activity against the Hcβtre. Fecal samples were extracted from the supernatants of 10% (w/v) suspension of sample and PBS with 50 μg/ml soybean trypsin inhibitor (Sigma-Aldrich). Nasal washes were collected by intubation of the trachea to access the nasopharyngeal cavity. An ~1-inch long tygon tubing (internal diameter 0.010 inches, outside diameter 0.030 inches; Cole-Parmer) was attached to a 1.0-ml syringe, an approach used to avoid any blood contamination of the nasal washes. A total of 200 μl of PBS was inserted via the trachea, and the exudate from the nares was collected into microfuge tubes. Cells and debris were removed by centrifugation for 10 min at 10,000 × *g*. HRP-goat anti-mouse IgA, IgG, IgG1, IgG2a, and IgG2b (Southern Biotechnology Associates) or biotinylated rat anti-mouse IgE mAb (BD Pharmingen) plus HRP-goat anti-biotin Abs (Vector Laboratories) were used for detection. Following 90 min of incubation at 37°C and a washing step, the specific reactivity was determined by the addition of an enzyme substrate, ABTS (Moss) at 100 μl/well. Absorbance was measured at 415 nm on a Kinetics Reader model EL312 (Bio-Tek Instruments). Endpoint titers were defined as the highest reciprocal of dilution of sample giving an absorbance at OD_{415} above 0.100 OD units above negative controls after 1 h of incubation at 25°C. For total IgE ELISA, a similar protocol was followed as for Ag-specific ELISA already described, but wells were coated with rat anti-mouse IgE mAb (2.0 μg/ml; BD Pharmingen). After blocking, serial dilutions (beginning at 2^5) of individual serum were done and added to coated wells in duplicate and incubated overnight at 4°C. After washing wells, 2.0 μg/ml biotinylated rat anti-mouse IgE was incubated for 2 h at 37°C, washed, then incubated with 1/500 dilution of HRP-goat anti-biotin for 1 h at 37°C. Wells were developed, as described, and a specific amount of IgE was extrapolated from a standard curve derived from wells incubated with an IgE anti-trinitrophenyl mAb (BD Pharmingen).

Lymphocyte isolation

Lymphocytes were isolated from nasal passages, submaxillary glands, small intestinal lamina propria, Peyer's patches, mesenteric lymph nodes (LN), spleens, nasal-associated lymphoreticular tissue, and head and neck LN. Splenic, Peyer's patch, mesenteric LN, nasal-associated lymphoreticular tissue, and head and neck LN mononuclear cell suspensions were obtained by conventional methods using dounce homogenization (21, 22) and yielding >95% viability by trypan blue exclusion. To isolate mononuclear cells from nasal passages and submaxillary glands, the tissues were minced and suspended in medium containing 300 U/ml *Clostridium histolyticum* type IV collagenase (Worthington Biochemical), followed by two 30-min digestions at 37°C or a single digestion for 45 min, respectively, in spinner flasks. The small intestinal lamina propria samples were isolated, as previously described (21, 22). After incubation, the digestion mixtures were passed through Nitex mesh (Fairview Fabrics) to remove undigested tissues. Mononuclear cells were subjected to Percoll (Amersham Biosciences) density gradient centrifugation, and the cells were interfaced between 40 and 75% Percoll (21, 22). Viability of >95% was noted for lymphocytes isolated from each tissue, as determined by trypan blue exclusion.

B cell Ab ELISPOT

Ab-forming cells (AFC) were enumerated using IgA and IgG Ag-specific ELISPOT assays similar to those previously described (21–23). Mixed cellulose ester membrane-bottomed microtiter plates (MultiScreen-HA; Millipore) were coated with 5.0 μg/ml recombinant Hc BoNT/A in sterile PBS. For total IgA or IgG AFC determinations, wells were coated with 5 μg/ml goat anti-mouse IgA or IgG (Hc-specific) Abs (Southern Biotechnology Associates) in sterile PBS overnight at 25°C. The plates were blocked at 37°C for 2 h in CM. A total of 100 μl of cells from each tissue at varying concentrations was added to the wells, and the plates were incubated at 37°C overnight. Cells were removed, and the plates were washed, as previously described (21–23). For detection of AFC responses, 100 μl of 1.0 μg/ml goat anti-mouse IgG and IgA-HRP conjugates (Southern Biotechnology Associates) were added to the wells, and the plates were incubated overnight at 4°C. After washing, the wells were developed with 100 μl of AEC (Moss), and the reaction was allowed to continue until spots developed (~30 min). The reaction was stopped with H_2O, the plates were allowed to dry overnight, and AFC were enumerated by counting under a low-power dissecting microscope (Leica).

Cytokine ELISPOT

Groups of BALB/c mice (5–10/group) were euthanized 3 wk after the last immunization to collect spleens. Total splenic, head and neck LN, and mesenteric LN mononuclear cells (5 × 10^6/ml) were resuspended in CM, and restimulated with 10 μg/ml recombinant Hc BoNT/A, OVA (tissue-culture grade; Sigma-Aldrich), or media in the presence of 10 U/ml human IL-2 (PeproTech) for 2 days at 37°C. Cells were washed and resuspended in CM. Stimulated lymphocytes were then evaluated by IFN-γ-, IL-4-, IL-5-, IL-6-, and IL-10-specific ELISPOT assays, precisely as previously described (22, 24).

BoNT intoxication challenge and mouse neutralization assay

To assess the protective value of the Hcβtre vaccine, BALB/c mice were immunized nasally with equimolar doses of Hcβtre or Hcβtre-Ad2F with or without CT as adjuvant, as previously described. For the first experiment, one group of mice was immunized nasally with 2 μg of BoNToxoid/A (List Biological Laboratories) plus 2 μg of CT on days 0, 7, 14, and 21. Mice were monitored for Ab titers to Hcβtre, Hc BoNT/A, and BoNT/A. On day 63 or day 96, mice were challenged i.p. with ~20,000 or 2000 LD_{50} BoNT/A (2 × 10^8 mouse LD_{50}/mg, Lot no. A121004-01; Metabiologics), respectively, in 200 μl of PBS containing 0.2% gelatin, as previously described (23). Mice were monitored hourly for the first 6 h and then daily for signs of morbidity, including difficulty breathing and lack of mobility. Mice were euthanized when signs of morbidity were observed.

To determine whether nasal immunization with Hcβtre immunogens was able to induce protective Abs in mucosal secretions against BoNT/A, we picked a mouse neutralization assay using BoNT/A complex (3.8 × 10^7 mouse LD_{50}/mg, batch no. MA0305, lot no. A121005-01; Metabiologics). In this assay, filter-sterile fecal extracts from naive or day 21 samples were combined with BoNT/A complex (1/2 final dilution of fecal extract) and incubated at room temperature for 30 min before i.p. injection into naive BALB/c mice. As a positive control, some of the naive fecal extracts were spiked with immune sera from mice immunized nasally with Hc BoNT/A plus CT at a final dilution of 1/110. If the anti-Hcβtre Abs in the fecal extracts were able to neutralize BoNT/A in the complex, the mice would not exhibit BoNT/A intoxication. If the fecal extracts did not contain BoNT/A-neutralizing Abs, the mice would exhibit signs of BoNT/A toxicity, including shortness of breath and immobility. Mice were monitored twice daily for up to 5 days. Morbid mice were euthanized in accordance with the Institutional Animal Care and Use Committee of both institutions.

Statistical analysis

An ANOVA, followed by Tukey's method, was used to evaluate differences between variations in Ab titers, Ab AFC, and cytokine-forming cell (CFC) levels and discerned to the 95% confidence interval. The Kaplan-Meier method (GraphPad Prism; GraphPad) was applied to obtain the survival fractions following intoxication with BoNT/A or the BoNT/A complex. Using the Mantel-Haenszel log rank test, the *p* value for statistical differences between surviving BoNT/A challenge and Hcβtre plus CT, Hcβtre-Ad2F plus CT, Hcβtre alone, Hcβtre-Ad2F alone, or BoNToxoid plus CT vaccinated mice or fecal extracts derived from vaccinated and naive mice were discerned at the 95% confidence interval.

Results

Recombinant Ad2F exhibits cell-binding activity

We hypothesized that the addition of a mucosal-targeting ligand, i.e., Ad2F, would possibly enhance vaccine uptake. To test for this response, several recombinant proteins were produced for this study (Fig. 1*A*). The Hc fragment of the *C. botulinum* neurotoxin A (Hc BoNT/A) is a small molecule subdomain corresponding to the binding domain for Hc referred to as the β-trefoil (Hcβtre) structure. A fusion protein between the Hcβtre and the Ad2F referred to as the Hcβtre-Ad2F was also assessed. Finally, a fusion between the Red2 fluorescent protein and Ad2F referred to as Red2-Ad2F was used. The recombinant Hc, Hcβtre, and Hcβtre-Ad2F migrated with the expected molecular mass of 58, 27.8, and 50 kDa, respectively, as determined by SDS-PAGE (Fig. 1*B*) or 60 kDa for Red2-Ad2F, as assessed by Western blot (Fig. 1*C*). To assess whether the Ad2F could bind to the mucosal epithelium, binding studies to human HeLa and 293A cells were performed using the Red2-Ad2F (Fig. 1*D*). The Red2-Ad2F was able to bind to both HeLa and 293A cells, but not the recombinant Red2. Neither Red2-Ad2F nor Red2 were able to bind to mouse L cells, suggesting that the Ad2F was indeed functional. To test whether this protein could bind to mouse epithelium, BALB/c mice were nasally dosed with Red2-Ad2F and after 90 min, nasal passages were evaluated for protein binding. As depicted in Fig. 1*E*, the

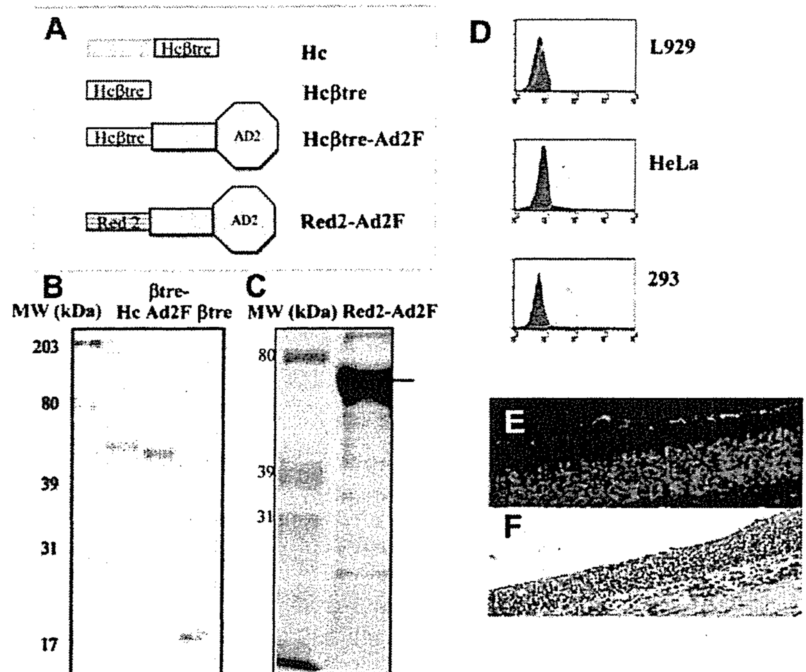

FIGURE 1. Construction of novel BoNT/A vaccines using the β-trefoil (βtre) structure of the BoNT/A Hc adapted with and without the adhesin Ad2F. *A*, Schematic depictions of BoNT/A Hc, Hcβtre/A (Hcβtre), Hcβtre genetically fused to Ad2F (Hcβtre-Ad2F), and the Red2 fluorescent protein genetically fused to Ad2F (Red2-Ad2F). *B*, Coomassie blue stained 12% SDS-PAGE of the Hcβtre vaccines: Hc (~58.8 kDa), Hcβtre-Ad2F (~50 kDa), and Hcβtre (~27.8 kDa). *C*, The Red2-Ad2F migrates with the expected molecular mass of ~60 kDa as detected by Western blot analysis using a polyclonal rabbit anti-Red2 Ab. *D*, FACS analysis of Red2-AdF binding to human epithelial HeLa and 293A cells (open histograms), but not to L929 fibroblasts. *E*, Red2-Ad2F binds to mouse nasal epithelium. BALB/c mice were given 50 μg of Red-Ad2F nasally, and 90 min later, nasal passages were harvested for frozen sections and evaluated for Red2 fluorescence (on apex of turbinate cells) together with DAPI nuclear (blue) stain. *F*, An adjacent section was cut for H&E staining.

