

Clostridium difficile Toxin A Carboxyl-Terminus Peptide Lacking ADP-Ribosyltransferase Activity Acts as a Mucosal Adjuvant

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The receptor binding domains of the most potent mucosal adjuvants, bacterial toxins and plant lectins, are organized in repeat units to recognize specific sugar residues. The lectin-like structure of the C-terminal region of *Clostridium difficile* toxin A prompted us to investigate the mucosal adjuvant properties of a nontoxic peptide corresponding to amino acids 2394 to 2706 (TxA_{C314}). We compared TxA_{C314} adjuvant activity to those of cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin subunit B (EtxB) coadministered orally or nasotracheally with poor peptide antigens (keyhole limpet hemocyanin [KLH] and hen egg lysozyme [HEL]). Levels of anti-KLH-specific serum immunoglobulin G (IgG) and IgA as well as that of mucosal IgA were significantly higher in animals immunized orally with TxA_{C314} plus KLH than with KLH alone, CT plus KLH, or EtxB plus KLH. Following intranasal immunization with TxA_{C314} plus HEL, levels of serum- and mucosa-specific antibodies were comparable to those induced by coadministering HEL with CT or EtxB. The TxA_{C314} adjuvant effect following oral, but not intranasal, immunization was dose dependent. The analysis of the subclasses of anti-KLH-specific IgG isotypes and the cytokines released from splenocytes of immunized mice challenged in vitro with KLH indicates the induction of a mixed Th1/Th2-type immune response, with prevalence of the Th1 branch. We conclude that TxA_{C314} enhances immune responses against mucosa-coadministered foreign antigens and represents a promising mucosal adjuvant, especially because its ability to stimulate mixed Th1/Th2 responses with a strong a Th1 component is extremely worthwhile against intracellular pathogens.

The vast majority of human and animal pathogens colonize and invade the host through mucosal surfaces. Therefore the induction of strong and persistent immune responses in these districts represents an attractive and rational approach to developing protective vaccines. Antigen administration through systemic routes generally stimulates sustained systemic immunity, but it does not guarantee effective mucosal immune responses (31). Indeed, following mucosal administration of non-replicating antigens, local immune responses are quite weak and short-lived. The physicochemical barriers at the mucosal surfaces that impair antigen transport across the epithelium, preventing a direct interaction with immune cells, and the low reactivity of the mucosal immune system are the leading causes of weak responses triggered by mucosal vaccines (36). Thus, a variety of strategies including live bacterial vectors, biodegradable microparticles, liposomes, and mucosal adjuvants are under investigation to enhance the responses to mucosally delivered antigens (48, 55).

A few molecules are highly immunogenic when delivered to the mucosal immune system; indeed, some of these proteins are also able to enhance the immune response against coadministered antigens. Most of these highly immunogenic molecules are proteins or glycoproteins that share an interesting structural feature: all present lectin-like properties. These molecules possess a modular organization with at least one non-

catalytic domain that reversibly binds to a specific mono- or oligosaccharide (47, 48). For example, the most powerful mucosal antigens/adjuvants identified to date, cholera toxin (CT) secreted by *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxin (LT), feature a lectin-like structure with five modules (B subunit) that bind to oligosaccharide residues on membrane receptors (1, 46). In keeping with this view, it has recently been reported that plant lectins, also showing a modular structure, such as mistletoe and *Lycopersicon esculentum* lectins, are potent mucosal immunogens and enhance the immune response to coadministered antigens (25, 50).

Clostridium difficile, the causative agent of hospital-acquired diarrhea, produces two large exotoxins, toxin A and toxin B (4). Both toxins have intracellular targets, as they specifically glucosylate Rho, Rac, and Cdc42 at threonine 37 using UDP-glucose as the cosubstrate, causing changes in cell morphology and physiology (21). Injection of purified toxin A into closed intestinal loops elicits an acute inflammatory diarrhea with massive fluid secretion, mucosal damage, and a prominent neutrophil infiltration driven by the release of proinflammatory cytokines (4). On the basis of its amino acid sequence, *C. difficile* toxin A can be roughly divided into enzymatic, translocation, and binding domains (8). Indeed the N terminus of the toxin includes a putative nucleotide binding domain and bears the catalytic activity, whereas the middle part of the molecule has a small hydrophobic region, assumed to be involved in membrane translocation (13). The carboxy terminus of toxin A is characterized by 38 continuous repeat units, occupying 853 amino acid (aa) residues out of a total of 2,710 aa (40). This region is the putative receptor binding domain of

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the molecule for it specifically recognizes a trisaccharide moiety and causes rabbit erythrocyte agglutination. Moreover, antibodies directed against this portion of the molecule neutralize toxin A toxicity both *in vitro* and *in vivo* (28, 30).

The paucity of adjuvants for human mucosal vaccines, because the toxicity of CT and LT limits their clinical application (38), prompted us to investigate whether the nontoxic receptor binding domain of *C. difficile* toxin A displayed any adjuvant activity against antigens coadministered via the oral or nasotracheal route. The hypothesis behind this study was that the carboxyl-terminal region of toxin A, consisting of identical repeat units, presents a striking structural homology with the most powerful mucosal adjuvants known. To test our hypothesis, we measured the immune responses to poor mucosal antigens (keyhole limpet hemocyanin [KLH] and hen egg lysozyme [HEL]) coadministered to mice, by the oral and nasotracheal routes, with a recombinant nontoxic fragment of toxin A (TxA_{C314}).

MATERIALS AND METHODS

Cloning, expression, and purification of recombinant toxin A fragment. Genomic DNA was extracted by standard procedures from *C. difficile* strain VPI 10463 (American Type Culture Collection, Manassas, Va.) cultured under anaerobic conditions for 48 h in brain heart infusion broth (Gibco). A 942-bp DNA fragment encoding the carboxyl-terminus region of toxin A (bp 7338 to 8280, inclusive) was amplified by PCR using a proofreading thermostable polymerase (Vent polymerase; New England Laboratories, Beverly, Mass.). Primers were based on the published sequence from strain VPI 10463 (GenBank accession no. M30307). A 5' sense primer for the 27-bp coding sequence of the 9-aa epitope of the hemagglutinin of influenza virus type A (HA) was synthesized to fuse the epitope coding sequence in frame with that for toxin A. The PCR amplification (35 cycles) involved melting the DNA strand at 94°C for 20 s, annealing at 64°C for 30 s, and extension at 72°C for 30 s. The amplified toxin A sequence was ligated into the pGEM easy vector (Promega) to generate the plasmid pGEM/TxA_{C314}. For expression of the TxA_{C314} recombinant protein, the cloned toxin A coding sequence was excised with BamHI-EcoRI restriction sites engineered in the PCR primers and was inserted into similarly digested pGEX (Pharmacia). The coding sequence for the TxA_{C314} fragment was fused in frame with the glutathione *S*-transferase (GST) binding protein-encoding sequence to generate the construct named pGEX/TxA_{C314}. Integrity of all recombinant constructs was verified by sequence analysis.

