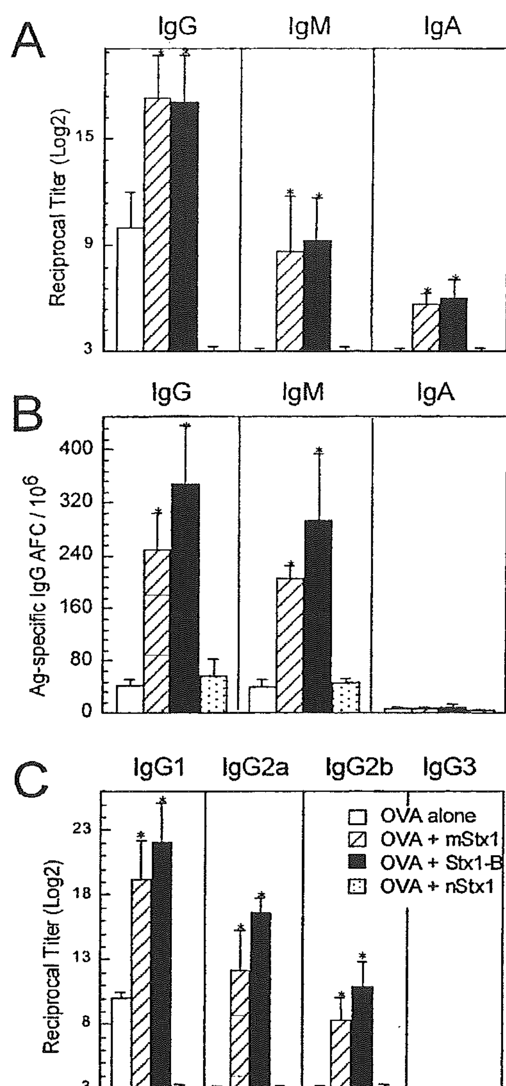

FIG. 3. Induction of OVA-specific antibody responses by coadministered Stx1 derivatives. Mice were subcutaneously immunized with OVA plus mStx1, nStx1, or StxB1. Specifically, C57BL/6 mice were subcutaneously immunized with 100 μg of OVA plus 10 μg of the Stx1 mutant (E167R/R170L; mStx1) (hatched bars), 50 ng of nStx1 (dotted bars), or 10 μg of StxB1 (black bars) as an adjuvant or with OVA alone (white bars) on days 0 and 14. OVA-specific serum IgG, IgM, and IgA Ab (A) and splenic OVA-specific antibody-forming cell (AFC) (B) responses were determined by ELISAs and ELISPOT assays, respectively. Furthermore, OVA-specific IgG subclass Ab responses (C) were also analyzed by ELISAs. Serum samples were collected on day 21 and examined for OVA-specific Abs and OVA-specific IgG subclass Ab responses by ELISAs. Mononuclear cells were isolated from the spleens of subcutaneously immunized mice on day 21 and examined by Ag-specific ELISPOT assays. *, $P < 0.05$ compared with mice immunized with OVA alone. The results are expressed as means ± SEM from a total of three separate experiments, each of which used five or six mice per group.

When $CD4^+$ T cells isolated from the spleens of mice subcutaneously immunized with OVA plus mStx1 or StxB1 were restimulated with OVA in vitro, increased proliferative responses were seen (Fig. 4A). In contrast, essentially no Ag-specific $CD4^+$ T-cell proliferation occurred in splenic $CD4^+$ T cells isolated from mice given OVA alone or OVA plus nStx1 (Fig. 4A). These results further demonstrate that mStx1 and StxB1 are potent adjuvants for the induction of OVA-specific $CD4^+$ T cells in vivo.

In a subsequent experiment, Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-6, and IL-10) cytokine production by antigen-specific $CD4^+$ T cells was analyzed at the protein level (Fig. 4B). Increased levels of both Th1 and Th2 cytokines were noted in cultures containing splenic $CD4^+$ T cells from mice subcutaneously immunized with OVA plus mStx1 or StxB1 (Fig. 4B), while nStx1 enhanced the production of only selected Th2 cytokines, including IL-5, IL-6, and IL-10, but not IL-4 and IFN-γ production (data not shown). Splenic $CD4^+$ T cells from mice given OVA alone produced low levels of IFN-γ, IL-5, IL-6, and IL-10 but did not produce IL-4. Taken together, these results show that the subcutaneous administration of OVA plus StxB1 or mStx1 as an adjuvant induces antigen-specific Th1 (e.g., IFN-γ)- and Th2 (e.g., IL-4)-type cytokine responses, which in turn account for the generation of OVA-specific IgG2a and IgG1 Ab responses, respectively, in serum.

