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Protective Immunity against *Clostridium difficile* Toxin A Induced by Oral Immunization with a Live, Attenuated *Vibrio cholerae* Vector Strain

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Clostridium difficile **causes pseudomembranous colitis through the action of Rho-modifying proteins, toxins A and B. Antibodies directed against *C. difficile* toxin A prevent or limit *C. difficile*-induced colitis. We engineered plasmid pETR14, containing the *hlyB* and *hlyD* genes of the *Escherichia coli* hemolysin operon, to express a fusion protein containing 720 amino acid residues from the nontoxic, receptor-binding, carboxy terminus of *C. difficile* toxin A and the secretion signal of *E. coli* hemolysin A. We introduced pETR14 into *Vibrio cholerae* and found that the toxin A-HlyA fusion protein was secreted by a number of *V. cholerae* strains and recognized by both monoclonal and polyclonal anti-*C. difficile* toxin A antibodies. We introduced pETR14 into an attenuated *V. cholerae* strain, O395-NT, and inoculated rabbits orally with this construct. Colonization studies disclosed that the *V. cholerae* vector containing pETR14 was recoverable from rabbit ilea up to 5 days after oral inoculation. Vaccination produced significant systemic anti-*C. difficile* toxin A immunoglobulin G and anti-*V. cholerae* vibriocidal antibody responses. Vaccination also produced significant protection against toxin A in an ileal loop challenge assay, as assessed by determination of both fluid secretion and histological changes. These results suggest that the hemolysin system of *E. coli* can be used successfully in *V. cholerae* vector strains to effect secretion of large heterologous antigens and that a *V. cholerae* vector strain secreting a nontoxic, immunogenic portion of *C. difficile* toxin A fused to the secretion signal of *E. coli* HlyA induces protective systemic and mucosal immunity against this toxin.**

Clostridium difficile is the causative agent of pseudomembranous colitis and as such is associated with significant morbidity, mortality, and cost (25, 36, 42). There is currently no effective vaccine against *C. difficile* disease, and measures to prevent acquisition of the organism are of limited effectiveness (10, 23, 38). *C. difficile* causes pseudomembranous colitis through the action of two large toxins that modify Rho proteins, with subsequent disruption of the actin cytoskeleton of intestinal epithelial cells (8). Toxin A appears to initiate intestinal damage, to result in mucosal disruption, and to permit full cytotoxicity of toxin B (36). The carboxy-terminal one-third of toxin A is comprised of approximately 800 amino acid residues and is essential for binding of toxin A to trisaccharide receptors on human intestinal epithelial cells (9, 29, 36, 51, 56, 58). Antibodies directed against toxin A prevent toxin binding, neutralize secretory and inflammatory effects, and limit or prevent clinical disease (2, 6, 22, 26–28, 31, 62). Antibodies which specifically bind epitopes at the carboxy terminus of toxin A prevent holotoxin binding and abrogate subsequent cytotoxic events (6, 12, 35, 63). A vaccine that produces immunity to toxin A in the intestinal lumen might prevent or reduce clinical illness following infection with toxigenic *C. difficile* and might be useful in patients at high risk of this infection.

Vibrio cholerae has many attributes that make it an attractive vaccine delivery vehicle for inducing mucosal immunity against heterologous antigens. *V. cholerae* is a noninvasive organism that induces potent and long-lasting mucosal and systemic humoral responses (20, 32, 47). *V. cholerae* is well studied at the molecular level, and attenuated vaccine strains have been developed and shown to be both safe and immunogenic in humans (24, 33, 40, 43, 54, 60). *V. cholerae* vector strains have also been used to successfully induce mucosal and systemic immune responses to heterologous antigens (1, 4, 5, 48). Since the extracellular secretion of heterologous antigens can increase the immunological response induced against such antigens (19, 30), and since *V. cholerae* secretes the nontoxic B subunit of cholera toxin (CTB) to the cell supernatant, small heterologous epitopes have been attached to CTB in successful attempts to achieve extracellular secretion of heterologous epitope-CTB fusion proteins in *V. cholerae* strains (3, 48, 50, 52). However, the small size of the heterologous epitopes that can be attached to but still allow secretion of CTB has limited the scope of the subsequent immune response directed against the heterologous antigen of interest.

Another mechanism of protein secretion by bacteria is exemplified by the hemolysin export system of *Escherichia coli*. Extracellular secretion of hemolysin A (HlyA) to the supernatant is dependent on a dedicated secretion system, including HlyB-HlyD-TolC, which recognizes the approximately 40 to 60 carboxy-terminal amino acid residues of HlyA to effect secretion of the hemolysin (13, 14, 17, 18, 37, 59, 61). Heterologous antigens that contain the HlyA secretion signal are secreted by the hemolysin system in *E. coli* and in *Salmonella typhimurium* (15, 57).

We adapted this system to obtain secretion of relatively large heterologous antigens in *V. cholerae*. In this study, we fused a 720-amino-acid protein representing the majority of the car-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype[a]	Reference or source
V. cholerae		
O395-NT	Serotype O1, classical, Ogawa, O395 Δ*ctxAB*; Km^r Sm^r	40
O395-N1	O1, classical, Ogawa, O395 Δ*ctxA*; Sm^r	40
569B	O1, classical, Inaba, wild type	39
CA401	O1, classical, Inaba, wild type; Sm^r	53
C6709	O1, El Tor, Inaba, wild type; Sm^r	4, 55
Peru2	C6709 Δ*attRS1*; Sm^r	4, 55
Bahrain2	O1, El Tor, Ogawa, E7946 Δ*attRS1*; Sm^r	55
Bengal2	Serotype O139, M010 Δ*attRS1*; Sm^r	60
E. coli JM105	*thi rpsL endA sbcB15 hsdR4 supE* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lac1*^{q} *lacZ* ΔM15]; Sm^r	Pharmacia P-L Biochemicals Inc., Milwaukee, Wis.
Plasmids		
pCD11	4.7-kbp *Pst*I fragment of *C. difficile* 10463 chromosomal DNA encoding the carboxy-terminal 2/3 of toxin A, cloned in pBR322; fragment contains an internal *Pst*I site; Tet^r	46
pMOhly1	Plasmid encoding the hemolysin operon of *E. coli*, with an internal deletion of *hlyA* such that nucleotides for the amino-terminal 34 amino acids are fused with nucleotides for the carboxy-terminal 61-amino-acid secretion signal, at a unique *Nsi*I site; Ap^r	15
pETR14	2.1-kbp *Pst*I fragment from 3′ end of insert of pCD11, encoding the nontoxic carboxy-terminal 1/3 of *C. difficile* toxin A, inserted in the *Nsi*I site of pMOhly1, in frame between the amino and carboxy termini of HlyA; Ap^r	This study

