Proteolytic Activity of the *Bacteroides fragilis* Enterotoxin Causes Fluid Secretion and Intestinal Damage In Vivo

RICHARD J. OBISO, JR.,* DAVID M. LYERLY, ROGER L. VAN TASSELL, AND TRACY D. WILKINS

Department of Biochemistry and Anaerobic Microbiology, Virginia Polytechnic Institute
and State University, Blacksburg, Virginia 24061-0305

Received 10 April 1995/Returned for modification 23 May 1995/Accepted 17 July 1995

Strains of *Bacteroides fragilis* that produce an enterotoxin have been implicated in diarrheal disease in farm animals and humans during the past decade. Our laboratory has purified and characterized this enterotoxin as a single polypeptide (M_r , ~20,000). Recently, we used PCR to clone and sequence the enterotoxin gene from *B. fragilis* and showed that it exhibits significant homology with extracellular metalloproteases. Further studies showed that the purified enterotoxin has protease activity. To further characterize the role of this enterotoxin in diarrheal disease, we studied the histological and pathological effects of highly purified *B. fragilis* enterotoxin in lamb, rabbit, and rat ligated intestinal loops. When the enterotoxin was injected into ligated ileal and colonic loops, there was significant tissue damage and subsequent fluid accumulation. The fluid response in the ileum was greater in lambs than in rabbits and rats, whereas the fluid response in the colon was greater in rabbits than in lambs and rats. Analysis of the intestinal fluid elicited by the enterotoxin revealed an accumulation of chloride and sodium as well as albumin and total protein. Histological examination revealed mild necrosis of epithelial cells, crypt elongation, villus attenuation, and hyperplasia. There was extensive detachment and rounding of surface epithelial cells and an infiltration of neutrophils. Enterotoxin activity was inhibited by the metal chelators EDTA and 1,10-phenanthroline; to some degree, the enterotoxin activity could be reconstituted by the addition of zinc to the chelated enterotoxin. Our results indicate that the enterotoxin elicits a significant fluid response subsequent to tissue damage in the small and large intestine. These data further support the idea that this enterotoxin is an important virulence factor in *B. fragilis*-associated diarrhea.

Bacteroides fragilis is a gram-negative, nonsporulating anaerobic rod that constitutes about 1 to 2% of the normal flora of humans and animals (19). This organism is an important clinical pathogen and is the most commonly isolated anaerobe from human clinical specimens. It has been associated with abscesses, soft tissue infections, and bacteremias (32). In the mid-1980s, L. L. Myers et al. (21) reported that some strains of *B. fragilis* produced an enterotoxin that could be detected in ligated lamb ileal loops. Since then, enterotoxigenic *B. fragilis* strains have been implicated as the cause of diarrhea in calves (2, 26), piglets (4, 22), foals (23), rabbits (20), and more recently, humans (27–29). Several animal models, including neonatal rabbits and neonatal germ-free pigs, have been established to study the pathogenicity of enterotoxigenic strains (8, 24, 25). Together, the results from these studies support the role of enterotoxigenic *B. fragilis* in gastrointestinal disease in humans and animals.

The results of studies of *B. fragilis* in the 1980s indicated that an enterotoxin was responsible for the activity elicited by enterotoxigenic strains, but characterization of the enterotoxin was hindered by the lack of an in vitro assay. Research on the enterotoxin was greatly facilitated by the development of a tissue culture assay. Weikel et al. showed that culture filtrates from the enterotoxigenic strains caused rapid morphological changes in human colon carcinoma cell lines, particularly HT-29, that were characterized by a decrease in monolayer resistance and dissociation of F-actin (35).

The enterotoxin has been purified from culture supernatants and characterized as a single polypeptide (M_r , ~20,000) (33). Examination of the DNA sequence of the *B. fragilis* enterotoxin gene revealed a signature zinc-binding consensus motif characteristic of such matrix metalloproteases as human fibroblast collagenase IV and *Pseudomonas aeruginosa* elastase. These observations were confirmed by showing that the enterotoxin is a protease; this protease hydrolyzes actin, tropomyosin, fibrinogen, myosin, and gelatin but not fibronectin, elastin, and albumin (17). Metalloproteases, including many that are endogenously produced by the colonic microflora in the human intestine, are ubiquitous in nature. Thus, the identification of the *B. fragilis* enterotoxin as a metalloprotease is particularly intriguing in view of the fact that host proteases and other proteases from the endogenous microflora have not been shown to be enterotoxic.

