Bacillus anthracis Edema Toxin Acts as an Adjuvant for Mucosal Immune Responses to Nasally Administered Vaccine Antigens

The Journal of Immunology

This information is current as of August 9, 2022.

Alexandra Duverger, Raymond J. Jackson, Frederick W. van Ginkel, Romy Fischer, Angela Tafaro, Stephen H. Leppla, Kohtaro Fujihashi, Hiroshi Kiyono, Jerry R. McGhee and Prosper N. Boyaka

References This article **cites 63 articles**, 42 of which you can access for free at:
http://www.jimmunol.org/content/176/3/1776.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Bacillus anthracis Edema Toxin Acts as an Adjuvant for Mucosal Immune Responses to Nasally Administered Vaccine Antigens[1]

Alexandra Duverger,* Raymond J. Jackson,* Frederick W. van Ginkel,[2]* Romy Fischer,*
Angela Tafaro,* Stephen H. Leppla,[‡] Kohtaro Fujihashi,*[†] Hiroshi Kiyono,[†§]
Jerry R. McGhee,* and Prosper N. Boyaka[3]*

Anthrax edema toxin (EdTx) is an AB-type toxin that binds to anthrax toxin receptors on target cells via the binding subunit, protective Ag (PA). Edema factor, the enzymatic A subunit of EdTx, is an adenylate cyclase. We found that nasal delivery of EdTx enhanced systemic immunity to nasally coadministered OVA and resulted in high OVA-specific plasma IgA and IgG (mainly IgG1 and IgG2b). The edema factor also enhanced immunity to the binding PA subunit itself and promoted high levels of plasma IgG and IgA responses as well as neutralizing PA Abs. Mice given OVA and EdTx also exhibited both PA- and OVA-specific IgA and IgG Ab responses in saliva as well as IgA Ab responses in vaginal washes. EdTx as adjuvant triggered OVA- and PA-specific CD4^{+} T cells which secreted IFN-γ and selected Th2-type cytokines. The EdTx up-regulated costimulatory molecule expression by APCs but was less effective than cholera toxin for inducing IL-6 responses either by APCs in vitro or in nasal washes in vivo. Finally, nasally administered EdTx did not target CNS tissues and did not induce IL-1 mRNA responses in the nasopharyngeal-associated lymphoepithelial tissue or in the olfactory bulb epithelium. Thus, EdTx derivatives could represent an alternative to the ganglioside-binding enterotoxin adjuvants and provide new tools for inducing protective immunity to PA-based anthrax vaccines. *The Journal of Immunology,* 2006, 176: 1776–1783.

*B*acillus anthracis expresses the protective Ag (PA),[4] the lethal factor (LF), and the edema factor (EF), which combine to form two AB-type toxins (1–3). The combination of PA and EF leads to the formation of anthrax edema toxin (EdTx), while lethal toxin (LeTx) results from the combination of PA and LF. The PA subunit targets cells via the anthrax toxin receptor (ATR) 1, which resembles the tumor endothelial marker 8 (4), and the related ATR2, which is similar to the capillary morphogenesis gene 2 (5). The EF is an adenylate cyclase which increases intracellular cAMP levels (6–8) and induces edema (6). Most previous studies have focused on LeTx-induced death and alterations in APC functions in susceptible macrophages (Mϕ) (9–11) and dendritic cells (12). Anthrax toxin-fusion protein deriva-

tives consisting of PA and the N-terminal domain of LF (LF$^{1–254}$) have been used to deliver Ags into the cytosol for presentation via MHC class I molecules and induction of CTL responses (13–16). A recent report suggested that PA may not be needed for intracellular delivery of proteins by the LF N-terminal fragment (17). Intradermal coimmunization with a DNA plasmid encoding the N-terminal fragment of LF, which shares homology with the N-terminal fragment of EF, was reported to induce higher anti-PA Ab responses than immunization with a single plasmid encoding PA (18). Although EdTx was reported to induce accumulation of cAMP in lymphocytes (19) and suppress T cell activation (20), little is known about the effect of EdTx on adaptive immune responses.

Cholera toxin (CT) and the related heat labile toxin I (LT-I) of *Escherichia coli* are AB-type toxins made of pentameric-binding B subunits and enzymatic A subunits with ADP-ribosyl transferase activities (21–23). The B subunits of CT and LT-I bind to GM1 gangliosides on target cells (24), while the more promiscuous B subunit of LT-I also exhibits affinity for GM2 and asialo-GM1 (25–27). CT and LT-I are the best described mucosal adjuvants and both promote mucosal secretory IgA (S-IgA) and plasma Ab responses to coadministered vaccine Ags. Unfortunately, the watery diarrhea induced by these toxins precludes their use as oral adjuvants in humans. In addition, major safety concerns relative to the potential of nasal enterotoxins to target CNS tissues have been reported (28, 29). Thus, nasal enterotoxin could damage CNS tissues in large part through their ADP-ribosyl transferase activity following binding of the B subunit to the promiscuous gangliosides expressed on cells of the CNS (30, 31).

We investigated whether an EdTx derivative could act as a mucosal adjuvant like the enterotoxin CT and LT-I, and promote S-IgA and systemic Ab responses to nasally coadministered vaccine

Ags. We also queried whether this regimen would enhance immunity to the binding B subunit PA itself and perhaps provide an extra bonus for anthrax immunity. We further examined whether the receptor specificity of EdTx would lead to the accumulation of this toxin into CNS tissues after nasal delivery.

Materials and Methods

Mice

Female C57BL/6 mice, 6–7 wk of age, were obtained from Charles River Laboratories and were 9–12 wk of age when used in these experiments. All studies were performed in accordance with both National Institutes of Health and University of Alabama at Birmingham institutional guidelines to avoid pain and distress.

Immunization

Mice were nasally immunized three times at weekly intervals with 100 μg of OVA (Sigma-Aldrich) alone, OVA plus 5 μg of rPA only, or OVA plus EdTx (5 μg of rPA together with 5 μg of rEF). The rPA was purified from cultures of a recombinant strain of *B. anthracis* as previously described (32). The EF was obtained from List Biological Laboratories (product no. 173) and was produced in a recombinant strain of *B. anthracis* using an expression plasmid constructed by S. H. Leppla. This EF protein contains a S447N mutation and was shown to display ~20-fold less enzymatic activity than the native EF (33). Controls included mice nasally immunized with OVA plus 1 μg of CT (List Biological Laboratories). Mice were lightly anesthetized and given 12.5 μl of vaccine/nostril. Blood and external secretions (fecal extracts, vaginal washes, and saliva) were collected as previously described (34, 35).

Evaluation of OVA- and PA-specific Ab isotypes and IgG subclass responses

Previously described ELISA was used to assess anti-OVA and anti-PA Ab levels in plasma and external secretions (34, 35). Briefly, microtiter plates were coated with OVA (1 mg/ml) or PA (5 μg/ml). The IgM, IgG, or IgA Abs were detected with HRP-conjugated goat anti-mouse μ-, γ-, or α-H-chain-specific antisera (Southern Biotechnology Associates). Biotin-conjugated rat anti-mouse IgG1 (clone A85-1; 0.5 μg/ml), IgG2a (clone R19-15; 0.5 μg/ml), IgG2b (clone R12-3; 0.5 μg/ml), or IgG3 (clone R40-82; 0.5 μg/ml) mAbs and HRP-conjugated streptavidin (BD Pharmingen) were used to measure IgG subclass responses. The color was developed with the addition of ABTS substrate (Sigma-Aldrich), and the absorbance was measured at 415 nm. End-point titers were expressed as the log$_2$ dilution giving an OD$_{415}$ of $\geq$0.1 above those obtained with nonimmunized control mouse samples.

