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The Cholera Toxin-Derived CTA1-DD Vaccine Adjuvant Administered Intranasally Does Not Cause Inflammation or Accumulate in the Nervous Tissues[1]

Anna M. Eriksson, Karin M. Schön, and Nils Y. Lycke[2]

Although highly effective, the use of GM1-receptor binding holotoxins as nasal mucosal adjuvants has recently been cautioned due to the risk for their accumulation in the brain and other nervous tissues. Therefore we have explored the efficacy of the CTA1-DD adjuvant for its ability to enhance nasal immune responses in mice. We found that despite the lack of a mucosal binding element, the B cell-targeted CTA1-DD molecule was an equally strong adjuvant as cholera toxin (CT). The potency of CTA1-DD was not a result of endotoxin contamination because more than a 50-fold higher dose of LPS was needed to achieve a similar enhancement. Moreover, the adjuvant effect was TLR4-independent and absent in mutant CTA1-E112K-DD, lacking enzymatic activity. The CTA1-DD adjuvant augmented germinal center formations and T cell priming in the draining lymph nodes, and contrary to CT, promoted a balanced Th1/Th2 response with little effect on IgE Ab production. CTA1-DD did not induce inflammatory changes in the nasal mucosa, and most importantly did not bind to or accumulate in the nervous tissues of the olfactory bulb, whereas CT bound avidly to the nervous tissues. We believe that the nontoxic CTA1-DD adjuvant is an attractive solution to the current dilemma between efficacy and toxicity encountered in CT-holotoxin adjuvant or *Escherichia coli* heat-labile toxin-holotoxin adjuvant strategies and provides a safe and promising candidate to be included in future vaccines for intranasal administration. ***The Journal of Immunology*****, 2004, 173: 3310–3319.**

Mucosal vaccines for nasal administration are highly warranted (1). However, the choice of adjuvant has proven crucial for the development of nonliving mucosal vaccines (2–4). Cholera toxin (CT)[3] and the closely related *Escherichia coli* heat-labile toxin (LT) are perhaps the most powerful and best studied mucosal adjuvants in experimental use today, but when exploited in the clinic their potential toxicity and association with cases of Bell's palsy (paralysis of the facial nerve) have led to their withdrawal from the market (5–9). In addition, studies in mice have shown that CT and LT can accumulate in the olfactory nerve and bulb when given intranasally (i.n.), a mechanism which is dependent on the ability of the B subunits of CT or LT to bind ganglioside-receptors present on all nucleated mammalian cells (10–12). Because we do not know whether such interactions occur in humans there is great need to identify alternative ways that circumvent toxicity, but which retain the potent adjuvant functions of CT or LT. Although less toxic mutants of CT and LT have been engineered with substantial adjuvant function, we believe that such molecules still carry a significant risk of causing adverse reactions (13–15). Especially when considering that the adjuvanticity of CT and LT appears to be a combination of the ADP-ribosyltransferase activity of the A subunit and the ability to bind ganglioside receptors on the target cells (6, 16, 17). Thus, identifying a dose of mutant holotoxin that meets with the need for a desired immunoenhancing effect in the clinic without toxic side effects may prove a cumbersome and difficult task.

We believe that a better solution to this dilemma of efficacy vs toxicity could be found in the CTA1-DD molecule that we have developed (18). This unique adjuvant is based on the enzymatically active A1 subunit of CT combined with a dimer of an Ig-binding element from *Staphylococcus aureus* protein A (19, 20). Hereby the molecule avoids binding to all nucleated cells, which could result in unwanted reactions and exploits fully the CTA1 enzyme in the holotoxin. Accordingly, all previous studies have found that CTA1-DD is nontoxic and has retained excellent immunoenhancing functions (18, 21, 22). When given systemically CTA1-DD provides comparable adjuvant effect to that of intact CT, greatly augmenting both cellular and humoral immunity against specific immunogens coadministered with the adjuvant (18, 22). It also functions as a mucosal adjuvant and should be safe because it is devoid of the B subunit and cannot bind to ganglioside receptors (18, 21). However, previous studies have not addressed the safety aspect of CTA1-DD when used as a nasal vaccine adjuvant. Also, whether the lack of receptor-binding impinges on the efficacy and quality of CTA1-DD as an i.n. adjuvant has not been previously investigated in detail. The CTA1-DD molecule binds exclusively to the Ig receptor on both naive and memory type B cells, whereas Fc receptors are not necessary for the adjuvant function (21). By contrast, CT binds to most nucleated cells, including specialized microfold and dendritic cells (DCs) located in close proximity to the lumen of the nasal-associated lymphoid tissue (NALT), a property that would appear to favor efficacy and

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[3] Abbreviations used in this paper: CT, cholera toxin; CTA, CT A subunit; rCTB, recombinant CT B subunit; LT, *E. coli* heat-labile toxin; GM1, monosialoganglioside; i.n., intranasally; DC, dendritic cell; NALT, nasal-associated lymphoid tissue; CLN, cervical lymph node; EU, endotoxin unit; GC, germinal center; ABC, avidin-biotin complex; TT, tetanus toxoid; cOVA, chicken OVA.

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function in CT above that of CTA1-DD (23–25). The NALT is considered the gateway for induction of mucosal immune responses following nasal vaccine administration (26, 27).

Nasal administration of mucosal vaccines is much preferred before oral or rectal administration, and provides strong mucosal as well as systemic immunity (26). Despite this, relatively few studies have addressed the mechanisms responsible for induction of immunity following i.n. immunization (28–31). The NALT has been well characterized both anatomically and functionally, but little is known about the interaction of adjuvants with cells of the innate immune system of the NALT. Adjuvants are usually microbial products, e.g., CFA, CpG DNA, lipoproteins, or LPS and they activate innate immune responses through binding to pattern recognition receptors, such as TLRs on APCs (32, 33). TLR activation usually elicits an inflammatory response, and there appears to be a direct relationship for many adjuvants between the degree of inflammation induced and the adjuvant potency (34–36). LPS-endotoxin binds to TLR4 and is a well-known contaminant of protein preparations, especially when recombinant technology is used. It is potentially present also in vaccines, which could greatly affect their immunogenicity (37, 38). Whether such endotoxin contamination in i.n. vaccines impacts on the immune response or is a confounding factor in the evaluation of the immunomodulating effect of mucosal adjuvants is largely unknown. Furthermore, whether inflammation is part of the mechanism by which i.n. adjuvants work has been poorly investigated (31). This is particularly interesting in the light of recent findings with DC, which clearly ascribe both pro- and anti-inflammatory functions to CT (39), and our previous finding that CTA1-DD does not cause inflammation (18, 40). Inflammation and a barrier disruption of the nasal mucosal membrane could have negative effects, i.e., augmenting the risk of toxic side effects, such as the accumulation of ganglioside-binding adjuvants and coadministered proteins in the olfactory nerve and brain following i.n. immunization (11, 41–43). The present study was undertaken to compare, in detail, the mechanisms of action of CTA1-DD and CT as i.n. mucosal adjuvants.

Materials and Methods

Mice

BALB/c mice and C57BL/6 mice were obtained from B&K Universal AB (Sollentuna, Sweden). C3H/HeN and C3H/HeJ mice were obtained from Charles River Breeding Laboratories (Sulzfeld, Germany). BALB/c and C3H/HeN mice are normal responders to endotoxin (LPS), whereas C3H/HeJ are hyporesponsive (44, 45). The mice were kept under pathogen-free conditions at the Department of Experimental Biomedicine, Göteborg University (Göteborg, Sweden). Sex-matched animals were used at 6–12 wk of age.