Induction of neutralizing antibody responses to tetanus toxin by subcutaneous immunization with the toxoid vaccine and Stx1 derivatives. Since the subcutaneous administration of OVA plus mStx1 or StxB1 elicited Ag-specific IgG and IgM Ab responses, we next determined whether vaccine Ag-specific Abs supported by the Stx1 derivatives were protective. Initially, we determined whether the subcutaneous administration of tetanus toxoid (TT) with mStx1 or StxB1 could induce TT-specific Ab responses. Mice subcutaneously immunized with TT plus >10 μg of mStx1 or StxB1 showed significant TT-specific serum IgM, IgG, and IgA Ab responses. In contrast, low Ab responses were detected after immunization with TT alone (Fig. 5A). In the next experiment, we determined if these Abs were also protective. Mice given TT plus Stx1 derivatives or TT alone were challenged with a lethal dose (130 LD_{50}s) of tetanus toxin and then monitored for paralysis and death. As expected, subcutaneous immunization with TT plus Stx1 derivatives provided complete protection. In contrast, TT alone provided no protection in mice against the paralysis and death that normally occur within 2 days of the administration of tetanus toxin (Fig. 5B). These findings indicate the effectiveness of TT-specific IgG Abs in serum induced by subcutaneously coadministered Stx1 derivatives.

DISCUSSION

B7-1 and B7-2 have been shown to be essential costimulatory molecules for the initial activation of $CD4^+$ T cells (21, 23, 24). With our experiments, we sought to determine the effect of Stx1 derivatives on the expression of such costimulatory molecules. Immature BMDCs and splenic DCs were used to help map the early events occurring after the administration of Stx1 derivatives and to determine the extent of the ability of those derivatives to initiate primary T-cell responses. Stx1 derivatives provided two different types of immunoregulation signals to DCs. First, StxB1 and mStx1 were shown in our in vitro and in vivo studies to enhance the activation of BMDCs and splenic DCs by augmenting MHC class II, CD80, CD86,

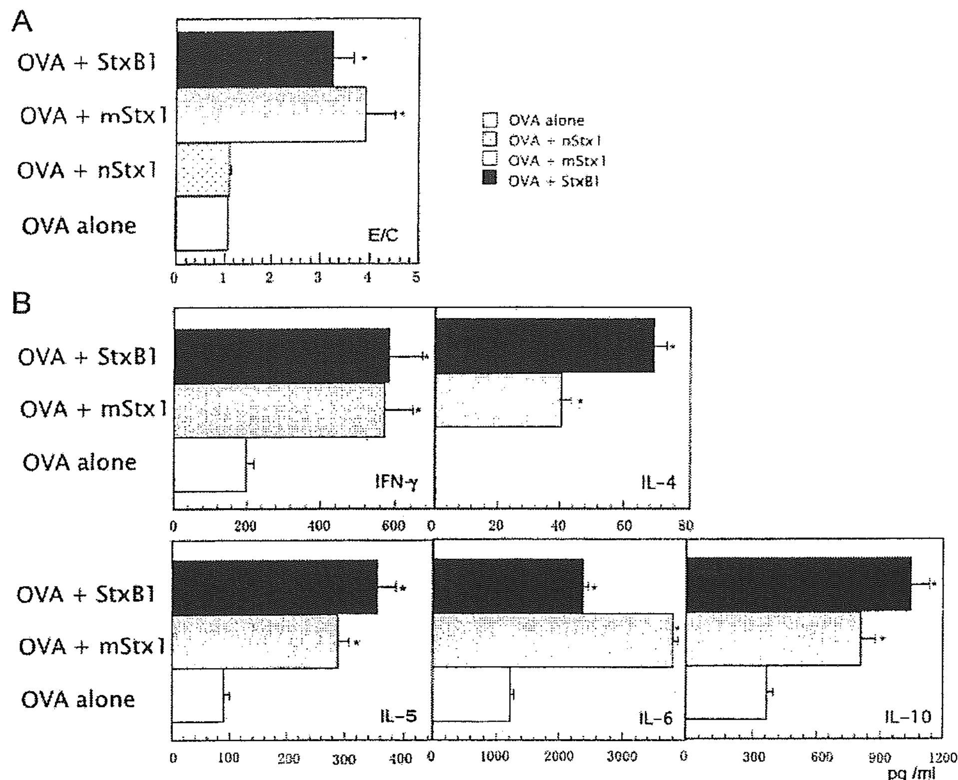

FIG. 4. Analysis of OVA-specific $CD4^+$ T-cell responses induced by coadministered Stx1 derivatives. OVA-specific $CD4^+$ Th-cell proliferative responses (A) and Th1 and Th2 cytokine synthesis (B) by $CD4^+$ T cells isolated from the spleens of mice subcutaneously immunized with OVA plus 10 μg of mStx1 (shaded bars), 50 ng of nStx1 (dotted bars), or 10 μg of StxB1 (black bars) or with OVA alone (white bars) were examined. Purified splenic $CD4^+$ T cells were cocultured at a density of 2×10^6 cells/ml with 1 mg/ml of OVA and with T-cell-depleted, irradiated splenic feeder cells (4×10^6 cells/ml) in complete medium containing rIL-2 (10 U/ml) for 3 days for proliferation assays and 5 days for cytokine synthesis measurements. A control culture consisting of the splenic $CD4^+$ T cells of naïve mice, feeder cells, and rIL-2 (10 U/ml) resulted in the incorporation of 230 ± 42 cpm of [^{3}H]thymidine. Culture supernatants were harvested and then analyzed for the synthesis of secreted cytokines by the use of appropriate cytokine-specific ELISAs. The minimum detection levels for the individual cytokines detected were as follows: IFN-γ, 9.4 pg/ml; IL-4, 7.8 pg/ml; IL-5, 15.6 pg/ml; IL-6, 15.6 pg/ml; and IL-10, 15.6 pg/ml. The results are expressed as means of the stimulation indexes ± SEM or pg/ml ± SEM from a total of three experiments using five or six mice per group. *, $P < 0.05$ compared with mice immunized with OVA alone. The results for OVA-specific $CD4^+$ T-cell proliferative responses (A) are expressed as E/C (experimental, stimulated value/control, nonstimulated value).