Total and Ag-specific IgE Abs

Total IgE Ab levels were determined by a BD OptEIA Set Mouse IgE (BD Pharmingen), according to instructions from the manufacturer. To prevent interference in the assay, serial dilutions of immune plasma were previously depleted of IgG by overnight incubation in Reacti-Bind Protein G-Coated Plates (Pierce). To detect Ag-specific IgE, the microtiter plates were coated with OVA (1 mg/ml) or PA (5 μg/ml). Serial dilutions of plasma were then added, IgE was detected with the biotinylated anti-mouse IgE Abs, and titers were determined as described above.

Macrophage toxicity assay to assess anti-PA-neutralizing Abs

The protective effects of PA-specific Abs were determined using a previously described assay (35) that measures their capacity to protect the J774 Mϕ cell line from LeTx (9, 11). Briefly, J774 Mϕ (5 $\times$ 10^4 Mϕ/well) were added to 96-well, flat-bottom plates. After 12 h of incubation, plasma or external secretions samples were added together with LeTx (400 ng/ml PA plus 40 ng/ml LF) and incubated for an additional 12 h as described elsewhere (31). Viable Mϕ were evaluated after addition of MTT (Sigma-Aldrich) (36).

Effect of EdTx on APCs in vitro

J774 Mϕ (5 $\times$ 10^4 cells/ml) or freshly isolated mesenteric lymph node or spleen cells from C57BL/6 mice were incubated in the presence of PA only (5 μg/ml), EF only (5 μg/ml), EdTx (PA + EF; 5 μg/ml), or CT (1 μg/ml). Forty-eight hours later, culture supernatants were collected for evaluation of cytokine responses. Cells were collected and stained for 30 min at 4°C with FITC- or PE-conjugated mAbs (BD Pharmingen). After three washing steps and fixation in 2% paraformaldehyde, the expression of activation and costimulatory molecules was analyzed by flow cytometry.

In vitro restimulation of Ag-specific CD4$^+$ T cells and cytokine-specific ELISA

T cells were isolated from spleen and cervical lymph nodes (CLNs) and restimulated in vitro as previously described (34, 35, 37) with OVA (1 mg/ml) or PA (20 μg/ml) in RPMI 1640 medium containing 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, 5 $\times$ 10^{-5} M 2-ME, 1 mM sodium pyruvate, and 10% FCS. The Th1 and Th2 cytokines in culture supernatants were determined by cytokine-specific ELISA as previously described (34, 35, 38). The mAb couples were from BD Pharmingen. Standard curves were generated using murine rIFN-γ, rIL-5, rIL-6, and rIL-10, rIL-12 (R&D Systems); rIL-2 and rIL-13 (BD Pharmingen); and rIL-4 (Pierce). The ELISAs were capable of detecting 5 pg/ml for IL-2, IL-4, and IL-5; 15 pg/ml for IFN-γ; 50 pg/ml for IL-13; 100 pg/ml for IL-6, IL-12; and 200 pg/ml for IL-10.

Tracking studies

The PA and CT were labeled with acridinium using an acridinium C$_2$ NHS ester labeling kit (Assay Designs). The specific activity of PA and CT used in the tracking studies were 2.11 $\times$ 10^7 relative light units (RLU)/ng and 2.06 $\times$ 10^7 RLU/ng, respectively. Mice were given the acridinium-labeled compounds by instilling 5-μl quantities into each nare. Twelve and 24 h following immunization, mice were sacrificed and the olfactory nerves and epithelium (ON/E), olfactory bulbs (OB) and brain (B) were removed as previously described (30). Each tissue was weighed and 200 μl of Cellytic MT lysis buffer (Sigma-Aldrich) was added per 10 mg wet weight of tissue. The ice-cold tissues were homogenized (20,000 rpm for 15–20 s) using a Tissue Tearor (Biospec Products) and frozen at -20°C. After thawing, the homogenates were centrifuged at 10,000 $\times$ g for 10 min and the supernatants were tested for light activity in triplicate in 96-well Microlite 2 plates (Thermo Labsystems). Nonimmunized mouse tissues served as controls.

Nasal EdTx-induced cytokine responses in vivo

Mice were given PBS, EdTx or CT by instilling 5-μl quantities into each nare. Twelve and 24 h later, mice were sacrificed and nasal washes were collected in 500 μl of PBS and the cytokine content was analyzed by ELISA. The nasopharyngeal-associated lymphoepithelial tissue (NALT), CLN, ON/E, and OBs were removed and subjected to real time RT-PCR (Lightcycler; Roche). The cytokine mRNA levels are expressed as crossing points (CP) or the cycle at which the fluorescence rises appreciably above the background fluorescence as determined by the Second Derivative Maximum Method (Roche Molecular Biochemicals LightCycler Software). The formula mRNA = $2^{(-(\text{CPcytokine} - \text{CP}\beta\text{-actin}))}$ corrects for differences in cDNA concentration between the starting templates of cytokine and housekeeping (i.e., β-actin) genes (39). The simplified formula $20 - (\text{CP}_{\text{cytokine}} - \text{CP}_{\beta\text{- actin}})$ was used to express relative cytokine mRNA responses in tissues of mice given nasal EdTx.

Statistics

The results are expressed as the mean $\pm$ 1 SD. Statistical significance (∗, $p \leq 0.05$) was determined by Student's *t* test and by ANOVA followed by the Fisher least significant difference test. For statistical analysis, cytokine levels below the detection limit were recorded as one-half the detection limit (e.g., IFN-γ = 7.5 pg/ml).

Results

EdTx promotes plasma Ab responses to nasally coadministered Ags

We first examined whether the adenyl cyclase EdTx, which acts through the ATR, would enhance Ab responses to mucosally administered protein Ags. Coadministration with 1 μg of EdTx (1 μg of PA and 1 μg of EF) enhanced OVA-specific plasma IgG Ab responses (Fig. 1*A*). Higher Ab responses were seen in mice given OVA and 5 μg of PA and 5 μg of EF although the titers failed to reach the statistical difference (Fig. 1*A*). The binding of EdTx to its receptor alone did not significantly contribute to the observed adjuvant activity, because neither PA alone (Fig. 1*B*) nor EF alone (data not shown) significantly increased OVA-specific IgG or promoted OVA-specific IgA Ab responses. Coimmunization with PA and EF (i.e., EdTx) sharply increased OVA-specific IgG and induced high levels of IgA Abs (Fig. 1*B*). The EdTx-induced IgG subclass responses consisted mainly of IgG1 and IgG2b Abs (Fig. 1*C*). Plasma samples collected 1 wk after two nasal immunizations