[a] Ap^r, ampicillin resistant; Sm^r, streptomycin resistant; Tet^r, tetracycline resistant.

boxy terminus of *C. difficile* toxin A to the secretion signal of *E. coli* HlyA and expressed this toxin A-HlyA fusion protein in conjunction with HlyB and HlyD in *V. cholerae*. We used this system to induce a mucosal and systemic immune response to *C. difficile* toxin A in rabbits, after oral inoculation with a *V. cholerae* vector strain, and showed that this immune response protected against a subsequent luminal challenge with toxin A.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. All strains were maintained at −70°C in Luria-Bertani broth (LB) medium (49) containing 15% glycerol. Streptomycin (100 μg/ml), tetracycline (25 μg/ml), and ampicillin (100 μg/ml) were added as appropriate. Cultures were grown at 37°C with aeration. Quantitative cultures were performed on thiosulfate-citrate-bile salts-sucrose plates or LB agar plates containing appropriate antibiotics.

Genetic methods. Isolation of plasmid DNA, restriction enzyme digestion, and agarose gel electrophoresis were performed according to standard molecular biological techniques (49). DNA sequencing was performed at the Massachusetts General Hospital, Department of Molecular Biology, DNA Sequencing Core Facility, using ABI Prism DiTerminator Cycle sequencing with AmpliTaq DNA polymerase FS and an ABI377 DNA sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, Calif.).

Plasmids were transformed into *E. coli* strains by standard techniques or were electroporated into *V. cholerae*, using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) according to the manufacturer's protocol, modified for electroporation into *V. cholerae* as previously described (16). Electroporation conditions were 2,500 V at 25-μF capacitance, producing time constants of 4.6 to 4.8 ms.

DNA restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase were used according to manufacturer's specifications. Restriction-digested DNA fragments were separated on 1% agarose gels, and required fragments were cut from the gel under UV illumination and recovered with GenElute agarose spin columns (Supelco Inc., Bellefonte, Pa.).

Expression and localization of toxin A-HlyA. To detect the toxin A-HlyA fusion protein expressed by *E. coli* and *V. cholerae*, we concentrated supernatants of various strains by immunoprecipitation with anti-*C. difficile* toxin A antibodies and detected immunoreactive proteins by Western blotting. Five-milliliter overnight cultures of JM105, JM105(pETR14), O395-NT(pMOhly1), and O395-NT(pETR14) were pelleted at 3,000 rpm for 10 min at 4°C (Sorvall Table Top RT6000B refrigerated centrifuge; Dupont Instruments, Wilmington, Del.). Supernatants were recovered and preabsorbed with 50 μl of a 1:1 (vol/vol) suspension of agarose-protein A beads (Sigma Chemical Co., St. Louis, Mo.) in Tris-buffered saline (pH 8.0)–0.05% Tween 20–1% bovine serum albumin (TBS-T-BSA) plus 50 μl of a 1:1 (vol/vol) suspension of agarose-mouse immunoglobulin G (IgG) beads (Sigma) in the same buffer. After agitation in an orbital shaker for 1 h at 4°C, the beads were pelleted at 3,000 rpm for 30 s. Recovered supernatants were combined with 5 μl of mouse monoclonal anti-*C. difficile* toxin A antibody PCG-4 (3.5 mg/ml; TechLab, Inc., Blacksburg, Va.). After an overnight incubation at 4°C, 100 μl of a 1:1 (vol/vol) of agarose-protein A beads in TBS-T-BSA was added, and mixing was allowed to continue at 4°C for an additional 6 h. Samples were spun, and supernatants were discarded. Pelleted beads were washed and resuspended in 50 μl of 1× Laemmli sample buffer (2% sodium dodecyl sulfate, 10% glycerol, 0.05 M Tris-HCl [pH 6.8], 0.01% bromophenol blue). Resuspended pellets were boiled for 5 min, treated with 5% β-mercaptoethanol, incubated at 37°C for 1 h, reboiled for 2 min, and run on a sodium dodecyl sulfate–10% polyacrylamide gel. Separated proteins were transferred onto a Hybond ECL nitrocellulose membrane (Amersham Life Sciences, Buckinghamshire, England) with a semidry blotting apparatus (Hoefer Scientific Instruments, San Francisco, Calif.). Immunoreactive proteins were visualized with sequential incubation with goat anti-*C. difficile* toxin A antibody (1:2,000; TechLab) in phosphate-buffered saline (PBS)–0.05% Tween 20 (PBS-T) and rabbit anti-goat IgG antibody–horseradish peroxidase conjugate (Southern Biotechnology Associates, Inc., Birmingham, Ala.) followed by development with the Amersham ECL Western blotting analysis system.

To prepare various cell fractions, 3-ml overnight cultures were spun at 14,000 rpm for 10 min (Eppendorf model 5415C centrifuge; Brinkmann Instruments, Inc., Westbury, N.Y.). The supernatant was recovered as the supernatant fraction, and periplasmic extracts of the pelleted cells were prepared as previously described (21). After extraction of the periplasm, cells were washed with 1 M Tris-HCl (pH 8.0) and recovered by spinning for 5 min at 14,000 rpm. Cell pellets were frozen at −70°C for 30 min, thawed, and resuspended in 1 M Tris-HCl (pH 8.0). Cell lysis was completed by sonication for three 5-s bursts at 60% intensity (Biosonik Sonicator; Bronwill Scientific, Rochester, N.Y.). Samples were then divided in half. One portion was retained as combined cytoplasmic and membrane fractions; for the other portion, membranes were pelleted at 14,000 rpm for 10 min at 4°C, and the supernatant of this spin was recovered as the cytoplasmic fraction. Estimation of the amount of toxin A-immunoreactive material in the membrane fraction was calculated as the difference between immunoreactive material in the combined cytoplasmic and membrane fractions and that in the cytoplasmic fraction alone.