To express the recombinant proteins in *E. coli*, recombinant constructs were transformed into *E. coli* BL21(DE3). Small-scale (20-ml) and large-scale (1,000-ml) cultures of the recombinant clone were grown in Luria-Bertani medium containing ampicillin to reach an optical density at 600 nm of 0.9 to 1.0. The expression of the plasmid-encoded recombinant protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cultures were harvested by centrifugation (5,000 × *g* for 15 min) after 1 to 16 h of incubation. Preliminary experiments using small-scale cultures showed that maximal expression of the recombinant protein was achieved after 4 to 6 h of incubation following IPTG addition. Cells were placed in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and then lysed with three 45-s bursts of ultrasonic power. Cellular debris was removed by centrifugation (12,000 × *g* for 15 min), and the soluble fractions were recovered and sterilely filtered through 0.2-μm-pore-size filters (Amicon).

The recombinant toxin A fragments were purified by affinity chromatography using a glutathione-Sepharose 4B resin (Pharmacia Biotech.). Cellular extracts containing the GST fusion proteins were chromatographed in buffer (50 mM Tris containing 1% Triton X-100 and 0.3 M NaCl) to allow binding to the resin, and, following extensive washing (10 resin bed volumes), the recombinant toxin fragments were removed by overnight digestion with thrombin (1 U, 20-μl bed volume). The integrity, purity, and concentrations of the recombinant proteins were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and a micro-Bradford test using the commercial Bio-Rad protein assay (Fig. 1). After a step of affinity chromatography using an endotoxin-removing gel (Pierce), the levels of endotoxin contamination in the recombinant protein preparations were determined to be less than 0.01 IU/ml (*Limulus* amoebocyte assay; BioWhittaker), which corresponds to less than 0.1 pg of

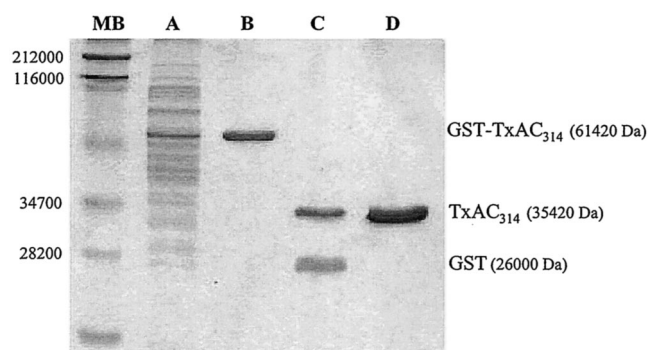


FIG. 1. SDS-PAGE analysis of purified TxA_{C314HA}. *E. coli* BL21(DE3) carrying the plasmid pGEX/TxA_{C314} was grown for 6 h at 37°C following induction with IPTG; cells were then collected by centrifugation and lysed. Soluble proteins were subjected to affinity chromatography purification on a glutathione-Sepharose 4B resin and then separated through SDS-12% PAGE gels stained with Coomassie blue. Lane A, total cell lysate following 6 h of induction with IPTG and before affinity column purification; lane B, recombinant protein (GST-TxA_{C314}) obtained following affinity chromatography of cell lysate on glutathione-Sepharose 4B resin and collected by addition of reduced glutathione; lane C, purified GST and TxA_{C314} obtained following digestion of recombinant GST-TxA_{C314} with thrombin; lane D, purified recombinant TxA_{C314} obtained by overnight digestion with thrombin (1 U/20-ml resin bed volume) of cell lysate purified and immobilized on glutathione-Sepharose 4B resin; lane MW, molecular mass markers (apparent molecular masses are shown in daltons).

lipopolysaccharide delivered per mouse (7). As expected (13, 40), the recombinant TxA_{C314} peptide was devoid of cytotoxic effects, as determined by cell morphology following incubation *in vitro* with CHO cells up to concentrations of 1 mg/ml (data not shown).

Mucosal adjuvants and antigens. CT and HEL were obtained from Sigma, and endotoxin-free KLH was purchased from Clontech. *E. coli* heat-labile enterotoxin subunit B (EtxB) was expressed and purified to homogeneity in our laboratory, as previously described (27).

Mucosal immunization. Male BALB/c mice were purchased from Charles River (Oderzo, Italy) and used at 6 to 8 weeks of age. Animals were housed in groups of four under controlled humidity and temperature conditions and were given free access to commercial rodent food and water. Experiments were carried out by following the guidelines recommended by the Institutional Animal Care and Use Committee of the University of Padua. Mice were bled 1 week prior to the first immunization and then on days 0 and 14 were immunized either orally or intranasally. Each experiment was performed two to four times for a total of 8 to 12 mice per experimental condition. For oral immunization, mice received 100 μl of sterile PBS containing KLH (5 mg) alone or supplemented with TxA_{C314} (0.1 to 10 μg), CT (10 μg), or EtxB (10 μg) via curved oral dosing needles. In a series of experiments TxA_{C314} (10 μg) was preincubated *in vitro* for 1 h at 22°C with an anti-toxin A antiserum and then coadministered with KLH by oral gavage. For intranasal immunization, mice were dosed through fine tips attached to a pipette. The antigen (HEL, 5 mg) was administered in a total volume of 30 μl of PBS (15 μl per nostril) supplemented with TxA_{C314} (0.1 to 10 μg), CT (10 μg), or EtxB (10 μg).

Collection of blood and feces. Blood samples were collected on days 13 and 35 after the primary immunization by bleeding from the retro-orbital plexus. Blood was left at room temperature for 2 h and centrifuged, and serum was collected and stored at -20°C. Feces (two or three pellets) were collected on the same days and immediately placed in preweighed microcentrifuge tubes containing ice-cold PBS (1:10 ratio) and a protease inhibitor mixture (5 mM phenylmethylsulfonyl fluoride, 5 μg of aprotinin and leupeptin/ml). Samples were then vortexed vigorously, insoluble material was removed by centrifugation (7,000 × *g* for 15 min), and clear supernatants were collected and individually stored at -20°C until used.

Detection of specific antibodies by ELISA. Enzyme-linked immunosorbent assays (ELISA) were set up to obtain measurements of specific murine immunoglobulin G (IgG), IgA, and IgG subtype responses. Sera and feces were diluted in the appropriate buffer and used to determine the antibody levels. High-bound-