Red2 fluorescence was associated with the nasal epithelium on its luminal surface.

Induction of BoNT-specific mucosal IgA and plasma IgG

To test whether Ad2F could efficiently deliver Hcβtre to the mucosa, mice were immunized nasally on days 0, 7, and 14. A kinetic analysis was performed and showed that the Ad2F could effectively stimulate a rapid secretory IgA (sIgA) Ab response to the Hcβtre, whereas nasal immunization with Hcβtre alone could not (Fig. 2*A*). Only weak plasma IgG anti-Hcβtre Ab titers were elicited with Hcβtre-Ad2F and none with Hcβtre alone. Thus, in subsequent studies, all immunizations were conducted with CT as mucosal adjuvant. Adjuvanted doses of Hcβtre-Ad2F resulted in a rapid onset of sIgA Ab titers when compared with onset for adjuvanted dosages of Hcβtre, which was delayed by 2 wk (Fig. 2*A*). In a similar fashion, adjuvanted doses of Hcβtre-Ad2F also resulted in a rapid onset of plasma IgG titers when compared with onset for dosages of Hcβtre plus CT, which was delayed by 1 wk (Fig. 2*B*). Differences in plasma IgG1 and IgG2a, but not IgG2b responses, were observed between the two immunization groups (Fig. 3*B*). Hcβtre-specific IgE and total IgE levels were also measured during peak Ab responses on day 28. Mice nasally immunized with Hcβtre, Hcβtre-Ad2F, or Hcβtre-Ad2F plus CT failed to show Hcβtre-specific IgE (Fig. 3*C*); in the group of mice nasally dosed with Hcβtre plus CT, only one in 10 mice showed an IgE titer of 2^6.

To determine whether immune sIgA anti-Hcβtre Ab titers were present in nasal washes, samples were collected on day 35 postprimary immunization. Mice given nasal Hcβtre-Ad2F plus CT showed significantly higher levels of sIgA in nasal washes when compared with mice given Hcβtre plus CT as adjuvant (Fig. 3*A*). From these studies, we showed that Hcβtre is immunogenic, and its immunogenicity can be advanced when genetically fused to Ad2F.

Nasal immunization with Hcβtre-Ad2F enhances IgA AFC in nasal passages and submaxillary glands

To determine the distribution of Ag-specific B cells following nasal vaccination, an Ab ELISPOT was performed on lymphoid tissues for Hcβtre-Ad2F plus CT or Hcβtre plus CT-treated mice (Fig. 4). Nasal immunization with Hcβtre-Ad2F plus CT stimulated a pronounced number of Ag-specific IgA AFC in nasal passages and submaxillary glands when compared with mice dosed with the adjuvanted Hcβtre ($p < 0.001$) (Fig. 4*A*). Ag-specific IgA

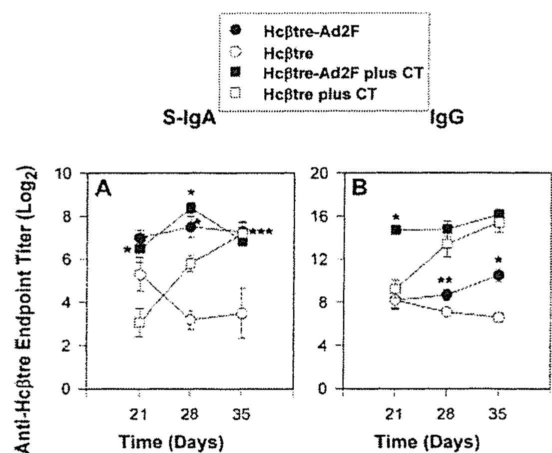

FIGURE 2. The addition of the mucosal-targeting ligand, Ad2F, enhances time for mucosal Ab production. BALB/c mice nasally immunized on days 0, 7, and 14 with Hcβtre or Hcβtre-Ad2F, either alone or in combination with CT, and fecal IgA (*A*) and plasma IgG (*B*) Ab titers against the Hcβtre were determined by standard ELISA methods. In the absence of CT, nasal immunization with Hcβtre-Ad2F elicited a robust and rapid onset of secretory IgA (sIgA) Abs, whereas mice nasally dosed with Hcβtre only produced a weak sIgA Ab response. Both groups produced weak plasma IgG anti-Hcβtre titers. Upon CT coadministration, a rapid onset and sustained sIgA and plasma IgG Ab titers were produced when nasally vaccinated with Hcβtre-Ad2F, but this rapid onset was not evident with the Hcβtre-dosed mice. Thus, for the remaining studies, only responses in adjuvanted mice were evaluated. Data represent the mean ± SEM (n = 10 mice). *, p = 0.001; **, p = 0.015; and ***, p = 0.02 represent the statistical differences in sIgA and IgG anti-Hcβtre levels between mice given the same vaccine with CT as adjuvant compared with mice dosed with their respective vaccine without CT.

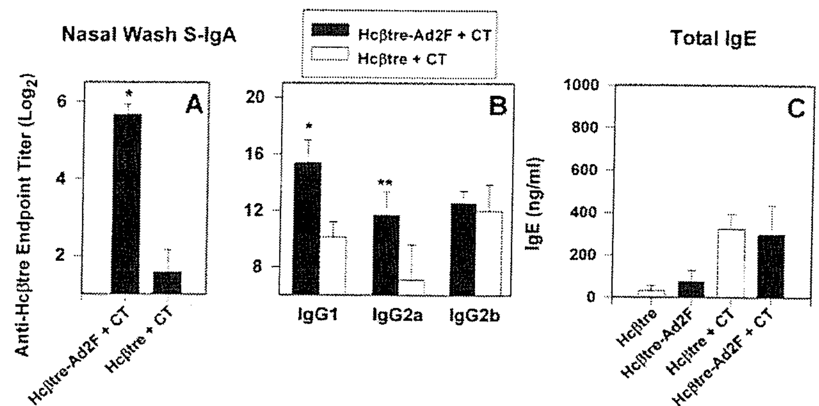

FIGURE 3. Nasal immunization with Hcβtre-Ad2F and CT shows increased secretory IgA (sIgA) (*A*) anti-Hcβtre titers with significant differences in plasma IgG1 and IgG2a titers (*B*) but no changes in total IgE levels (*C*). Data represent mean ± SEM (n = 10) sampled at day 35 postprimary immunization or sampled at day 28 for total IgE levels. Only one of 10 mice from the Hcβtre plus CT-dosed group showed a plasma IgE anti-Hcβtre titer of 2^6; none of the other groups showed a Hcβtre-specific IgE titer. *, $p < 0.001$ and **, $p = 0.003$ represent the differences in sIgA, IgG1, and IgG2a Ab titers between mice nasally dosed with Hcβtre-Ad2F plus CT and mice dosed with Hcβtre plus CT, respectively.

AFC were also significantly increased in Peyer's patches ($p < 0.05$). The remaining Ag-specific IgA AFC response for the small intestinal lamina propria, spleen, and nasal-associated lymphoreticular tissue was the same for both groups. Evaluation of Ag-specific IgG AFC showed only subtle changes in small intestinal lamina propria, Peyer's patches, spleen, nasal passages, and nasal-associated lymphoreticular tissue (Fig. 4*C*).

Nasal immunization with Hcβtre-Ad2F plus CT as adjuvant elicits a mixed Th cell response

To learn what are the supportive Th cells for the observed Ag-specific B cell responses, adjuvanted Hcβtre-Ad2F-dosed or Hcβtre-dosed mice were evaluated for CFC. Lymphocytes from spleen, head and neck LN, and mesenteric LN were cultured and assayed for the production of IFN-γ, IL-4, IL-5, IL-10, and IL-13. Following 48 h of stimulation, lymphocytes were analyzed by cytokine ELISPOT method. Although the responses varied among the tissues examined, for the most part, a mixed Th cell phenotype was observed when triggered with Hc BoNT/A. In the spleen, IFN-γ, IL-4, and IL-5 CFC were induced to equivalent levels for both dosing groups, but increased numbers of IL-10- and IL-13-producing cells were detected in the Hcβtre-Ad2F-dosed mice (Fig. 5*A*). In the head and neck LN, IL-4, IL-5, and IL-10 were not significantly different between either dosing group; however, IFN-γ ($p < 0.001$) and IL-13 ($p < 0.05$) were particularly enhanced in Hcβtre-Ad2F-dosed mice (Fig. 5*B*). In the mesenteric LN, IL-5 and IL-10 were similar for both groups; however, Hcβtre-Ad2F-dosed mice exhibited significantly less IFN-γ ($p < 0.011$), IL-4 ($p < 0.05$), and IL-13 ($p < 0.001$) CFC (Fig. 5*C*).

Nasal vaccination confers protection against systemic BoNT intoxication

Two challenge studies with BoNT doses that would cause intoxication in humans were conducted using immunized mice. In the first experiment, groups of BALB/c mice were nasally dosed with equimolar amounts of Hcβtre (25 μg) and Hcβtre-Ad2F (50 μg) with CT (2 μg). One control group was given BoNToxoid/A (2 μg) with CT, and another control group was left unimmunized. Mice were immunized four times at weekly intervals and assessed for immune titers on day 57 postprimary immunization. ELISA were performed using Hcβtre, Hc BoNT/A, or native BoNT/A as coating Ags (Fig. 6*A*). Both groups of mice dosed with either Hcβtre or Hcβtre-Ad2F showed elevated Ab endpoint titers against all three Ags; however, the Hcβtre-Ad2F-dosed group showed a slightly reduced titer against Hc BoNT/A. Mice immunized with BoNToxoid showed poor Ab reactivity against the immunizing immunogen, as well as to Hcβtre and Hc BoNT/A (Fig. 6*A*). On day 63, mice were challenged i.p. with 20,000 mouse LD_{50}, which is equivalent to 1.0 human LD_{100} (3), and both the Hcβtre-dosed or Hcβtre-Ad2F-dosed groups showed complete protection (Fig. 6*B*). In addition, neither group showed signs of BoNT intoxication. Surprisingly, the mice immunized with the BoNT/A toxoid failed to show any protective efficacy and succumbed to intoxication as quickly as naive mice (all within 3 h).