We quantified the amount of toxin A protein in various cellular fractions by using an enzyme-linked immunosorbent assay (ELISA) standardized with intact toxin A. Briefly, 96-well microtiter plates were coated with mouse anti-*C. difficile* toxin A monoclonal antibody PCG-4 (100 ng/well; TechLab) in 50 mM carbonate buffer (pH 9.6) and blocked with PBS–1% bovine serum albumin (PBS-BSA; Sigma). Serial dilutions of various cell fractions in PBS-T (undiluted to 1:2,185) were applied to the wells, and the plates were incubated at room temperature overnight and then washed in PBS-T. A 1:4,000 dilution of polyclonal goat anti-*C. difficile* toxin A antiserum (TechLab) in PBS-T was then applied. After a 12-h incubation at room temperature, plates were washed with PBS-T, a 1:6,000 rabbit anti-goat IgG antibody–horseradish peroxidase conjugate (Southern Bio-

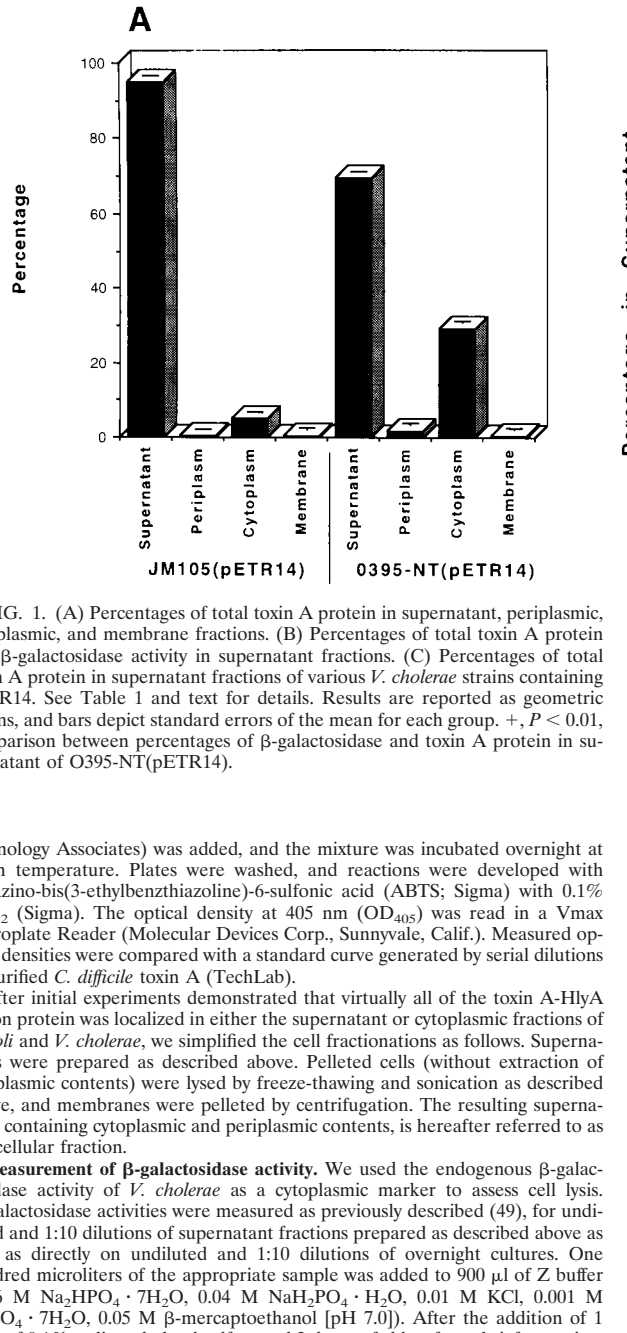

FIG. 1. (A) Percentages of total toxin A protein in supernatant, periplasmic, cytoplasmic, and membrane fractions. (B) Percentages of total toxin A protein and β-galactosidase activity in supernatant fractions. (C) Percentages of total toxin A protein in supernatant fractions of various *V. cholerae* strains containing pETR14. See Table 1 and text for details. Results are reported as geometric means, and bars depict standard errors of the mean for each group. +, $P < 0.01$, comparison between percentages of β-galactosidase and toxin A protein in supernatant of O395-NT(pETR14).

technology Associates) was added, and the mixture was incubated overnight at room temperature. Plates were washed, and reactions were developed with 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS; Sigma) with 0.1% H_2O_2 (Sigma). The optical density at 405 nm (OD_{405}) was read in a Vmax Microplate Reader (Molecular Devices Corp., Sunnyvale, Calif.). Measured optical densities were compared with a standard curve generated by serial dilutions of purified *C. difficile* toxin A (TechLab).

After initial experiments demonstrated that virtually all of the toxin A-HlyA fusion protein was localized in either the supernatant or cytoplasmic fractions of *E. coli* and *V. cholerae*, we simplified the cell fractionations as follows. Supernatants were prepared as described above. Pelleted cells (without extraction of periplasmic contents) were lysed by freeze-thawing and sonication as described above, and membranes were pelleted by centrifugation. The resulting supernatant, containing cytoplasmic and periplasmic contents, is hereafter referred to as the cellular fraction.

Measurement of β-galactosidase activity. We used the endogenous β-galactosidase activity of *V. cholerae* as a cytoplasmic marker to assess cell lysis. β-Galactosidase activities were measured as previously described (49), for undiluted and 1:10 dilutions of supernatant fractions prepared as described above as well as directly on undiluted and 1:10 dilutions of overnight cultures. One hundred microliters of the appropriate sample was added to 900 μl of Z buffer (0.06 M $Na_2HPO_4 \cdot 7H_2O$, 0.04 M $NaH_2PO_4 \cdot H_2O$, 0.01 M KCl, 0.001 M $MgSO_4 \cdot 7H_2O$, 0.05 M β-mercaptoethanol [pH 7.0]). After the addition of 1 drop of 0.1% sodium dodecyl sulfate and 2 drops of chloroform, brief vortexing, and a 37°C incubation for 5 min, 0.2 ml of *o*-nitrophenyl-β-D-galactopyranoside in 0.1 M sodium phosphate buffer (pH 7.0) (4 mg/ml; Sigma) was added, and samples were incubated at 37°C until yellow. Reactions were stopped with 0.2 ml of 1 M sodium carbonate. Samples were spun at 14,000 rpm for 5 min, and the OD_{420} and OD_{550} were read. The percentage of β-galactosidase activity in supernatant versus whole cell fractions was compared with the percentage of toxin A protein present in supernatant and cellular fractions measured in parallel.