Although some histological studies have shown the effects of enterotoxigenic strains of *B. fragilis* and crude toxin preparations in experimental animal models, there have been no detailed studies on the action of the highly purified enterotoxin in the intestine, particularly in the colon, where the organism resides. Our study was undertaken to answer the following questions. First, does the intestinal response elicited by the purified enterotoxin resemble the responses that have been described in animals infected with enterotoxigenic strains? Second, is the purified enterotoxin active in the colon, where it is produced? Third, do reagents that inactivate the cell-rounding activity in situ and proteolytic activity in vitro also inactivate the enterotoxin activity in vivo?

To answer these questions, we used the ligated loop assay with lambs, rabbits, and rats. Our results support the hypothesis that the enterotoxin is responsible for *B. fragilis*-associated

* Corresponding author. Mailing address: Department of Biochemistry and Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0305. Phone: (703) 231-5094. Fax: (703) 231-7126. Electronic mail address: robiso@mail.vt.edu.

diarrhea. The enterotoxin causes extensive damage to the intestine, and the proteolytic activity is responsible for this effect.

MATERIALS AND METHODS

Reagents. *Clostridium difficile* toxin A was purified by a previously described method (16). *P. aeruginosa* elastase was kindly supplied by Ali Azghani (The University of Texas Health Center, Tyler, Tex.) and USB (Amersham Biochemical, Arlington Heights, Ill.). All other reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.).

***B. fragilis* strains.** *B. fragilis* VPI 13784, VPI 2553, and ATCC 43860 were grown anaerobically according to standard methods described in the Virginia Polytechnic Institute Anaerobe Laboratory Manual (11). Fresh chopped meat cultures were used to inoculate prereduced, anaerobically sterilized, brain heart infusion broth. The brain heart infusion cultures were incubated at 37°C for 24 h. Five milliliters of each culture was diluted 1:10 in sterile phosphate-buffered saline (PBS), centrifuged at $12,000 \times g$ for 15 min at 4°C, and washed twice with 50 ml of sterile PBS. The washed cells were stored under CO₂ at 4°C until they were assayed with tissue culture cells and in loops.

Preparation of crude culture filtrate. *B. fragilis* VPI 13784 was grown anaerobically in 2 liters of brain heart infusion broth at 37°C for 24 h by a previously described method (33). The culture was centrifuged at $12,000 \times g$ for 30 min at 4°C, and the supernatant (300 ml) was filtered through a 0.2- μ m-pore-size membrane. The filtrate was placed in a dialysis bag (Spectrapor membrane tubing 12,000 MWCO; Baxter Diagnostics, McGraw Park, Ill.) and concentrated 30 times with polyethylene glycol. The concentrated filtrate was passed through a 0.45- μ m-pore-size membrane and stored at -30°C until it was assayed.

Preparation of purified enterotoxin. Highly purified *B. fragilis* enterotoxin was prepared from culture filtrates of VPI 13784 by a previously described method (33). Briefly, the enterotoxin was purified by sequential ammonium sulfate precipitation, ion-exchange chromatography on Q-Sepharose, hydrophobic chromatography on phenyl-agarose, and high-resolution ion-exchange chromatography with a Mono Q column (Pharmacia, Piscataway, N.J.). Purification of the enterotoxin was monitored by its characteristic rounding of HT-29 cells (ATCC HTB 38) and hydrolysis of rabbit skeletal muscle actin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (17). The enterotoxin was homogeneous, as was shown by crossed immunoelectrophoresis, SDS-PAGE, as well as SDS-capillary electrophoresis. Purified toxin was frozen at -30°C immediately after elution from the Mono-Q column to assure maximal biological activity.