and/or CD40 expression. Since previous research has already shown that the strong expression of MHC and costimulatory molecules on antigen-presenting cells is associated with a high level of T-cell activation (4, 6), StxB1 and mStx1, with their demonstrated ability to enhance the expression of MHC and/or costimulatory molecules, must lead to an enhanced $CD4^+$ T-cell response. Second, certain Stx derivatives were shown to induce TNF-α, which has been shown to play a role in the immune regulation of B lymphocytes and the maturation of DCs (34, 49). Among the derivatives, StxB1 proved to be the most potent inducer of TNF-α. With the two distinct immunoenhancing signals noted above, nontoxic forms of Stx1 derivatives have been demonstrated by our study to be strong candidates as adjuvants to enhance antigen-specific T-cell and B-cell immune responses.

Our in vitro studies demonstrated that the maturation of in vitro BMDCs was enhanced by Stx1 derivatives, especially StxB1. Our results also showed that T-cell proliferation and cytokine production by Ag-specific $CD4^+$ T cells were augmented by StxB1-treated BMDCs. To examine whether the series of immunoenhancing events triggered by Stx1 derivative-treated BMDCs in vitro also reflected the in vivo situation, we also performed a series of in vivo experiments. Our findings revealed that DC maturation occurred after the administration of the Stx1 derivatives. Furthermore, we demonstrated that a mutant form of Stx1 (E167Q/R170L; mStx1) and the B subunit of Stx1 (StxB1) show potential as novel adjuvants for the induction of antigen-specific systemic Th and B-cell immune responses. The subcutaneous coadministration of nontoxic StxB1 or mStx1 as an adjuvant with a protein Ag resulted in the induction of high IgG anti-OVA Ab responses in serum. When these two distinct forms of nontoxic Stx1 derivatives were subcutaneously coadministered, the derivatives elicited both $CD4^+$ Th1- and Th2-type responses via the mixed production of Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-6, and IL-10) cytokines. The Stx1 derivative supported mixed (Th1 and Th2) cytokine synthesis, reflecting the generation of OVA-specific IgG1, followed by IgG2a, in the systemic compartment. Similarly, Stx1 derivative molecules were seen to support the generation of Ag-specific Th1- and Th2-type $CD4^+$ T cells in vitro.