Expression and purification of fusion proteins

For the production of fusion proteins, *E. coli* TG-1 cells transformed with the expression vectors for the CTA1-DD or the CTA1-E112K-DD fusion proteins were grown in 250 ml flasks overnight in 2xYT medium (yeast extract and trypton) with 50 mg/ml kanamycin at 37°C. After culture, the cells were collected by centrifugation and the fusion proteins, produced as inclusion bodies, were solubilized by treatment with 6 M guanidine-HCl. After addition of distilled water to allow refolding, the fusion proteins were purified by affinity chromatography on IgG Sepharose (Pharmacia Biotech, Uppsala, Sweden) as described (46) and stored in 0.5 M HAc at 4°C until use. Adjuvant preparations were routinely tested for the presence of endotoxin by end-point chromogenic *Limulus* amebocyte lysate methods (LAL Endochrome, Charles River Endosafe, Charleston, SC). Endotoxin levels were below 8000 endotoxin units (EU)/mg in CTA1-DD preparations and below 7200 EU/mg in CTA1-E112K-DD preparations.

ADP-ribosyltransferase activity

ADP-ribosyltransferase activity was determined using the NAD:agmatine assay as described earlier (18, 47). Briefly, the ADP-ribosyltransferase activity was determined by assaying 2-fold dilutions of CTA1-DD, CTA1-E112K-DD, or CT and assessing the ADP-ribosylagmatine formation through incorporation of [U-^{14}C]adenine. Each sample contained 50 mM sodium phosphate (pH 7.5), 100 μM GTP, 5 mM $MgCl_2$, 100 mM [U-^{14}C]adenine-labeled NAD (Amersham, Little Chalfont, U.K.), 10 mM agmatine, 0.1 mg/ml chicken OVA (cOVA), and the respective proteins. After 3 h at 30°C, 10-μl samples were transferred to Ag1-X4 columns (Bio-Rad, Hercules, CA). Eluates containing [U-^{14}C]adenine-labeled ADP-ribosylagmatine were collected for determination of radioactivity. We observed that CTA1-DD exhibited enzymatic activity that was around 10–20% of that of the CT holotoxin. Based on this observation 20 μg per dose of CTA1-DD was used throughout this study, and was compared with a 10 times lower dose of CT. The enzymatically inactive mutant, CTA1-E112K-DD, failed to demonstrate ADP-ribosyltransferase activity even at the highest doses tested.

Biotinylation

CTA1-DD, CT, and recombinant CT B subunit (rCTB) were biotinylated following dialysis in sodium bicarbonate buffer. The dialyzed proteins were mixed with D-Biotinoyl-ϵ-aminocaproic acid-*N*-hydroxysuccinimide ester according to the manufacturer's instructions (Roche, Bromma, Sweden) for 2 h at room temperature, followed by extensive dialysis against PBS. Binding ability of the biotinylated adjuvants to IgG and GM1, compared with unlabelled material, was assessed by ELISA as described (18).

Ags and immunizations

Tetanus toxoid (TT, 1000 EU/mg) was purchased from SBL Vaccine (Solna, Sweden). CT (230 EU/mg) was obtained from List Biological Laboratories (Campbell, CA). rCTB was the kind gift from professor J. Holmgren (Department of Medical Microbiology and Immunology, University of Göteborg, Göteborg, Sweden). cOVA (52 EU/mg) and pure LPS from *E. coli* serotype 055:B5 (5.5×10^6 EU/mg) were purchased from Sigma-Aldrich (St. Louis, MO). BALB/c mice were immunized i.n. or i.p. with 10 μg of TT and/or 50 μg of cOVA in the presence or absence of adjuvant. For studying Ab production and T cell responses, five to six mice per group were immunized twice i.p. or three times i.n. at 10-day intervals. The mice were sacrificed 8 days after the final immunization, and serum, bronchoalveolar lavage and/or the cervical lymph nodes (CLN) were collected.

Ab determinations

Analysis of anti-TT-specific Abs in serum and bronchoalveolar lavage was performed with ELISA as described (18). Briefly, 96-well ELISA plates (Nunc, Roskilde, Denmark) were coated with 3 μg/ml TT in PBS. After blocking with 0.1% BSA/PBS, serum samples were added at a 1/100 dilution, and serial 3-fold dilutions were performed in corresponding subwells. For bronchoalveolar lavage, samples were added at a 1/5 dilution, and diluted for serial 2-fold dilutions. Bound total Abs were visualized with HRP-conjugated rabbit anti-mouse Ig Abs (DAKO, Älvsjö, Sweden) at 1/300 dilution, followed by *o*-phenylenediamine substrate (1 mg/ml) in citrate buffer, pH 4.5, containing 0.04% H_2O_2. Isotype-specific Abs were determined using anti-mouse IgG1-, IgG2a-, or IgA-specific alkaline phosphatase-conjugated Abs at 1/500 dilution (Southern Biotechnology Associates, Birmingham, AL) followed by phosphatase substrate (1 mg/ml; Sigma-Aldrich) in ethanolamine buffer, pH 9.8. IgG1- and IgG2a-Ab production were also determined using biotinylated mAbs at 1/200 dilution (BD Pharmingen, San Diego, CA) for detection, followed by Streptavidin-alkaline phosphatase (DAKO). For analysis of total IgE content in serum, soft 96-well ELISA plates (Dynatech Laboratories, Chantilly, VA) were coated with rat anti-mouse IgE (Serotec, Oxford, U.K.). After blocking, serum samples were added at a 1/100 dilution and serially diluted in the plate. The concentration of IgE in the samples was compared with an IgEκ standard curve of known concentrations (BD Pharmingen). IgE Abs were determined using rat anti-mouse IgE biotin (Serotec) followed by Extravidin-Peroxidase (Sigma-Aldrich). *o*-Phenylenediamine substrate was used for development as previously described. The reactions were read in a Multiskan MS spectrophotometer (Labsystems, Stockholm, Sweden). Titers were calculated using the linear part of the curve and were defined as the interpolated value, giving rise to an absorbance of 0.4 above background. The mice were analyzed individually and $\log_{10}$ titers were expressed as means ± SEM of five to six mice per group. IgE content in serum was correlated to the standard curve of known concentrations and was expressed in nanograms per milliliter for individual mice.

In vitro Ag stimulation

Single cell suspensions of CLN were obtained by passing the tissue through a nylon mesh. Erythrocytes were lysed with ammonium chloride and the cell suspensions were washed three times in HBSS (Life

Technologies, Paisley, Scotland). The cells were resuspended at a final concentration of 10^6 cells/ml and cultured in triplicates of 200-μl aliquots in round-bottom 96-well tissue culture plates (Nunc) in Iscove's medium containing 10% FCS and additives, either alone or with 10 μg/ml TT, 2 mg/ml cOVA, or 1 μM CTA1-DD.

Proliferation assay. Proliferation to recall Ag was assessed after 72 h of culture, by the addition of 1 μCi/well [^{3}H]thymidine (Amersham) for the last 6 h of culture. Cell-bound DNA was harvested on filter mats and [^{3}H]thymidine incorporation was measured in a beta-scintillation counter. Data were expressed as mean cpm ± SEM.