Analysis of *B. fragilis* toxin activity. To ensure equivalent levels of biological activity, purified enterotoxin preparations and samples to be injected into ligated loops were diluted to a concentration of 1 μ g/ml and assayed by the cell-rounding assay (33). HT-29 cells (ATCC HTB 38) were grown and maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum according to standard tissue culture procedures. For the cell-rounding assay, we harvested stock cultures grown with a nonenzymatic cell dissociation solution at 37°C under 5% CO₂ in 100-mm-diameter plates, washed the cells from the plate surface, diluted them in McCoy's medium, and allocated 125 μ l of the diluted suspensions into 96-well microtiter plates. The cells were allowed to settle, attach, and grow for 1 week prior to the assay. The day before the assay, the culture wells were replaced with fresh medium at the desired volume (either 100 μ l or 133 μ l). Samples whose titers were to be determined were diluted two- or threefold in sterile PBS, pH 7.4, and 100 μ l or 67 μ l of the sample was added to the tissue culture wells with thorough mixing. The plates were incubated for 4 h at 37°C under 5% CO₂, after which time they were examined for the characteristic cell-rounding effect. Cell-rounding titers were expressed as the reciprocal of the highest dilution of enterotoxin that caused more than 50% cell rounding. A cell-rounding unit (CRU) was defined as the lowest amount of toxin that elicits a positive response, i.e., more than 50% cell rounding.

Inhibition and reconstitution studies. The purified *B. fragilis* enterotoxin (5 μ M, based on an estimated molecular weight of 20,000) was incubated with 10 mM Na₄EDTA or 4 mM 1,10-phenanthroline for 1 h at 37°C in 10 mM PBS, pH 7.4. The solution was then dialyzed for 48 h at 4°C against PBS. For reconstitution experiments, the chelated enterotoxin was incubated with 100 μ M zinc chloride for 1 h at 37°C, and this was followed by dialysis for 24 h at 4°C against PBS. The chelated and reconstituted enterotoxin preparations were stored at -30°C.

Protein assay. Protein concentrations were determined with the Pierce Coomassie Protein Assay Kit (Pierce, Rockford, Ill.) in a procedure recommended by the manufacturer. Bovine serum albumin was used as a standard for determining protein concentrations. A₅₉₅ was measured with a Beckman DU-70 spectrophotometer.

Ligated loop assays. Adult male and female New Zealand White rabbits (2.5 to 4.1 kg), 2-week-old male and female Western Cross lambs (3.9 to 4.5 kg), and adult female Sprague-Dawley rats (170 to 200 g) were used in all studies. Lambs were fasted for 12 h, and rabbits and rats were fasted overnight. All animals were given water ad libitum. The animals were anesthetized with ketamine (Fort Dodge Inc., Fort Dodge, Iowa) and diazepam (Elkins-Sinn, Cherry Hill, N.J.). In the lamb ileum, the intestine was ligated proximal to the ileocecal junction to give loops of 5 cm in length. In the rabbit, ileal loops were begun 5 to 8 cm proximal

to the sacculus rotundas and also were 5 cm in length. In the rats, ileal loops began proximal to the ileocecal junction and were 2 cm in length. The colonic loops started approximately 12 cm cranial to the spiral colon in lambs, 5 cm distal to the appendix in rabbits, and 1 cm distal to the cecum in rats. Samples were injected into the ligated loops, and the injection site was tied off to prevent leakage of the sample. Blank loops were placed between each injected loop to serve as noninjected spacer loops. After injection, the exposed intestine was placed back into the body cavity and the muscle and skin incisions were sutured. After a designated incubation period, the lambs and rabbits were sacrificed with Buthanesia solution (Schering-Plough, Kenilworth, N.J.) and the rats were sacrificed in a carbon dioxide chamber. The volume (milliliters) of accumulated fluid and the length (centimeters) of the loops were measured, and a ratio of volume to length was determined for each loop. The accumulated fluid recovered was kept on ice for analysis. Tissue samples approximately 2 cm in length were collected and placed in 10% neutral buffered formalin for histological analysis.

Fluid and histological analysis. Collected fluid samples were centrifuged ($2,000 \times g$ for 10 min at 4°C), and the supernatant was analyzed for electrolytes (Na⁺, Cl⁻, and K⁺), total protein, albumin, and pH with an Ektachem 700 multiple analyzer (Kodak, Rochester, N.Y.). Tissues were embedded in paraffin, sectioned with a rotary microtome (Reichert-Jung, Heidelberg, Germany), and stained with hematoxylin (Fisher Scientific, Pittsburgh, Pa.) and eosin (Fisher Scientific) for examination by light microscopy. Fluid analysis and tissue processing were done at the facilities at the Virginia-Maryland Regional College of Veterinary Medicine Histopathology Laboratory (Blacksburg, Va.).