Oral inoculation of rabbits with *V. cholerae* vectors. Orogastric colonization of rabbits was performed as previously described (7). *V. cholerae* O395-NT (pETR14) and O395-NT(pMOhly1) were grown overnight at 37°C in LB medium containing ampicillin, pelleted, washed in PBS, and resuspended in fresh LB medium to a final concentration of 10^{10} CFU per ml. Male New Zealand White rabbits (approximately 2.5 kg, 9 to 11 weeks old; Millbrook Farm, Amherst, Mass.) were fasted overnight and sedated. Gastric acid was neutralized with 50 mg of cimetidine per kg of body weight given intravenously at time zero. Ten milliliters of a 1:1 (vol/vol) 5% $NaHCO_3$–*V. cholerae* inoculum in LB was given by gastric tube 20 min later. At 60 min, 1 ml of tincture of opium was given intraperitoneally. For rabbits receiving cholera toxin (CT) as an immunoadjuvant, 15 μg of CT (List Biological Laboratories Inc., Campbell, Calif.) was added to the oral inoculum. Vaccinated rabbits (two or three in each group) received two oral inocula, the first on day 0 and the second on day 14. Blood was drawn on days 0, 14, 21, and 28. Bile was obtained on day 28.

A separate cohort of rabbits was orally inoculated once with *V. cholerae* O395-NT(pETR14) to measure intestinal colonization and retention of the plasmid in vivo. Washed ilea from these rabbits were cultured to quantitate the presence of the *V. cholerae* vector and of pETR14 after oral inoculation. Four rabbits received normal water and two rabbits received water containing ampicillin (1 mg/ml). Beginning on day 2, individual rabbits from each group were appropriately anesthetized and then sacrificed with Fatal-Plus (Vortech Pharmaceuticals, Dearborn, Mich.). A 10-cm segment of ileum (beginning 10 cm proximal to the mesoappendix and moving cephalad) was removed, opened, washed in PBS, weighed, and homogenized in LB medium by using a tissue grinder (Corning Inc., Corning, N.Y.); dilutions of the homogenate were plated on LB medium containing streptomycin (7, 44, 45). After overnight incubation, colonies were replica plated onto thiosulfate-citrate-bile salts-sucrose plates to confirm their identity as *V. cholerae* and onto LB medium containing ampicillin to measure retention of plasmid pETR14. Plasmid preparations from represen-

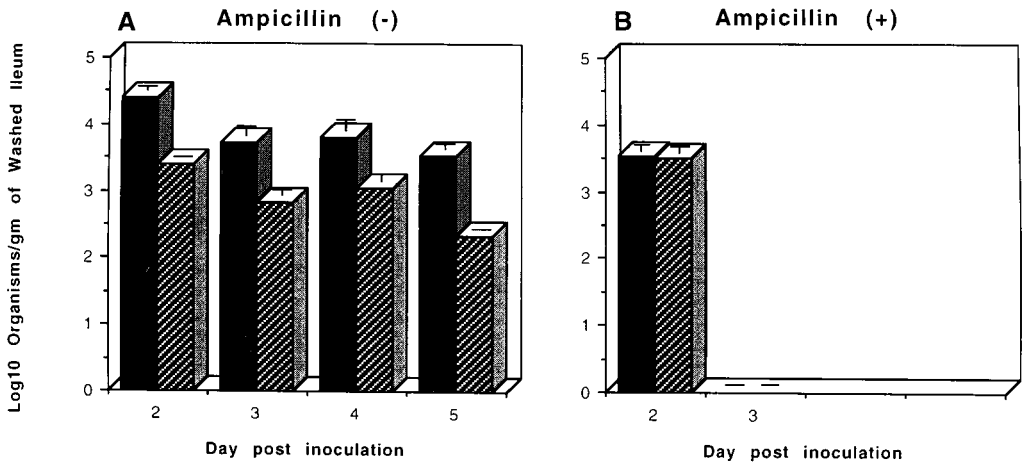

FIG. 2. Ileal colonization after oral inoculation with *V. cholerae* O395-NT(pETR14) in rabbits treated with water without ampicillin supplementation (A) or water supplemented with 1 mg of ampicillin per ml (B). Solid columns represent the geometric mean numbers of recovered *V. cholerae* vector, O395-NT per gram of ileum. Striped columns represent the number of recovered *V. cholerae* vector still containing plasmid pETR14 per gram of ileum. Bars represent standard errors of the mean for each group.

tative ampicillin-resistant colonies of *V. cholerae* were examined to confirm the presence of pETR14.

Measurement of serum vibriocidal antibodies. Serum vibriocidal antibody titers were measured in a microassay as follows. The endogenous complement activity of test sera was inactivated by heating sera at 56°C for 1 h. Fifty-microliter serial twofold dilutions (1:25 to 1:25,600) of test sera and PBS were placed in wells of sterile 96-well tissue culture plates. Fifty microliters of a 10^8-CFU/ml culture of *V. cholerae* O395-NT in PBS with 22% guinea pig complement (Gibco BRL Life Technologies, Gaithersburg, Md.) was added to each serum dilution, and plates were incubated at 37°C for 1 h. Then 150 μl of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) was added to each well, and plates were incubated at 37°C for 2.5 h. The OD_{600} was then measured. The titer was calculated as the dilution of serum causing a 50% reduction in optical density compared with wells containing preimmune (day 0) serum.

Serum and biliary antibody responses to *C. difficile* toxin A. Each well of 96-well microtiter plates was coated with 100 ng of mouse monoclonal anti-*C. difficile* toxin A antibody PCG-4 (TechLab). After overnight incubation at room temperature and washing, purified *C. difficile* toxin A in carbonate buffer (pH 9.6) (100 ng/well; TechLab) was added. Plates were incubated overnight, washed, and blocked with PBS-BSA. Duplicate, serial dilutions of rabbit sera (1:25 to 1:492,075) from days 0, 14, 21, and 28 following inoculation were incubated overnight. A 1:1,000 goat anti-rabbit IgG–alkaline phosphatase conjugate (Sigma) in PBS-T–2% dried milk was added, and plates were incubated overnight. Reactions were developed with 2 mg of *p*-nitrophenyl phosphate (Amresco Inc., Solon, Ohio), per ml, diluted in 1 M Tris-HCl (pH 8.0). The OD_{405} was measured kinetically. Plates were read for 5 min at 19-s intervals, and the maximum slope for an optical density change of 0.2 U was reported as milli-optical density units per minute. The end dilution was defined as the highest dilution of serum producing a kinetic reading that was statistically greater ($P < 0.05$) than that of preimmune (day 0) serum.