FIGURE 1. Adjuvant effect of EdTx for nasally coadministered Ag. *A*, Effect of EdTx dose on OVA-specific Ab responses. Mice were immunized three times at weekly intervals with 100 μg of OVA only or OVA plus 1 or 5 μg of EdTx (5 μg of PA plus 5 μg of EF). *B*, Role of EdTx component in the adjuvanticity. *C*, Plasma levels of OVA-specific IgG subclass Abs were determined 1 wk after the last immunization. *D*, Plasma samples were collected 1 wk after the second immunization (day 14) and examined for their content of total and OVA-specific IgE Ab levels. Mice were immunized three times at weekly intervals with 100 μg of OVA only (□), OVA plus 5 μg of PA (▨), or OVA plus 5 μg of EdTx (5 μg of PA plus 5 μg of EF) (■). Plasma levels of OVA-specific Abs were determined 1 wk after the last immunization. The results are expressed as the reciprocal log$_2$ titers ± one SD and are from three (*A*) or five experiments (*B–D*) and four mice/group. ∗, $p < 0.05$.
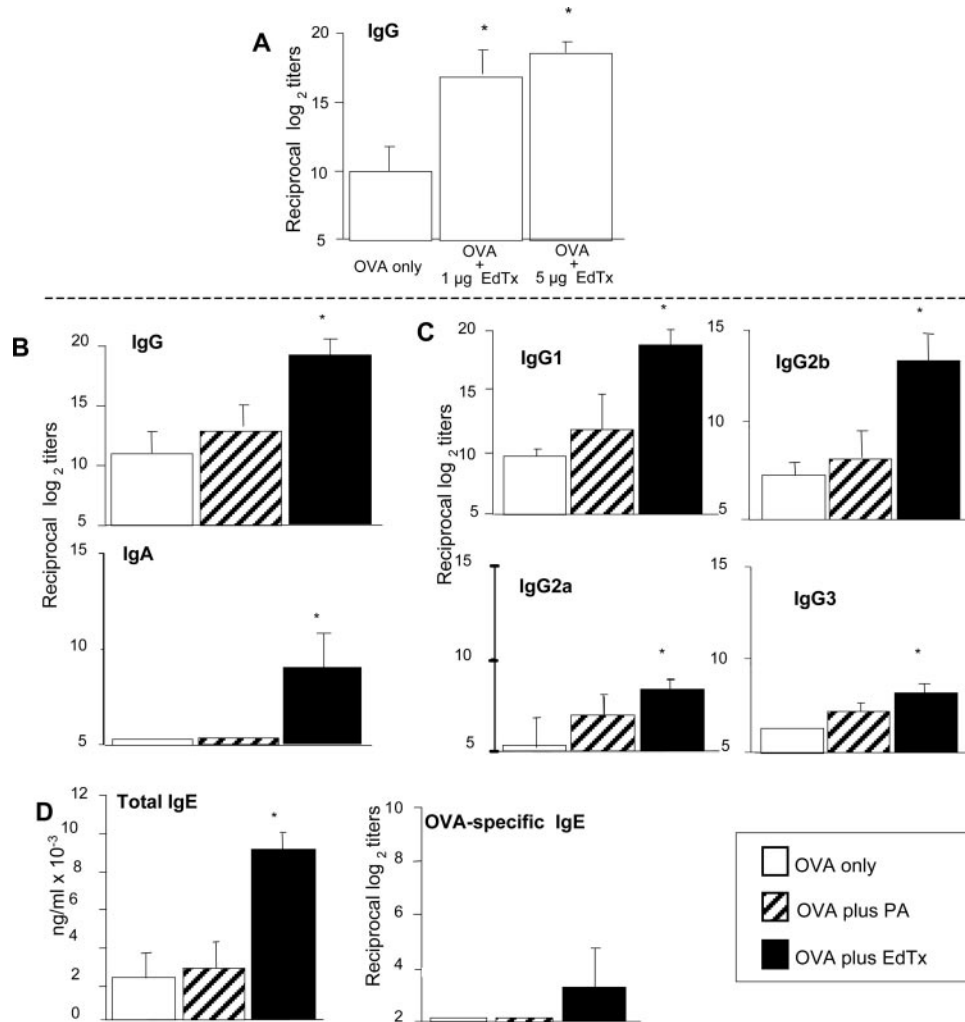
with OVA and EdTx contained elevated levels of total IgE Abs and low but significant OVA-specific IgE Abs that were not detected in mice nasally immunized with OVA only or OVA plus PA (Fig. 1*D*).

EdTx enhances PA-specific plasma Ab responses

In addition to acting as adjuvants for mucosally coadministered Ags, CT and LT-I are good immunogens that induce high Ab responses to their respective cell ganglioside-binding B subunits (40–43). Thus, we next addressed whether EdTx could enhance immunity to its binding PA subunit. Low levels of PA-specific IgG and IgA Abs were seen in the plasma of mice that received 5 μg of PA. Interestingly, PA-specific IgG and IgA Ab responses were both significantly increased in mice given EdTx (5 μg of PA plus 5 μg of EF), indicating that the enzymatic EF subunit also enhanced PA-specific Ab responses (Fig. 2*A*). PA-specific IgE Abs were not detected in mice that received PA without EF. However, coadministration of EF induced PA-specific IgE Ab levels (Fig. 2*A*), which were significantly higher than OVA-specific Abs in the same mice (Fig. 1*C*). The EdTx-induced PA-specific IgG subclass responses also predominantly consisted of IgG1 and IgG2b Abs followed by IgG2a and IgG3 (Fig. 2*B*).

EdTx induces PA- and OVA-specific Abs in saliva and vaginal secretions

Because nasal administration of EdTx enhanced systemic Ab responses to both the coadministered protein (i.e., OVA) and the binding B subunit (i.e., PA) itself, we next ascertained whether EdTx also induced PA- and OVA-specific IgA and IgG Abs in saliva and mucosal IgA Ab responses in other external secretions. OVA-specific IgA Abs were not detected in the saliva or the vaginal secretions of mice given OVA only or OVA plus PA (Fig. 3*A*). In contrast, the saliva of mice that received EdTx exhibited both OVA- and PA-specific IgA and IgG Abs (Fig. 3*A*). In addition, PA- and OVA-specific IgA Abs were detected in the vaginal washes of mice immunized with OVA plus EdTx (Fig. 3*B*).

Previous studies have shown that CT as a nasal adjuvant could induce a broad mucosal Ab response with PA-specific IgA Abs in the oral cavity (saliva), gastrointestinal (fecal extracts), and genitourinary (vaginal washes) tracts (35). In contrast to CT, the adjuvant activity of EdTx failed to induce high OVA-specific IgA Abs in fecal extracts (Fig. 3*C*). High amounts of PA (i.e., 40 μg) were needed for CT as a nasal adjuvant to induce anti-PA IgA Abs in fecal extracts (35). Increasing the dose of PA to 40 μg failed to enhance PA- or OVA-specific IgA Abs in fecal extracts (data not shown), suggesting that different mechanisms are involved in the mucosal adjuvanticity of CT when compared with EdTx.