A second challenge study was performed using 10-fold less BoNT/A for the challenge. As described with our immunogenicity studies, mice were given only three doses of vaccine at weekly intervals. BALB/c mice were nasally dosed with 25 μg of Hcβtre or 50 μg of Hcβtre-Ad2F in the absence or presence of 2 μg of CT. The control group was only given three nasal doses of CT. On day 82, sera were collected and evaluated for IgG Abs specific for

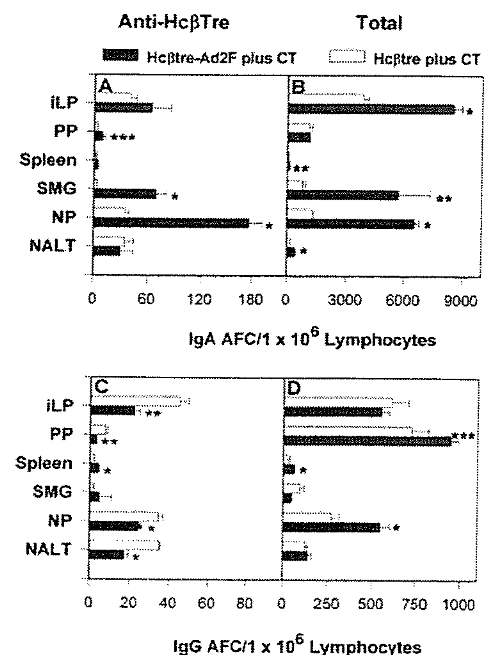

FIGURE 4. Nasal immunization with Hcβtre-Ad2F plus CT as adjuvant enhances the number of IgA AFC in nasal passages (NP), submaxillary glands (SMG), and Peyer's patches (PP) (*A*) when compared with Hcβtre plus CT, but showed slightly less IgG AFC response in nasal passages, Peyer's patches, and small intestinal lamina propria (iLP) (*C*). B cell ELISPOT was conducted 4 wk postprimary immunization and Hc BoNT/A-specific (*A* and *C*) and total AFC were measured (*B* and *D*). Data are expressed as mean ± SEM of three separate experiments of AFC/1 × 10^6 lymphocytes. *, $p \leq 0.001$; **, $p \leq 0.016$; ***, $p < 0.05$.

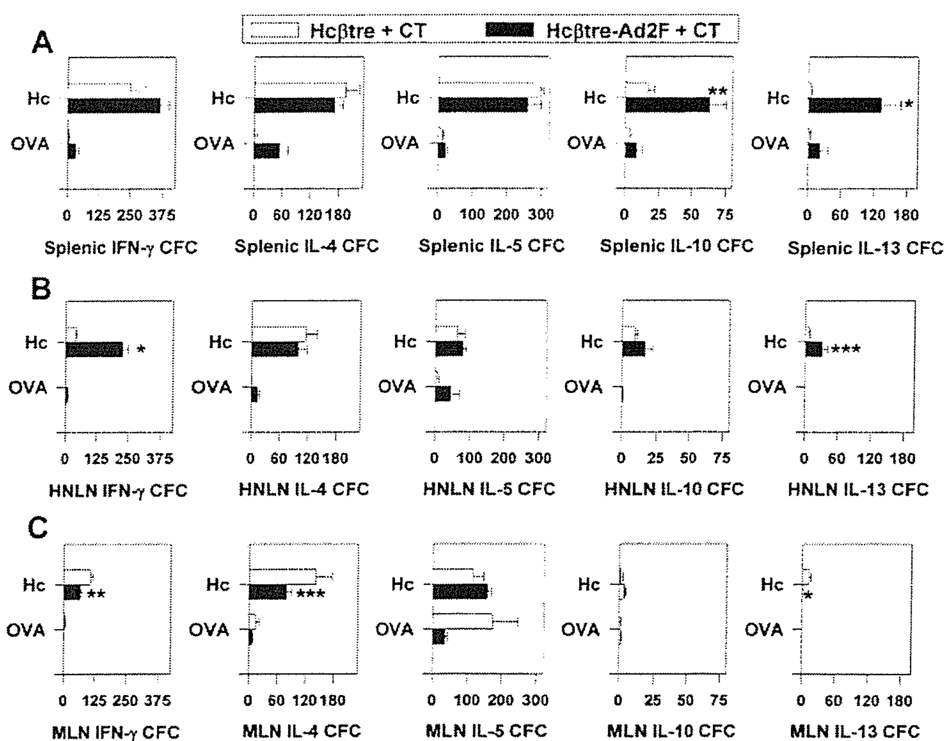

FIGURE 5. Mucosal targeting enhances head and neck LN IFN-γ and IL-13 production following nasal immunization with Hcβtre-Ad2F. Three weeks after their final immunization, Hcβtre-Ad2F plus CT-dosed and Hcβtre plus CT-dosed mice were evaluated for CFC responses in the spleen (*A*), head and neck LN (HNLN) (*B*), and mesenteric LN (MLN) (*C*) by a cytokine ELISPOT method. Immune lymphocytes were isolated and cultured with Hc BoNT/A (Hc), OVA, or media only for 2 days, and then evaluated for IFN-γ, IL-4, IL-5, IL-10, and IL-13 CFC. Data shown are mean ± SEM of CFC/1 × 10^6 lymphocytes from a total of three experiments. *, $p < 0.001$; **, $p \leq 0.011$; ***, $p < 0.05$.

Hc BoNT/A and Hcβtre. Each of the vaccinated groups showed elevated Ab titers to Hc BoNT/A and Hcβtre, which were significantly greater ($p < 0.001$) than the Ab titers in the CT only immunized group (Fig. 7*A*). On day 96, mice were challenged i.p. with ~2000 LD_{50}. Most of the CT-dosed mice succumbed to the BoNT/A intoxication within 4 h, and none survived (Fig. 7*B*). Mice dosed with Hcβtre or Hcβtre-Ad2F alone succumbed to BoNT intoxication within 2 h. Mice nasally immunized with Hcβtre plus CT and Hcβtre-Ad2F and CT survived the ~2000 LD_{50} lethal challenge. Thus, these studies showed that immunization with the Hcβtre, either alone or fused to Ad2F, could elicit neutralizing Abs that protect against systemic lethal challenge.

Mucosal Abs protect against lethal challenge with the BoNT/A complex

To determine whether mucosal secretions contain neutralizing Abs can passively protect against BoNT/A, an in vivo mouse neutralization assay was used with the BoNT/A complex. The BoNT/A complex was used in these studies to mimic natural exposure to BoNT. In this assay, sterile-filtered fecal extracts were combined with 6.5 LD_{50} of the BoNT/A complex, and incubated at room temperature for 30 min before i.p. injection into naive BALB/c mice. Sterile fecal extracts were prepared from mice immunized with Hcβtre-Ad2F plus CT or Hcβtre plus CT, from naive mice, or from naive samples spiked with immune anti-Hcβtre plasma. All of the mice given the naive fecal extracts succumbed within 24 h (Fig. 8). Mice given the naive fecal extract containing the added anti-Hcβtre Abs were protected against challenge with the BoNT/A complex ($p = 0.005$). Likewise, all mice given the fecal extracts from Hcβtre-Ad2F plus CT-dosed mice survived, and 75% of the mice given fecal extracts from mice dosed with Hcβtre and CT survived the challenge with the BoNT/A complex ($p = 0.005$). These collective results showed the feasibility of nasal immunization with our novel BoNT/A immunogens to induce neutralizing Abs in mucosal secretions that protect against intoxication with BoNT or BoNT complex.

Intramuscular immunization with Hcβtre or Hcβtre-Ad2F elicits elevated Ab titers

To assess how the Ad2F-targeting molecule may enhance the immunogenicity of the Hcβtre, i.m. immunization studies with

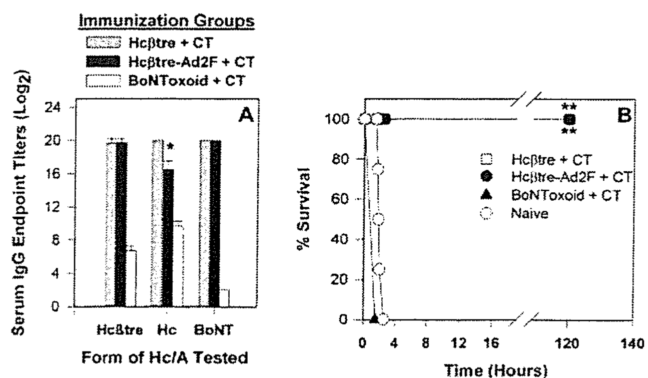

FIGURE 6. Nasal immunization with adjuvanted Hcβtre-Ad2F or Hcβtre, but not with BoNToxoid/A, confers complete protection in BALB/c mice subsequently challenged i.p. with 20,000 LD_{50} BoNT/A on day 63 postprimary immunization. Immunizations were conducted on days 0, 7, 14, and 21, and the groups were subdivided as follows: Group 1 (n = 3 mice) given 25 μg of Hcβtre plus CT (2 μg); Group 2 (n = 4 mice) given 50 μg of Hcβtre-Ad2F (to ensure that equivalent amount of Hcβtre was given); Group 3 (n = 3 mice) given 2 μg of BoNToxoid/A; and Group 4 (n = 4 mice) was left as naive. *A*, On day 57, plasma IgG titers from each group were measured against Hcβtre, BoNT/A Hc, or native BoNT/A. The mice dosed with adjuvanted Hcβtre showed a greater endpoint titer against Hc BoNT/A than mice dosed with Hcβtre-Ad2F; however, both dosing groups showed similar endpoint titers against native BoNT/A. *, p = 0.002. *B*, On day 63, mice were challenged i.p. with 20,000 mouse LD_{50} and monitored for survival for 5 days. Both the adjuvanted Hcβtre-dosed and Hcβtre-Ad2F-dosed mice showed a 100% survival rate. **, $p < 0.05$.

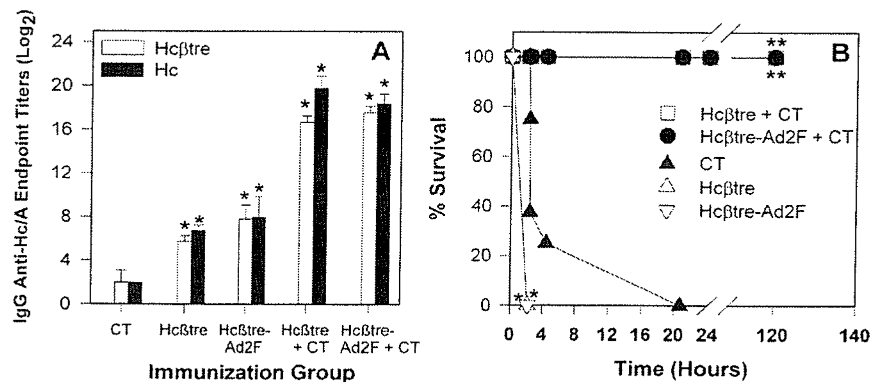

FIGURE 7. Nasal immunization with adjuvanted Hcβtre-Ad2F or Hcβtre confers complete protection in BALB/c mice subsequently challenged i.p. with 2000 LD_{50} BoNT/A on day 96 postprimary immunization. Immunizations were conducted on days 0, 7, and 14, and the groups were subdivided as follows: Group 1 ($n = 5$ mice) given 25 μg of Hcβtre plus CT (2 μg); Group 2 ($n = 5$ mice) given 50 μg of Hcβtre-Ad2F (to ensure equivalent amount of Hcβtre was given) plus CT (2 μg); Group 3 ($n = 8$ mice) given 2 μg of CT only; Group 4 ($n = 5$ mice) given 25 μg of Hcβtre; and Group 5 ($n = 5$ mice) given 50 μg of Hcβtre-Ad2F. *A*, On day 84, plasma IgG titers from each group were measured against Hcβtre and BoNT/A Hc, and equivalent endpoint titers were obtained between groups 2 and 3 and between groups 4 and 5. Plasma IgG titers from each group were significantly greater than titers in the control CT-dosed group. *, $p < 0.001$. *B*, On day 96, mice were challenged i.p. with 2000 mouse LD_{50} and monitored for survival for 5 days. Both the adjuvanted Hcβtre-dosed and Hcβtre-Ad2F-dosed mice showed a 100% survival rate, unlike the mice dosed with Hcβtre or Hcβtre-Ad2F alone, which did not. *, $p \leq 0.002$ and **, $p = 0.015$.