Cytokine assay. Culture supernatants from in vitro unstimulated and TT-stimulated cells were analyzed for the presence of IFN-γ, IL-4, and IL-10 using the Bio-Plex Unmixed Multiplex Cytokine Assay (Bio-Rad) according to the manufacturer's instructions after 96 h of culture. The assay was read on a Luminex 100 (Austin, TX), and analyzed using the Bio-Plex Manager software. The mean cytokine concentration in supernatants from TT-stimulated cells above background from unstimulated samples was expressed in picograms per milliliter ± SEM.

Histology

BALB/c mice were immunized with PBS with or without CTA1-DD or CT. At 24 h after one single, or three intranasal immunization, the heads of the mice with facial skin stripped were severed from the body along the line between the upper and lower jaws. The heads were fixed with 4% formaldehyde, decalcified, and embedded in paraffin. Transverse sections, at about the eye level, were stained by conventional histological procedures using H&E.

Immunohistochemistry

CLN, NALT, nasal mucosa, and olfactory bulb were dissected from mice as described below, and embedded in Tissue Tek OCT compound (Sakura, Zoeterwoude, the Netherlands). Tissues were immediately frozen in liquid nitrogen and stored at −70°C. The 7-μm thick frozen tissue sections were prepared on microslides using a Zeiss cryostat (Zeiss, Cambridge, U.K.). Cryostat sections were then fixed in acetone, air dried, rinsed in PBS, and blocked with normal horse serum (1/20) for 15 min (for immunohistochemical staining using peroxidase substrate endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 5 min before serum blockade). Some sections were incubated with biotinylated adjuvants or Abs for 1 h. Others came from mice already exposed to biotinylated product. These slides were analyzed by incubation with avidin-biotin complex (ABC) Vectastain (Vector Laboratories, Burlingame, CA) for 30 min in a humidified chamber, rinsed, and incubated with rabbit peroxidase anti-peroxidase Abs (DAKO). The slides were rinsed again and the color reaction was developed for 10 min using amino-ethylcarbazole substrate (Vector). The slides were counterstained with Mayer hematoxylin. Some sections were incubated for 30 min with fluorescently labeled Abs.

Inflammatory markers. Mice i.n. immunized with TT alone or in combination with CTA1-DD or CT were killed 24 h after immunization. Cryostat transverse sections of the mouse heads, fixed in acetone, were prepared as earlier described, but from decalcified mouse heads snap frozen in Tissue Tek OCT compound. The sections were stained with biotinylated anti-Gr-1 Ab (BD Pharmingen) and analyzed as earlier described, or fluorescently labeled with CD11b-PE (BD Pharmingen) in combination with FITC-labeled peanut (*Arachis hypogaea*) hemagglutinin (Sigma-Aldrich).

Tissue distribution. NALT were dissected using a method described by Wu et al. (48). Briefly, the palate was cut with curved scissors along the teeth from front to rear, and was then carefully dissected from the underlying bone tissue. The NALT still attached to the palate were then quickly embedded and frozen. Alternatively, the NALT tissue was studied in transverse cryosections of the mouse head as previously described. NALT and CLN from naive BALB/c mice and mice immunized with biotinylated CTA1-DD or CT were sectioned and analyzed as described. Sections from naive mice were preincubated with biotinylated CTA1-DD (20 μg/ml), CT (20 μg/ml), or PBS as a control for 1 h before incubation with ABC.

Germinal center formation. BALB/c mice were immunized i.n. with TT with or without the CTA1-DD adjuvant, the enzymatically inactive CTA1-E112K-DD, the CT holotoxin, or increasing doses of LPS. The mice were sacrificed 14 days later. The cryosections were double-labeled with Texas Red-conjugated anti-IgM (Southern Biotechnology Associates) and FITC-labeled GL7 Ab (BD Pharmingen) for 30 min in a humidified chamber, to detect germinal center (GC) formation.

Olfactory bulb accumulation. The brains from naive C57/BL6 mice or mice i.n. immunized with biotinylated CTA1-DD (10 μg) or rCTB (10 μg) were dissected out, 24 h after immunization, by separating the skull in a sagittal plane along the parietal and frontal bone plates. This was followed by the removal of both the frontal and nasal bone plates to gain access to the brain and the olfactory bulbs. Tissue Tek OCT compound embedded brains were frozen, and the olfactory bulbs were sectioned and analyzed with ABC Vectastain as earlier described. Sections from naive mice were preincubated with biotinylated CTA1-DD (20 μg/ml), rCTB (20 μg/ml), or with PBS as a control for 1 h before incubation with ABC.

FACS analysis

For FACS analysis of the in vitro binding ability of biotin-labeled CTA1-DD and CT to NALT cells, NALT from naive BALB/c mice were dissected by removing the palate as previously described. The NALT lymphoid cells were then carefully dissociated from the palate by gentle vortexing. The cell suspension was washed in BSA/PBS and the volume was adjusted to 1×10^7 cells/ml. Aliquots of 100 μl of cells/tube were incubated on ice for 30 min with either biotinylated CTA1-DD or CT (2 μg/1×10^6 cells). BSA/PBS was used as a control. After thorough washing, the cells were incubated on ice for another 30 min with anti-IgDa-FITC (BD Pharmingen) and PE-conjugated streptavidin (DAKO). Analysis was performed using a FACScan analyzer (BD Biosciences, San Jose, CA). Gates were set on live cells, excluding dead cells and debris by forward and side scatter light emission. Fluorescence profiles were analyzed by dot plot representations.

Statistical analysis

We used the nonparametric Mann-Whitney *U* test for analysis of significance. A value of $p < 0.05$ was considered to be significant. All Ab groups were also compared by ANOVA.

Results

CTA1-DD and CT are equally immunoenhancing despite completely different binding properties to cells of the innate immune system

CT and LT are perhaps the most potent mucosal adjuvants we know of today, but their inherent toxicity precludes clinical use (12, 49). Therefore, we have developed a unique adjuvant, CTA1-DD, which retains the enzymatic activity of CT, but is devoid of the ganglioside-binding element of the holotoxin (18, 22, 40). We evaluated the efficacy and safety of CTA1-DD as a nasal vaccine adjuvant and compared it with the intact CT. We predicted that because of their different properties in receptor-binding ability to cells of the innate immune system we would find CT to be far more effective than CTA1-DD as an i.n. mucosal adjuvant, especially when considering that CT could bind to all nucleated cells whereas CTA1-DD would bind only B cells (6, 18, 21, 50). Surprisingly, however, we found that the adjuvant effect of CTA1-DD was comparable to that of CT, when doses of roughly similar ADP-ribosylating activity were used (Fig. 1). We also made the observation that CTA1-DD and CT were equally efficient when given i.n. as they were when used i.p. (Fig. 1). Importantly, i.n. immunizations with the two adjuvants gave similar enhancement of mucosal IgA responses in bronchoalveolar lavage, demonstrating that CTA1-DD can fully replace CT as a mucosal vaccine adjuvant (Fig. 1*B*).

Because the result was unexpected we initiated experiments to control for binding of CTA1-DD and CT to cells of the innate immune system in the NALT. Following nasal administration of biotinylated CT or CTA1-DD we could trace the molecules to the nasal cavity (Fig. 2). At 1 h post-administration we detected both molecules in the mucosa and later at 6 h we also found labeled material in the CLN in both the CT and CTA1-DD groups (Fig. 2). Next we isolated cells from the NALT following nasal administration of the biotinylated molecules and analyzed these by FACS. However, we repeatedly failed to detect cells carrying either CT or CTA1-DD, even though we tried at 0.5, 1, 6, and 24 h after treatment. Therefore, we reverted to frozen sections of NALT, which were labeled ex vivo with biotinylated CT or CTA1-DD for detection of possible receptor interactions that would explain the presence of both molecules in the sections of NALT and CLN.