EdTx induces anti-PA-neutralizing Abs

Nasal immunization with OVA and PA resulted in low levels of neutralizing Abs that were seen only in the plasma (Table I). Mice immunized with OVA and EdTx exhibited significantly higher titers of neutralizing Abs in the plasma than mice immunized with OVA plus PA (Table I). More interestingly, saliva of mice given
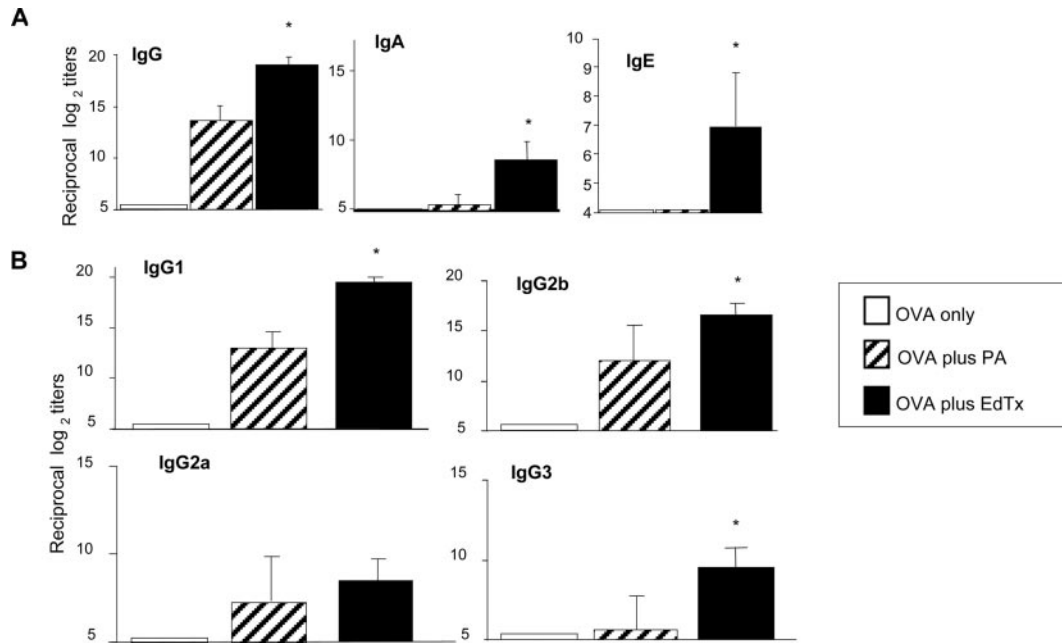
FIGURE 2. Plasma PA-specific Ab responses following nasal immunization with EdTx as adjuvant. Mice were immunized three times at weekly intervals with 100 μg of OVA only ($\square$), OVA plus 5 μg of PA ($\boxtimes$), or OVA plus 5 μg of EdTx (5 μg of PA plus 5 μg of EF) ($\blacksquare$). Plasma PA-specific IgE Ab responses were determined in samples collected at day 14, and plasma PA-specific Ab isotype (*A*) and IgG subclass responses (*B*) were determined 1 wk after the last immunization. The results are expressed as the reciprocal $\log_2$ titer $\pm$ one SD from five separate experiments and four mice per group per experiment. $*$, $p < 0.05$

OVA and EdTx contained significant levels of anti-PA neutralizing Abs (Table I). No significant neutralizing Abs were detected in vaginal washes (Table I).

Ag-specific T cell cytokine responses following nasal immunization with EdTx as adjuvant

To characterize CD4$^+$ Th cell cytokine pathways associated with EdTx-induced immunity, we examined the pattern of cytokines secreted by OVA- and PA-specific CD4$^+$ T cell after a 5-day in vitro restimulation. Spleen CD4$^+$ T cells from mice immunized with OVA and EdTx secreted mixed Th1- and Th2-type cytokines after in vitro restimulation with OVA or PA as indicated by high levels of IFN-γ (Th1) but also IL-5, IL-6, and IL-13 in culture supernatants (Table II). IL-4 levels were below the limit of detection. The same profile of cytokine responses was seen in the culture supernatant of CLN CD4$^+$ T cells restimulated under the same conditions (data not shown).

Effect of EdTx on cytokine secretion and costimulatory molecule expression by Mϕ, in vitro

We assessed the direct effects of EdTx or CT on the expression of costimulatory molecules (i.e., CD40 and CD86) as well as cytokine secretion by J774 Mϕ to address whether EdTx regulates APC functions. Consistent with previous reports (44–46), CT induced high levels of IL-6 responses in treated cells (Fig. 4*A*) and increased costimulatory molecule expression (Fig. 4*B*) by Mϕ in vitro. The IL-6 responses after EdTx stimulation were ~10-fold lower than those measured in culture supernatants of J774 Mϕ cultured in the presence of CT (Fig. 4). However, EdTx up-regulated the expression of both CD40 and CD86 by J774 Mϕ to the same extent as the mucosal adjuvant CT. These findings were further confirmed on T cell-depleted spleen cells (data not shown).

Nasal PA does not target CNS tissues

We next explored the possibility that EdTx could target the ON/E or other CNS tissues and induce inflammatory responses. Fig. 5*A*
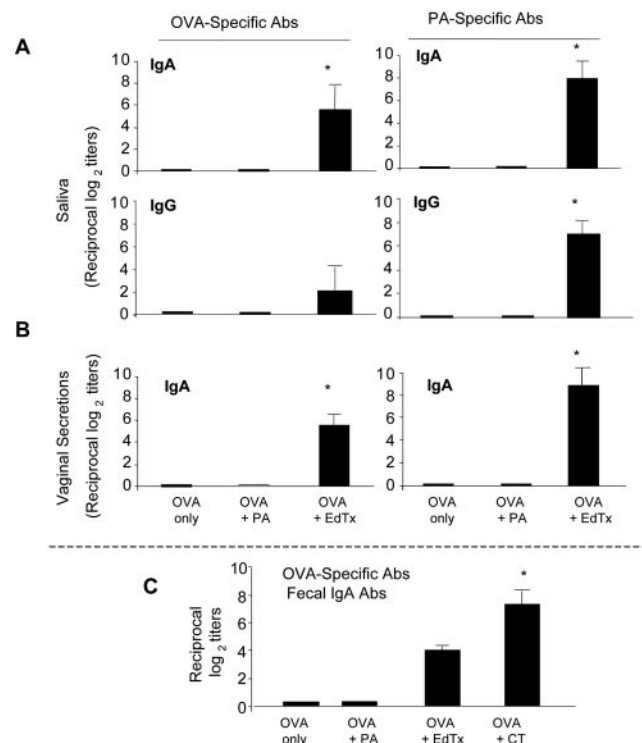
FIGURE 3. Mucosal IgA Ab responses following nasal immunization with EdTx as adjuvant. Mice were immunized three times at weekly intervals with 100 μg of OVA only, OVA plus 5 μg of PA, or OVA plus 5 μg of EdTx (5 μg of PA plus 5 μg of EF). Saliva (*A*) and vaginal washes (*B*) were collected 2 wk after the last immunization. The Ab levels are expressed as the reciprocal $\log_2$ titer $\pm$ 1 SD from five separate experiments and four mice per group per experiment. Fecal extracts (*C*) were collected 2 wk after the last immunization with either OVA only, OVA plus PA, OVA plus EdTx, or OVA plus CT. The IgA Ab levels were expressed as the reciprocal $\log_2$ titer $\pm$ 1 SD from three separate experiments and four mice per group per experiment. $*$, $p < 0.05$

Table I. *Neutralizing anti-PA Abs produced in response to nasal administration of EdTx protect Mϕ against the lethal effect of LeTx in vitro*[a]

Immunization	Neutralizing Ab Titers (1/Dilution)		
	Plasma	Saliva	Vaginal Washes
OVA only	BD	BD	BD
OVA plus PA	$8,192 \pm 256*$	BD	BD
OVA plus EdTx	$131,072 \pm 1,024*$	$10 \pm 3*$	3 ± 2

[a] Serial dilutions of each sample were added to J774 Mϕ cultures in the presence of LeTx. The neutralizing titers were determined as the last dilution yielding an MTT OD equal to twice the background value. Results are shown as neutralizing Ab titers and are expressed as the reciprocal dilutions titers $\pm$ 1 SE of three separate experiments and five mice per group per experiment. BD, Below detection level; *, $p < 0.05$.

illustrates the failure of PA to accumulate in the ON/E 24 h following nasal delivery of 5 μg of PA. In addition, there was no PA detectable in the OBs or brain and only minor amounts (< 0.15 ng/10 mg tissue) were observed in the NALT, CLN, or spleen 24 h after nasal delivery (data not shown). Nasal PA given together with EF (EdTx) did not increase PA accumulation in olfactory or brain tissues (Fig. 5A). In contrast to PA or EdTx, nasal delivery of a 0.5-μg dose of CT resulted in significant accumulation in the ON/E which was further increased when the CT dose was 10-fold higher (Fig. 5A). We also found that nasal CT, but not EdTx, up-regulated IL-1 mRNA levels in the ON/E and in the NALT suggesting that CT targeted these two sites (Fig. 5B). Finally, mice given nasal CT, but not those given nasal EdTx, exhibited high levels of IL-6 in nasal washes (Fig. 5C).