Hcβtre or Hcβtre-Ad2F with and without CT adjuvant were performed. BALB/c mice were dosed with equimolar amounts of Hcβtre vaccine given alone or together with 1.0 μg of CT adjuvant on days 0, 7, and 14. Ab titers were assessed weekly and showed that two doses of Hcβtre-Ad2F were sufficient to produce elevated IgG Ab titers when compared with mice similarly dosed with Hcβtre by day 14 (Fig. 9*A*). In fact, the Ab titers induced by Hcβtre-Ad2F remained significantly greater through day 28. The addition of adjuvant modestly improved the IgG Ab response induced by Hcβtre-Ad2F. In addition, the Hcβtre-Ad2F plus CT group showed enhanced IgG Ab titers when compared with the Hcβtre plus CT group (Fig. 9*A*). Secretory IgA Ab titers were induced by all groups, but clearly mice dosed with Hcβtre-Ad2F with or without CT showed the greatest sIgA response peaking on day 21 (Fig. 9*B*), but these Ab responses subsequently deteriorated to sIgA levels obtained in mice immunized with Hcβtre or Hcβtre plus CT. Thus, these data show that the inclusion of the Ad2F molecule improves the immunogenicity of Hcβtre when given parenterally even in the absence of adjuvant.

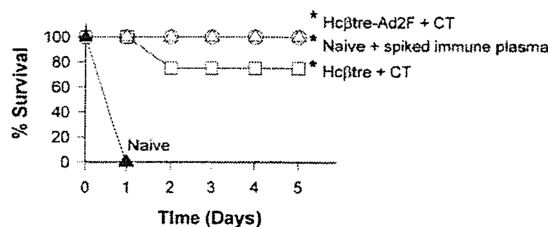

FIGURE 8. Abs in mucosal samples are protective against challenge with the BoNT/A complex. Day 21 mucosal samples were evaluated for their BoNT-neutralizing capacity using a mouse neutralization assay. Filter-sterilized soluble fecal extracts (diluted 1/2) from Hcβtre-Ad2F plus CT dosed ($n = 4$ mice) (○) and Hcβtre plus CT dosed ($n = 4$ mice) (□), from naive mice ($n = 8$) (▲), or from naive mice, but spiked with immune mouse plasma ($n = 7$) (△) were reacted with ~6.5 LD_{50} BoNT/A complex for 30 min, then were injected i.p. into naive BALB/c mice. All mice treated with naive fecal extract and the BoNT/A complex became moribound within 1 day, and all of the mice given the naive fecal extract spiked with immune plasma survived. Complete protection was obtained in all mice treated with the BoNT/A complex plus fecal extracts from mice nasally immunized with Hcβtre-Ad2F plus CT. This survival fraction was not statistically different from mice given fecal extracts from Hcβtre plus CT only. *, $p = 0.005$ for survival fractions from treatment groups compared with mice given naive fecal samples.

Discussion

The current vaccine to prevent botulism is a pentavalent BoNT-oxoid comprised of toxoids for five serotypes (A, B, C, D, and E)

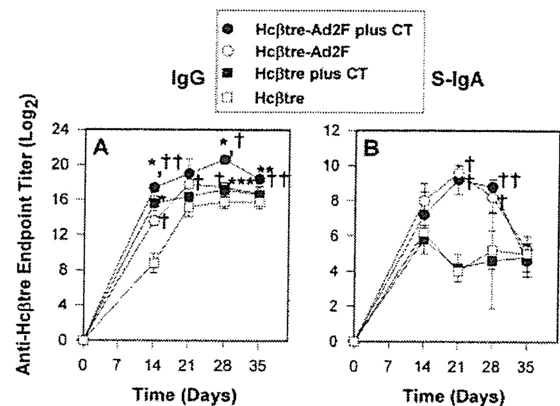

FIGURE 9. Parenteral immunization with Hcβtre-Ad2F enhances serum IgG and mucosal IgA Ab responses to Hcβtre. BALB/c mice i.m. immunized on days 0, 7, and 14 with Hcβtre or Hcβtre-Ad2F, either alone or in combination with CT. Plasma IgG (*A*) and fecal IgA (*B*) Ab titers against the Hcβtre were determined by standard ELISA methods. In the absence of CT, i.m. immunization with Hcβtre-Ad2F elicited a robust and rapid onset of IgG and sIgA Abs, whereas mice i.m. immunized with Hcβtre produced a delayed IgG and a weak sIgA Ab response. By day 35, both groups had equivalent IgG and sIgA anti-Hcβtre titers. Upon CT coadministration with Hcβtre-Ad2F or Hcβtre, a rapid onset of plasma IgG Ab titers were produced, but mice vaccinated with Hcβtre-Ad2F showed greater Ab responses. SIgA Ab titers were greater in the Hcβtre-Ad2F plus CT-dosed group than titers in mice similarly dosed with Hcβtre. Thus, these studies show that targeting with Ad2F enhances Ab responses to Hcβtre. Data represent mean ± SD ($n = 5$ mice). *, $p \leq 0.001$; **, $p = 0.005$; and ***, $p = 0.024$ represent the statistical differences in sIgA and IgG anti-Hcβtre levels between mice given the same vaccine with or without CT. †, $p \leq 0.001$ and ††, $p \leq 0.009$ represent differences between mice given Hcβtre-Ad2F or Hcβtre or given Hcβtre-Ad2F plus CT or Hcβtre plus CT.

adsorbed to alum. In addition, there is a second monovalent vaccine for BoNT/F (3). Although the conventional vaccine is efficacious, it bears a number of disadvantages, and an improved vaccine could allay these limitations. First, the BoNToxoids used for the preparation of the pentavalent vaccine are impure, with as little as 10% toxoid present in the vaccine (7). As a result, the vaccinee is exposed to irrelevant Ags unnecessary for eliciting protection. Second, the process of formalin inactivation of BoNT to produce toxoids chemically modifies the toxin resulting in a considerable loss of tertiary structure (7), thus reducing the number of viable antigenic and protective epitopes expressed by native toxins (7, 8). This latter point was evident in our study in which commercially prepared toxoid failed to elicit elevated Ab titers to either Hc BoNT/A, Hcβtre, or the native BoNT/A. Moreover, the residual formalin present in the vaccine is very painful to the recipient (25) and may deter compliance with the regular boosts required. The immunization protocol is rather elaborate, with vaccinations required at 0, 2, and 12 wk, a booster at 12 mo, and additional boosters every 2 years to maintain the desired level of immunity. Consequently, a new vaccine with conformationally preserved epitopes may lessen the number of immunizations and require less frequent boosters to maintain protective immunity. Third, some formalin remains in the vaccine to keep the toxin in the denatured form (19, 26). This result is problematic because formalin is carcinogenic (27–29), thus making its presence in a vaccine less desirable. Fourth, there are safety considerations for producing native toxins for formalin inactivation, resulting in higher costs associated with preparing the toxoid vaccine (25). Fifth, natural botulism, as well as illicit dissemination of BoNT (3), would almost surely occur via mucosal surfaces, and a vaccine developed for mucosal delivery would be advantageous to induce secretory IgA, as well as systemic, IgG Ab responses (23). This additional level of protection is far superior to that of the conventional pentavalent BoNT vaccine not optimized to include mucosal anti-BoNT immunity. A final disadvantage is that, although traditionally BoNTs have been grouped as one of seven serotypes, it has become apparent that each serotype is actually composed of a family of closely related subtypes in which the primary sequence of each BoNT/A subtype can vary by as much as 32% (30). Therefore, it would be advantageous to produce a vaccine that can preserve as many of the neutralizing and cross-neutralizing epitopes among serotypes and serotype variants.

In an effort to develop an alternative BoNT vaccine with improved efficacy, we adopted a two-prong strategy using BoNT/A as a vaccine prototype. We and others have recently identified the Hc containing the cell-binding domain in the C terminus as a potential vaccine candidate (11, 31) that contains a β-trefoil structure (18). The Hcβtre is a structure common to all serotypes (18), and this motif is even repeated in the progenitor toxin complex (32). This Hcβtre domain has been implicated in previous studies using a peptide analysis approach to determine the protective epitopes for Hc BoNT (9, 10). In the first study (9), three Hc regions in BoNT/A were identified for eliciting protection: BoNT/$A_{455-661}$, BoNT/$A_{780-939}$, and BoNT/$A_{1150-1289}$, conferring 78, 29, and 75% protection, respectively, after challenge with two LD_{99} dosed. In the second study, the synthetic peptide corresponding to Hc BoNT/$A_{1230-1253}$ was ~40% protective in mice against a lethal BoNT/A challenge (10). Thus, it appeared from these studies that, at a minimum, peptides encompassing a portion of the Hcβtre were protective. In addition, mAbs to Hc BoNT/A, some of which contain epitopes within the β-trefoil structure, have also been shown to be protective (10, 30).

The second attribute of our approach is the addition of a mucosal-targeting molecule Ad2F. Replication-deficient adenovirus vectors have widely been used in vaccination regimens (33, 34), and these vectors have been proven efficacious in their ability to stimulate protective immune responses (35, 36). However, as with any vaccine platform, concerns arise as to whether repetitive application of these vectors can limit their use (33, 34). Similar concerns are applicable with our methods, as well; however, again their application may be beneficial in a prime-boost strategy involving vaccination with the Hcβtre, both with or without Ad2F. It was evident from this study that the recombinant fusion protein with Ad2F could bind to the mouse nasal epithelium enabling uptake of the Hcβtre-Ad2F vaccine. Its use rapidly stimulated both mucosal IgA and systemic IgG Abs when Ad2F was present. Because nasal immunization with Hcβtre ultimately developed to similar anti-Hcβtre Ab titers as with Hcβtre-Ad2F, subsequent immunizations may only require Hcβtre. These studies will be pursued. When tested parenterally, our results showed that the inclusion of Ad2F-binding moiety enhanced plasma IgG and fecal IgA responses. This finding is of interest because i.m. immunization in the absence of CT adjuvant showed elevated IgG Ab titers as early as after two doses of Hcβtre-Ad2F and remained elevated at least until day 35 postprimary immunization. Although the coadministration of CT adjuvant clearly enhanced the IgG Ab titer, this increase was, at best, 9.2-fold. Additional studies will be taken to discern whether more vaccine can enhance this Ab response in the absence of adjuvant or whether the amount of adjuvant can be reduced. Such study will require evaluation of more suitable adjuvants approved for human use. How Ad2F is improving the systemic IgG response after i.m. immunization, at this point, can be speculated. Ad2F binds the coxsackie-adenovirus receptor (reviewed in Refs. 37, 38) and is expressed in a variety of tissues (39, 40). Thus, the parenteral delivery of Hcβtre-Ad2F may be taken up more efficiently because of coxsackie-adenovirus receptor expression. Alternatively, the Ad2F may enhance the immunogenicity of Hcβtre. Moreover, the Hcβtre also induced elevated Abs alone, albeit, lesser than Hcβtre-Ad2F. However, mucosal IgA Abs were induced to greater levels when using the Hcβtre-Ad2F than when using Hcβtre, although both showed only low mucosal Ab levels after day 35, suggesting long term mucosal memory was not induced. Nonetheless, these results show the feasibility of using either Hcβtre-Ad2F or Hcβtre in parenteral immunization paradigm.