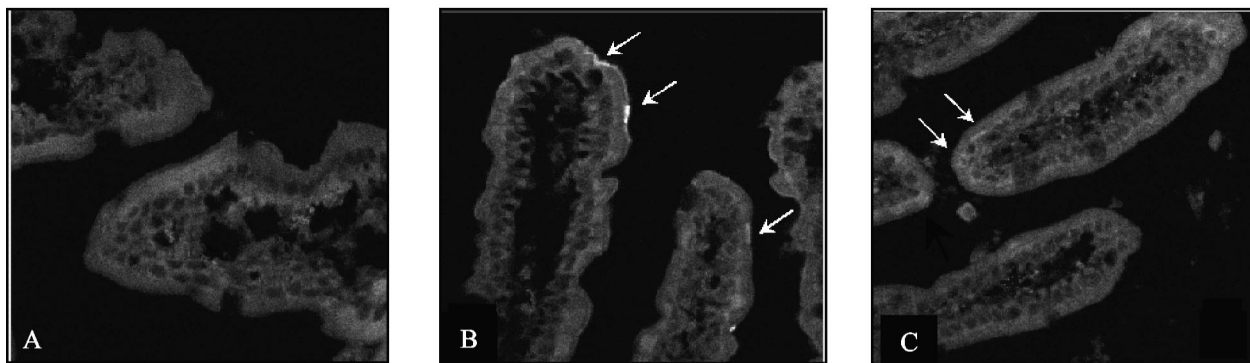


FIG. 2. Binding of TxA_{C314} to enterocytes in mouse intestine. Closed ileal loops were injected with buffer, either alone or containing 20 μg of TxA_{C314} . After 10 to 120 min the animals were sacrificed; loops were removed, fixed in 4% buffered paraformaldehyde, and placed in embedding medium for frozen tissue specimens; and 10- μm -thick longitudinal sections were cut. To determine TxA_{C314} binding to intestinal epithelial cells, sections were incubated with a rabbit polyclonal antibody against the HA epitope fused at the N terminus of TxA_{C314} . The immunocomplexes were detected with a FITC-conjugated donkey anti-rabbit IgG, and then sections were analyzed with a Leica TCS-NT/SP2 confocal microscope with a 40 \times objective. (A) Absence of signal in a loop injected with buffer. (B) Representative section obtained from an ileal loop incubated for 10 min with TxA_{C314} . With the anti-HA antibody, it is possible to detect the presence of a strong signal at villus tips, indicating the presence of TxA_{C314} bound to the epithelium. (C) Representative section obtained from an ileal loop 120 min after injection with TxA_{C314} . Following incubation with a rabbit antibody against the HA epitope fused to TxA_{C314} it is possible to observe the presence of a faint signal within the cytoplasm of the epithelial cells.

capacity microtiter plates (Corning-Costar, Cambridge, Mass.) were coated with 100 μl of the appropriate antigen solution/well (10 $\mu\text{g}/\text{ml}$) in 0.1 M carbonate-bicarbonate buffer (pH 9.6) and incubated for 16 h at 4°C. After being washed (PBS containing 0.1% Tween 20 [PBS-T]), plates were blocked with 4% bovine serum albumin in PBS-T (90 min at 22°C) prior to sample addition (2 h at 22°C). Following washing (three times) with PBS-T, plates were incubated with biotinylated antiserum (anti-IgG, anti-IgA, anti-IgG1, or anti-IgG2a) for 1 h at 22°C and then streptavidin-labeled peroxidase was added (1 h at 22°C) prior to the addition of developing solution (3,3',5,5'-tetramethylbenzidine; microwell peroxidase substrate; Sigma). The reaction was stopped by addition of 100 μl of 0.2 M H_2SO_4 , and the optical density at 450 nm was determined with an ELISA plate reader (LX300; Epsom Diagnostic). Optimal working dilutions of anti-IgG (1:4,000) and anti-IgA (1:2,000) biotinylated capture antibodies were determined after preliminary assays, whereas working dilutions of anti-IgG subtype antisera were as recommended by the manufacturer (Southern Biotechnology Associated, Inc.). To assess the level of specific anti-KLH and anti-HEL antibodies on each plate, a reference curve was constructed by coating microtiter plate wells with known amounts of purified mouse IgG, IgA, IgG1, and IgG2a (range, 250 to 3.0 ng/ml; ICN). The serum and fecal concentrations of specific anti-HEL and anti-KLH antibodies were determined by subtracting the mean values obtained in nonimmune animals (background) from those obtained in each experimental (immune) mouse. Then the concentrations of specific anti-HEL and anti-KLH antibodies were expressed as micrograms per milliliter.

Induction and measurement of cytokine production. Spleens were removed aseptically 35 days after the primary immunization, and single-cell suspensions were prepared by forcing the tissue homogenates through a wire mesh (2 mm). Red blood cells were removed by density gradient centrifugation (3). Mononuclear cells were washed twice in the culture medium (RPMI medium supplemented with 20 mM 2-mercaptoethanol, 100 IU of penicillin/ml, 100 μg of streptomycin/ml, and 10% heat-inactivated fetal calf serum), seeded at $10^6/\text{ml}$, and then restimulated in triplicate with the appropriate antigen (50 $\mu\text{g}/\text{ml}$) for 72 h in a humidified 5% CO_2 incubator at 37°C. Positive (phytohemagglutinin at 5 $\mu\text{g}/\text{ml}$) and negative (cells with culture medium only) controls were run in each experiment. Following incubation, cultures were harvested by centrifugation and the supernatants were collected, fractionated, and stored at -80°C. The levels of interleukin-2 (IL-2), gamma interferon (IFN- γ), IL-10, and IL-4 were measured by commercially available ELISAs, running each sample in duplicate (Biosource, Camarillo, Calif.).

Confocal immunofluorescence studies. To assess binding of TxA_{C314} to intestinal epithelial cells, closed 3- to 4-cm-long ileal loops (one for each animal) were prepared in overnight fasting mice as previously described (3). Ileal loops were then injected with 50 μl of 50 mM Tris buffer, either alone or containing 20 μg of TxA_{C314} . The abdomen was then closed, and animals were left on a heating pad to keep the body temperature at 37°C. Animals were sacrificed at various time points (10 to 120 min), and the loops were removed, washed in PBS, and

fixed in 4% buffered paraformaldehyde for 60 min. Tissues were placed in embedding medium for frozen tissue specimens, and longitudinal sections (10 μm thick) were cut with a cryostat. To determine TxA_{C314} binding to intestinal epithelial cells, sections were washed in PBS (twice for 5 min) and then incubated for 10 min in 50 mM NH_4Cl in PBS to quench reactive aldehydes. Nonspecific protein binding was blocked with 10% donkey serum in PBS for 1 h at 22°C. Sections were then incubated (1 h at 22°C) with a rabbit polyclonal antibody (Santa Cruz Biotechnology) against the HA epitope genetically fused at the N terminus of TxA_{C314} . Sections were then washed three times with PBS-T at room temperature and then incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (1:200 dilution) for 1 h at 22°C. Finally, sections were washed extensively, and slides were mounted, with a drop of 1-mg/ml *N*-propylgallate (Sigma) to reduce photobleaching, in 90% glycerol-PBS. Sections were then analyzed and photographed with a Leica TCS-NT/SP2 confocal microscope using a 40 \times objective. Images were digitally stored with Leica software. As a control, slides were incubated with nonimmune rabbit IgG.

Statistics. Data are expressed as the means \pm standard errors. Statistical analysis was performed using analysis of variance, and Bonferroni's test was used to test for significance between groups. Statistical significance was considered for *P* values <0.05.

RESULTS

TxA_{C314} binds to enterocytes in mouse intestine. We first determined whether TxA_{C314} was able to bind to mucosal epithelial cells *in vivo*. As shown in Fig. 2, following injection in mouse ileal loops, TxA_{C314} readily bound to villus tips. The pattern of TxA_{C314} binding to intestinal villi *in vivo* was quite similar to the distribution described for toxin A binding to the rabbit ileum *in vitro* (15). TxA_{C314} binding was evident within 10 min following injection into the ileal loops, and after 2 h it was possible to observe the peptide inside the epithelial cells (Fig. 2).