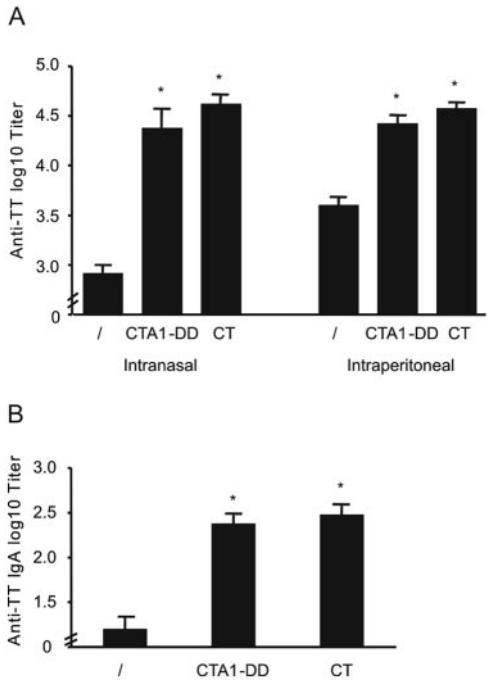

FIGURE 1. CTA1-DD and CT exhibit comparable adjuvant function given i.n. BALB/c mice were given three i.n. or two i.p. immunizations with 10 μg of TT, with or without CTA1-DD (20 μg) or CT (2 μg). Mice were sacrificed 8 days after the final immunization. Sera (*A*) and bronchoalveolar lavage (*B*) were collected and analyzed for specific anti-TT total Ig (serum) or IgA (lavage) Ab content, respectively. Mean anti-TT $\log_{10}$ titers $\pm$ SEM are given for groups of five to six mice. The data are representative of at least three separate experiments giving similar results; *, $p < 0{,}05$ vs TT alone.

Whereas CT bound extensively to all cells of the NALT, including nerve cells adjacent to this tissue, binding of CTA1-DD was not detected (Fig. 2, *H* and *I*). When cells in single cell preparations from NALT were exposed to the biotinylated molecules and analyzed by FACS for binding of CT or CTA1-DD, we observed that CTA1-DD bound exclusively to B cells whereas CT bound all cells from NALT (Fig. 2, *K* and *L*) or CLN (data not shown). Thus, CTA1-DD appeared to access the NALT and CLN compartments despite being able to bind only B cells. By contrast, CT avidly bound to all cells of the NALT, including B cells and DC. Nevertheless, both molecules were potent adjuvants given i.n., suggesting that the ability to broadly interact with distinct receptors on cells of the NALT was not necessary for a strong enhancing function.

The adjuvant effect is independent of endotoxin

To exclude confounding factors that could have accounted for the unexpectedly strong adjuvant activity of CTA1-DD, we analyzed whether a possible endotoxin contamination could have affected the adjuvant function, especially because LPS is known to act through TLRs supposedly present in NALT. We reasoned that such an interaction could well explain the potent effect of CTA1-DD given i.n. Although endotoxin contamination has consistently been low in preparations of CTA1-DD (18, 22), we wanted to secure that even minute amounts of endotoxin did not influence the adjuvant function. To this end mice were immunized with TT and CTA1-DD, or different concentrations of LPS from *E. coli*. In these experiments, the endotoxin activity of the CTA1-DD preparation, according to the *Limulus* method, corresponded to 0.02 μg of purified LPS per dose of protein. We found that doses above 0.1 μg of LPS were required to detect a significant augmenting effect on serum anti-TT responses following three i.n. immunizations (Table I). A dose at least 50-fold higher than the highest contaminating concentration in CTA1-DD, i.e., 1 μg of LPS, was needed to enhance the serum anti-TT response to the same level as that stimulated by the CTA1-DD adjuvant (Table I). Moreover, a mutant molecule, CTA1-E112K-DD, enzymatically inactive and therefore lacking adjuvant function (22), but which contained the same amount of contaminating endotoxin as CTA1-DD, failed completely to enhance serum anti-TT Ab responses (Fig. 3*A*). Finally, because the contaminating endotoxin still could have had an additive effect on the adjuvanticity of CTA1-DD, we also evaluated the enhancing effect in TLR4-disrupted, C3H/HeJ (LPS hyporesponsive), or wild-type C3H/HeN mice. However, again we found no reduction of the adjuvant effect of CTA1-DD in these mice compared with wild-type mice, demonstrating that the adjuvant mechanism of CTA1-DD was independent of endotoxin (Fig. 3*B*).

Intranasal immunization with CTA1-DD does not cause inflammatory changes in the nasal mucosa

A local inflammatory reaction to CTA1-DD could have influenced the adjuvant function, e.g., by disrupting the mucosal barrier, which could have augmented the uptake and immunogenicity of coadministered Ag. We prepared tissue sections of the nasal cavity at various time points after administration of the adjuvants. However, we observed no histological changes indicative of inflammation or membrane barrier disruption in any of the mice, not even after three immunizations (Fig. 4). Furthermore, immunohistochemical staining of the sections with CD11b- and Gr-1-specific mAbs, which detect macrophages and neutrophils, respectively, did not reveal signs of inflammation as compared with sections from mice given TT i.n. alone without adjuvant (data not shown). In fact, none of the groups exhibited mucosal inflammation following even three i.n. immunizations with CTA1-DD or CT (Fig. 4). Increasing the dose of CTA1-DD given i.n. from 20 μg to as high as 125 μg did not seem to have adverse effects on the mice (our unpublished observation). Thus, in agreement with our earlier work CTA1-DD appeared to be a safe and nontoxic adjuvant that did not trigger an inflammatory response after nasal administration (18, 40).

GC formations are augmented after i.n. immunizations with the CTA1-DD adjuvant

Because the efficacy of CTA1-DD as a mucosal adjuvant did not depend on any of the potentially confounding factors that we had investigated so far, we turned to analyzing the CLN, hoping to find evidence of adjuvant function that could be compared with CT. Strikingly, we found that both CTA1-DD and CT greatly enhanced GC-reactions in CLN, clearly suggesting that there were local effects of the adjuvants following nasal administration (Fig. 5, *B* and *D*). This effect appeared to be unique for the ADP-ribosyltransferase active molecules, CTA1-DD and CT, because the inactive CTA1-E112K-DD mutant failed to promote GC development (Fig. 5*C*). Interestingly, low (0.1 μg) nonadjuvant active doses of LPS failed to promote GC reactions, whereas adjuvant active doses ($\geq$1 μg/dose) gave enlarged GC (Fig. 5, *E* and *F*). Thus, local adjuvant activity after nasal administration seemed to be best reflected in the GC reactions in the CLN.