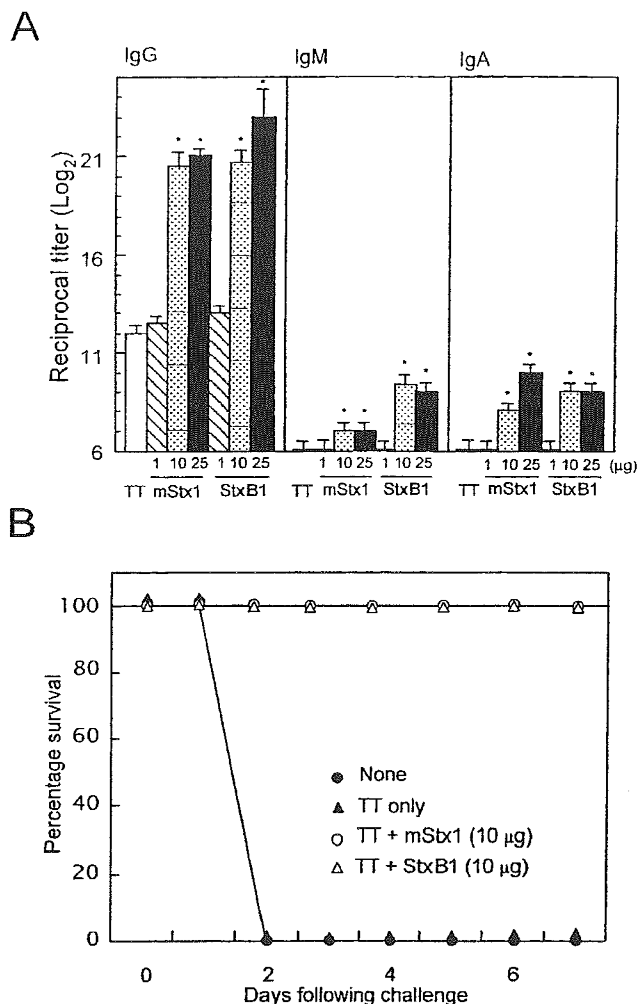

FIG. 5. Induction of TT-specific serum IgG, IgM, and IgA antibody responses by coadministered Stx1 derivatives (A) and protection against fatal challenge with tetanus toxin (B). Mice were subcutaneously immunized with TT plus mStx1, nStx1, or StxB1. Specifically, C57BL/6 mice were subcutaneously immunized with 100 μl of TT plus 1 μg of Stx1 mutant (E167R/R170L; mStx1) or 1 μg of StxB1 (hatched bars), 10 μg of Stx1 mutant or 10 μg of StxB1 (dotted bars), or 25 μg of Stx1 mutant or 25 μg of StxB1 (black bars) as an adjuvant or with TT alone (white bars) on days 0 and 14. Serum samples were collected on day 21 and examined for TT-specific Ab responses by ELISA. One week after the last immunization, mice were challenged on day 21 by the subcutaneous injection of 130 LD_{50}s of tetanus toxin in 0.5 ml of PBS including 0.2% gelatin. *, $P < 0.05$ compared with mice immunized with OVA alone. The results are expressed as means ± SEM from a total of three separate experiments, each of which used five or six mice per group.

In addition to Stx1 derivatives, pertussis toxin (37) and LT (47) have been shown to potentiate a similarly mixed Th1- and Th2-type response.

In a separate study, LT-treated BMDCs, like StxB1-treated cells, were shown to enhance CD80, CD86, MHC class II, and CD40 expression (unpublished data). It has been shown that the up-regulation of CD80, CD86, MHC class II, or TNF-α and/or IL-12 production most closely correlates with the adjuvant activity of toxin-based immunomodulatory molecules (8, 53). Although many details of the molecular mechanisms behind the enhancement of Th1- and Th2-type responses by Stx derivatives remain to be elucidated, the present study has demonstrated that the Stx derivatives (e.g., StxB1 and mStx1) can be grouped as Th1- and Th2-inducing adjuvants. For an investigation of the molecular mechanisms underlying the adjuvanticity of mStx1 and StxB1, one possible experiment would be to examine and compare the Th1- and Th2-type pathway induced by mStx1 and that promoted by StxB1. The induction of signaling molecules such as T-bet, GATA-3, c-Maf, and SLAT by mStx1 and StxB1 could be compared, since these molecules have been shown to be associated with Th1- or Th2-cell differentiation (19, 27, 46).

Our results revealed that 50 ng of nStx1 did not induce serum IgG Ab responses to a coadministered Ag, indicating that nStx1 does not possess adjuvant activity. This observation is consistent with a previous study that showed that nStx1 does not possess adjuvant activity when given orogastrically (43). In contrast, all mice given 10 μg of mStx1 or StxB1 as an adjuvant generated systemic antigen-specific IgG responses (Fig. 4). Since a lower concentration of nStx1 was used in the in vivo experiment, one can consider that the administration of a higher dose may lead to the induction of the antigen-specific immune responses seen with mStx1 and StxB1. To this end, we subcutaneously coadministered higher doses of nStx1 (e.g., 100 to 150 ng) to mice, and all of those mice died (data not shown). These findings further demonstrate that nontoxic forms such as mStx1 and StxB1 possess adjuvant activities when administered at high doses, while the adjuvanticity of the native form cannot be assessed at these high doses due to its toxicity. In addition, mStx1 and StxB1 do not have the damaging side effects of nStx1. Previous studies have reported that Stxs induce necrosis via their RNA *N*-glycosidase activity (39) but that, in contrast, the Stx1 mutant and StxB1 (2) are much less toxic or nontoxic in terms of their inhibitory effects on protein synthesis, their cytotoxicity, and their lethality to mice compared with native forms of Stx1 (2, 33). nStx1 may signal the induction of cell death instead of immune enhancement. In contrast, the nontoxic forms, mStx1 and StxB1, provide appropriate activation signals for the induction of $CD4^+$ Th1- and Th2-type responses via the expression of CD80, CD86, and MHC class II on DCs, leading to the generation of IgG1 and IgG2a Ab responses to the coadministered Ag.

It is well established that CT is an effective adjuvant for the induction of antigen-specific mucosal IgA and systemic IgG and IgA Ab responses to coadministered protein Ags (9). CT preferentially induces Ag-specific Th2-type $CD4^+$ T-cell responses via the high-level synthesis of IL-4 and IL-5 (28). However, the enterotoxin possesses ADP-ribosyltransferase activity, which causes severe diarrhea and is thus unsuitable for use in humans (41). Therefore, several studies have investigated the potential adjuvant effect of the B subunit as a nontoxic derivative of CT. Highly purified recombinant CT-B has been shown to be ineffective as an adjuvant compared with the holotoxin for the induction of Ag-specific IgA and IgG immune responses (25, 54). However, it should be noted that two recent studies provided contradictory results, showing that nasally coadministered recombinant CT-B provided mucosal adjuvant activity (13, 48). It is interesting that StxB1 possesses adjuvant activity. Since the membrane ligand molecules of