Adjuvant effect of TxA_{C314} on KLH-specific serum antibody responses following oral immunization. Low levels of KLH-specific IgG were detected after a single dose of orally administered antigen in 4 of 12 mice immunized with CT plus KLH or EtxB plus KLH and in 8 of 12 mice immunized with TxA_{C314} plus KLH. None of the animals that received only KLH showed a detectable antibody response. As expected, 3 weeks

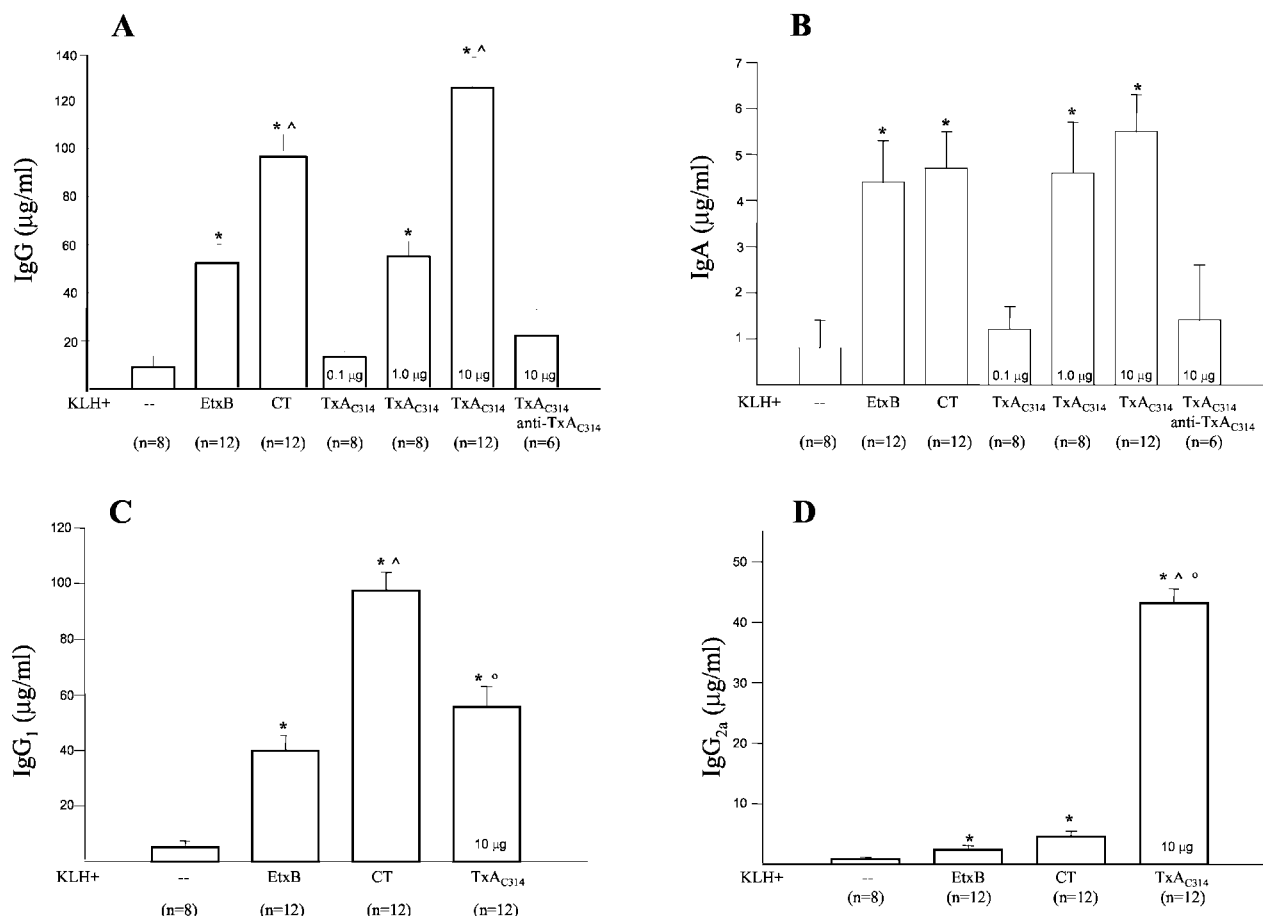


FIG. 3. Levels of systemic KLH-specific antibodies in mice immunized by oral gavage. BALB/c mice (8 to 12 per group) were immunized orally on days 0 and 14 with KLH (5 mg) alone or supplemented with TxA_{C314} (0.1 to 10 µg), CT (10 µg), or EtxB (10 µg). At day 35, blood was collected by retro-orbital bleeding and serum concentrations of IgG (A), IgA (B), IgG₁ (C), and IgG_{2a} (D) reactive to KLH were measured by ELISA. In some experiments TxA_{C314} (10 µg/mouse) was preincubated in vitro with anti-*C. difficile* toxin A antiserum (anti-TxA_{C314}) and then coadministered by oral gavage with KLH. The level of specific antibodies reacting to KLH was determined by preparing a reference curve by using mouse IgG, IgA, IgG₁, or IgG_{2a} and subtracting the background from each sample (nonimmune mice [$n = 8$]). Results are means \pm standard errors. *, $P < 0.01$ versus KLH alone; ^, $P < 0.01$ versus KLH plus EtxB, °, $P < 0.01$ versus KLH plus CT.

after the second immunization dose, low levels of KLH-specific serum IgG were detected in mice immunized orally with KLH alone (Fig. 3). However, anti-KLH-specific serum IgG significantly increased in mice immunized with CT plus KLH or EtxB plus KLH and also with TxA_{C314} plus KLH (Fig. 3). As shown in Fig. 3, at doses of 10 µg/mouse TxA_{C314} was 2.4-fold ($P < 0.01$) more effective at enhancing the anti-KLH-specific immune response than EtxB and slightly more effective (1.26-fold increase; $P \geq 0.05$) than CT. TxA_{C314} at doses of 0.1 µg/mouse did not show any significant adjuvant activity but were as effective as 10 µg of EtxB at 1 µg/mouse (Fig. 3). However, we could not test doses of TxA_{C314} higher than 10 µg/mouse since it was not possible to obtain more-concentrated peptide solutions because of a certain degree of insolubility.

Following two oral doses, KLH-specific serum IgA was detected in mice coadministered KLH with CT, EtxB, or TxA_{C314}. As shown in Fig. 3, the highest levels of anti-KLH-specific IgA were detected in mice that received 10 µg of TxA_{C314} or CT as the adjuvant, whereas slightly lower levels

were observed in mice dosed with EtxB as the adjuvant ($P \geq 0.05$).

To determine whether the TxA_{C314}-mediated adjuvant effect required binding to mucosal epithelial cells, we preincubated TxA_{C314} with a mouse polyclonal antibody raised against the carboxy terminus region of *C. difficile* toxin A, before coadministration with KLH. This antibody was effective at neutralizing toxin A-mediated toxic effects in vivo and in vitro (data not shown). As shown in Fig. 3, preincubation of TxA_{C314} with the neutralizing antibody completely abolished the TxA_{C314}-mediated adjuvant effect.