Serum IgA specific for toxin A was similarly measured, using a 1:1,000 dilution of goat antiserum against rabbit IgA antibody (α-chain specific; Sigma), followed by a 1:1,000 dilution of rabbit anti-goat IgG–alkaline phosphatase conjugate (Sigma). Reactions were developed and read as described above. IgA antibody responses in bile were measured in quadruplicate at a 1:100 dilution in PBS-T in 96-well microtiter assay plates previously coated with PCG-4 and toxin A. A 1:1,000 dilution of goat antiserum against rabbit IgA (Sigma) in PBS-T–2% milk was added, followed by a 1:2,000 dilution of rabbit anti-goat IgG antibody–alkaline phosphatase conjugate. Plates were developed and read as described above.

Protective efficacy against challenge with *C. difficile* toxin A. Creation of ileal loops in vaccinated and control rabbits was performed essentially as previously described (1, 11, 27, 34, 41). Rabbits were appropriately anesthetized on day 28 following oral inoculation. After shaving and prepping of the abdominal wall, the abdominal cavity was opened with a single midline incision. Intestines were mobilized, and the duodenum was ligated with silk ties. Serial 10-cm distal small intestinal segments were then created with ties. Short, 2-cm intervening intestinal segments were used as spacers between the 10-cm intestinal segments. Mesenteric vessels and vascular arcades were avoided. One-milliliter aliquots containing 1 or 5 μg of *C. difficile* toxin A (TechLab) in PBS-BSA were instilled into the lumens of serial 10-cm intestinal segments. One-milliliter aliquots of PBS-BSA or CT (10 μg; List) in PBS-BSA were used as negative or positive controls, respectively. Each sample was tested in duplicate in each animal. The intestines were then replaced within the abdominal cavity, and the incision was closed. Animals were returned to their cages, and analgesia was administered to ensure comfort. After 12 h, anesthetized animals were sacrificed, and each ileal loop segment was removed, grossly inspected, and weighed, and its length was measured. Weight (grams)-to-length (centimeters) ratios were calculated for each intestinal segment. Intestines were preserved in 10% formalin, and histological examinations were performed on hematoxylin-and-eosin-stained sections of paraffin-embedded tissues.

Statistics and graphics. Data were plotted by using CA-Cricket Graph software (Computer Associates, Garden City, N.Y.), and statistical significance was analyzed with a two-tailed *t* test for the comparison of means.

RESULTS AND DISCUSSION

Construction of pETR14. A 2,160-bp *Pst*I fragment, encoding the majority of the carboxy-terminal one-third of *C. difficile* toxin A, was recovered from pCD11 and ligated into the compatible *Nsi*I site of pMOhly1 so that the coding sequence for toxin A was in frame with the coding sequences of the upstream 34 amino acids and downstream 61 amino acids of HlyA, to generate plasmid pETR14. Plasmid pETR14 also encodes *E. coli hlyB* and *hlyD*. The construction was verified by restriction digestion and sequencing at the junctions of the insert. pETR14 was introduced into *E. coli* JM105 and various *V. cholerae* strains, including *V. cholerae* O395-NT, a vaccine strain of classical Ogawa *V. cholerae* O395 in which both copies of *ctx* have been deleted.

Expression and localization of the toxin A protein encoded by pETR14. We anticipated that the toxin A-HlyA fusion protein encoded by pETR14 would be secreted to the supernatant in the presence of HlyB and HlyD. To test this hypothesis, supernatants from *E. coli* and *V. cholerae* strains were immunoprecipitated with anti-toxin A antibodies, and proteins were visualized by Western immunoblotting with anti-toxin A antisera. Supernatants of JM105(pETR14) contained an immunorective protein of approximately 89 kDa (the estimated molecular mass of the intact toxin A-HlyA fusion), while supernatants of O395-NT(pETR14) contained a somewhat smaller, approximately 82-kDa immunoreactive protein, suggesting proteolytic processing of the fusion during or after secretion by *V. cholerae* (data not shown). The toxin A-containing protein in the supernatant of O395-NT(pETR14) was larger than the toxin A fragment itself (79 kDa), suggesting that at least some of the HlyA sequences were retained after secretion. No proteins

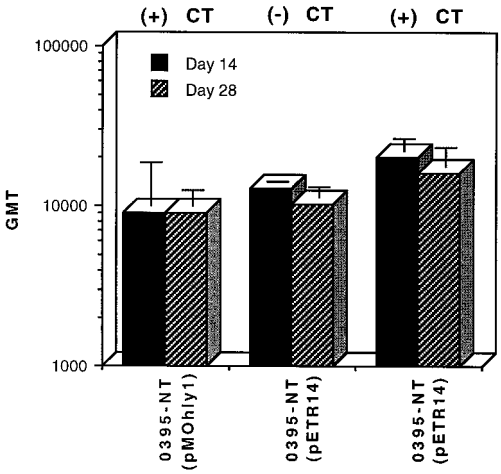

FIG. 3. GMTs of vibriocidal antibody responses on days 14 and 28 following oral inoculation of rabbits with *V. cholerae* vector strains with or without 15 μg of CT orally coadministered as an immunoadjuvant. Bars represent standard error of the mean for each group.