StxB1 (e.g., globotriaosylceramid [Gb3] and globotetraosylceramide [Gb4]) are completely different from those of CT-B and LT-B (e.g., GM1), biological stimulation signals provided by StxB1 via Gb3 and Gb4 could be more effective than those transmitted by CT-B and GM1. Several cell surface receptors, including Ig, transferrin receptor, FcγR, and DEC-205, can mediate endocytosis and effective antigen presentation (16, 29, 35). Furthermore, glycosphingolipids, including GM1, have also been implicated as sites for the delivery of immunity-enhancing signals (40). Our present findings suggest that Gb3 may also mediate the effective endocytosis of and antigen presentation by DCs. Therefore, the A subunits of the toxins may not be necessary for immunoenhancing activity, unlike those of other known AB_5 toxins such as CT and LT. In this study, StxB1 possessed a costimulatory molecule-enhancing activity, while CT-B fails to induce either CD80 or CD86 expression on B cells or macrophages (1, 53). To elucidate the relationship between the increased expression of costimulatory molecules and the binding of StxB1 to its receptor, Gb3, we examined whether the signals for the enhancement of costimulatory molecules by StxB1 can be blocked by treatment of the receptors for StxB1. After a treatment of Gb3, costimulatory molecule expression was blocked (unpublished data). This means that the signaling pathway via the receptor for StxB1 plays a role in the enhancement of costimulatory molecule expression. In addition, it should be noted that it is possible that the A subunit of Stx1 is more responsible for toxicity than for adjuvanticity.

The nStx1 treatment of BMDCs was associated with some increase in OVA-specific $CD4^+$ T-cell proliferative as well as Th1/Th2 cytokine responses (Fig. 2). However, nStx1 did not induce any up-regulation of CD80, CD86, or MHC class II and did not enhance the secretion of TNF-α or IL-12 (Fig. 1 and Table 1). When the nStx1-treated BMDCs were treated with CD80- and/or CD86-blocking antibodies, the levels of $CD4^+$ T-cell proliferation and Th1/Th2 cytokine secretion were not altered. However, when mStx1- or StxB1-treated BMDCs were similarly treated with blocking antibodies specific for CD80 and CD86, the antigen-specific $CD4^+$ T-cell responses were inhibited (unpublished results). These findings suggest that the ability of nStx1 to stimulate the proliferation and cytokine secretion of T cells does not stem from the up-regulation of costimulatory molecules.

Another interesting biological characteristic of nStx1 is that its in vivo administration resulted in the down-regulation of all of the surface molecules associated with lymphocyte stimulation on splenic DCs (Table 2). To this end, nStx2 has been shown to reduce the number of splenic $CD4^+$ and $B220^+$ cells when it is administered to mice (44). Thus, nStx1 may exert at least two negative influences on lymphocytes, namely, it can down-regulate costimulatory molecule expression or even result in death. A separate study lent support to this view by showing that approximately 25% of BMDCs underwent apoptosis and necrosis after exposure to nStx1 in vitro, while no such cell death was seen after exposure to nontoxic Stx1 derivatives such as mStx1 and StxB1 (data not shown).

In summary, our findings have provided new evidence that nontoxic Stx1 derivatives (e.g., mStx1 and StxB1) can effectively induce costimulatory molecules (CD80 and CD86) and/or MHC class II on DCs. This study has further shown that nontoxic forms of Stx1 derivatives, including mStx1 and StxB1, possess adjuvant activity and can elicit Ag-specific $CD4^+$ Th1- and Th2-type responses for the subsequent induction of antigen-specific IgG1 and IgG2a Ab responses following subcutaneous immunization with a protein Ag. The nontoxic Stx derivatives can be considered promising new candidates for effective and safe adjuvants.

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All experiments described herein were approved by the local authorities. All procedures were done in agreement with National Institutes of Health guidelines for the handling of laboratory animals.

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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.

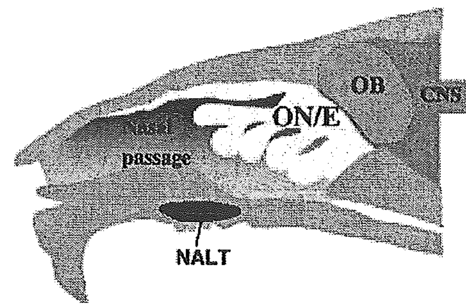

FIG. 1. Anatomy of the murine nasal tract and CNS. Indicated are the locations of the the ON/E, the NALT, and the OB in the nasal tract and adjacent CNS.

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.