KLH-specific IgG subclass patterns. The levels of KLH-specific IgG₁ and IgG_{2a} were significantly higher in mice immunized with KLH plus CT, EtxB, or TxA_{C314} than in mice immunized with KLH alone (Fig. 3). KLH-specific IgG₁ levels were 1.7-fold higher in mice immunized with CT plus KLH than in animals dosed with TxA_{C314} plus KLH ($P < 0.01$). In contrast, anti-KLH-specific IgG_{2a} levels were significantly ($P < 0.01$) higher in mice immunized orally with TxA_{C314} plus

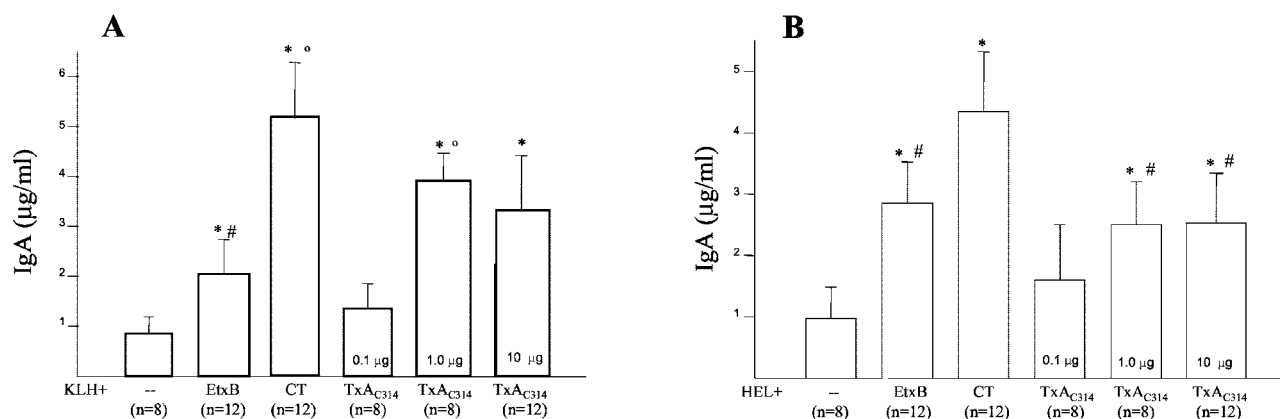


FIG. 4. Fecal anti-KLH- and anti-HEL-specific antibodies. BALB/c mice (8 to 12 per group) were immunized on days 0 and 14 orally with KLH (5 mg) or intranasally with HEL (5 mg) either alone or supplemented with TxAc₃₁₄ (0.1 to 10 µg), CT (10 µg), or EtxB (10 µg). Three weeks after the second inoculation, feces were collected and anti-KLH- (A) or anti-HEL- (B)-specific IgA levels were measured by ELISA. Results are means \pm standard errors. *, $P < 0.01$ versus KLH (A) or HEL (B) alone; °, $P < 0.05$ versus KLH plus EtxB; #, $P < 0.05$ versus HEL plus CT.

KLH than in mice immunized with CT or EtxB and KLH (9.7- and 18.5-fold increases, respectively; Fig. 3).

Adjuvant effect of TxAc₃₁₄ following oral immunization on KLH-specific mucosal IgA responses. Specific anti-KLH IgA was detected at low levels in the feces of a few mice immunized by oral gavage with KLH alone (Fig. 4). However, following oral immunization with CT plus KLH, EtxB plus KLH, and TxAc₃₁₄ plus KLH, specific anti-KLH IgA was present at significantly higher concentrations in the feces (Fig. 4). Furthermore, 1 µg of TxAc₃₁₄ was as effective as 10 µg of CT and significantly more effective than 10 µg of EtxB to release mucosal KLH-specific IgA.

Adjuvant effect of TxAc₃₁₄ following intranasal immunization on HEL-specific antibody responses. To determine whether the protocol of immunization influenced the outcome of antibody production, we evaluated the immune responses triggered following nasotracheal immunization using HEL as the test antigen. Intranasal administration of HEL alone induced the appearance of low levels of specific serum IgG only after the second immunization dose; however the coadministration of either CT, EtxB, or TxAc₃₁₄ with HEL triggered significant specific antibody responses (Fig. 5). As shown in Fig. 5, it was not possible to observe a dose-response effect in the serum antibody response obtained by coadministering HEL with TxAc₃₁₄, since TxAc₃₁₄ was effective only at 10 µg/mouse.

Following intranasal immunization, significant levels of HEL-specific serum IgA were detected in mice that received CT, EtxB, or TxAc₃₁₄ and HEL, but not HEL alone. As shown in Fig. 5, the highest levels of HEL-specific IgA were detected in mice that received 10 µg of either CT or TxAc₃₁₄ as the adjuvant, whereas slightly lower levels were measured with EtxB as the adjuvant (not significant).

HEL-specific IgG subclass patterns. As expected, the levels of anti-HEL-specific IgG1 and IgG2a were significantly higher in mice immunized with HEL plus CT, EtxB, or TxAc₃₁₄ than in mice immunized with HEL alone (Fig. 5). Anti-HEL-specific IgG1 and IgG2a levels in mice immunized intranasally with CT plus HEL and TxAc₃₁₄ plus HEL were comparable (Fig. 5). Indeed, immunization with CT plus HEL or TxAc₃₁₄

plus HEL stimulated the production of anti-HEL-specific IgG1 levels that were significantly ($P < 0.05$) higher than IgG2a levels (Fig. 5).

Adjuvant effect of TxAc₃₁₄ following intranasal immunization on HEL-specific mucosal IgA responses. Following intranasal immunization with HEL alone (Fig. 4) anti-HEL IgA in feces was barely detectable. However, following intranasal immunization with CT plus HEL, EtxB plus HEL, or TxAc₃₁₄ plus HEL, specific anti-HEL IgA was present at significant levels in the feces (Fig. 4). Coadministration of HEL with 10 µg of CT was significantly more effective ($P < 0.05$) than EtxB or TxAc₃₁₄ in stimulating the release of anti-KLH-specific mucosal IgA. Indeed, EtxB and TxAc₃₁₄ showed a similar adjuvant activities (Fig. 4).

Anti-CT- and -TxAc₃₁₄-specific antibody responses. Anti-CT- and anti-EtxB-specific serum IgG was detected, as expected, in animals following oral administration of 10 µg of CT or EtxB (Fig. 6A) and at an even higher level following intranasal administration (Fig. 6). Indeed, following oral or intranasal coadministration of TxAc₃₁₄ with either KLH or HEL the levels of anti-TxAc₃₁₄ IgG in the serum were only barely detectable (Fig. 6). Similar results were obtained by measuring the levels of specific IgA in the feces of animals following either oral or intranasal immunization (data not shown).