Our study showed that the Hcβtre is strongly immunogenic when delivered nasally. As was evident, the Hcβtre stimulated 100% protection to mice when challenged i.p. with either 20,000 or 2,000 LD_{50}. This immunity was found to be long lasting as noted by the day 96 challenge. In addition, we found that the Abs within mucosal secretions were protective. Using a mouse neutralization assay, sterile fecal extracts protected against 6.5 LD_{50} of the BoNT/A complex. Intoxication with the BoNT complex is 100-fold more toxic than purified BoNT (41, 42), when using the gastric route of exposure. This BoNT complex would most likely represent the form of toxin dissemination in bioterrorism. Our results suggest that the protective value of these mucosal Abs, at a minimum, approximates the lower end protection (2,000 LD_{50}) found with systemic Abs. It also shows that our vaccine formulation is better than using BoNToxoid as a nasal vaccine immunogen for the induction of protective humoral immunity in mucosal secretions. For example, fecal extracts from 75 to 100% of mice nasally immunized with Hcβtre plus CT or Hcβtre-Ad2F plus CT, respectively, protected mice against a lethal BoNT/A complex challenge. However, when a similar assay was performed with fecal extracts from mice nasally immunized with BoNToxoid, only one-third of the mice were protected against one LD_{50} of BoNT/A complex (23). Clearly, this evidence showed that immunization

with the Hcβtre is protective and is a better immunogen than the toxoid.

Although no differences in protection were noted between mice dosed with the Hcβtre or the Hcβtre-Ad2F, it is important to note that the addition of the Ad2F acted as a mucosal accelerant in which anti-Hcβtre sIgA, and plasma IgG Ab titers were more quickly induced. Although Hcβtre-Ad2F given without adjuvant was able to induce some Ab titers to the Hcβtre, elevated plasma IgG Ab titers did not become elevated as when in the presence of an adjuvant. It may be more advantageous to give Hcβtre-Ad2F without adjuvant as a booster, rather than to initiate the Ab response, and thus lessen the need for mucosal adjuvants. Studies are currently addressing this possibility. The use of the Hcβtre immunogen may also be beneficial to elicit a polyclonal antiserum to neutralize against BoNT intoxication. Because a portion of the neutralizing epitopes are retained in the Hcβtre (9–11), Abs directed against this cell-binding domain may even be comparable to the use of mAbs.

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Disclosures

The authors have no financial conflict of interest.

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Innate Immunity in the Mucosal Immune System

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Abstract: The mucosal immune system is equipped with unique innate and acquired defense mechanisms which provide a first line of protection against ingested and inhaled infectious agents. Peyer's patches (PPs) and nasopharynx-associated lymphoid tissue (NALT) have been shown to be important inductive sites for the initiation of the acquired phase of antigen-specific immune responses. In addition, the mucosal innate immune system acts as both a physical and an immunological boundary, playing a key role in the sensing and eliminating of pathogens and in the creating of symbiosis. The mucus layer covering the mucosal epithelium acts as a first physical and biochemical barrier. An additional layer of physical protection against microorganisms is provided by a tightly interlaced cell-to-cell network of epithelial cells and intraepithelial lymphocytes. Various antimicrobial peptides produced by the epithelium and secreted into the mucosal lumen can directly kill the invading pathogenic bacteria. Finally, Toll-like receptors (TLRs) associated with the mucosal compartment have been shown to recognize the pathogen-associated molecular patterns (PAMPs) of a variety of pathogenic and commensal microorganisms. Therefore, a greater understanding of the immunological progression from mucosal innate to acquired immune systems should facilitate the development of new generation of mucosal vaccines to prevent and control infectious diseases.

INTRODUCTION

The common mucosal immune system (CMIS) consists of two immunologically important sites, termed "inductive" and "effector" [1]. In the gut, the antigens including food antigens, bacteria and virus, are taken up by M cells (Membranous/Microfold cells) located in the follicle-associated epithelium (FAE) covering the inductive sites such as Peyer's patches (PPs) [2] and isolated lymphoid follicles (ILFs) [3]. The professional antigen-presenting cells (APCs) such as dendritic cells (DCs) are located in the pocket of M-cells, in the subepithelial dome region under the FAE or in the interfollicular regions where antigen processing and presentation are executed for the induction of antigen-specific Th1 or Th2 cells as well as IgA-committed B cells [1, 4]. Antigen-specific IgA-committed B cells migrate from inductive sites to effector sites such as the lamina propria region of the intestinal tract, where they are activated by Th2 cell-derived IgA-enhancing cytokines such as IL-5 and IL-6 and then differentiated into plasma cells secreting dimeric/polymeric forms of IgA [1]. These IgA antibodies then bind to poly Ig receptors (pIgR) produced by epithelial cells and are transported as secretory IgA (S-IgA) into intestinal secretions [1]. Unlike the exclusively systemic immune responses induced by injections, mucosal modes of vaccine delivery generate antigen-specific immune responses in both mucosal and systemic immune compartments [1]. Thus, the concept of mucosal immunization is naturally attractive for the development of new generation of vaccines for the control of emerging and re-emerging infectious diseases.

In addition to the role played by these well-characterized gut-associated lymphoid tissues (GALT) such as PPs and ILFs in antigen sampling and in the subsequent generation of antigen-specific immune responses, our findings as well as those of other researchers indicate the presence of GALT-independent antigen-uptake systems for the induction of antigen-specific immune responses [5-7]. It has been suggested that CD18-expressing phagocytes such as DCs located at the intestinal villi are involved in the dissemination of the invasion gene (SPI1)-deficient *S. typhimurium* from the intestinal tract to systemic compartments such as the spleen [5]. In addition, it has been shown that intestinal DCs are capable of opening the tight junction that characterizes intestinal epithelium and of extending dendrites to the lumen side to sample bacterial antigen directly [6]. Our recent finding demonstrated that villous M cells located at the intestinal villi act as novel antigen entry sites, just as do M cells located in the FAE of PPs [7]. Furthermore, villous M cells were found to be intact even in several PP-deficient mice including *in utero* lymphotoxin β receptor (LTβR)-Ig-treated, LTαR$^{-/-}$, TNFα$^{-/-}$ LTα$^{-/-}$, and inhibition of differentiation 2 (Id2) $^{-/-}$ mice. These data suggest that villous M cells and intestinal DCs represent a novel gateway for the initiation of GALT-independent mucosal immune responses [5-7]. Our previous studies have lent support to the presence of such a GALT-independent gateway by showing that antigen-specific immune responses are induced in the absence of PPs and/or ILFs following oral immunization [8, 9]. Armed with both GALT-dependent and -independent pathways, the mucosal immune system is well-

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equipped to provide a highly effective first line of defense against invading pathogens and to effectively generate protective immunity.

In addition to the acquired phase of immunity, the mucosal immune system harbors a series of physical, chemical and biological innate molecules (Table 1). For example, the mucosal surface is covered by a thick layer of mucous consisting of two major mucin molecules (MUC2 and 3) which provide physical and chemical barriers [10-14]. In addition, the cell-to-cell adhesion mechanisms among mucosal epithelia represent another strong physical barrier against pathogens. Of these sealing mechanisms, the molecules forming tight junctions, such as occludin and claudin-family molecules, are key players in the caulking of intercellular space [15-19]. Furthermore, the defensin family of antimicrobial molecules mainly expressed by Paneth cells located at the intestinal crypt have been shown to kill infectious microorganisms [20]. Considerable attention has been focused on the characterization of the recently discovered Toll-like receptor (TLR) family in the innate immunity system. TLRs have been shown to be essential receptors, capable of recognizing pathogen-associated molecular patterns (PAMPs) with their common pathogen-associated molecules such as lipopolysaccaride (LPS) and peptidoglycan [21, 22]. This review will focus on the contribution made by the mucus layer, adhesion molecules, TLRs and antimicrobial peptides to the first line of defense against pathogens provided by mucosal innate immunity.

VISCOELASTIC SHEET FORMATION BY MUCINS AS A FIRST LINE OF DEFENSE

The large mucosal surface area is covered by a thick layer of mucus with viscoelastic properties. One of the primary functions of the layer is to provide a physical and biological protective barrier against infectious microorganisms [23] (Fig. 1). In addition, mucus also has the important secondary role of ensuring in maintaining an appropriate concentration of antibodies at the mucosal surface by preventing antigen-specific IgA antibodies from being physically carried away [24]. Mucins (MUC) in the mucosal layer are broadly categorized as being either "soluble" or "membrane" forms. Soluble types of MUC gene (MUC5AC, 5B and 6) except MUC2 are forming a cluster at chromosome 11p15.5 [25]. Currently, eleven types of human epithelial cell-associated mucins (MUC1-4, 5AC, 5B, 6-8, 11 and 12) have been identified [26]. In the intestine, MUC2 and MUC3 [10-14] are predominantly expressed. These intestinal MUCs conserve up to fifty percent of their amino acid composition, including high levels of proline, threonine and/or serine (PTS) residues [10]. The PTS residue regions of monomeric MUCs are highly glycosylated because of the abundant binding to *O*-linked oligosaccharides [27].

Of the members of the MUC family, MUC2 has been the most extensively characterized as an example of the soluble form, and MUC3 as an example of the membrane form [10-14]. MUC2 is a dominant intestinal mucus-formation molecule which is abundantly expressed by goblet cells located at the intestinal villous epithelium [23, 24]. The molecule consists of more than 5100 amino acids [23, 24], with tandem repeats rich in threonine (Thr) and serine (Ser) and abundant binding to *O*-linked oligosaccharides [23, 24, 27]. The carbohydrate content is estimated to be up to 90% of the total molecular weight. In contrast, the N- and C- terminal regions of the MUC2 molecule, termed disulfide-rich domains or D-domains, are poorly glycosylated, but rich in cysteine (Cys) residues which polymerize by disulfide bonds to form a dimeric and/or a polymeric mucus layer. Polymerization increases the molecular weight of monomeric MUC2 from ~ 1.5×10^6 Da to ~ 10^7 Da [23, 24]. Like MUC2, MUC3, one of the membrane-type mucin molecules, is also highly expressed in the intestine [12] and has an extracellular Thr/Ser-rich domain bound to *O*-linked oligosaccharides [12]. Unlike

Table 1. Uniquness of Innate Immunity at Mucosa

Molecule	Special Features	Roles		
		Physical	Chemical	Immunological
Mucins (MUC)	Intestine: MUC2 (soluble form), MUC3 (membrane form)	+	+	-
Occlaudin/Claudin	Formation of tight junction among IECs	+	-	-
E-cadherin	Formation of intermediate junction among IECs and heterophilic junction with $\alpha_E\beta_7$ integrin between IEC and IEL	+	-	-
Ep-CAM	Formation of intermediate junction among IECs and homophilic junction between IEC and IEL	+	-	-
MICA/CD1d	Non-classical MHC class I Activation of $\gamma\delta$IELs	-	-	+
TLR	Recognition of PAMPs Intestinal epithelium: Inhibition of expression at cell surface	-	-	+
NOD	Cytoplasmic receptor for PGN	-	-	+
Defensins	Killing pathogenic microorganisms Immunomodulatory activity	-	+	+