CTA1-DD augments both Th1 and Th2 responses

As the magnitude of the serum anti-TT- or GC-responses were equally enhanced when either CTA1-DD or CT were used, we

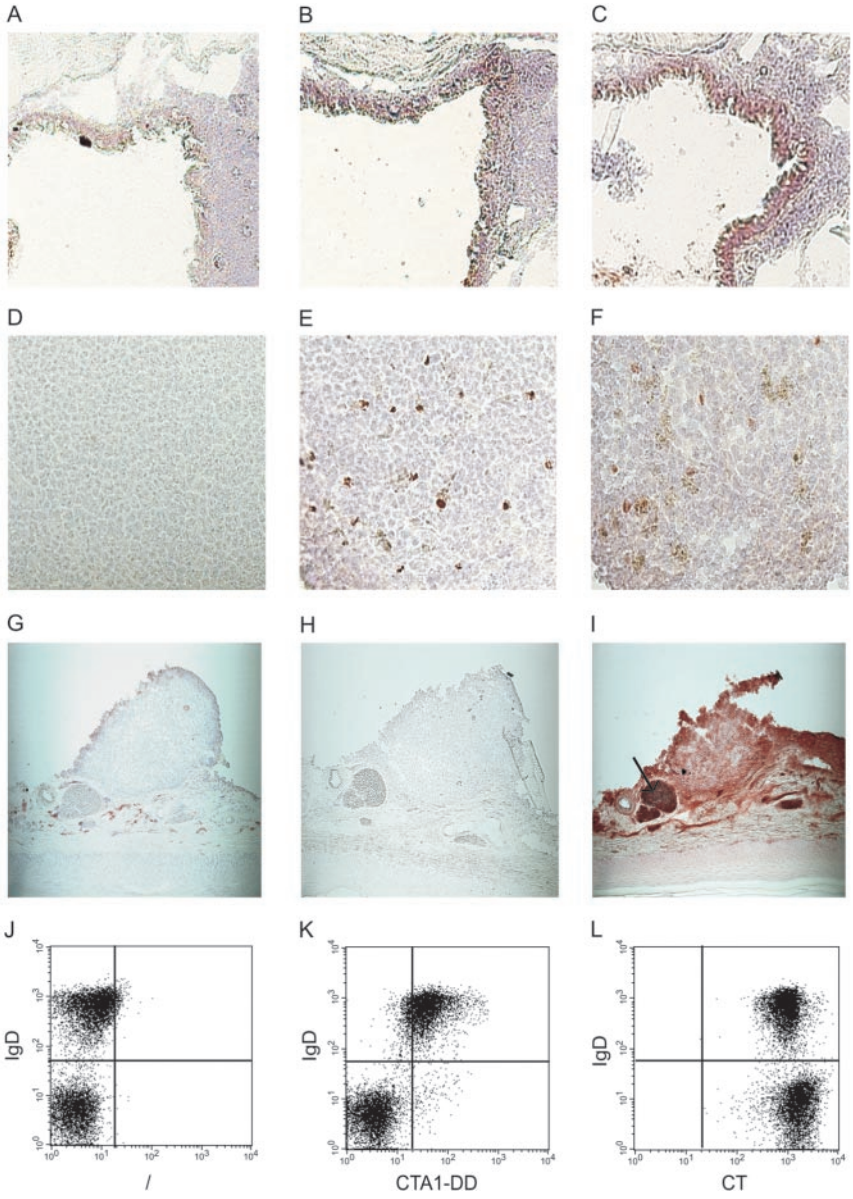

FIGURE 2. Distinct differences in cellular interactions of CTA1-DD and CT in the NALT. Biotinylated CTA1-DD (*B* and *E*) or CT (*C* and *F*) at 20 and 2 μg doses, respectively, was given to mice i.n., and the deposition of the molecules in NALT and CLN was monitored at various time points. PBS-treated mice were used as controls (*A* and *D*). *A–C*, Presence of CTA1-DD (*B*) and CT (*C*) in the nasal mucosa 1 h after administration. *D–F*, Distribution of CTA1-DD (*E*) and CT (*F*) in the CLN 6 h after nasal administration. *G–L*, Ex vivo demonstration of the binding ability of biotinylated CTA1-DD (*H* and *K*) or CT (*I* and *L*) to sections, or isolated cells, from the NALT. Negative controls (*G* and *J*) are shown. *G–I*, Binding of CTA1-DD (*H*) and CT (*I*) to the NALT after direct application to tissue sections from naive mice. Binding of CT to nervous tissue adjacent to the NALT is indicated with an arrow. *J–L*, FACS analysis of the ability of biotinylated CTA1-DD (*K*) or CT (*L*) to bind to IgD-positive B cells from the NALT. All tissue sections were analyzed with a Vectastain ABC kit, followed by rabbit peroxidase anti-peroxidase Abs and amino-ethylcarbazole substrate and counter stained with Mayer hematoxylin.

evaluated whether the adjuvant effects also were qualitatively similar. An extended analysis of serum anti-TT Ab responses following i.n. immunization revealed that CTA1-DD promoted increased IgG1 as well as IgG2a anti-TT Ab levels, whereas CT enhanced IgG2a levels less strongly (Fig. 6). Together with our finding of a dramatic increase in serum total IgE levels in CT-treated mice, but not in CTA1-DD adjuvant treated, we had reason to believe that CT stimulated Th2 differentiation more strongly than CTA1-DD (Fig. 6*B*). Upon direct examination of T cells isolated from the CLN of i.n. immunized mice, we observed greatly enhanced proliferation and cytokine production to recall Ag in vitro in both the CT- and CTA1-DD-treated groups (Fig. 7). In this model both TT and OVA were used as i.n. immunogens and both Ags elicited similar T cell priming. Whereas CTA1-DD stimulated strong IFN-γ as well as IL-4 and IL-10 production, CT promoted relatively more IL-4 and IL-10 and less IFN-γ (Fig. 7*B*). Therefore, CTA1-DD stimulated a better balanced induction of Th1 and Th2 immunity, whereas the CT effect was skewed toward a Th2 type.

CTA1-DD does not bind or accumulate in the CNS

Recently, major concern was raised against the use of CT or LT in mucosal vaccines for nasal administration (7, 8, 51). Investigations in mice had revealed extensive accumulation of CT, or proteins admixed with CT, in the olfactory nerve and bulb following i.n. immunizations (10–12). The mechanism for this accumulation appeared to be dependent on the ability of the B subunit of the holotoxins to bind to the GM1 ganglioside receptor present on all

Table I. *Pure LPS with comparable endotoxin activity as the contaminating endotoxin in the CTA1-DD adjuvant preparation has no immunoenhancing activity*[a]

Adjuvant	Endotoxin Concentration (EU/dose)	Anti-TT $\log_{10}$ Titer
None	10	3.1 ± 0.3
LPS (μg)		
0.01	70	3.1 ± 0.4
0.1	560	3.4 ± 0.4
1	5,500	4.2 ± 0.1*
10	55,000	4.5 ± 0.2*
CTA1-DD	≤160	4.3 ± 0.1*

[a] TT-specific total Ig Ab content in sera from BALB/c mice given three intranasal immunizations with 10 μg of TT with or without the CTA1-DD adjuvant (20 μg) or different concentrations of LPS from *E. coli.*

* $p < 0.05$ vs. TT alone.