Adjuvant effect of TxAc₃₁₄ following oral immunization on KLH-specific cellular responses. As shown in Fig. 7, spleen lymphocytes obtained from mice immunized orally with only KLH exhibited a modest release of IFN- γ , IL-2, IL-4, and IL-10 following in vitro challenge with KLH, as opposed to the more robust cytokine release observed in cells isolated from mice dosed with CT plus KLH, EtxB plus KLH, or TxAc₃₁₄ plus KLH. Exposure of spleen cells purified from mice immunized with TxAc₃₁₄ plus KLH produced significantly more IL-2 and IFN- γ following in vitro exposure to KLH than exposure of cells obtained from mice immunized with CT plus KLH or EtxB plus KLH (Fig. 7). Indeed, IL-4 and IL-10 secretion was significantly enhanced in cells purified from mice immunized with CT plus KLH or EtxB plus KLH and challenged in vitro with KLH but was only slightly evident in splenocytes obtained from mice immunized with TxAc₃₁₄ plus KLH (Fig. 7). Indeed,

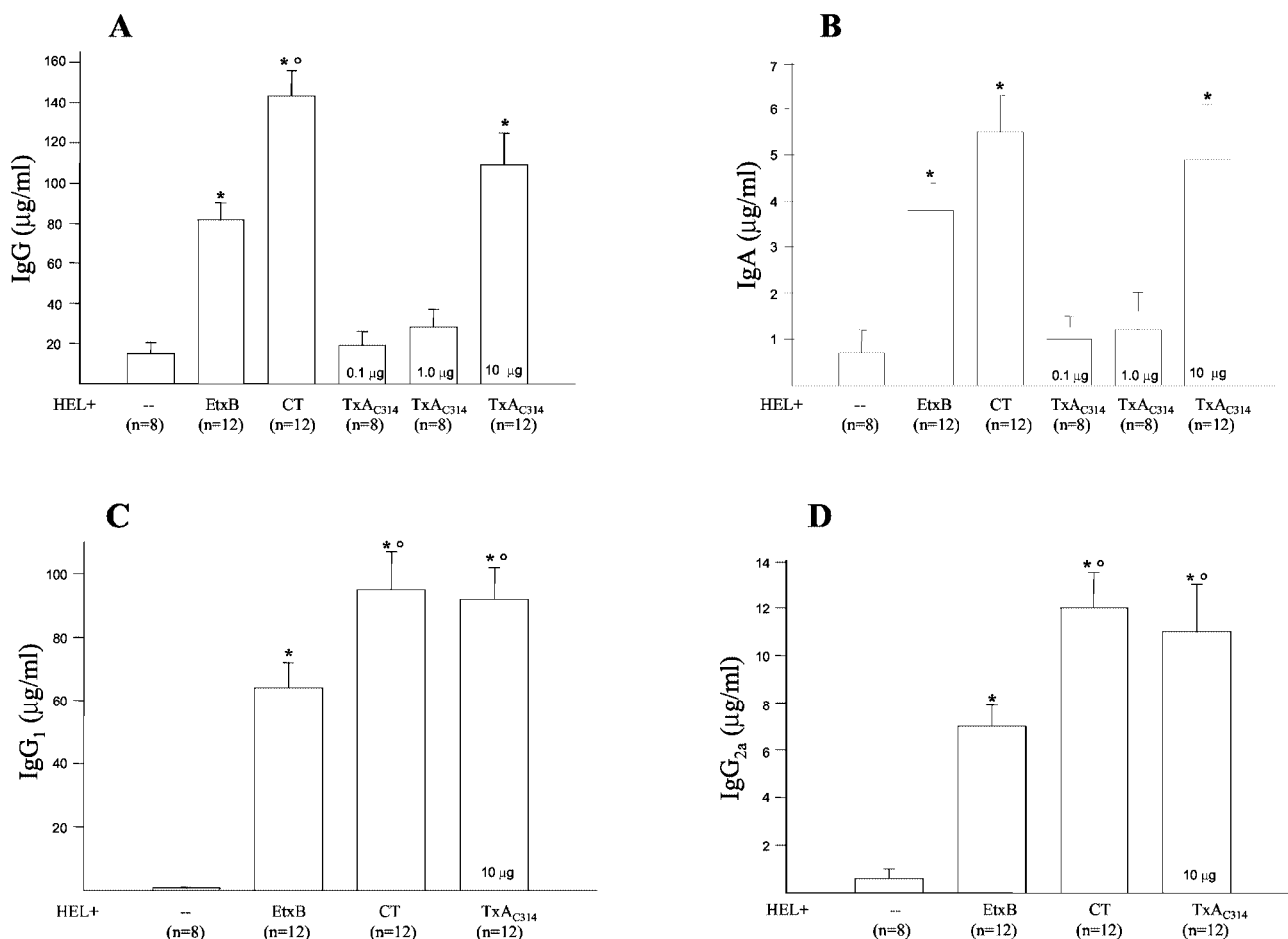


FIG. 5. Levels of systemic anti-HEL-specific antibodies in mice immunized intranasally. BALB/c mice (8 to 12 per group) were immunized intranasally on days 0 and 14 with HEL (5 mg) alone or supplemented with TxA_{C314} (0.1 to 10 µg), CT (10 µg), or EtxB (10 µg). At day 35 blood was collected by retro-orbital bleeding and serum concentrations of IgG (A), IgA (B), IgG₁ (C), and IgG_{2a} (D) reactive to HEL were measured by ELISA. The level of specific antibodies reacting to HEL was determined by preparing a reference curve with mouse IgG, IgA, IgG₁, or IgG_{2a} and subtracting the background from each sample (nonimmune mice [$n = 8$]). Results are means \pm standard errors. *, $P < 0.01$ versus HEL alone; °, $P < 0.05$ versus HEL plus EtxB.

phytohemagglutinin stimulation induced a robust production of the cytokines in all experimental groups (data not shown).

DISCUSSION

The growing body of knowledge on the immune system and human pathogens makes it possible to develop new recombinant vaccines. However, the development of effective mucosal vaccines is still jeopardized by the poor immunological properties of nonreplicating antigens and the lack of efficient delivery systems and mucosal adjuvants (14). To date the most effective mucosal adjuvants are CT and LT and their genetically detoxified mutant versions, which are used as vaccine carriers and immunomodulators (38). Here we report that a recombinant peptide, the nontoxic carboxy-terminus domain of *C. difficile* toxin A, demonstrates robust adjuvant properties when coadministered with poor antigens via mucosal routes. The profile of antigen-specific IgG subclasses and of cytokine release from immune splenocytes challenged in vitro with the

test antigens used in this study is compatible with the induction of a mixed, Th1/Th2-type immune response.

C. difficile toxin A, a large clostridial toxin, acts at microgram concentrations in the rodent gut to stimulate fluid secretion, disrupt intestinal integrity, and trigger the release of proinflammatory mediators (4). Indeed, systemic administration of a few nanograms of toxin A holotoxin results in animal death (29). The amino acid sequences of *C. difficile* toxins suggest the existence of three functional domains within a single large peptide rather than the classical AB structure, with specialized binding and catalytic subunits, of the vast majority of cytotoxins acting on intracellular targets (42). Thus the catalytic domain, located at the N terminus of the molecule, is essential for cell rounding and death (18). Microinjections of *C. difficile* toxins into fibroblasts or the direct expression of the catalytic domain (aa 1 to 556) in cells mimics the morphological and physiological changes caused by exposure to the holotoxin (18). However, the role of the UDP-glucosyltransferase catalytic domain in intestinal inflammation and cytokine release from immune