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

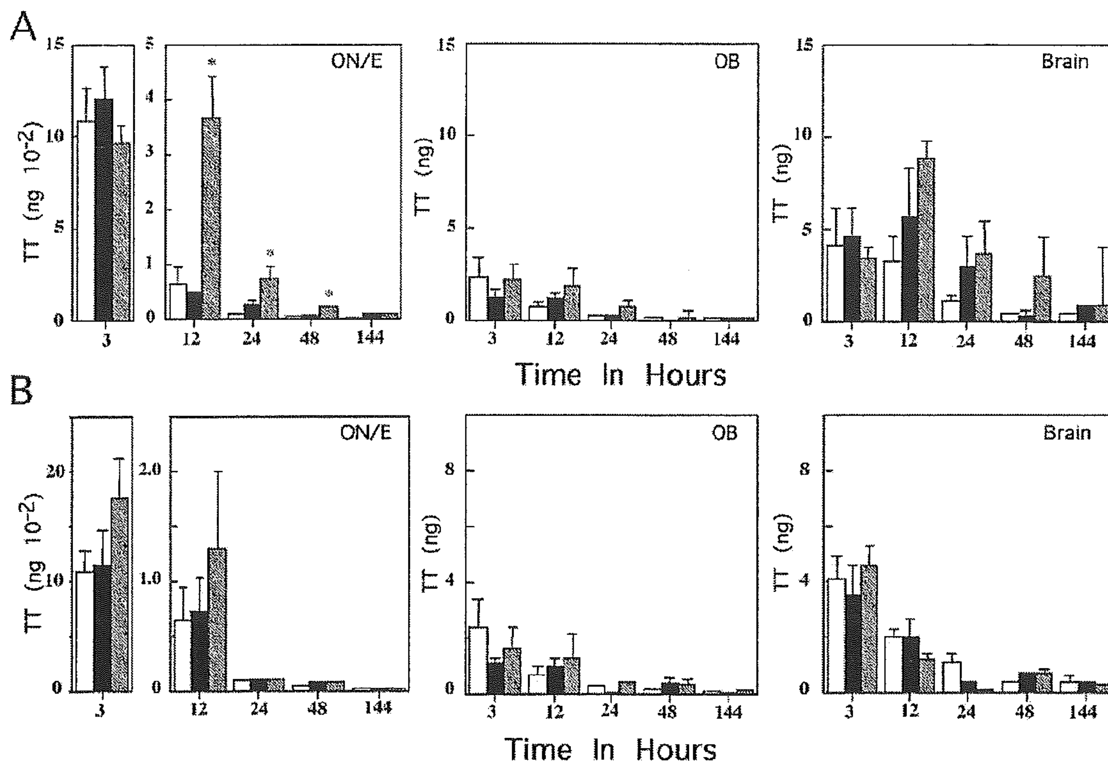

FIG. 2. Comparison of nCT- and mCT- (A) with nLTh-1- and mLTh-1-induced uptake (B) of ^{125}I-TT into olfactory and neuronal tissues. (A) Distribution of ^{125}I-TT in the ON/E, OB, and brain after nasal application of ^{125}I-TT alone (open bars) or in combination with 10 μg of mCT (solid bars) or 1.0 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