Discussion

The enterotoxins CT and LT-1, which deliver their ADP ribosyl transferase A subunit via ganglioside targeting, are well-recognized adjuvants for induction of mucosal immunity to coadministered Ags (40–42). The CTA1-DD molecule which targets CT-A to B cells was also shown to be an effective mucosal adjuvant (31, 47, 48). EdTx delivers its adenylate cyclase EF subunit into target cells following binding of PA on its membrane receptors, the ATRs. It has been recently reported that ATR1/TEM8 is expressed by epithelial cells (49). However, previous studies have shown that PA binds more effectively to the basolateral membrane of polarized epithelial cells (50), suggesting that ATRs may not be expressed at the apical membrane of these cells. In this study, we show that EdTx promotes both systemic and mucosal adaptive immunity to nasally coadministered Ags and enhances PA-specific Ab responses significantly above levels achieved by administration of PA without EF. We also show that ATR targeting by EdTx did

not lead to the accumulation of this adjuvant into olfactory and CNS tissues.

Anthrax toxin derivatives have been evaluated as molecular syringes for intracellular delivery of peptides (13, 51) or protein Ags (14–16) for presentation via the MHC class I pathway and induction of cytotoxic CD8$^+$ T cells. Our results show that in vivo delivery of anthrax EdTx provides necessary signals for induction of mucosal and systemic immunity to coadministered protein Ags. Although data summarized in this manuscript only referred to nasal delivery of EdTx, we also found that EdTx is an adjuvant for protein Ags coinjected i.p. in both C57BL/6 and BALB/c mice (data not shown). Studies with the ganglioside-targeting enterotoxins CT and LT-I (52–55) and derivatives including their chimeras (34, 56) or the B cell-targeting CTA1-DD (31, 57) have demonstrated the importance of receptor binding for controlling the immune responses induced by these ADP-ribosylating adjuvants. Thus, the adjuvant activity of CT appears to be more dependent on IL-4 and CD4$^+$ Th2 cell cytokines (52, 53). In contrast, the more promiscuous LT-I, which binds GM1 gangliosides like CT but also asialo-GM1 and -GM2 gangliosides, promotes a broader spectrum of responses with CD4$^+$ Th cells producing both IFN-γ and Th2-type cytokines (34, 54). The adjuvant activity of nasal EdTx appears to involve Ab and T cells responses that resemble those induced by LT-I rather than CT. Thus, EdTx induced CD4$^+$ T cells secreting both IFN-γ and Th2-type cytokines. Further, EdTx as an adjuvant promoted only modest levels of OVA-specific IgE (log$_2$ titers = 3) when compared with those seen after nasal immunization with CT (i.e., log$_2$ titers = 8). Although it has been suggested that ATRs may not be expressed at the apical membrane of epithelial cells (50), there is no information to date on the relative expression of ATRs on immune cells and potential cellular targets of nasally administered EdTx. We have shown here that PA alone or as a component of EdTx does not target olfactory tissues and do not induce IL-1-specific mRNA in ON/E. It is also important to note that EdTx promotes a similar profile of serum Ab responses than CTA1-DD which targets B cells (31).

The mechanisms underlying the induction of mucosal immunity and S-IgA Ab responses by bacterial toxins remain only partially understood. Studies over the past two decades have shown a role for CT-induced cytokines on its mucosal adjuvanticity. Thus, CT was shown to induce both IL-6 and IL-1 secretion by epithelial cells and APCs (44, 58–60). Both IL-1 and IL-6 were later shown to be adjuvants for systemic immunity to nasally coadministered protein vaccines (38, 61) and IL-1 was also able to promote mucosal IgA Ab responses (61). Other factors thought to contribute to the adjuvanticity of CT include its ability to up-regulate the expression of MHC (44) and costimulatory molecules (45, 46, 62).

Table II. *Ag-specific CD4$^+$ T cell cytokine responses induced by nasal EdTx as an adjuvant*[a]

Immunization	In vitro Stimulation	Cytokines (pg/ml)			
		IFN-γ	IL-5	IL-6	IL-13
OVA only	None	156 ± 30	BD	36 ± 1	20 ± 2
	OVA	$1320 \pm 49*$	$41 \pm 1*$	$175 \pm 10*$	36 ± 6
	PA	189 ± 10	BD	31 ± 1	20 ± 0
OVA plus EdTx	None	267 ± 37	BD	31 ± 1	20 ± 0
	OVA	$9666 \pm 1524*$	$418 \pm 85*$	$386 \pm 16*$	$188 \pm 29*$
	PA	$16550 \pm 2557*$	$1340 \pm 160*$	$192 \pm 28*$	$406 \pm 78*$

[a] Spleen CD4$^+$ T cells were isolated 21 days after the initial immunization and restimulated in vitro with OVA (1 mg/ml) or PA (20 μg/ml). Culture supernatants were collected after 5 days and cytokines evaluated by ELISA. The results are expressed as the mean $\pm$ 1 SE and are representative of four separate experiments. The same profile of responses was seen with CD4$^+$ T cells isolated from corresponding cervical lymph nodes. BD, Below detection levels; *, $p < 0.05$

FIGURE 4. Effects of EdTx on cytokine secretion and costimulatory molecule expression by Mφ. The J774 Mφ were cultured for 48 h in the presence of PA (5 μg/ml), EF (5 μg/ml), EdTx (5 μg/ml PA + 5 μg/ml EF), or CT (1 μg/ml). A, Cytokine secretion was analyzed in culture supernatants, and results were expressed as mean ± 1 SD of four separate experiments (∗, $p < 0.05$). B, Cells were analyzed by flow cytometry for expression CD40 and CD86. Results are representative of four separate experiments.
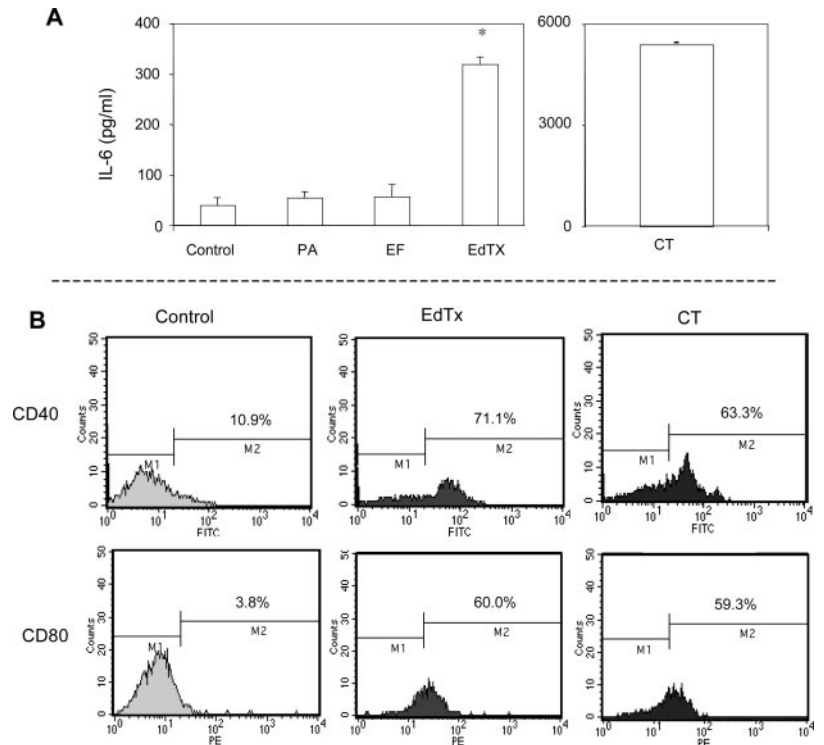
We have shown that EdTx stimulates IL-6 secretion by Mφ cultures and enhances the expression of costimulatory molecules. We should stress that IL-6 levels induced by EdTx were ∼10-fold lower than those seen in Mφ cultures stimulated with the same dose of CT. The lower stimulatory effect of EdTx for IL-6 secretion was further confirmed in vivo where nasal delivery of CT but not EdTx induced IL-6 secretion in nasal washes. It is unlikely that