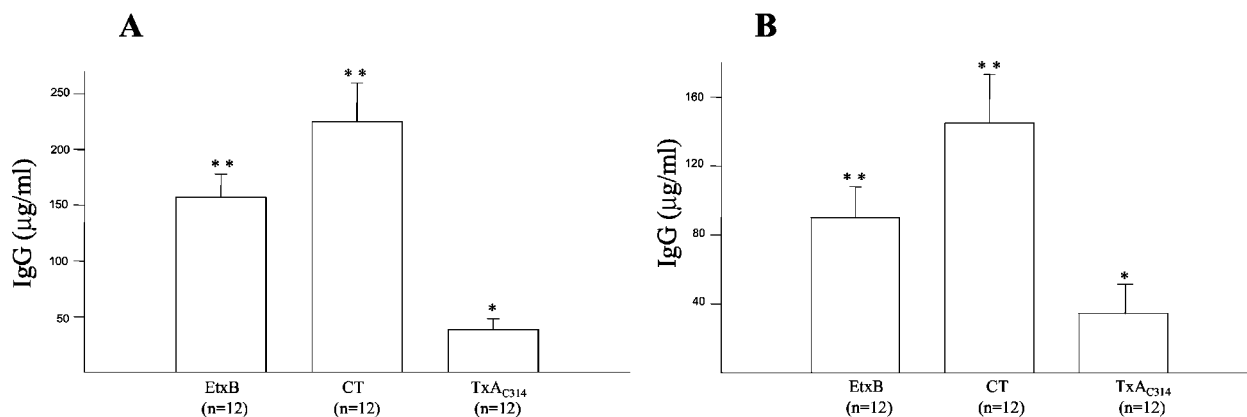


FIG. 6. Levels of systemic anti-CT, anti-EtxB, and anti-TxA_{C314} in mice immunized by oral gavage or nasotracheally. BALB/c mice (8 to 12 per group) were immunized twice orally with KLH (5 mg) or intranasally with HEL (5 mg) supplemented with 10 µg of either TxA_{C314}, CT, or EtxB or with just buffer, and after 35 days blood was collected by retro-orbital bleeding. Following oral gavage (A) or intranasal (B) immunization, specific IgG anti-CT, anti-EtxB, and anti-TxA_{C314} in serum were measured by ELISA. The level of specific antibodies was determined by preparing a reference curve with mouse IgG and subtracting the background from each sample (nonimmune mice [$n = 8$]). Results are means \pm standard errors. **, $P < 0.01$ versus control; *, $P < 0.05$ versus control.

cells is not clear since mitochondrial oxygen radical generation, NF- κ B activation, and IL-8 gene up-regulation appear to precede holotoxin-mediated Rho glucosylation (17). Whereas the carboxy terminus domain of *C. difficile* toxin A shows a lectin-like structure and mediates toxin binding to membrane receptors and erythrocyte agglutination (13, 28), recombinant peptides comprising the C terminus region of toxin A lack significant inflammatory or cytotoxic effect in vivo and in vitro (16, 30, 52). Although apparently devoid of intrinsic toxic and proinflammatory activity, the receptor binding portion of toxin A may still activate biological responses. In fact, membrane receptor clustering is an event known to trigger a variety of intracellular signaling pathways. Thus, antibody-mediated CD3 and T-cell receptor ligation or CT B-subunit-induced clustering of GM1 gangliosides on immune cells activates signal transduction cascades, cytokine release, and lymphocyte proliferation (9, 45). Indeed, we have observed in preliminary experiments that TxA_{C314}, as well as anti-CD3 antibodies, stimulates IL-2 and IFN- γ release from normal mouse spleen cells in vitro (I. Castagliuolo and M. Sardina, unpublished observation), strongly suggesting that the carboxy terminus domain of *C. difficile* toxin A exhibits specific direct biological activities.

An intriguing common feature of powerful mucosal antigens and adjuvants is their hetero-oligomeric structure, composed of structurally and functionally distinct catalytic and binding subunits (44, 46). CT and LT, as well as mistletoe lectins, possess a catalytic domain and a carbohydrate recognition domain able to bind simultaneously several specific membrane receptors. Recent studies aimed at developing new effective mucosal adjuvants have generated nontoxic mutant CT and LT retaining mucosal immunogenicity and adjuvant activity (38). Thus, it is now evident that the enzymatic ADP ribosylation activity in CT and LT is not absolutely required to exert the adjuvant activity whereas the carbohydrate-binding domain can act as a mucosal adjuvant per se (38).

Our data suggest that the TxA_{C314} peptide is an effective mucosal adjuvant following either oral and nasotracheal ad-

ministration. This is quite interesting since oral adjuvants have to survive the harsh gastric environment and avoid destruction from pancreatic proteases. Accordingly, bacterial toxins are generally more effective mucosal adjuvants following nasotracheal administration than following oral administration (38, 54) (Fig. 3 to 5). However, it has recently been reported that several plant lectins are stable in the rodent gut and retain the ability to interact with the mucosal epithelium following oral feeding (11). In addition, certain clostridial toxins, including *C. difficile* toxins, retain their biological activity following oral administration (22, 28). Indeed, *C. difficile* toxin A and the peptide TxA_{C314} are extremely resistant to protease digestion in vitro (2; I. Castagliuolo and V. De Filippis, unpublished observation). Interestingly, *C. difficile* toxins, like other bacterial toxins, undergo pH-dependent structural changes probably required to escape from the intracellular endocytic compartment in order to exert the full biological activity (41). Thus, exposure to the gastric pH may induce conformational changes that favor transmembrane translocation of TxA_{C314}, enhancing its biological activity.

C. difficile toxin A, like other ADP-ribosylating toxins, must bind to intestinal epithelial cells in vivo to exert its adjuvant activity. It is possible that the differences in the immune responses elicited by CT, EtxB, and TxA_{C314} are due, at least in part, to different properties for binding to the gastrointestinal and nasopharyngeal mucosa (39, 46). Indeed, the sugar binding properties can confine lectins to specific cell populations, such as M cells in the Peyer's patches or the follicle-associated epithelium in nasopharyngeal tissue (5, 24). Thus, the repeating units in the toxin A carboxy terminus recognizing the trisaccharide Gal α 1-3Gal β 1-4GlcNAc cause binding to rodent intestinal epithelial and immune cells (23). The fate of *C. difficile* toxins following receptor binding in vivo is not known; however, in vitro the toxin binds to and translocates across the plasma membrane within a few minutes to reach its intracellular target(s) (13, 16). Specific antigen binding and uptake by epithelial cells constitute an essential, although probably not sufficient, first step in eliciting an immune response following