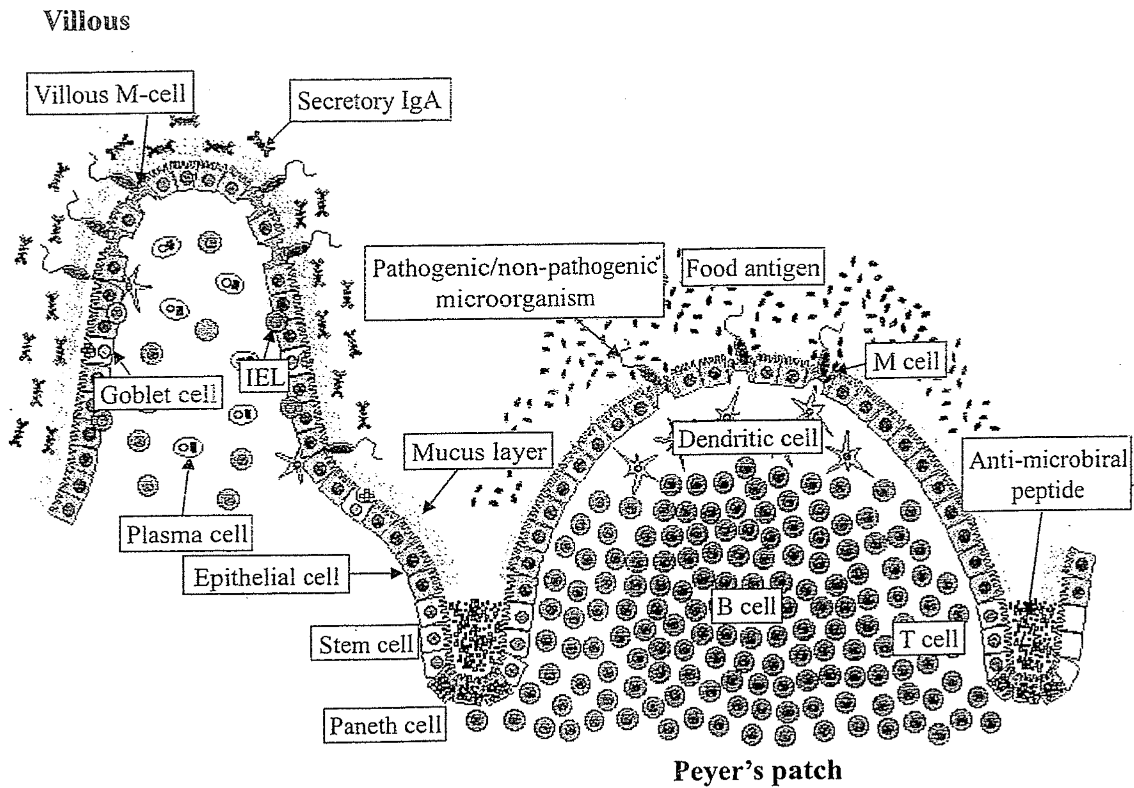

Fig. (1). Mucosal immunity of intestinal tract. M-cells located at FAE region of Peyer's patchs and villous M-cells uptake the antigen including pathogenic/non-pathogenic microorganisms and food antigens to initiate the induction of GALT dependent and independent antigen-specific acquired immunity, respectively. In addition, intestinal villi are covered by thick mucus layer and anti-microbial peptides are secreted by Paneth cells to defend the mucosal compartment as a part of innate immunity.

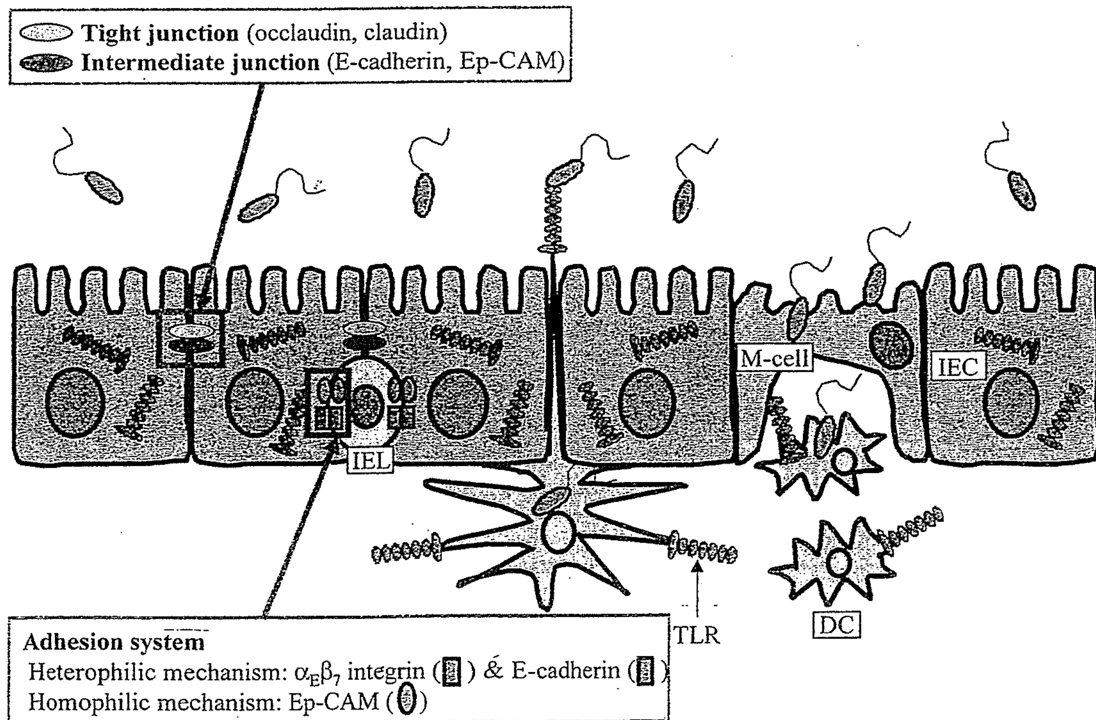

Fig. (2). Epithelial barrier in the mucosal immune system. IECs and IELs as well as among IECs are tightly welded by adhesion molecules, such as occlaudin, claudin, E-cadherin and Ep-CAM to provide physical barrier as a part of the mucosal innate immune system. The mucosal innate immune system also possesses unique TLR expression. For example, IECs do not express TLR at the cell surface but cytoplasmic region dissimilar to antigen presenting cells (APCs), such as DCs, to avoid execution of unnecessary immunological stimulation.

MUC2, however, MUC3 is abundantly expressed by epithelial as well as by goblet cells [12]. In situ hybridization analysis indicates that MUC2 develops in humans at 9 weeks of gestation between primordial villi containing the developing crypt. At 27 weeks, when the intestinal epithelium is fully differentiated, the expression profile shifts from embryonic to adult. In contrast, MUC3 mRNA is expressed at a very early stage (as early as 6.5 weeks of gestation) in the primitive gut endoderm before cytodifferentiation of the epithelium. Hence, MUC3 may not only serve to form a mucus layer acting as a physical barrier against invading pathogens, but it may also play other key roles such as regulating cytodifferentiation [13].

At least 5 types of cells of epithelial lineage (absorptive cells, goblet cells, enteroendocrine cells, Paneth cells and M cells) are present in the intestine [2, 28]. Recently, it was reported that Math1 (a basic helix-loop-helix transcription factor) is essential for the differentiation of secretory cells (goblet cells, enteroendocrine cells and Paneth cells) [28]. In contrast, Hes1 [Hairy and Enhancer-of-split (HES)-type proteins] has been reported to be an important molecule for enterocyte differentiation [29]. It is possible that the differentiation of precursors of epithelial lineage into epithelial cells could be related to the creation of symbiosis. As mentioned above, it is well known that goblet cells are major producers of different mucin family molecules. The colonization of commensal microorganisms in the gut has been shown to stimulate goblet cell development and function. As expected, the number of goblet cells is decreased in germ-free (GF) mice [30, 31], leading to a thinner mucus layer than in conventional (CV) mice [30]. In addition, mucus composition, particularly in terms of carbohydrate, is significantly different between GF and CV animals [30]. Goblet cells located at the cecal mucosa in CV but not GF mice strongly reacted with *Ulex europaeus* agglutinin I (UEA1), which has specificity for α-L-fucose, as well as *Dolichos biflorus* agglutinin (DBA), which specifically reacts with α-D-GalNAc [30]. In addition, it was reported that microbe-derived factors induced the secretion of mucin from goblet cells [32-34]. For example, cholera toxin (CT) of *Vibrio cholerae*, which is a well-known mucosal immunomodulator, can induce the secretion of mucin from HT29-18N2 and HT29/B6, which are highly differentiated cells from the human goblet cell line HT29 [32, 33]. Listeriolysin O from *Listeria monocytogenes* has also been shown to induce the exocytosis of mucin in HT29-MTX [34]. However, the precise molecular mechanisms for toxin-induced mucus expression in intestinal goblet cells remain poorly understood. Furthermore, proinflammatory cytokines such as IL-1 and TNF-α have been shown to induce mucin expression in the LS180 cell line of human intestinal carcinoma [35]. These data suggest that mucin expression depends on many biologically active factors produced by both the microorganisms and the host immune system to successfully form and maintain the mucosal mucus barrier protecting the host from infection.

In contrast to the thick mucus layer covering the intestinal epithelium, the mucosal layer at inductive sites like the follicle-associated epithelium (FAE) of PPs and ILFs is thin, creating a gateway environment which allows easy access to lumenal antigens [36]. In fact, M cells are mainly localized at the FAE region of PPs and do not express the MUC2 gene [36]. The terminus of carbohydrate on MUC2 is highly fucosylated [37], which is strongly reacted with UEA1 [38]. Interestingly, UEA1 also specifically reacts with PP associated-M-cells in mice [39-41]. Our recent study further showed that villous M cells are developed independently from PPs, but are also recognized by UEA-1 [7]. It is still not understood that the fucosylated molecule(s) but not MUC2 expressed by both PP associated- and villous M cells is functionally important in mucosal innate and acquired immunity. In either case, for the expression of MUC, FAE of PPs and ILFs, especially M cells, may be under a different umbrella of the regulatory system than other epithelial regions. It is also interesting to propose that the MUC requirements of PP associated-M cells and villous M cells may differ, since they are associated with distinctively different immunological environments, the former located on the organized lymphoid structure [2] and the latter situated in the diffused connective tissue of the intestinal tract [7]. In any case, the correlation between the formation of a viscoelastic sheet-based MUC innate immunity and the induction pathway of mucosal acquired immune system must be further elucidated if we are to understand the cross-communication(s) between mucosal innate and acquired immunity for the initiation of active and quiescent immune responses to mucosal antigens (e.g., protection and symbiosis, respectively).