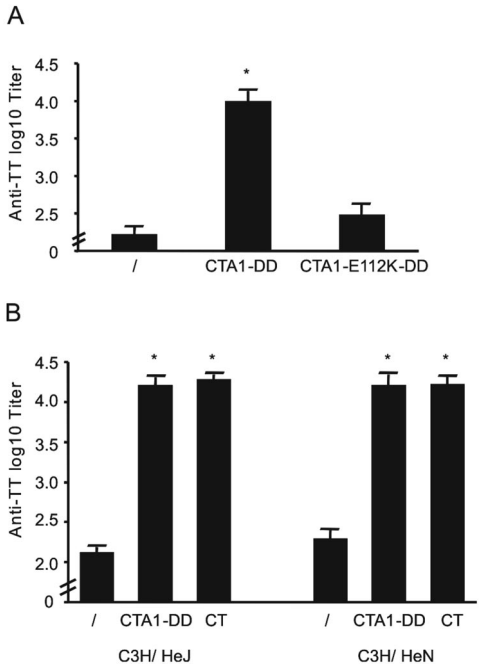

FIGURE 3. Endotoxin has no effect on the adjuvant function of CTA1-DD. *A*, BALB/c mice were given three i.n. immunizations with 10 μg of TT alone or together with CTA1-DD (20 μg) or the enzymatically inactive CTA1-E112K-DD mutant (20 μg). *B*, Endotoxin hyporesponsive (HeJ) or sensitive (HeN) mice were given three i.n. immunizations with 10 μg of TT in the absence or presence of CTA1-DD (20 μg) or CT (2 μg). The mice were sacrificed 8 days after the final immunization and individual sera were collected and analyzed for specific anti-TT total Ig Ab content. Mean anti-TT $\log_{10}$ titers $\pm$ SEM are given for groups of three to six mice. The data are representative of at least three separate experiments giving similar results; $*$, $p < 0{,}05$ vs TT alone.

nucleated cells, including nerve cells (25, 50). CTA1-DD, devoid of the B subunit, was therefore compared with CT for its ability to bind and accumulate in the olfactory nerve and bulb after nasal administration. For comparison, we gave either biotinylated CTA1-DD or rCTB and analyzed the degree of accumulation of adjuvant molecules in sections of olfactory bulb taken 24 h after a single immunization. Immunohistochemical analysis revealed strong binding of rCTB to the nervous tissue of the olfactory bulb (Fig. 8*A*). By contrast, no detectable CTA1-DD was found in any of the mice (Fig. 8*B*). Moreover, when labeled CTA1-DD or rCTB was applied directly onto tissue sections of olfactory bulb from naive mice, only rCTB was detected, demonstrating that CTA1-DD does not bind GM1 ganglioside receptors, and therefore is unlikely to accumulate in nervous tissues (Fig. 8, *C* and *D*). Thus, CTA1-DD is a potent nontoxic adjuvant that safely can be used in mucosal vaccines for nasal administration with no risk of accumulation in the nervous system.

Discussion

The present study unequivocally documents that CTA1-DD is a highly efficient and safe mucosal adjuvant that greatly augments T cell and B cell responses following intranasal immunizations. Unlike ganglioside-receptor binding CT or LT, or their less toxic mutants, CTA1-DD did not bind or accumulate in the olfactory nerve or CNS following intranasal application. No adverse effects on the nasal mucosa were observed, in agreement with our previous observation, indicating that CTA1-DD exerts adjuvant function in the absence of an inflammatory response (18, 40). Thereby, CTA1-DD constitutes a rare type of immunomodulating agent that is not proinflammatory, but rather promotes the expression of effective costimulation (18). The effect is restricted to the innate and adaptive immune system and does not appear to impinge on other organ system, such as the nervous tissues. Importantly, not even very high doses (125 μg) of CTA1-DD given i.n. demonstrated toxic side effects. By contrast, CT in doses >2.5 μg has been shown to give significant inflammation in the lung, but only minimal inflammatory responses in the nasal passage (31). The key element in our adjuvant is the enzyme, whereas the DD-dimer is responsible for reducing toxicity by targeting CTA1 to the APC, and thus is essential for the function of CTA1-DD (22). Of note, doses of CT that host an ADP-ribosylating activity comparable to the high doses of CTA1-DD tested i.n. are lethal to mice (52, 53).

We could only detect direct binding of CTA1-DD to B cells of the NALT. In contrast, CT will bind to all cells, including epithelial cells and possibly also Ag sampling microfold cells overlaying the NALT. Despite this restricted binding, the effect of CTA1-DD as a mucosal adjuvant was most impressive. It appears that CT and CTA1-DD, or unrelated proteins, can be taken up from the nasal

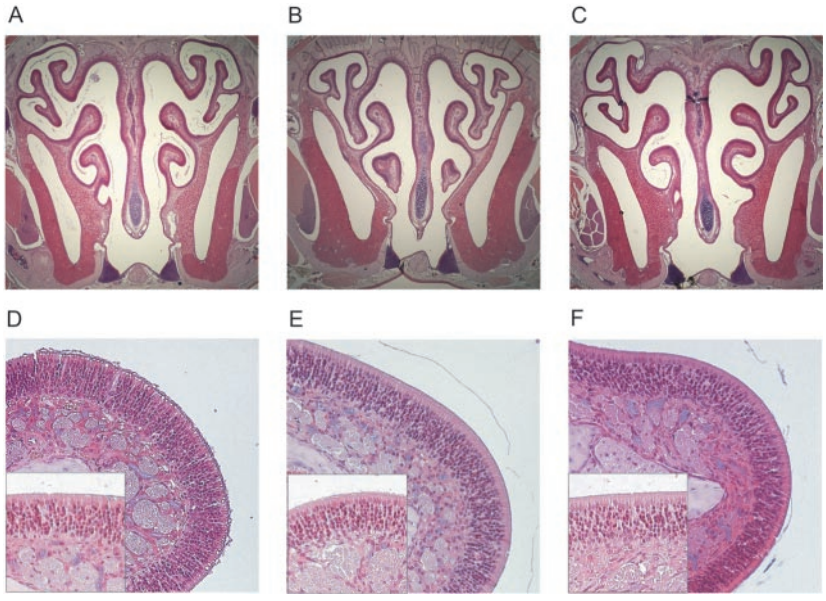

FIGURE 4. Intranasal immunization with CTA1-DD does not cause histological changes in the nasal cavity. Frontal cross-sections of the nasal cavity from mice, taken 24 h after nasal administration of PBS (*A* and *D*), 20 μg of CTA1-DD (*B* and *E*) or 2 μg of CT (*C* and *F*). A whole view of the nasal passage is shown in *A–C*. *D–F*, An enlarged view of the nasal mucosa is shown. Sections at the level of the eyes were prepared and the tissues were stained with H&E to assess the degree of inflammation. Similar tissue sections of the nasal mucosa, taken from mice after three i.n. immunizations, are inserted into *D–F*.