the reduced ability of EdTx to induce IL-6 (and IL-1) could alone explain the polarized Ag-specific mucosal IgA Abs responses when compared with those generally induced by CT (34, 46, 63) or LT as adjuvants (34, 46, 54, 56). The role of the adenylate cyclase activity for the adjuvanticity remains to be elucidated. In this regard, the EF used in our studies contains a S447N mutation that could account for its recently reported 20-fold lower ability to
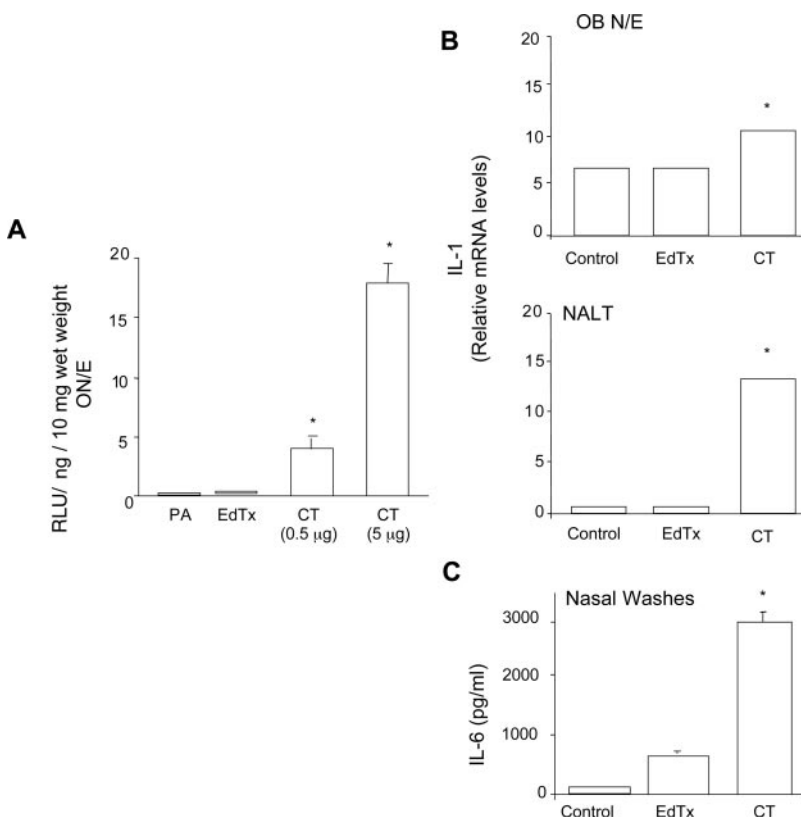

FIGURE 5. A, Tracking uptake into olfactory and CNS tissues. Groups of mice were given acridinium-labeled PA (5 μg), EdTx (5 μg of PA plus 5 μg of EF), or CT (0.5 or 5 μg) by the nasal route. A, Twenty-four hours later, mice were sacrificed and the ON/E and OBs were collected, homogenized, and tested for light activity as described in *Materials and Methods*. Results are expressed as mean RLU per nanogram of individual tissues ± 1 SD and are from two separate experiments with three mice per group (∗, $p < 0.05$). B, IL-1 mRNA analysis by real-time RT-PCR. Results are expressed as mean relative mRNA levels from triplicate assays performed with pooled tissues (three mice per group) and are representative of three separate experiments (∗, $p < 0.05$). C, Analysis of IL-6 levels in nasal washes by ELISA. Results are expressed as picograms per milliliter ± 1 SD of IL-6 levels in individual nasal washes and are from two experiments with three mice per group. ∗, $p < 0.05$ when compared with controls given PBS.

induce cAMP when compared with recombinant native EF protein (33). Several mutants of both CT and LT-I, which are devoid of ADP-ribosyl transferase activity, were shown to retain their mucosal adjuvant activity and the ability to promote high mucosal IgA Ab responses. In another system, the mucosal adjuvant activity of CTA1-DD was reported to require both effective B cell targeting and the ADP ribosyl transferase activity (57). Thus, further studies are warranted to establish the mechanisms of mucosal adjuvanticity of EdTx.

Enterotoxin adjuvants are also potent immunogens which induce elevated immune responses to their binding subunits (41, 43). A major finding of this study resides in the fact that EdTx is a potent immunogen and that the presence of EF increased anti-PA responses above levels achieved after administration of PA alone. We have previously reported that high levels of PA-specific Abs could be detected in mouse plasma following nasal immunization with PA doses of up to 25 μg when CT was used as adjuvant (35). The same studies showed that 40 μg of PA/dose were needed to promote PA-specific mucosal IgA Abs. The results summarized here indicated that ATR targeting with much lower doses of PA (i.e., 5 μg) and EF allows the induction of PA-specific S-IgA Abs. Interestingly, increasing the dose of PA given with EF to 40 μg did not increase the levels of PA-specific mucosal IgA Abs, suggesting that codelivery of EF achieved optimal S-IgA Ab responses with low nasal dose of PA. Although, nasal immunization with CT or LT-I as adjuvant primarily promotes IgA Ab responses in the respiratory and genitourinary tracts, low but significant responses are consistently seen in the gastrointestinal tract. PA was previously reported to deliver a functionally active CT-A subunit into mammalian cells using an LF$^{1-254}$-CT-A fusion protein (64). Therefore, it will be interesting to examine whether the ATR-mediated cellular targeting, the adenylate cyclase, and possibly other activities of EF govern the polarized PA-specific mucosal IgA Ab responses induced by EdTx as adjuvant.

In summary, we have shown that ATR targeting of an adenylate cyclase subunit provides an effective strategy for enhancing the immune response to nasal vaccines. The EdTx as nasal adjuvant does not target the olfactory or other CNS tissues and thus could represent a safer alternative to ganglioside-binding adjuvants. In addition, EdTx very efficiently promoted anti-PA Ab responses both in saliva and in plasma and this could have important implications for improving the efficacy of current PA-based anthrax vaccines.

Disclosures

The authors have no financial conflict of interest.