FORMATION OF CELLULAR JUNCTION NETWORK AT MUCOSAL EPITHELIUM

Underneath the mucus blanket is the mucosal epithelium, a layer of epithelial cells acting as a part of the first line of defense and as an important physical and immunological barrier against pathogens or non-self antigens. The mucosal epithelial cells present a formidable barrier because they are tightly welded together by a number of adhesion mechanisms (Table 1, Fig. 2). Occlaudin has been identified as a molecule composing the tight junctions located at the most apical and lateral regions of the epithelial cell layer [15]. In addition, the claudin family, which is not homologous to occlaudin, can form the tight junction together with occlaudin [16, 17]. In fact, L-fibroblasts lacking internal occlaudin or claudin family expression gained cell-adhesion activity when claudin-1, -2 and -3 genes were introduced [42]. Intestinal epithelium expresses high levels of occlaudin and of four types of claudin (claudin-2, -3, -4 and -5) [43]. Interestingly, claudin-4 is highly expressed at the dome region of the FAE in PPs, where M-cells are located [44]. In addition, the C-terminal region of *Clostridium perfringens* enterotoxin (C-CPE) specifically binds to claudin-4 and induces the breakdown of the tight junction [45]. Therefore, it is reasonable to speculate that certain pathogenic microorganisms can successfully initiate invasion by breaking the tight junction-based mucosal barrier. At the same time, these tight junction-targeting molecules produced by pathogens can also induce mucosal immunity.

E-cadherin, another member of adhesion molecule expressed by epithelial cells, can form intermediate junctions. E-cadherin-mediated homophilic adhesion is regulated in a Ca^{2+}-dependent manner [46]. E-cadherin forms a dimer on the lateral surface of the epithelial cells which acts as a zip-

per structure binding it to the adjacent epithelial cells [47, 48]. In addition, E-cadherin links cytoskeleton-related molecules, such as β-catenin, to form the strong intermediate junction [47, 48]. Epithelial cell adhesion molecule (Ep-CAM), which regulates independently of Ca^{2+}, also shows a homophilic adhesive function among epithelial cells [49, 50]. The adhesive structure mediated by Ep-CAM in epithelium neighbors that mediated by E-cadherin, and these molecules both form the intermediate junction [49, 50]. It is interesting to speculate that the redundant expression of both Ca^{2+}-dependent E-cadhedrin and Ca^{2+}-independent Ep-CAM is built into the physical bridging system between epithelial cells as a way to ensure proper physical sealing in the volatile environment of the intestinal tract.

In addition to mucosal epithelial cells, high numbers of lymphocytes termed intraepithelial lymphocytes (IELs) are also situated in the mucosal epithelium and, together with the epithelial cells, contribute to the formation of the immunological barrier [1]. The majority of IELs are $CD3^+$ T cells bearing either αβ or γδ T cell receptors (TCR) and approximately 80% of the $CD3^+$ T cells belong to the CD8 subset using either αβ heterodimeric or αα homodimeric chains [1]. These $CD8^+$IELs have been shown to possess cytotoxic activity and are thus thought be key players in the immediate elimination of pathogen-infected epithelial cells [1]. It was suggested that $CD4^-CD8\alpha\alpha^+$ IELs bearing both αβ TCR and γδ TCR developed from cryptopatches (CPs) which are thought to be the nesting site for the thymus-independent pathway, while $CD4^+CD8^-$ and $CD4^-CD8\alpha\beta^+$ IEL development was thymus-dependent [51]. Currently, however, the development of thymus-independent IELs remains a subject of intense debate. Very recently it was reported that IELs expressing αβ TCR (αβIELs) containing $CD4^-CD8\alpha\alpha^+$ IELs developed from immature $CD4^+CD8^+$ thymocytes [52]. Furthermore, it has been suggested that isolated lymphoid follicles (ILFs), one of the inductive site networks of the mucosal immune system, may also develop from CPs [53].

In mice, an IEL is found every sixth epithelial cell and so a large number of T lymphocytes are located in the intestinal epithelium [1]. Unlike systemic T cells, which mainly express αβ TCR, the expression of TCR in IELs is almost equally divided between αβ (αβIELs) and γδ (γδIELs) [54]. Because IELs are adjacent to intestinal epithelial cells (IECs), cell-to-cell communications between IECs and IELs are important to the assembly and maintenance of the epithelial barrier. It is easily conceivable that IECs and IELs depend on each other for their development and subsequent coexistence at the mucosal epithelium. For example, IECs are capable of producing IL-7 [55] and IL-15 [56], which are important for the stimulation and development of γδIELs, while γδIELs express keratinocyte growth factor (KGF), which stimulates the growth of IECs [57]. Furthermore, IECs express non-classical MHC class I, such as MICA and CD1d [58-60]. The expression of MICA in IECs is induced by various stresses, such as heat shock, oxidative stress and bacterial or viral infection [58]. On the other hand, NKG2D, which is an essential receptor of MICA, is mainly expressed by NK cells and T cells including IELs [61]. It has been suggested that the interaction of MICA/NKG2D between IECs and IELs might be important for the activation of γδIELs because NKG2D-expressing $V\delta1^+$γδIELs react with MICA expressed by IECs [62]. In addition, IECs spontaneously express CD1d and have the ability to present glycolipid antigens, such as α-galactosylceramide, to CD1d-restricted T cells located at intestinal villi [59, 60].

Furthermore, γδIELs have been shown to possess unique features for the regulation of the mucosal immune system. Because $TCR\delta^{-/-}$ mice induce significantly lower levels of antigen-specific immune responses in both mucosal and systemic sites than do wild-type mice following oral immunization with antigen and mucosal adjuvant, γδIELs are presumed to be involved in the induction and regulation of antigen-specific IgA responses [63]. In addition, γδIELs have been shown to regulate oral tolerance, which is an essential suppressive mechanism of the mucosal immune system responsible for creating a symbiosis between the host and commensal antigens [64].

Although cross-communicaton between IECs and IELs has been a focus of research, little is currently known about the underlying cellular and molecular mechanisms of physical cell-to-cell interactions between IECs and IELs. It has been recently reported that $\alpha_E\beta_7$ integrin mediates T-cell adhesion to epithelial cells through its binding to the E-cadherin that is selectively expressed on epithelial cells [65-67]. In fact, the number of IELs decreased but did not disappear in α_E integrin-deficient mice [68]. These data suggest the presence of additional adhesive mechanisms underlying the physical co-dependency of IECs and IELs.

To further explore the adhesion mechanisms connecting IECs and IELs, we established several monoclonal antibodies (mAbs) which reacted to the basolateral surface of the intestinal epithelium. One Ep-CAM-specific mAb reacted with the cell wall of IELs as well as the basolateral surface of IECs. In contrast, lamina propria lymphocytes (LPLs), which mainly contain $CD4^+$ T cells and IgA-committed B cells, did not react with the mAb. These data strongly indicate that Ep-CAM plays a role in the adhesiveness of IECs and IELs [69] (Fig. 2). In addition, it was very recently reported that IELs also expressed junctional adhesive molecules such as occludin and E-cadherin [70]. These data suggest that the mucosal epithelium is able to create and maintain the unique immunological intestinal barrier *via* elegant cell-to-cell interaction mechanisms which ensure a high degree of adhesiveness between IECs and IELs.

TOLL-LIKE RECEPTOR FAMILY FOR MUCOSAL PROTECTION AND SYMBIOSIS

In 1996, Lemaitre *et al.* reported that Toll was the essential receptor for host defense against fungus infection in *Drosophila*, which is equipped with only innate immunity [71]. In mammals, the homologous molecule is the Toll-like receptor (TLR), which was found in 1997 to be a pathogen-associated molecule pattern (PAMP) recognition molecule [72]. Therefore, we now have at least two categories of antigen recognition systems for the recognition and elimination of invading infectious microorganisms and non-self pathogens, namely TLR for innate immunity, and T cell receptors (TCR) and B cell receptors (BCR or immunoglobulin) for acquired immunity. Currently, 11 types of TLR have been identified and their role in the host innate immune system has been characterized [22]. The cytoplasmic regions of

TLRs strongly resemble those of the IL-1 receptor family, so this region has been termed the Toll/IL-1 receptor (TIR) domain [21, 22]. In contrast, the extracellular domain of each receptor harbors distinct characteristics, with IL-1 receptors possessing an immunoglobulin-like domain and TLRs having leucin-rich repeats [21, 22]. This fact reflects the biological uniqueness of the immune system in which the immunobiologically distinct receptors share the cytoplasmic signaling molecule for the subsequent stimulation of innate immunity- associated cytokines (e.g., TNF-α, IFN-γ and IL-12) which will send the activation signal that initiates acquired immunity. The TLR signaling pathway induces the expression of inflammatory cytokines such as TNF-α and IL-12 *via* myeloid differentiation primary-response protein 88 (MyD88), an adaptor molecule containing the TIR domain at the C-terminal and the death domain at the N-terminal region [73]. MyD88 recruits IL-1R-associated kinase 4 (IRAK4) at the death domain and induces the IRAK4-mediated phosphorylation of IRAK1. TNF-receptor-associated factor 6 (TRAF6) is also recruited and associated with activated IRAK1 and then activates both mitogen-activated protein (MAP) kinase, leading to the translocation of activator protein-1 (AP-1), and the inhibitor of nucleus factor-κB (IκB) kinase complex, resulting in the activation of nucleus-factor-κB (NF-κB) [21, 22, 73]. In contrast, TLR3 and TLR4 induce interferon β (IFN-β) and IFN-inducible genes *via* an MyD88- independent pathway [74]. TIR-domain-containing adaptor proteins inducing IFN-β (TRIF), alias TIR-domain-containing adaptor molecule (TICAM-1), is essential to the MyD88-independent pathway. TRAF6- and TRAF-family-associated NF-κB activator (TANK)-binding kinase 1 (TBK1), which is associated with the C-terminal region of TRIF, induces activation of IFN-regulatory factor 3 (IRF3) and NF-κB, respectively [21, 22, 74].

TLRs recognize PAMP such as lipopolysaccharide (LPS) from gram-negative bacteria, peptidoglycan (PG) from gram-positive bacteria, as well as bacterial nucleic acids and flagellin [22]. TLR1, TLR2 and TLR6 are essential receptors for the recognition of lipopeptides including PG [75, 76], TLR4 for the recognition of LPS [77], TLR5 for the recognition of flagellin [78], and TLR9 for the recognition of CpG motif of DNA [79]. For virus-associated PAMP, TLR3 is an essential receptor for viral double strand RNA [80], while TLR7 and TLR8 are implicated in the recognition of virus-derived single strand RNA [81]. These different TLRs can be divided into two groups based on the location of their expression in innate immunity affiliated cells. TLR1, 2, 4 and 6 are expressed on the cell surface and recognize bacterial cell compartments, while TLR3, 7, 8 and 9 are expressed in the cytoplasmic region and recognize the bacterial/viral nucleic acids after internalization of the respective PAMP by the cell [22].

TLRs are mainly expressed by antigen-presenting cells (APCs) such as dendritic cells (DC) or macrophages (Mφ). Following pathogen invasion and subsequent antigen uptake by phagocytosis, APCs process antigen and present the peptide on MHC class II to naïve CD 4^+ T cells to initiate antigen-specific acquired immunity. At the same time, the TLR-mediated signaling pathway induces the expression of costimulatory molecules including CD80 and CD86 on APCs to form a cooperative activation complex with TCR and MHC class II during the antigen presentation for maximum stimulation in the initiation of acquired immunity [22]. Therefore, the process of TLR-mediated PAMP recognition and the subsequent signaling cascade are thought to bridge innate and acquired immunity in the creation of protective immune responses against infection.