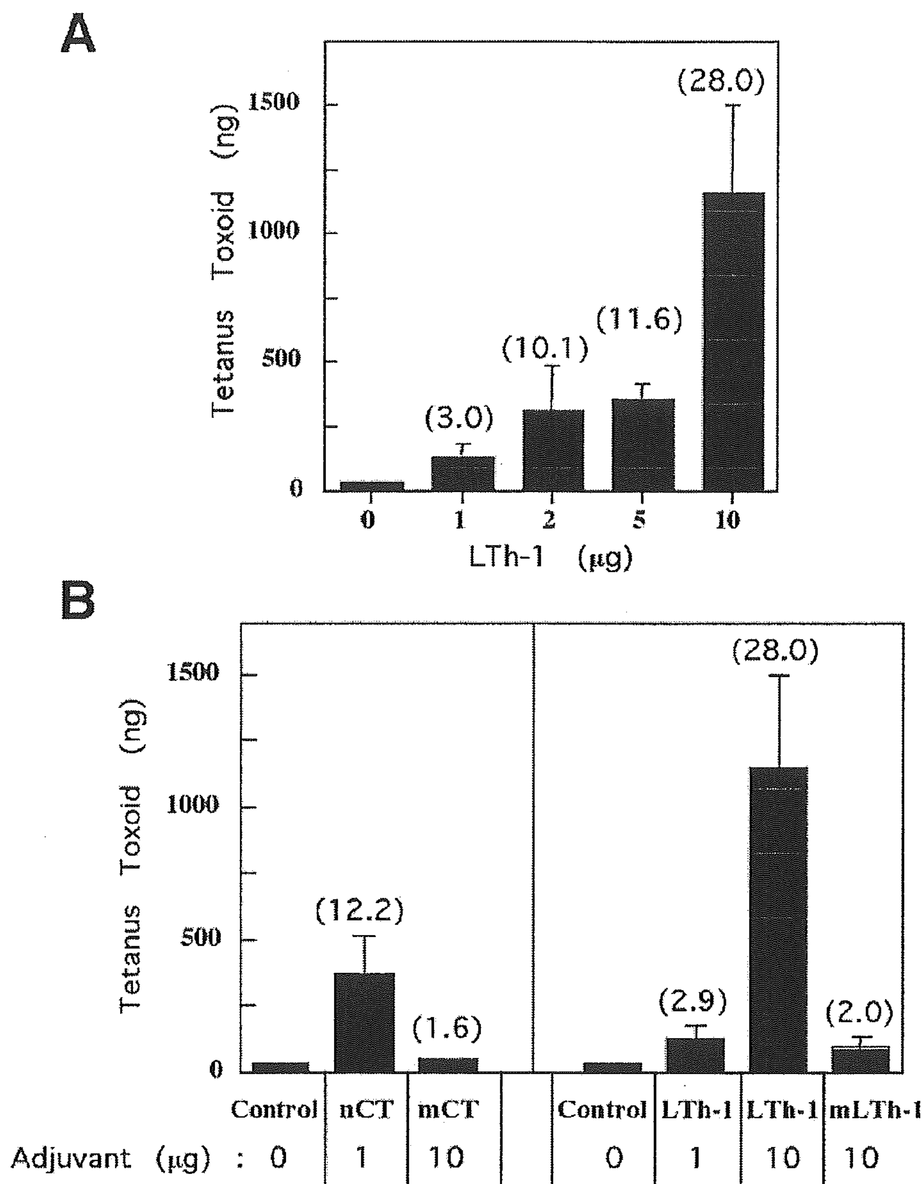

FIG. 3. Enterotoxin-dependent redirection of ^{125}I-TT into the ON/E. Various doses of nLTh-1, i.e., 0, 1, 2, 5, and 10 μg, were combined with 20 μg ^{125}I-TT and applied nasally. The ON/E were collected 12 h after application to assess redirection of the protein. (A) Indicated is the increase (n-fold) 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

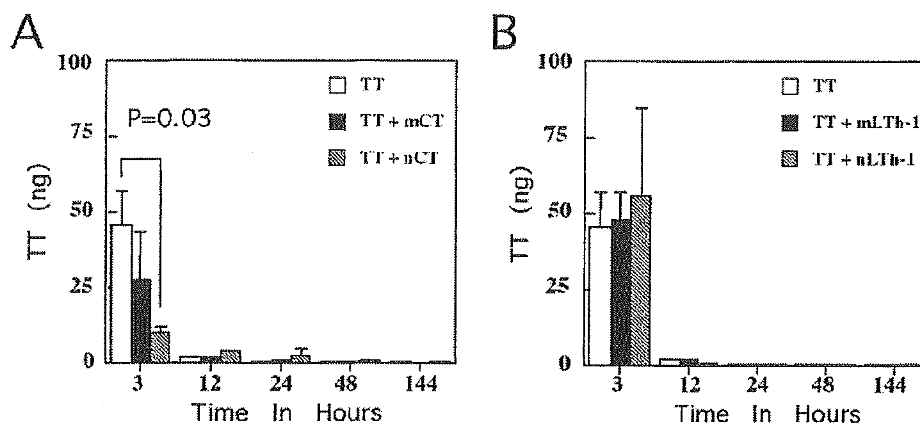

FIG. 4. Comparison of nCT- and nLTh-1- with mCT (E112K)- and mLTh-1 (E112K)-induced uptake of ^{125}I-TT into NALT. Distribution of ^{125}I-TT in NALT is expressed as TT (ng) accumulation per organ. A total of 20 μg of ^{125}I-TT alone or in combination with 10 μg of mCT or 1.0 μg of nCT (A) or ^{125}I-TT 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*.

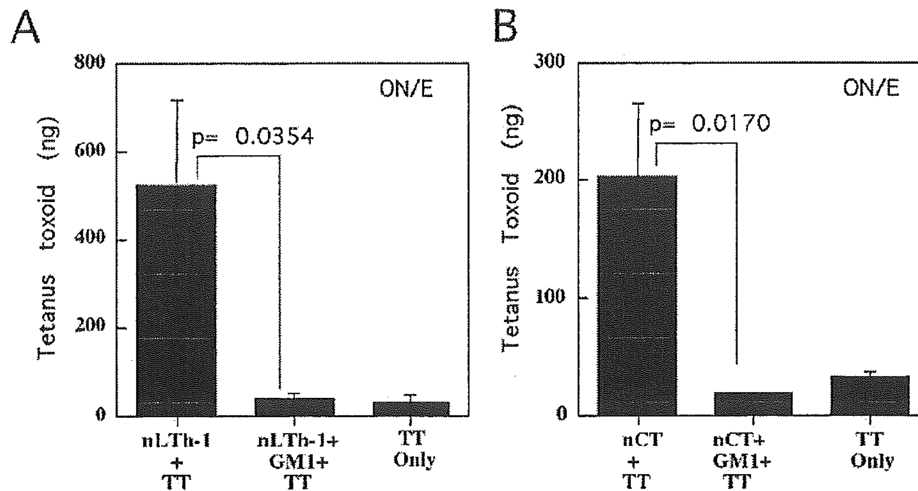

FIG. 5. Influence of blocking the GM1 binding site on nLTh-1 and nCT on tissue distribution of coadministered ^{125}I-TT in the ON/E after nasal application. nLTh-I (A) and nCT (B) were preincubated with a 15-fold molar excess of GM1 for 30 min at room temperature prior to nasal application together 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