References

1. Mock, M., and A. Fouet. 2001. Anthrax. *Annu. Rev. Microbiol.* 55: 647–644. ‘
2. Collier, R. J., and J. A. Young. 2003. Anthrax toxin. *Annu. Rev. Cell Dev. Biol.* 19: 45–70.
3. Moayeri, M., and S. H. Leppla. 2004. The roles of anthrax toxin in pathogenesis. *Curr. Opin. Microbiol.* 7: 19–24.
4. Bradley, K. A., J. Mogridge, M. Mourez, R. J. Collier, and J. A. Young. 2001. Identification of the cellular receptor for anthrax toxin. *Nature* 414: 225–229.
5. Scobie, H. M., G. J. Rainey, K. A. Bradley, and J. A. Young. 2003. Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. *Proc. Natl. Acad. Sci. USA* 100: 5170–5174.
6. Leppla, S. H. 1982. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc. Natl. Acad. Sci. USA* 79: 3162–3166.
7. Guo, Q., Y. Shen, N. L. Zhukovskaya, J. Florian, and W. J. Tang. 2004. Structural and kinetic analyses of the interaction of anthrax adenylyl cyclase toxin with reaction products cAMP and pyrophosphate. *J. Biol. Chem.* 279: 29427–29435.
8. Shen, Y., N. L. Zhukovskaya, Q. Guo, J. Florian, and W. J. Tang. 2005. Calcium-independent calmodulin binding and two-metal-ion catalytic mechanism of anthrax edema factor. *EMBO J.* 24: 929–941.
9. Singh, Y., S. H. Leppla, R. Bhatnagar, and A. M. Friedlander. 1989. Internalization and processing of *Bacillus anthracis* lethal toxin by toxin-sensitive and -resistant cells. *J. Biol. Chem.* 264: 11099–11102.
10. Friedlander, A. M., R. Bhatnagar, S. H. Leppla, L. Johnson, and Y. Singh. 1993. Characterization of macrophage sensitivity and resistance to anthrax lethal toxin. *Infect. Immun.* 61: 245–252.
11. Hanna, P. C., D. Acosta, and R. J. Collier. 1993. On the role of macrophages in anthrax. *Proc. Natl. Acad. Sci. USA* 90: 10198–10201.
12. Agrawal, A., J. Lingappa, S. H. Leppla, S. Agrawal, A. Jabbar, C. Quinn, and B. Pulendran. 2003. Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin. *Nature* 424: 329–334.
13. Ballard, J. D., R. J. Collier, and M. N. Starnbach. 1996. Anthrax toxin-mediated delivery of a cytotoxic T-cell epitope in vivo. *Proc. Natl. Acad. Sci. USA* 93: 12531–12534.
14. Goletz, T. J., K. R. Klimpel, N. Arora, S. H. Leppla, J. M. Keith, and J. A. Berzofsky. 1997. Targeting HIV proteins to the major histocompatibility complex class I processing pathway with a novel gp120-anthrax toxin fusion protein. *Proc. Natl. Acad. Sci. USA* 94: 12059–12064.
15. Brossier, F., M. Weber-Levy, M. Mock, and J. C. Sirard. 2000. Protective antigen-mediated antibody response against a heterologous protein produced in vivo by *Bacillus anthracis*. *Infect. Immun.* 68: 5731–5734.
16. Lu, Y., R. Friedman, N. Kushner, A. Doling, L. Thomas, N. Touzjian, M. Starnbach, and J. Lieberman. 2000. Genetically modified anthrax lethal toxin safely delivers whole HIV protein antigens into the cytosol to induce T cell immunity. *Proc. Natl. Acad. Sci. USA* 97: 8027–8032.
17. Kushner, N., D. Zhang, N. Touzjian, M. Essex, J. Lieberman, and Y. Lu. 2003. A fragment of anthrax lethal factor delivers proteins to the cytosol without requiring protective antigen. *Proc. Natl. Acad. Sci. USA* 100: 6652–6657.
18. Price, B. M., A. L. Liner, S. Park, S. H. Leppla, A. Mateczun, and D. R. Galloway. 2001. Protection against anthrax lethal toxin challenge by genetic immunization with a plasmid encoding the lethal factor protein. *Infect. Immun.* 69: 4509–4515.
19. Kumar, P., N. Ahuja, and R. Bhatnagar. 2002. Anthrax edema toxin requires influx of calcium for inducing cyclic AMP toxicity in target cells. *Infect. Immun.* 70: 4997–5007.
20. Paccani, S. R., F. Tonello, R. Ghittoni, M. Natale, L. Muraro, M. M. D'Elios, W. J. Tang, C. Montecucco, and C. T. Baldari. 2005. Anthrax toxins suppress T lymphocyte activation by disrupting antigen receptor signaling. *J. Exp. Med.* 201: 325–331.
21. Spangler, B. D. 1992. Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol. Rev.* 56: 622–647.
22. Gill, D. M. 1976. The arrangement of subunits in cholera toxin. *Biochemistry* 15: 1242–1248.
23. Gill, D. M., J. D. Clements, D. C. Robertson, and R. A. Finkelstein. 1981. Subunit number and arrangement in *Escherichia coli* heat-labile enterotoxin. *Infect. Immun.* 33: 677–682.
24. Heyningen, S. V. 1974. Cholera toxin: interaction of subunits with ganglioside GM1. *Science* 183: 656–657.
25. Holmgren, J., M. Lindblad, P. Fredman, L. Svennerholm, and H. Myrvold. 1985. Comparison of receptors for cholera and *Escherichia coli* enterotoxins in human intestine. *Gastroenterology* 89: 27–35.
26. Griffiths, S. L., R. A. Finkelstein, and D. R. Critchley. 1986. Characterization of the receptor for cholera toxin and *Escherichia coli* heat-labile toxin in rabbit intestinal brush borders. *Biochem. J.* 238: 313–322.
27. Fukuta, S., J. L. Magnani, E. M. Twiddy, R. K. Holmes, and V. Ginsburg. 1988. Comparison of the carbohydrate-binding specificities of cholera toxin and *Escherichia coli* heat-labile enterotoxins LTh-I, LT-IIa, and LT-IIb. *Infect. Immun.* 56: 1748–1753.
28. Couch, R. B. 2004. Nasal vaccination, *Escherichia coli* enterotoxin, and Bell's palsy. *N. Engl. J. Med.* 350: 860–861.
29. Mutsch, M., W. Zhou, P. Rhodes, M. Bopp, R. T. Chen, T. Linder, C. Spyr, and R. Steffen. 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N. Engl. J. Med.* 350: 896–903.
30. van Ginkel, F. W., R. J. Jackson, Y. Yuki, and J. R. McGhee. 2000. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. *J. Immunol.* 165: 4778–4782.
31. Eriksson, A. M., K. M. Schon, and N. Y. Lycke. 2004. The cholera toxin-derived CTA1-DD vaccine adjuvant administered intranasally does not cause inflammation or accumulate in the nervous tissues. *J. Immunol.* 173: 3310–3319.
32. Ramirez, D. M., S. H. Leppla, R. Schneerson, and J. Shiloach. 2002. Production, recovery and immunogenicity of the protective antigen from a recombinant strain of *Bacillus anthracis*. *J. Ind. Microbiol. Biotechnol.* 28: 232–238.
33. Cooksey, B. A., G. C. Sampey, J. L. Pierre, X. Zhang, J. D. Karwoski, G. H. Choi, and M. W. Laird. 2004. Production of biologically active *Bacillus anthracis* edema factor in *Escherichia coli*. *Biotechnol. Prog.* 20: 1651–1659.
34. Boyaka, P. N., M. Ohmura, K. Fujihashi, T. Koga, M. Yamamoto, M. N. Kweon, Y. Takeda, R. J. Jackson, H. Kiyono, Y. Yuki, and J. R. McGhee. 2003. Chimeras of labile toxin one and cholera toxin retain mucosal adjuvanticity and direct Th cell subsets via their B subunit. *J. Immunol.* 170: 454–462.
35. Boyaka, P. N., A. Tafaro, R. Fischer, S. H. Leppla, K. Fujihashi, and J. R. McGhee. 2003. Effective mucosal immunity to anthrax: neutralizing antibodies and Th cell responses following nasal immunization with protective antigen. *J. Immunol.* 170: 5636–5643.
36. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65: 55–63.
37. Lillard, J. W., Jr., U. P. Singh, P. N. Boyaka, S. Singh, D. D. Taub, and J. R. McGhee. 2003. MIP-1α and MIP-1β differentially mediate mucosal and systemic adaptive immunity. *Blood* 101: 807–814.