RESULTS

Previous studies have described the ability of enterotoxigenic strains of *B. fragilis* and culture filtrates from these strains to induce fluid responses in ligated ileal loops and to damage the intestinal mucosa in various animal species. This damage consisted of sloughing of surface epithelial cells along the intestinal mucosa, crypt hyperplasia, and mucosal attenuation (20-25). As part of our study, we examined the fluid response and tissue damage following the injection of the purified enterotoxin into ligated loops in the ileum and colon to determine if it damaged the mucosa in a similar manner.

***B. fragilis* enterotoxin purity and activity.** The purified enterotoxin yielded a single band with a molecular weight of 20,000 by SDS-PAGE and eluted as a single peak with a molecular weight of 20,000 by SDS-capillary electrophoresis. We monitored the biological activity of the purified preparations (diluted to 1 μ g) immediately before performing the ligated loop assay to ensure consistency among sample preparations and purification batches and also to determine if the titer correlated with the loop response. CRU were consistently between 2,084 and 2,187 CRU/ μ g at 4 h for native enterotoxin samples. Enterotoxin exposed to 100°C for 15 min was inactive. Enterotoxin treatment with EDTA or 1,10-phenanthroline reduced the titers to 128 or 196 CRU/ μ g at 4 h. Chelated enterotoxin that had been reconstituted with zinc had titers of 512 to 1,024 CRU/ μ g at 4 h. Other metalloproteases (*Clostridium histolyticum* collagenase, *Bacillus thermoproteolyticus* thermolysin, and *P. aeruginosa* elastase) did not cause HT-29 cells to become rounded at 4 h.

Histology. Injection of the purified enterotoxin resulted in a dose-dependent fluid response in ligated ileal loops in the lamb, rabbit, and rat (Fig. 1a). At a challenge dose of 1 μ g, the enterotoxin elicited a weak nonhemorrhagic fluid response in all animals and there was no hemorrhage noted in the mucosa. A challenge dose of 50 μ g, on the other hand, resulted in mild hemorrhage along the mucosal wall, and the fluid was weakly hemorrhagic. The hemorrhage at high doses was patchy in most instances. Fluid responses in the ileum were higher in the lamb than in the rabbit or rat at the 5-, 20-, and 50- μ g doses. The fluid responses in the ileum in all species were less than the fluid responses in the colonic loops. In all animals, the fluid was viscous. Enterotoxin that was heat inactivated at 100°C for 30 min did not elicit a fluid response, and there was no hemorrhage.

The histologic changes produced in the ileal loops by the

FIG. 1. Responses elicited by the *B. fragilis* enterotoxin in the ileum loops (a) and colon loops (b) in lambs, rabbits, and rats. Note that the y axis for panel b is different than that for panel a. Dose amounts are given on the x axis. PBS was used as a control.

enterotoxin at challenge doses of 20 µg are shown in Fig. 2. Tissue taken from lamb ileal loops treated with the enterotoxin (Fig. 2b) showed inflammation and necrosis of the villus epithelial cells. There was extensive exfoliation of surface epithelial cells; crypts were elongated and contained intensely stained basophilic epithelial cells. The villi were shortened, and there was an increase in the number of neutrophils. Some epithelial cells had pyknosis, but necrosis was limited to the tips of the villi. Tissue from lamb ileal loops challenged with *B. fragilis* VPI 13784 cultures and concentrated culture filtrate had similar but less extensive damage to the intestinal mucosa (data not shown). Rabbit ileal loops challenged with 20-µg doses of the enterotoxin are shown in Fig. 2d. The rabbit ileal loops had shortened villi and slight necrosis of the villus tips, with less exfoliation and hyperplasia than was observed in the lamb ileum. There also was an accumulation of neutrophils in the villi. Rat ileal loops challenged with 20-µg doses of the enterotoxin are shown in Fig. 2f. There was massive tissue damage in the rat ileal loops. There were shortened villi and necrosis of villus tips similar to but more apparent than that in the lamb enterotoxin-treated loops. There also was sloughing of surface epithelial cells and hemorrhage along the intestinal mucosa.

The fluid response elicited following the injection of the enterotoxin into colonic loops is illustrated in Fig. 1b. As was the case with the ileal loops, there was a dose-dependent response in all the animals. A challenge dose of 1 µg elicited a weak fluid response that was nonhemorrhagic. Challenge doses of 50 µg caused intense fluid responses in all animals that were characterized by mild hemorrhage along the intestinal mucosa and in the fluid. The volume-to-length ratios were higher in the

rabbit than in the lamb or rat. Heat-inactivated enterotoxin did not elicit any responses.