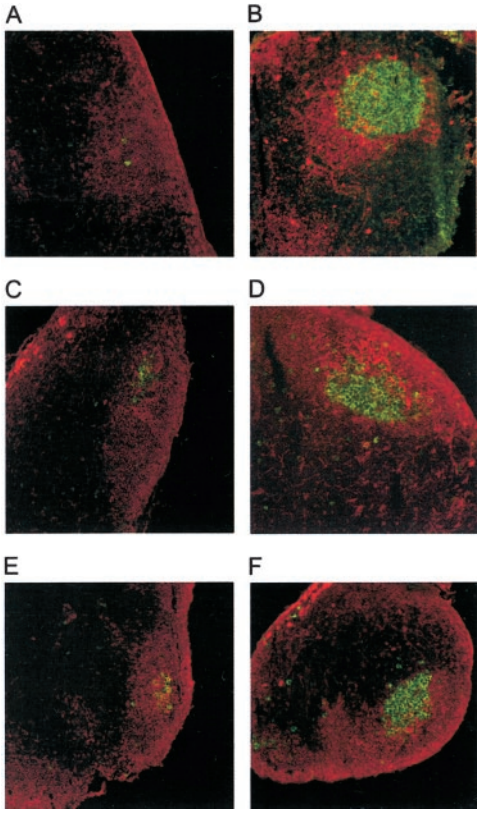

FIGURE 5. CTA1-DD strongly promotes GC formation following i.n. immunization. BALB/c mice were immunized i.n. with 10 μg of TT alone (*A*) or together with 20 μg of CTA1-DD (*B*), 20 μg of CTA1-E112K-DD (*C*), 2 μg of CT (*D*), or LPS from *E. coli* in a low dose (0.1 μg) (*E*), or a high dose (10 μg) (*F*). Mice were sacrificed 14 days postimmunization, and cryosections of the CLN were prepared and double labeled with Texas Red-conjugated anti-IgM (red) and FITC-labeled GL7 Ab (green), to detect GCs (yellow/green). This is one representative experiment of three giving similar results.

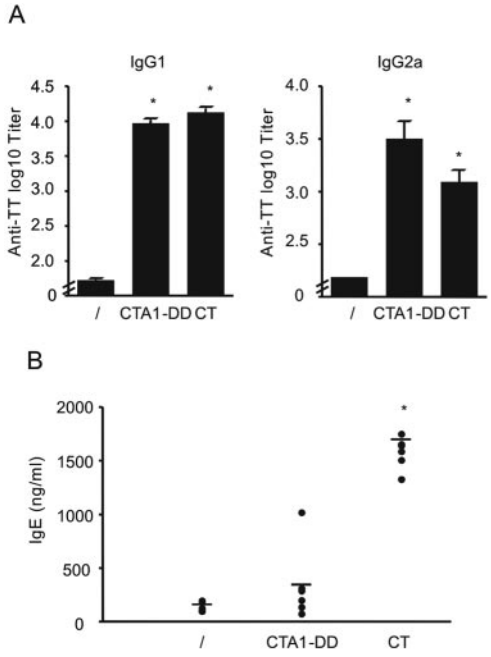

FIGURE 6. CTA1-DD provides a balanced Th1/Th2 Ab response. BALB/c mice were given three i.n. immunizations with 10 μg of TT, with or without CTA1-DD (20 μg) or CT (2 μg). The mice were sacrificed 8 days after the final immunization, and individual sera was collected and analyzed for specific anti-TT IgG1 or IgG2a Ab content (*A*). Values are given as mean anti-TT $\log_{10}$ titers $\pm$ SEM for groups of six mice. Total serum IgE levels were also determined (*B*). Values are expressed in nanograms per milliliter for individual mice. The data are representative of at least three separate experiments giving similar results; $*$, $p < 0{,}05$ vs TT alone. To confirm the result we also used isotype-specific mAbs for the analysis, which gave a similar distribution of IgG1 and IgG2a Abs in CTA1-DD- and CT-treated mice.

cavity into the NALT irrespective of their receptor-binding ability. This notion finds support in several other studies in which Ag uptake has been monitored after nasal application (29, 54, 55). Furthermore, the serum responses after i.n. immunizations were comparable to those obtained after i.p. injections, arguing for a very effective handling of Ag and adjuvant in the NALT. This explains why i.n. vaccination remains the preferred route for stimulating strong systemic immunity concomitantly with local mucosal immune responses (26). Because we failed to detect cells in the NALT carrying CTA1-DD or CT following intranasal administration, we do not know which cells were the targets for the adjuvants. However, we have direct evidence for the induction of a regional response as GC reactions were induced in the CLN following immunizations with both adjuvants. Noteworthy, all preparations with an i.n. adjuvant effect, including the high doses of LPS, stimulated significant GC reactions in the CLN. Whether Ag and adjuvant interacted with the very same cells of the innate immune system in the NALT or the CLN was not part of the present study. It can be assumed however, that CTA1-DD bound to B cells in the NALT, but whether the adjuvant effect was mediated by B cells or the DC cannot be assessed, as yet. Although CTA1-DD fully matched CT as an i.n. vaccine adjuvant, the qualitative effect on the T cell and B cell responses to Ag were not identical. This might suggest a difference in the target cell population. Cells exposed to CTA1-DD subsequently acting as APC, clearly gave a more balanced Th1/Th2 type of response, whereas CT demonstrated skewing toward a Th2 domination. The latter observation is in agreement with many previous studies, some of which have cautioned the use of holotoxin adjuvants because of the risk of developing IgE-mediated allergy (31). CT most probably acts through DC in the NALT, even though we have no experimental evidence for that at present, but recent findings by our group and others have demonstrated that DC in the spleen or lymph nodes are the main targets for CT in vivo (17, 56, 57). In addition, Porgador et al. (29) found that DC isolated from NALT exhibited enhanced MHC class I-restricted APC function following i.n. immunizations with CT adjuvant. Notwithstanding this result, the actual presence of adjuvant and/or Ag in the APC of NALT and CLN still awaits to be shown in most protocols used for i.n. immunizations (55). A simple explanation to the failures in demonstrating the presence of adjuvant in the APC of the NALT, may simply be that higher doses are required, or alternatively, that APC of the lower respiratory tract are the target cells, i.e., in analogy with observations made by other investigators (58). Although labeled CTA1-DD and CT was found in the CLN, and the fact that we observed GC in the CLN, would argue against the latter notion. Future experiments using more sensitive techniques could hopefully resolve this question.

Obviously CTA1-DD and CT could have differential effects on APC, and that could explain the difference in priming of Th1- and Th2-type immunity. One such factor is costimulation mediated by CD86, which has been claimed to promote the Th2 type more than the Th1 type of response (59, 60). Because we did not detect cells carrying CT or CTA1-DD in the NALT or CLN, this possibility was never tested. However, we recently found in vivo that CT

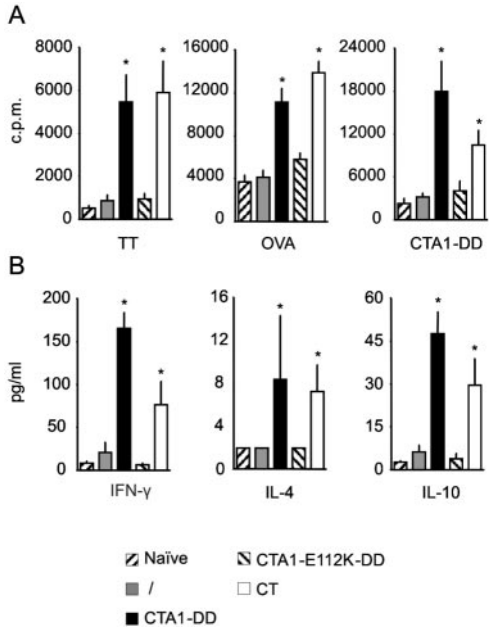

FIGURE 7. Intranasal immunization with CTA1-DD strongly augments T cell priming. BALB/c mice were given three i.n. immunizations with cOVA (50 μg) and TT (10 μg) with or without CTA1-DD (20 μg) (■), the enzymatically inactive CTA1-E112K-DD mutant (20 μg) (▨), or CT (2 μg) (□). Eight days after the final immunization, mice were sacrificed. Single cell suspensions of the CLN were prepared and the cells were cultured in the absence or presence of TT (10 μg/ml), cOVA (2 mg/ml), or CTA1-DD (1 μM). *A*, T cell proliferation after 72 h of stimulation as assessed by [^{3}H]thymidine uptake, expressed as mean cpm ± SEM for groups of six mice. *B*, Concentrations of indicated cytokines in culture supernatants following stimulation with TT for 96 h. Values are given as mean picograms per milliliter ± SEM of groups of six mice. Results (*A* and *B*) from two pooled experiments are shown; ∗, $p < 0.05$ vs TT alone.