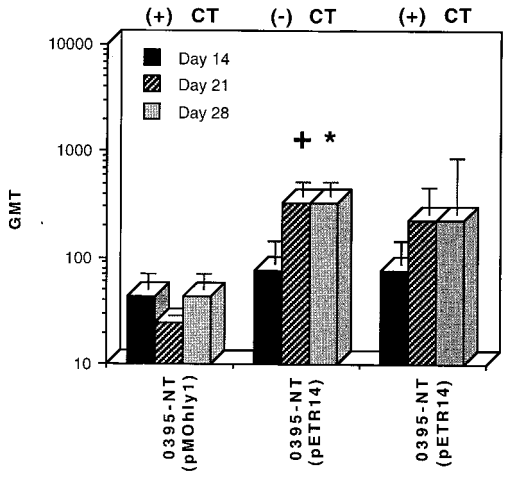

FIG. 4. Serum IgG antibody responses against *C. difficile* toxin A on days 14, 21, and 28 in rabbits that received two oral inoculations of the indicated strains on days 0 and 14, with or without 15 μg of CT orally. Results are reported as end GMTs; bars depict standard errors of the mean for each group. +, $P < 0.01$; *, $P < 0.05$, compared to group receiving O395-NT(pMOhly1) plus CT as a negative control.

reactive with anti-toxin A antibodies were immunoprecipitated from supernatants of strains JM105 and O395-NT(pMOhly1). To estimate the amounts of the toxin A-HlyA fusion proteins present in supernatants, we used an ELISA standardized with intact toxin A. In this assay, *E. coli* JM105(pETR14) produced approximately 50 to 250 ng of toxin A protein per ml of supernatant per OD_{600} of overnight culture, while *V. cholerae* O395-NT (pETR14) produced approximately 10 to 50 ng/ml/OD_{600}.

We isolated various cellular fractions from strains JM105 (pETR14) and O395-NT(pETR14) and localized total toxin A-immunoreactive material by ELISA. For the *E. coli* strain, approximately 95% of total toxin A protein was found in the supernatant, approximately 5% was found in the cytoplasm, and less than 1% was found in either the periplasmic or membrane fraction (Fig. 1A). For *V. cholerae* O395-NT(pETR14), approximately 70% of total toxin A protein was found in the supernatant, approximately 29% was found in the cytoplasm, less than 2% was found in the periplasm, and less than 1% was found in the membrane fraction. Simultaneous measurements of β-galactosidase and toxin A protein in cellular and supernatant fractions suggested that the presence of toxin A protein in the supernatant fraction did not reflect lysis of *V. cholerae* (Fig. 1B).

Plasmid pETR14 was introduced into a number of different *V. cholerae* strains to examine their ability to secrete the toxin A protein. Each of the strains containing pETR14 produced approximately 1 to 50 ng of total immunoreactive toxin A protein per ml of culture per OD_{600}. Surprisingly, however, there were substantial differences between strains in the percentage of total toxin A protein found in supernatant fractions compared with that retained in the cell (Fig. 1C). Sixty to 80% of the total toxin A protein was exported to the supernatant by two different derivatives of classical *V. cholerae* O395, as well as by the classical strain 569B (the parent of vaccine strain CVD103HgR) (33) and the *V. cholerae* O139 strain Bengal2. In contrast, virtually no toxin A protein was found in the supernatants of *V. cholerae* classical strain CA401 and the El Tor strains tested, despite similar overall production of total toxin A protein. Since the focus of this work was on developing a *V. cholerae* vector system for producing mucosal immunity to *C. difficile* toxin A, vaccine development was pursued with O395-NT, which localized the majority of the toxin A protein expressed from pETR14 to the cell supernatant. We have not yet further characterized the differences in secretion between various *V. cholerae* strains.

Intestinal colonization of rabbits following oral inoculation with O395-NT(pETR14). To investigate the duration of colonization of rabbit intestines with a *V. cholerae* vector strain containing pETR14, quantitative cultures of washed rabbit ilea were performed daily after oral inoculation of rabbits with O395-NT(pETR14) (Fig. 2). The *V. cholerae* vector strain was recoverable from rabbit ilea for at least 5 days after inoculation; approximately 1/10 of the recovered colonies retained plasmid pETR14 throughout this period (Fig. 2A). Ampicillin (1 mg/ml) was added to the water supply of vaccinated rabbits in an attempt to increase the percentage of the recovered vector strain retaining the plasmid. Interestingly, while virtually all of *V. cholerae* recovered from day 2 in the presence of

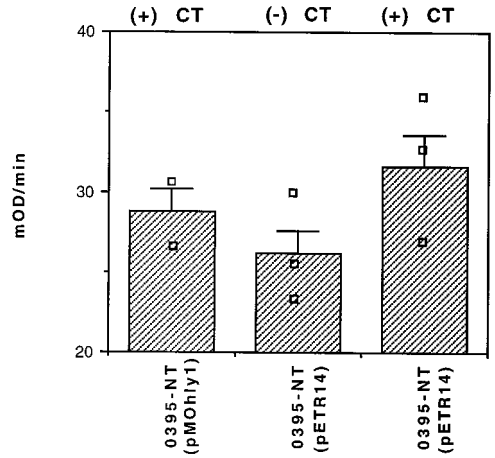

FIG. 5. Bile IgA antibody responses against *C. difficile* toxin A on day 28 in rabbits that received two oral inoculations of the indicated strains on days 0 and 14, with or without 15 μg of CT orally. Results were determined by kinetic ELISA; squares represent data points from individual animals. The geometric mean plus standard error of the mean for each group is shown. mOD, milloptical density units.