The histologic changes caused by the enterotoxin in lamb, rabbit, and rat colonic loops are shown in Fig. 3. In the lamb (Fig. 3b), there were extensive inflammation, necrosis, and exfoliation of the surface epithelial cells. Crypt hyperplasia and shortened intestinal lining were apparent. There was a large number of neutrophils throughout the full thickness of the intestinal wall, producing peritonitis. Large hemorrhages were noted in some areas of the injected loops, whereas other areas were unaffected. Hemorrhage also occurred in the rabbit colonic tissue (Fig. 3d), and there also were exfoliation and crypt hyperplasia. The surface epithelium was necrotic and shortened, and there were an abundant number of neutrophils and mild peritonitis. As was the case with the lamb colonic loops, some areas within the rabbit loops appeared unaffected. In the rat colonic loops (Fig. 2f), extensive intestinal damage by exfoliation of the surface epithelium through to the submucosa was noted. Some areas of the intestinal lining were hemorrhagic, and there was necrosis of the intestinal mucosa.

Chemical analysis of fluids elicited by the enterotoxin. Table 1 summarizes the fluid analyses after 18 h of fluid accumulation in the small and large intestinal loops in lambs and rabbits. The enterotoxin induced a significant net accumulation of sodium and chloride. Loops treated with enterotoxin also accumulated a significant amount of albumin and total protein.

As a control, we compared the fluid elicited with this enterotoxin with that elicited with *C. difficile* toxin A, a tissue-damaging enterotoxin that causes a viscous, hemorrhagic fluid response in ligated loops (14). Both enterotoxins caused an increase in sodium and chloride, and the accumulations of albumin and total protein in the ileum were similar for both toxins. Albumin was the major protein component, representing at least 25% of the total protein in the lamb and at least 70% in the rabbit. The fluids in the loops from lambs and rabbits exhibited banding patterns similar to those of sera from these species when they were analyzed by SDS-PAGE (data not shown). We did not test intestinal secretions from the rat ligated loops for electrolyte and protein secretion because of the sample volume required.

Analyses of other metalloproteases for enterotoxic activity. We investigated the activity of other bacterial metalloproteases in the lamb and rabbit intestinal loops. We tested *P. aeruginosa* elastase, *B. thermoproteolyticus* thermolysin, and *C. histolyticum* collagenase IV, all of which contain zinc-binding motifs nearly identical to the motif found in the *B. fragilis* enterotoxin (10). At challenge doses of 2,500 µg, elastase, thermolysin, and collagenase IV had volume-to-length fluid ratios of 0.32 ± 0.06 , 0.02 ± 0.08 , and 0.02 ± 0.10 , respectively, in lamb ileal loops. The ratios were 0.86 ± 0.12 , 0.71 ± 0.20 , and 0.02 ± 0.10 , respectively, in rabbit ileal loops. The fluids in the loops injected with the large quantities of elastase and thermolysin were not viscous or hemorrhagic. Elastase from *P. aeruginosa* caused sloughing of the surface epithelial cells in the lamb ileum, but the intestinal damage was minimal. In colonic loops in both the lamb and the rabbit, ratios with these metalloproteases were 0.11 ± 0.10 or lower and there was no hemorrhage.

Inhibition and reconstitution studies. The results of previous studies in our laboratory revealed that the enterotoxin is a metalloprotease (17). These observations were supported by our findings that the cell-rounding activity in situ and the proteolytic activity in vitro are inactivated by chelating agents and that the enterotoxin contains approximately one zinc atom per molecule of enterotoxin. To further investigate the role of zinc in the biological activity of the enterotoxin, studies to determine if the enterotoxic activity was inhibited by the che-

FIG. 2. Histologic changes produced in ileal loops by the enterotoxin at challenge doses of 20 μ g. The tissues were stained with hematoxylin and eosin. (a) Ileum from a lamb ligated loop treated with PBS control. The villi are long and visually normal. There are a few neutrophils apparent. (b) Enterotoxin-treated lamb ileum. There is extensive detachment and rounding of surface epithelial cells. Villi are necrotic and shortened, and an increased accumulation of neutrophils is visually apparent. (c) Normal rabbit ileum inoculated with PBS control. The villi are long and thin and lined with a uniform layer of tall columnar epithelial cells. (d) Rabbit ileum inoculated with purified enterotoxin. Epithelial cells are sloughed, erythrocytes are present, and villi are shortened. Mild necrosis of individual epithelial cells is apparent. (e) Normal rat ileum treated with PBS. (f) Rat ileum treated with purified enterotoxin. There are sloughing of epithelial cells, necrosis, and hemorrhage in the mucosa.