exposed DC express CD86, but not CD80, in the T cell zone, although both molecules were up-regulated in vitro (Ref. 56 and N. Y. Lycke, unpublished observations). It may thus be that CTA1-DD acts via up-regulation of CD80 in vivo, but does not suppress IL-12 or Th1 development, directly or indirectly, i.e., through enhancing RANTES, IL-1, IL-6, IL-10, and TGF-β production or inhibiting CD40 or ICAM-1, as CT appears to do with the APC (39, 57, 61). Whether CTA1-DD acts exclusively through B cells or also affects DC in vivo is currently not fully resolved. Preliminary experiments indicate that CTA1-DD can be taken-up by APC other then B cells, which also show enhanced APC-function. B cell-deficient mice have been found to react with enhanced T cell priming when CTA1-DD is used (A. M. Eriksson, unpublished observations). Whether B cells or DC act separately or interact for enhanced APC function in the presence of CTA1-DD in normal mice is thus poorly understood. Several possibilities exist, from cross-priming, with DC presenting Ag in the context of apoptotic CTA1-DD-exposed B cells, to actual interactions of CTA1-DD exposed DC and B cells in DC clusters in the lymph node or spleen. These and other possibilities will be investigated.

Both CT and CTA1-DD are potent adjuvants because the ADP-ribosylating enzyme is the key component allowing both molecules to interact with the innate immune system. However, binding is through different receptors and yet they exert comparable adjuvant functions based on their equimolar ADP-ribosylating activity. CT binds GM1-ganglioside receptors, whereas CTA1-DD binds to Ig receptors, the modulating effect being independent of Fc receptor expression (21). Thus, in the case of CTA1-DD and CT, the CTA1-enzyme is introduced into the target cell by different routes and mechanisms (6, 18, 21, 50). The mutant, CTA1-E112K-DD, does not act as an i.n. adjuvant, disclosing that it is the enzyme that is crucial and not the binding element. Nor did we find that contaminating endotoxin was contributing to the adjuvant effect. A study by Unger et al. (62) demonstrated recently that large amounts of LPS (25 μg) were required to break mucosal tolerance

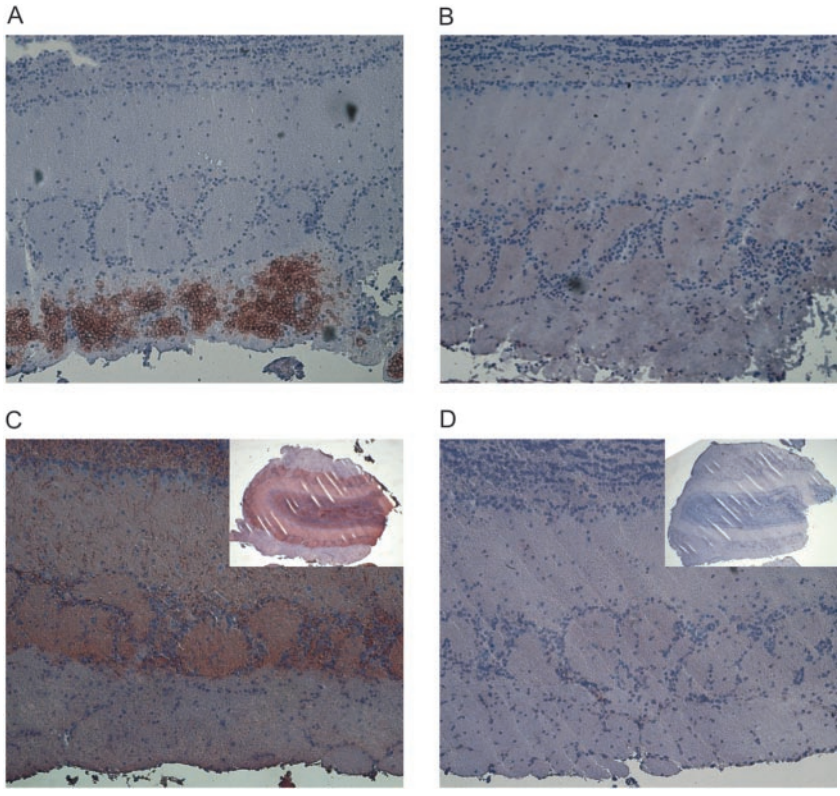

FIGURE 8. CTA1-DD does not bind to the nervous tissue of the olfactory bulbs, whereas strong binding was observed with CTB. Binding and accumulation of biotinylated CTB (*A* and *C*) or CTA1-DD (*B* and *D*) to the nervous tissue of the olfactory bulbs was analyzed 24 h after a single nasal administration of adjuvant (*A* and *B*) or after direct application to tissue sections from naive mice (*C* and *D*). Lower magnifications of *C* and *D* are inserted. The labeling was visualized with the Vectastain ABC kit followed by rabbit peroxidase anti-peroxidase Abs and amino-ethylcarbazole substrate, and the sections were counter stained with Mayer hematoxylin.

induction via the nasal route. Doses of 0.1–1 μg of endotoxin i.n. were reported to trigger TNF-α-dependent inflammation in mice and thus, endotoxin could have been assumed to influence the function of CTA1-DD (63). However, we found no difference in the overall ability of CTA1-DD to augment i.n. immune responses in TLR4-defective C3H/HeJ mice compared with that in wild-type mice. Accordingly, the stronger Th1-inducing effect of CTA1-DD relative to that of CT seems not to be an effect of endotoxin contamination, and thus, is independent of TLR4 signaling (64). These findings render support for the idea that the ADP-ribosyltransferase-mediated adjuvant function is unique and different from other microbial adjuvants, of which many elicit proinflammatory responses (33).

Somewhat at variance with our findings, Ohmura et al. (38) reported that the same mutant as we have used, CTE112K given i.n. as a holotoxin had potent adjuvant function. We cannot explain this difference, but the role of mutant CTA1 in the holotoxin does not involve enzymatic activity and thus, it would argue for some other modulating property, be it structural, or in any other way associated with the AB_5 complex (47). Of course, all holotoxin molecules would likely accumulate in the CNS because they are ganglioside binders, which could preclude clinical use if such accumulation had functional consequences (11, 25).

Immunization using the nasal route requires less Ag and adjuvant compared with an oral immunization. For vaccine manufacturers, these and other advantages have made it the route of choice (1). A commercial i.n. vaccine using the LT adjuvant was recently withdrawn from the market because of possible association with side effects such as rhinorrhea, headache, and most seriously facial paresis (Bell's palsy) (7–9, 51). In the present study we believe that we have documented beyond doubt that in mice the CTA1-DD adjuvant does not bind or accumulate in the olfactory nerve or the brain. In keeping with results of previous work, we found that rCTB bound and accumulated extensively in the olfactory nerve and brain. Thus, CTA1-DD appears to be a safe and efficient vaccine adjuvant candidate to be included in future nasal vaccines.

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