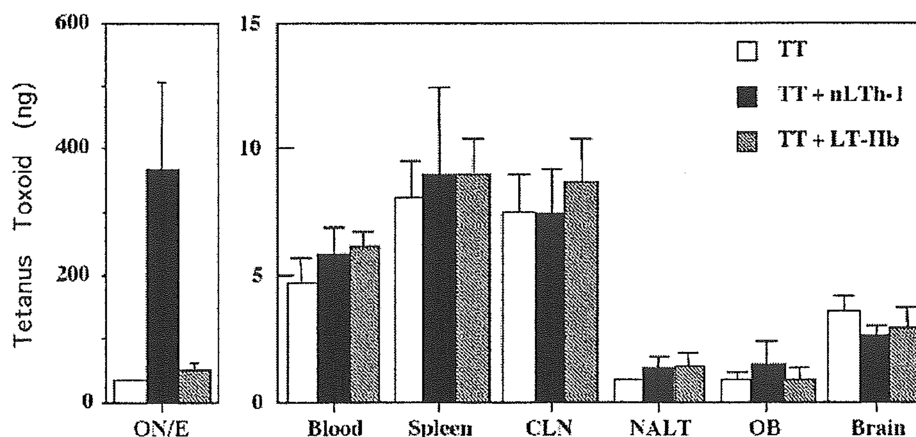

FIG. 6. Trafficking of ^{125}I-TT (20 μg) given nasally without or with nLTh-1 (5 μg) or LT-IIb (5 μg) as mucosal adjuvant. The uptake of ^{125}I-TT into the ON/E, OB, brain, NALT, CLNs, blood, and spleen is shown 12 h after nasal application. The results depicted are from five mice/group and are representative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

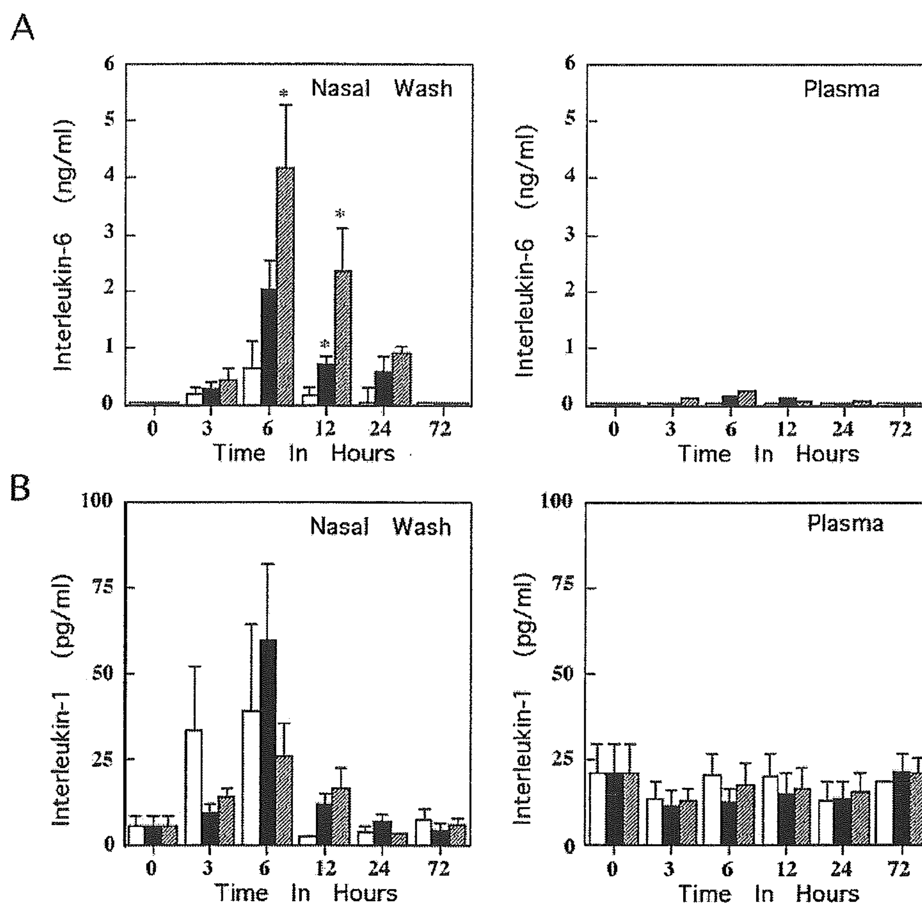

FIG. 7. Inflammatory cytokine expression in the nasal tract after nasal application of TT with or without mCT or nCT. Nasal washes were collected at 0, 3, 6, 12, and 24 h after nasal application of TT alone (open bars), TT and mCT (solid bars), or TT and nCT (hatched bars). The inflammatory cytokine 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