lating agents EDTA and 1,10-phenanthroline were performed. For the analysis, enterotoxin (20 μ g/ml) was treated with a molar excess of chelating agents, which was followed by dialysis. The ability of the enterotoxin to elicit fluid responses in the ileum or colon of lambs and rabbits was inhibited >90% by either EDTA or 1,10-phenanthroline, and there was no tissue damage. Chelated enterotoxin was reactivated by incubation with a molar excess of zinc. The reconstituted enterotoxin regained approximately 50% of its initial activity, as determined by the volume-to-length ratios. The tissue damage from reactivated enterotoxin was identical to that caused by native

enterotoxin, although there was less tissue damage from reactivated enterotoxin than with the native enterotoxin.

DISCUSSION

Metalloproteases occupy a number of roles in their ability to function as virulence factors for microorganisms. In some instances, they directly damage the tissue during the infection or inactivate endogenous factors that normally are involved in the regulation of the host response to the infection. The tissue-damaging proteases of *Serratia* species and *P. aeruginosa*, for

FIG. 3. Histologic changes produced in colonic loops by the enterotoxin at challenge doses of 20 μ g. The tissues were stained with hematoxylin and eosin. (a) Tissue section of a normal lamb colonic loop inoculated with PBS as a control. There is no damage to the afflicted areas, and the glands are intact. (b) The mucosa of a ligated colonic loop from a lamb inoculated with *B. fragilis* enterotoxin. There are extensive inflammation, necrosis, and exfoliation of surface epithelial cells. The mucosa is attenuated, and an increase in neutrophils is apparent. (c) Rabbit mucosa from the colon of a control (PBS-inoculated) loop. (d) Rabbit colonic mucosa injected with *B. fragilis* enterotoxin. There is exfoliation of epithelial cells. The epithelium is shortened and mildly hemorrhagic. (e) Rat colon treated with PBS as a control. (f) Rat colonic mucosa treated with the *B. fragilis* enterotoxin. The intestinal lining is hemorrhagic, and there is exfoliation of the surface epithelial cells. There is also necrosis, and the epithelium is shortened.

example, are responsible for the extensive tissue damage that occurs during eye and lung infections caused by these opportunistic pathogens (9, 12, 15, 36). In addition, these proteases activate host factors, such as endogenous protease inhibitors, that may exacerbate the disease through the action of nonspecific tissue-damaging proteases (e.g., those released from neutrophils) (5). Other examples include the tissue-damaging metalloproteases (e.g., collagenases) produced by *C. histolyticum* and *Legionella* spp. (7, 31). In other instances, metalloproteases may activate toxins produced by microorganisms. The hemagglutinating metalloprotease of *Vibrio cholerae*, for exam-

ple, nicks cholera toxin, converting it to an active form (1). Alternatively, metalloproteases may inactivate regulatory factors in the host, resulting in disease. Recently, the neurotoxins of *Clostridium botulinum* and *Clostridium tetani* have been identified as metalloproteases that cleave the peptidyl linkages of proteins, such as synaptobrevin and cellubrevin, which regulate neurotransmitters (6, 30). These findings demonstrate the diverse mechanisms by which metalloproteases act as virulence factors.

The results of our recent studies showing that the *B. fragilis* enterotoxin is a metalloprotease continues to broaden the role

TABLE 1. Fluid accumulation alterations caused by the *B. fragilis* enterotoxin (20 µg/ml) and *C. difficile* toxin A (20 µg/ml) in ligated lamb and rabbit small and large intestine loops at 18 h