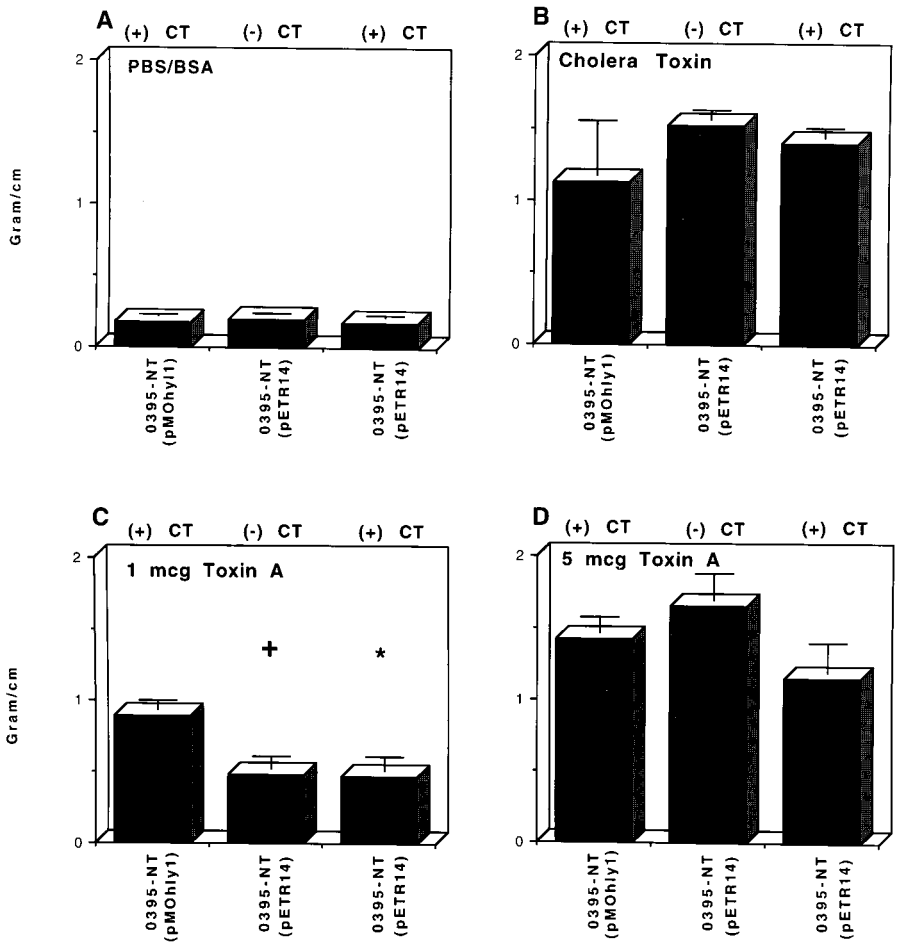

FIG. 6. Protection against *C. difficile* toxin A in an ileal loop challenge assay following oral inoculation with various strains on days 0 and 14, with or without 15 μg of CT orally. Fluid secretory responses in ileal loops (weight/length) were measured 12 h after intraluminal administration of PBS-BSA (A), 10 μg of CT (B), 1 μg of *C. difficile* toxin A (C), and 5 μg of toxin A (D). Results are reported as the geometric mean of the measured responses; bars represent the standard errors of the mean for each group. +, $P < 0.01$; ∗, $P < 0.05$, compared to vaccination with O395-NT(pMOhly1) plus CT as a negative control.

ampicillin contained pETR14 (Fig. 2B), the addition of ampicillin led to rapid clearance of the bacterium itself. On day 2, 10-fold less *V. cholerae* was recoverable in animals receiving ampicillin compared with those not receiving ampicillin, and by day 3, no *V. cholerae* was recoverable in the ampicillin-treated group. We elected to study oral inoculation of rabbits with O395-NT(pETR14) without ampicillin treatment, as a strategy to develop mucosal immunity to *C. difficile* toxin A. We also wished to assess the effect, if any, of adding CT as an immunoadjuvant to the oral inoculum. We gave two doses of vaccine on days 0 and 14 and compared results to rabbits inoculated with O395-NT(pMOhly1) plus CT as a negative control.

Serum vibriocidal antibody responses. Successful oral immunization of rabbits was demonstrated by the induction in all animals of appreciable vibriocidal antibody titers on days 14 and 28 (Fig. 3). No appreciable booster effect was seen after the second inoculation on day 14 or by the addition of CT as an immunoadjuvant.

Serum and bile antibody responses to *C. difficile* toxin A after vaccination. Rabbits inoculated with *V. cholerae* O395-NT(pETR14) developed significant serum IgG anti-*C. difficile* toxin A antibody responses on both day 21 and day 28 (Fig. 4). Although there was no overall effect of administering CT as an immunoadjuvant with the vaccine strain, the rabbit that developed the most prominent serum IgG anti-toxin A antibody responses (geometric mean titer [GMT], 1:2,085) had received O395-NT(pETR14) plus CT. There were no appreciable differences in serum IgA anti-toxin A antibody responses in vaccinated and control animals (data not shown).

The biliary IgA anti-toxin A antibody responses were highest in animals that received O395-NT(pETR14) and CT as an immunoadjuvant, although this difference did not reach statistical significance compared with the control group (Fig. 5).

Protective efficacy of vaccination against challenge with *C. difficile* toxin A. We used a *C. difficile* toxin A challenge in ligated rabbit ileal loops and measured both fluid secretory responses and histological changes, to assess the protective efficacy of vaccination. Fluid secretory responses of ileal loops to PBS-BSA (negative control) and 10 μg of CT (positive control) were similar in all animals, regardless of prior vaccination (Fig. 6A and B). Of note, the small doses of CT used as an immunoadjuvant in these experiments were not sufficient to evoke a protective anti-CT antibody response. Animals immunized with O395-NT(pETR14), with or without CT as an immunoadjuvant, were significantly protected against subsequent challenge with 1 μg of *C. difficile* toxin (Fig. 6C). When the challenge dose of toxin A was increased to 5 μg, significant protection by prior vaccination was not evident (Fig. 6D),