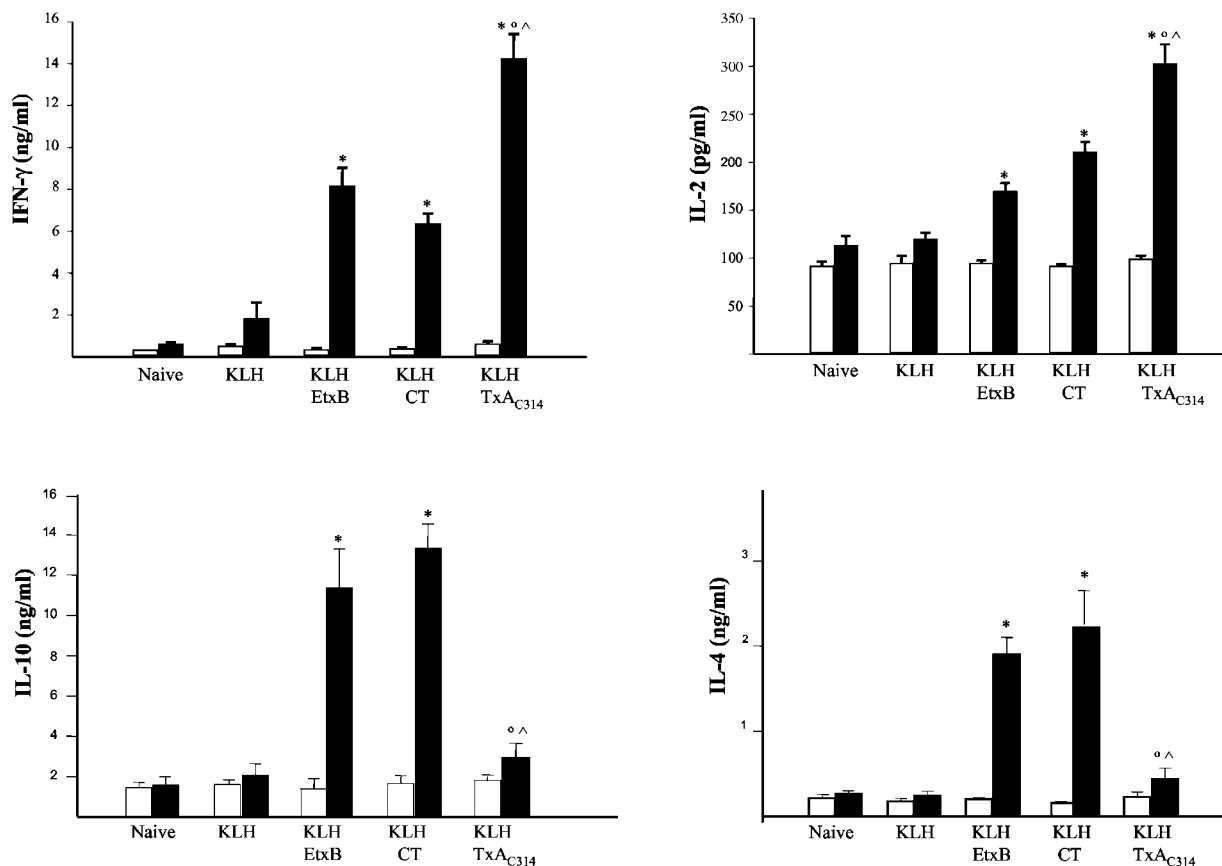


FIG. 7. Cytokine production of spleen cells isolated from mice immunized by oral gavage. BALB/c mice were immunized orally on days 0 and 14 with KLH (5 mg) alone or supplemented with TxA_{C314} (10 μ g), CT (10 μ g), or EtxB (10 μ g). On day 35 splenocytes were collected, seeded in culture (10⁶/ml), and in vitro restimulated with KLH (50 μ g/ml) (black bars) or left unstimulated (open bars). Culture supernatants collected after 72 h were assayed for cytokines IFN- γ , IL-2, IL-4, and IL-10 by ELISA. Results are means \pm standard errors from three to six mice per group. *, $P < 0.05$ versus the respective nonstimulated cells; °, $P < 0.05$ versus mice immunized with KLH plus EtxB; ^, $P < 0.05$ versus mice immunized with KLH plus CT.

antigen delivery via a mucosal route. Binding to membrane receptors of LT, CT, or other ADP-ribosylating toxins, such as pertussis toxin, is a prerequisite for immunogenicity of these toxins since it may facilitate receptor-mediated transepithelial uptake, possibly leading to entry into a nonproteolytic intracellular environment (6). Indeed, following binding to the epithelial brush border, plant lectins as well as CT are translocated across the mucosal epithelium to reach and, eventually, activate lamina propria lymphocytes (26, 51). Thus, weak antigens fused to carrier peptides, such as bacterial toxins, can be efficiently delivered to the mucosal immune system (7). We observed that TxA_{C314} readily binds to and penetrates intestinal enterocytes; however our time course experiments cannot determine whether TxA_{C314} can directly reach the submucosal lymphocytes through the intact epithelium.

As previously claimed, we observed that orogastric or intranasal coadministration of soluble weak antigens, such as KLH and HEL, with CT or EtxB resulted in the induction of a mixed Th1/Th2-type responses (20, 32, 37). This was supported by the cytokine profile, by the release of significant amount of Th1- (IFN- γ and IL-2) and Th2 (IL-4 and IL-10)-type cytokines, and by the production of both IgG subclasses (IgG1 and IgG2a) although the predominant production of serum IgG1 was de-

tected (Fig. 3 and 5). Our data indicate that mucosal immunization with antigens in combination with TxA_{C314} seems to induce also a mixed Th1/Th2-type immune response, as indicated by the production of both IgG subclasses (IgG1 and IgG2a). However, following oral immunization high levels of serum IgG2a (Fig. 3) are coupled to the release of Th1-type cytokines (Fig. 7). This complex and not well defined profile of the immune response is not completely unprecedented since, following mucosal immunization, Th1-type and Th2-type responses can be observed at different stages of strong immune responses and depend on specific antigen/adjuvant combinations (20, 34, 37, 43). The molecular mechanisms leading to a more prominent production of Th1- or Th2-type cytokines and different IgG isotypes are presently not completely known; however, we can speculate that they rely on the different effects of CT and TxA_{C314} on immune cells. Thus, CT induces intracellular cyclic AMP accumulation, leading to protein kinase A activation, whereas it fails to activate NF- κ B, enhances major histocompatibility complex class II-restricted antigen presentation in vitro, activates the Th2-type CD4⁺ lymphocyte population, down-regulates inflammatory reactions associated with autoimmune disorders, and induces CD8⁺ lymphocyte apoptosis (33). On the other hand, *C. difficile* toxins cause

intracellular Ca²⁺ influx, production of reactive oxygen radicals, and NF- κ B activation, which eventually trigger the release of a variety of Th1-type cytokines from immune and nonimmune cells (12, 35, 53).

The most widely experimentally used mucosal adjuvants, CT and LT, are strongly immunogenic by themselves and potentiate the immune response to bystander antigens through several mechanisms (19). Indeed, we observed that TxA_{C314}, although effective at enhancing the immune response against coadministered antigens, was poorly immunogenic by itself (Fig. 6). Although this is quite unusual for a mucosal adjuvant, recent studies have clearly demonstrated that cytokines (peptides or cDNA) can replace CT and LT in nasal and intragastric vaccination protocols (10, 49). Thus, mucosal administration of cytokines can enhance antigen presentation and promote humoral and cytotoxic T-lymphocyte-mediated immune responses. Therefore, we speculate that TxA_{C314}-mediated adjuvant activity relies primarily on the ability of TxA_{C314} to stimulate cytokine release from mucosal immune cells, although further studies are necessary to elucidate its mechanism of action.

In conclusion, bacterial toxins and plant lectins are the most powerful mucosal adjuvants known to date, but significant problems result from the toxic effects of their catalytic domain. In this study we report for the first time that the carboxy terminus region of *C. difficile* toxin A, TxA_{C314}, is an effective mucosal adjuvant following intragastric and intranasal administration. However further studies are required to prove that this peptide is devoid of significant toxic effects in vivo and that TxA_{C314} is able to stimulate protective immune responses against coadministered viral and bacterial antigens.

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