38. Boyaka, P. N., M. Marinaro, R. J. Jackson, S. Menon, H. Kiyono, E. Jirillo, and J. R. McGhee. 1999. IL-12 is an effective adjuvant for induction of mucosal immunity. *J. Immunol.* 162: 122–128.

39. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C_T)$ method. *Methods* 25: 402–408.

40. Elson, C. O., and W. Ealding. 1984. Cholera toxin feeding did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated protein antigen. *J. Immunol.* 133: 2892–2897.

41. Elson, C. O., and W. Ealding. 1984. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. *J. Immunol.* 132: 2736–2741.

42. Clements, J. D., N. M. Hartzog, and F. L. Lyon. 1988. Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. *Vaccine* 6: 269–277.

43. Elson, C. O., and M. T. Dertzbaugh. 1999. Mucosal adjuvants. In *Mucosal Immunology*. P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, and J. R. McGhee, eds. Academic Press, San Diego, pp. 817–838.

44. Bromander, A. K., M. Kjerrulf, J. Holmgren, and N. Lycke. 1993. Cholera toxin enhances alloantigen presentation by cultured intestinal epithelial cells. *Scand. J. Immunol.* 37: 452–458.

45. Cong, Y., C. T. Weaver, and C. O. Elson. 1997. The mucosal adjuvanticity of cholera toxin involves enhancement of costimulatory activity by selective up-regulation of B7.2 expression. *J. Immunol.* 159: 5301–5308.

46. Yamamoto, M., H. Kiyono, M. N. Kweon, S. Yamamoto, K. Fujihashi, H. Kurazono, K. Imaoka, H. Bluethmann, I. Takahashi, Y. Takeda, et al. 2000. Enterotoxin adjuvants have direct effects on T cells and antigen-presenting cells that result in either interleukin-4-dependent or -independent immune responses. *J. Infect. Dis.* 182: 180–190.

47. Agren, L. C., L. Ekman, B. Lowenadler, and N. Y. Lycke. 1997. Genetically engineered nontoxic vaccine adjuvant that combines B cell targeting with immunomodulation by cholera toxin A1 subunit. *J. Immunol.* 158: 3936–3946.

48. Agren, L., E. Sverremark, L. Ekman, K. Schon, B. Lowenadler, C. Fernandez, and N. Lycke. 2000. The ADP-ribosylating CTA1-DD adjuvant enhances T cell-dependent and independent responses by direct action on B cells involving anti-apoptotic Bcl-2- and germinal center-promoting effects. *J. Immunol.* 164: 6276–6286.

49. Bonuccelli, G., F. Sotgia, P. G. Frank, T. M. Williams, C. J. de Almeida, H. B. Tanowitz, P. E. Scherer, K. A. Hotchkiss, B. I. Terman, B. Rollman, et al. 2005. Anthrax toxin receptor (ATR/TEM8) is highly expressed in epithelial cells lining the toxin's three sites of entry (lung, skin, and intestine). *Am. J. Physiol.* 288: C1402–C1410.

50. Beauregard, K. E., S. Wimer-Mackin, R. J. Collier, and W. I. Lencer. 1999. Anthrax toxin entry into polarized epithelial cells. *Infect. Immun.* 67: 3026–3030.

51. Doling, A. M., J. D. Ballard, H. Shen, K. M. Krishna, R. Ahmed, R. J. Collier, and M. N. Starnbach. 1999. Cytotoxic T-lymphocyte epitopes fused to anthrax toxin induce protective antiviral immunity. *Infect. Immun.* 67: 3290–3296.

52. Marinaro, M., H. F. Staats, T. Hiroi, R. J. Jackson, M. Coste, P. N. Boyaka, N. Okahashi, M. Yamamoto, H. Kiyono, H. Bluethmann, et al. 1995. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. *J. Immunol.* 155: 4621–4629.

53. Vajdy, M., M. H. Kosco-Vilbois, M. Kopf, G. Kohler, and N. Lycke. 1995. Impaired mucosal immune responses in interleukin 4-targeted mice. *J. Exp. Med.* 181: 41–53.

54. Takahashi, I., M. Marinaro, H. Kiyono, R. J. Jackson, I. Nakagawa, K. Fujihashi, S. Hamada, J. D. Clements, K. L. Bost, and J. R. McGhee. 1996. Mechanisms for mucosal immunogenicity and adjuvancy of *Escherichia coli* labile enterotoxin. *J. Infect. Dis.* 173: 627–635.

55. Okahashi, N., M. Yamamoto, J. L. Vancott, S. N. Chatfield, M. Roberts, H. Bluethmann, T. Hiroi, H. Kiyono, and J. R. McGhee. 1996. Oral immunization of interleukin-4 (IL-4) knockout mice with a recombinant *Salmonella* strain or cholera toxin reveals that CD4$^+$ Th2 cells producing IL-6 and IL-10 are associated with mucosal immunoglobulin A responses. *Infect. Immun.* 64: 1516–1525.

56. Kweon, M. N., M. Yamamoto, F. Watanabe, S. Tamura, F. W. Van Ginkel, A. Miyauchi, H. Takagi, Y. Takeda, T. Hamabata, K. Fujihashi, et al. 2002. A nontoxic chimeric enterotoxin adjuvant induces protective immunity in both mucosal and systemic compartments with reduced IgE antibodies. *J. Infect. Dis.* 186: 1261–1269.

57. Agren, L. C., L. Ekman, B. Lowenadler, J. G. Nedrud, and N. Y. Lycke. 1999. Adjuvanticity of the cholera toxin A1-based gene fusion protein, CTA1-DD, is critically dependent on the ADP-ribosyltransferase and Ig-binding activity. *J. Immunol.* 162: 2432–2440.

58. Lycke, N., A. K. Bromander, L. Ekman, U. Karlsson, and J. Holmgren. 1989. Cellular basis of immunomodulation by cholera toxin in vitro with possible association to the adjuvant function in vivo. *J. Immunol.* 142: 20–27.

59. Bromander, A., J. Holmgren, and N. Lycke. 1991. Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages in vitro. *J. Immunol.* 146: 2908–2914.

60. McGee, D. W., C. O. Elson, and J. R. McGhee. 1993. Enhancing effect of cholera toxin on interleukin-6 secretion by IEC-6 intestinal epithelial cells: mode of action and augmenting effect of inflammatory cytokines. *Infect. Immun.* 61: 4637–4644.

61. Staats, H. F., and F. A. Ennis, Jr. 1999. IL-1 is an effective adjuvant for mucosal and systemic immune responses when coadministered with protein immunogens. *J. Immunol.* 162: 6141–6147.

62. Gagliardi, M. C., F. Sallusto, M. Marinaro, S. Vendetti, A. Riccomi, and M. T. De Magistris. 2002. Effects of the adjuvant cholera toxin on dendritic cells: stimulatory and inhibitory signals that result in the amplification of immune responses. *Int. J. Med. Microbiol.* 291: 571–575.

63. Yamamoto, S., H. Kiyono, M. Yamamoto, K. Imaoka, K. Fujihashi, F. W. Van Ginkel, M. Noda, Y. Takeda, and J. R. McGhee. 1997. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. *Proc. Natl. Acad. Sci. USA* 94: 5267–5272.

64. Sharma, M., H. Khanna, N. Arora, and Y. Singh. 2000. Anthrax toxin-mediated delivery of cholera toxin-A subunit into the cytosol of mammalian cells. *Biotechnol. Appl. Biochem.* 32(Pt. 1): 69–72.