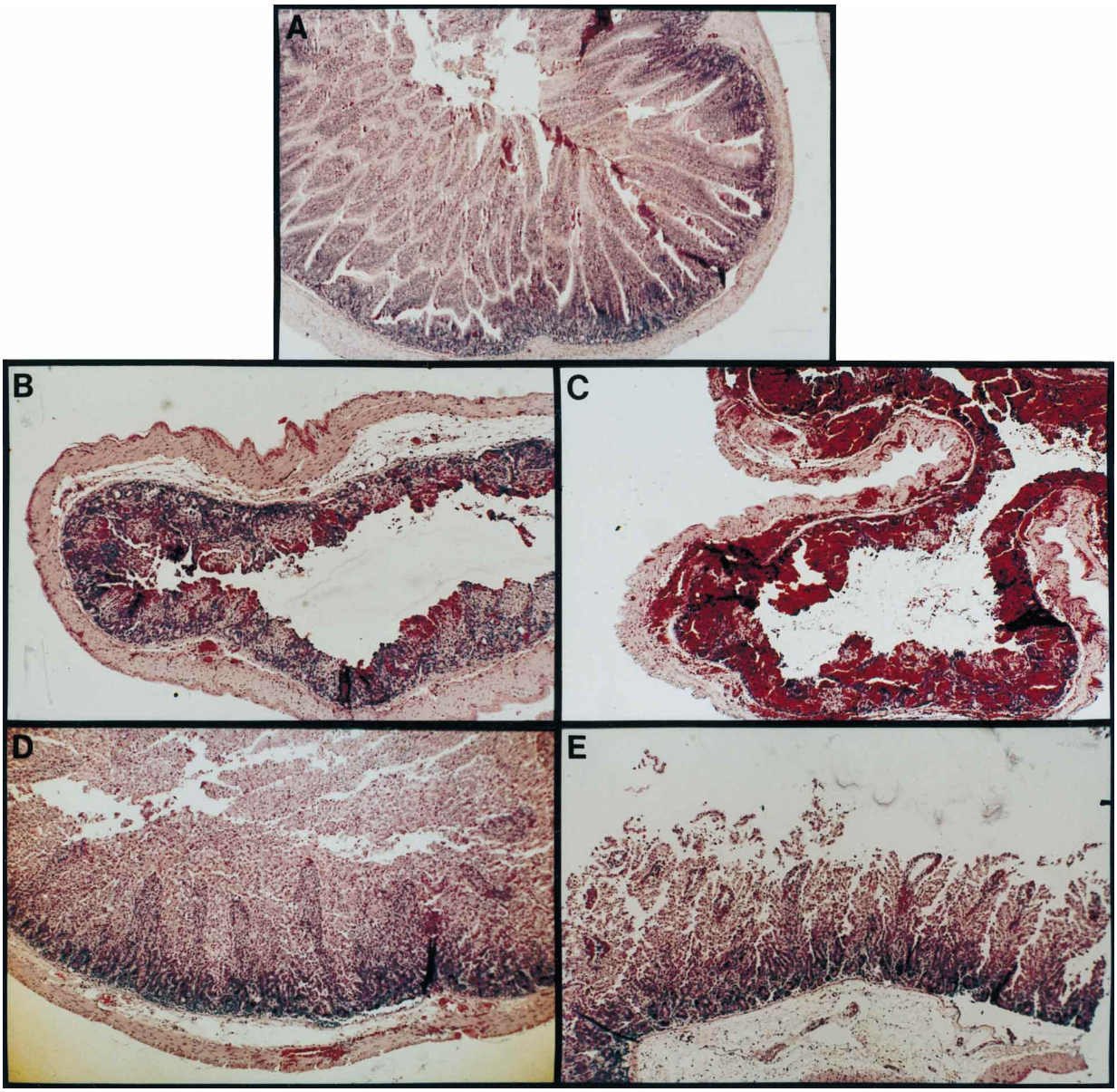

FIG. 7. Protection against histologic damage from *C. difficile* toxin A in an ileal loop challenge assay. Sections of ilea were recovered 12 h after intraluminal administration of PBS-BSA (A), 1 μg of *C. difficile* toxin A (B and D), and 5 μg of *C. difficile* toxin A (C and E). Intestinal segments shown in panels A, B, and C are from control animals that received *V. cholerae* O395-NT(pMOhly1) plus CT orally. Intestinal segments shown in panels D and E are from a vaccinated animal that received *V. cholerae* O395-NT(pETR14) plus CT orally. Magnification is ×40 for all panels.

although a slight decrease in the fluid secretory response in animals that received O395-NT(pETR14) plus CT coadministered as an immunoadjuvant was present.

To further assess protective efficacy against challenge with toxin A, histological examination was performed on intestinal segments from control animals and from the vaccinated animal with the most prominent anti-*C. difficile* toxin A serological response prior to challenge. Examination of ileal segments challenged with PBS-BSA showed normal intestinal architecture in all animals examined (Fig. 7A). Histological examination of ileal segments after challenge with CT showed no necrosis or cellular injury; however, there was splaying of intestinal villi, a finding consistent with the marked fluid secretory response induced by CT (data not shown). Histological findings in intestinal segments following challenge with *C. difficile* toxin A varied, depending on the dose of toxin A used and on the vaccination status of the examined animal. Intestinal segments from control animals challenged with toxin A disclosed marked histological changes. In control animals challenged with 1 μg of toxin A, there was severe villous necrosis with almost complete loss of villous height (Fig. 7B). Residual villous structures were markedly edematous and hemorrhagic. Multifocal crypt necrosis was present. The muscularis mucosae was intact, but prominent edema and vascular congestion were present in the submucosa. Following challenge with 1 μg of toxin A, intestinal segments from the vaccinated animal disclosed only partial villous necrosis, with preservation of over 50% of villous height (Fig. 7D). Although crypt areas containing mild architectural distortions were present, no deeper hemorrhagic or necrotic areas were observed.

Challenge of control animals with 5 μg of toxin A produced total villous necrosis in ileal loops (Fig. 7C). Edema and hemorrhage were prominent. Extensive crypt necrosis was present, with complete loss of crypts focally. Areas of focal necrosis were also present in the muscularis mucosae. The submucosa was markedly edematous, hemorrhagic, and necrotic. The muscularis externa contained extensive hemorrhage and pronounced separation of the longitudinal and circular muscular layers by edema and hemorrhage. In contrast, histological examination of intestinal segments following challenge of the vaccinated animal with 5 μg of toxin A disclosed only subtotal villous necrosis with villous hemorrhage (Fig. 7E). There were focal areas of crypt hemorrhage and necrosis; other structures were unremarkable. The histological changes observed in intestinal segments of the vaccinated animal following challenge with 5 μg of toxin A were felt to be equivalent to the histological changes observed in the intestinal segments of control animals following challenge with the fivefold-lower dose (1 μg) of toxin A. The fact that toxin A-induced intestinal damage was reduced but not eliminated in the vaccinated animal could be due to the high potency of even small amounts of toxin A in a closed ileal loop, perhaps in combination with suboptimal immunological responses to toxin A in vaccinated animals.

Our results suggest that large protein epitopes fused to the secretion signal of *E. coli* HlyA can be secreted by *V. cholerae* in the presence of HlyB and HlyD. We have used this system to induce protective immunity in rabbits against *C. difficile* toxin A following oral inoculation with a *V. cholerae* vector strain expressing a fusion protein which contains the carboxy terminus of *C. difficile* toxin A inserted between the amino and carboxy termini of HlyA. Further experiments to better understand the processing of such fusions in *V. cholerae* and to increase the immune responses against the *C. difficile* toxin A fragment expressed by a live *V. cholerae* vaccine vector are currently in progress.

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