The issue of TLR expression and immunological role at the mucosal epithelium is currently being actively investigated. The harsh environment of mucosal compartments such as the intestinal tract is totally different from the immunologically sterile or quiescent conditions found in the systemic compartments where most APCs are situated. Mucosal tissues are directly and continuously exposed to the outside environment as well as to abundant commensal microorganisms. In such circumstances, TLR may take on a different biological role, helping to establish symbiosis *via* the induction of tolerance to intestinal commensal bacteria. In fact, IECs have been shown to express low levels of TLRs and to poorly respond to microbial antigens [82-85]. Furthermore, IECs do not express MD-2, an associated molecule with an extracellular domain of TLR4, which is required for the recognition of LPS [82, 84]. MD-2 is also thought to be involved in the transport of TLR4 from the cytoplasmic region to the cell surface [86] and so its absence in IECs may account for their weak or absent expression of TLRs. Our findings also indicate that human corneal epithelial cells (CECs), which are directly exposed to the outside environment and reach a symbiotic condition, do not express TLR2 and TLR4 at the cell surface, though they normally express corresponding receptor-specific mRNA and proteins in the cytoplasmic region, and therefore are unresponsive to LPS and PGN [87]. In addition, they do not lead to activation *via* TLR signaling even when LPS is artificially introduced into the cytoplasm [87]. Taken together, these findings show that epithelial cells, which are heavily involved in the creation of symbiosis at mucosal compartments, manipulate the TLR family-mediated PAMP recognition system to avoid execution of unnecessary immunological stimulation for the activation of innate and subsequent acquired immunity [88] (Fig. 2).

It was recently reported that the nucleotide oligomerization domain family (NODs), which recognize γ-D-glutamyl-*meso*-diaminopimelic acid of PGN from gram-negative bacteria (the ligand of NOD1) and *N*-acetylmuramyl-L-ananyl-D isoglutamine of PGN from both gram-positive and -negative bacteria (the ligand of NOD2), were involved in the PAMPs recognition system [89, 90]. NODs, which are cytoplasmic pattern recognition receptors, are found on three distinct functional domains: the effector-binding domain (EBD), the alias caspase activation and recruitment domain (CARD), and the NOD domain and effector-binding domain [90]. Upon stimulation, NODs induce self-oligomerization and then engage in a homophilic CARD-CARD interaction with Rip-like interacting caspase-like apoptosis-regulatory protein kinase (RICK), which leads to the activation of NF-κB [91]. Though IECs express only low levels of TLR4 and show poor responsiveness to LPS [82-85], IECs have been shown to spontaneously express NOD1 and recognize enteroinvasive *E. coli* [92]. Taken together, these findings suggest that IECs cannot only adjust themselves to mucosal microbial environments by modulating TLR expression for the appropriate handling of LPS or PGN from commensal and/or

pathogenic microorganisms at the cell surface, but can also fully recognize and respond to a dangerous invasion by microorganisms.

MICROBICIDAL PEPTIDES AS PROSTRATING AND REGULATORY MOLECULES AT MUCOSAL SITES

As a part of the innate immune system, the host is equipped with an array of anti-microbial peptides produced by mucosal epithelium-associated cells including epithelial cells, Paneth cells and polymorphonuclear leukocytes (PMN). These anti-microbial peptides can immediately eliminate pathogens from the entry site. In vertebrates, a defensin family consisting of three forms of α-, β-, and θ-defensins has been well characterized as anti-microbial peptides. The α- and β- defensins are 3-4 kDa peptides containing three invariantly paired disulfide bonds [93]. Except for linear spacing and disulfide pairings, α- and β-defensins are similar in structure [93]. The expression pattern of the defensin family by PMN varies from species to species. For example, α-defensin is an important component of granules in human [94], rabbit [95] and rat PMNs [96, 97], while ovine [98] and murine [99] PMNs completely lack the peptide, instead containing another form of anti-microrbial peptides, cathelicidins, which have multiple biological roles regarding cell proliferation, migration and immune modulation in addition to microbial activity [100, 101]. In contrast, θ-defensin is expressed only by rhesus macaque PMNs [102, 103]. θ-defensin also differs from α- and β-defensins in structure, with its covalently closed circular polypeptide chains of 18 amino acids stabilized by three disulfide bonds [104]. It was very recently reported that θ-defensin can protect the host from infection caused by herpes simplex viruses [103]. In contrast with the expression of α- and θ-defensins by different species of PMN, β-defensin is widely expressed by the intestinal epithelial cells and thus thought to be one of the key innate immunity molecules associated with the mucosal immune system [105]. The mechanisms used by antimicrobial peptides of the defensin family in the killing of microorganisms are currently under active investigation and discussion [106]. It has been suggested that anti-microbial peptides may strongly bind to the outer leaflet of the cell membrane of microorganisms but not of plants and animals [106]. Antimicrobial peptides are cationic (positively charged) molecules, while the outer leaflet of microorganisms is heavily populated by lipids with more negatively charged phospholipid headgroups than those of eukaryotes. In addition, though no definitive conclusions regarding the killing mechanisms of microbial peptides have been drawn, many hypotheses have been reported, including the induction of the fatal depolarization of the normally energized bacterial membrane and the creation of physical holes whereby cellular components leak out [106].

It is also interesting to point out that defensins have been shown to possess immuno-modulatory activity and to enhance antigen-specific immune responses [107]. Co-administration of ovalbumin (OVA) and the human neutrophil peptide defensin resulted in the enhancement of antigen-specific Thl/Th2 cell and B cell immune responses following mucosal immunization [107]. Not only do the members of the defensin family provide direct anti-microbial activity as innate immunity molecules, they also can initiate acquired immunity *via* the augmentation of T and B cell interactions for the induction of antigen-specific immune responses (Table **1**).

Paneth cells located at the crypt region of the intestinal epithelium are granulated secretory epithelial cells that spontaneously secrete α-defensins (Fig. **1**). In mice, the α-defensins produced by Paneth cells are also termed cryptidins [108]. While only two types of α-defensins, HD-5 and HD-6, are found in the human intestinal crypt [109, 110], at least 17 isoforms of cryptidins have been characterized from the cDNA library of the murine intestinal crypt [111]. Purified cryptidin 1-6 has been reported to have anti-microbial activity *in vitro* [111-113]. The bacteriocidal activity of defensins was also demonstrated *in vivo* using gene-manipulated mice. An overexpression model using a transgenic system suggested that HD5 transgenic mice were more resistant to oral challenge with virulent *Salmonella typhimurium* than were conventional mice [114]. Paneth cells responded to bacterial stimulation by *S. typhimurium* and *E. coli* by inducing the secretion of cryptidin, but no such response was seen to eukaryotic stimulation [115]. It was also reported that Paneth cells induced degranulation (secretion of α-defensin) *via* Toll-like receptor (TLR) 9 after stimulation with CpG DNA, thereby increasing resistance against oral challenge with *Salmonella* [116]. Comprehensive analysis of TLR family expression indicated that Paneth cells expressed all types of TLR mRNA except TLR4 [92]. However, it is interesting to note that Paneth cells responded to LPS in C3H/HeJ mice despite the lack of TLR4 expression [117]. These data suggest that Paneth cells may harbor TLR-independent PAMP recognition mechanisms. It is interesting to postulate that the mucosal epithelium might be furnished with a not-as-yet identified PAMP recognition mechanism that is distinct from that observed with the professional antigen-presenting cells situated in systemic compartments. A greater understanding of Paneth cells and of the antimicrobial peptides they produce might lead to the discovery of unique recognition mechanisms for pathogenic microorganisms and of subsequent activation pathways for the production of microbicidal peptides.

In contrast to α-defensins, β-defensins are widely expressed in several mucosal tissues such as those in the intestinal, respiratory and urinary tracts [105]. In humans, six members of the β-defensin family, hBD-1 to -6, have been identified [118-122]. IECs constitutively express hBD-1 but not hBD-2; however, the latter defensin is expressed upon stimulation with inflammatory cytokine, IL-1α or infection with enteroinvasive bacteria [123]. The role of the PMAP recognition family of TLRs in the induction of anit-microbial peptides remains a matter of debate and conflicting results have been reported [87, 124-126]. The production of hBD-2 was not induced in IECs by stimulation with PGN or LPS unless specific genes for TLR2 and TLR6 or TLR4 were artificially transformed [124]. In addition, corneal epithelial cells (CECs) did not respond to microbial PMAP molecules [87], though they had previously been shown capable of responding to LPS [125]. In the case of the reproductive epithelium, vaginal epithelial cells (VECs) induce the expression of hBD-2 after stimulation with microbial PAMP antigens such as LPS and PGN, because, unlike IECs, VECs express TLR4 and TLR2, respectively [126]. Taken together,

these findings suggest that innate immune responses mediated *via* TLRs in the mucosal epithelium are heterogeneous and that a variety of external environments at local mucosa sites influence the expression and recognition mechanisms of TLRs in the epithelium. Therefore, individual anti-microbial peptides might be under the control of different antigen recognition and activation mechanisms offered by distinct populations of cells (e.g., PMN, Paneth cells, IECs, CECs and VECs) located at the mucosal epithelium.

SUMMARY

The mucosal immune system provides the first line of defense against infection caused by lumenal pathogens. The innate aspect of the mucosal immune system provides an effective surface barrier through multiple layers of harmonized physically and biologically effective effector molecules (Table 1). The mucus, which consists of the MUC molecules that form the viscoelastic gel layer, serves as a physical and biological barrier covering the mucosal epithelium. The mucus layer is also enriched with microbicidal peptides (innate immunity) and S-IgA (acquired immunity). The expression of MUC has been shown to be regulated by stimulation signals originating from microorganisms. In addition, the tightly knit mucosal epithelium, its cells joined together by a number of molecular structures (e.g., occlaudin, claudin, E-cadherin and Ep-CAM), bars intracellular entry by lumenal antigens. Furthermore, TLR-PAMP recognition mechanisms in the mucosal epithelium seem to be different from those of DCs and macrophages situated in the sterile conditions of the systemic compartment. These PAMP-recognition mechanisms peculiar to the epithelium can be disabled or impaired so as to promote symbiosis and prevent the unnecessary generation of danger signals. Taken together, these findings suggest that the mucosal innate immune system not only acts as a sensor of and barrier against infectious agents, but also serves to maintain the homeostasis in the mucosal tissues. Greater elucidation of the functional heterogeneity of the mucosal epithelium should facilitate the understanding of the dynamism of the mucosal innate immune system.

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

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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.**

Bacillus 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 derivatives 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

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[4] Abbreviations used in this paper: PA, protective Ag; LF, lethal factor; EF, edema factor; EdTx, anthrax edema toxin; LeTx, lethal toxin; ATR, anthrax toxin receptor; CT, cholera toxin; LT-I, heat labile toxin-I; S-IgA, secretory IgA; CLN, cervical lymph node; RLU, relative light unit; ON/E, olfactory nerves and epithelium; OB, olfactory bulb; Mϕ, macrophage; NALT, nasopharyngeal-associated lymphoepithelial tissue; CP, crossing point.

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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 ≥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 (5×10^4 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 (5×10^4 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, 5×10^{-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 2.11×10^7 relative light units (RLU)/ng and 2.06×10^7 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 × 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 = $2^{(-(CP_{cytokine} - CP_{\beta\text{-actin}}))}$ corrects for differences in cDNA concentration between the starting templates of cytokine and housekeeping (i.e., β-actin) genes (39). The simplified formula 20 − ($CP_{cytokine}$ − $CP_{\beta\text{- actin}}$) was used to express relative cytokine mRNA responses in tissues of mice given nasal EdTx.

Statistics

The results are expressed as the mean ± 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