Source of fluid	Treatment	Amt of fluid (ml/cm)	Amt of electrolyte (µeq/cm)			Amt of albumin (mg/cm)	Amt of total protein (mg/cm)	pH
			Na ⁺	Cl ⁻	K ⁺			
Lamb ileum	Control ^a	0.11 ± 0.08	39 ± 28	40 ± 24	2.4 ± 1.5	0.2 ± 0.2	0.3 ± 0.2	7.4 ± 0.2
	<i>B. fragilis</i> toxin ^a	1.90 ± 0.55 ^b	260 ± 88 ^b	190 ± 65 ^b	14 ± 2.4	19 ± 13 ^c	39 ± 17 ^c	6.8 ± 0.3
	<i>C. difficile</i> toxin A ^d	2.40 ± 0.61 ^b	137 ± 45 ^c	115 ± 41 ^c	ND ^e	31 ± 12 ^c	41 ± 14 ^c	7.6 ± 0.4
Lamb colon	Control ^a	0.15 ± 0.50	25 ± 6.3	28 ± 2.1	ND	0.5 ± 0.3	6.4 ± 5.1	6.2 ± 0.6
	<i>B. fragilis</i> toxin ^a	1.20 ± 0.15 ^c	150 ± 22 ^b	140 ± 26 ^c	ND	19 ± 9.2 ^c	68 ± 28 ^c	8.2 ± 0.2
	<i>C. difficile</i> toxin A ^d	1.92 ± 0.81 ^c	121 ± 15 ^b	88 ± 22 ^c	ND	ND	ND	ND
Rabbit ileum	Control ^a	0.10 ± 0.03	24 ± 0.9	37 ± 13	0.4 ± 0.3	0.6 ± 0.3	0.9 ± 0.5	8.5 ± 0.1
	<i>B. fragilis</i> toxin ^a	0.92 ± 0.28 ^c	110 ± 47 ^c	100 ± 50 ^c	5.5 ± 0.2	24 ± 18 ^c	33 ± 20 ^c	7.9 ± 0.3
	<i>C. difficile</i> toxin A ^d	2.67 ± 0.31 ^b	99 ± 32 ^c	78 ± 24	ND	38 ± 14 ^c	46 ± 16 ^c	7.4 ± 0.6
Rabbit colon	Control ^a	0.25 ± 0.37	32 ± 19	25 ± 15	ND	3.3 ± 1.8	3.5 ± 2.1	8.4 ± 0.1
	<i>B. fragilis</i> toxin ^a	3.20 ± 0.88 ^b	480 ± 95 ^b	310 ± 85 ^b	ND	35 ± 13 ^c	41 ± 12 ^c	8.7 ± 0.1 ^c
	<i>C. difficile</i> toxin A ^d	2.87 ± 0.51 ^b	176 ± 85 ^c	160 ± 67 ^c	ND	ND	ND	ND

^a Each value represents the mean ± standard deviation of one to two loops in four lambs or four rabbits.^b $P \leq 0.004$ for active toxin versus control.^c $P \leq 0.05$ for active toxin versus control.^d Each value represents the mean ± standard deviation of one to two loops in two lambs or two rabbits.^e ND, not determined.

of metalloproteases in disease. This enterotoxin is the first well-documented example of a metalloprotease that acts as a potent enterotoxin. There have been other reports of some metalloproteases causing an enterotoxic response in different animal models (3, 13). However, in these instances, the studies used crude toxin preparations or an approximately 1,000 times greater dose than that required with the *B. fragilis* enterotoxin. The fact that *B. fragilis* typically resides in the colon strengthens the potential role of this metalloprotease as a virulence factor in colonic disease.

The results of our studies show that the highly purified enterotoxin is active in the lamb, rabbit, and rat ileum and colon and elicits a dose-dependent response. The volume of fluid response, the level of viscosity, and the amount of hemorrhage increased proportionately at higher challenge doses. The purified enterotoxin increases the levels of sodium and chloride in the intestinal loop in all animal species. We suspect, however, that this increase in electrolytes is more likely due to the tissue damage rather than active transcellular chloride secretion.

In our studies, the ileal loop assay in the rabbit was more variable than the assay in lambs and the responses were weaker. These observations corroborate the results from previous studies indicating that enterotoxigenic strains and crude filtrates elicit a weaker response in rabbits than in lambs (24, 25). The weaker and more variable response in the rabbit ileum may be attributed to the physiology of the rabbit ileum, which also gives variable responses with cholera toxin (18). On the other hand, the rabbit colonic loop assay gave consistently higher volume-to-length ratios than that of colonic loops in lambs. In the rat, there was dramatically more tissue damage, with a similar or lower amount of fluid loss per centimeter, than in the rabbit or lamb. The more extensive damage may be, at least in part, because the rat lumen is narrower and the loop lengths were smaller than those of the lamb or rabbit intestine. The rat loop assay represents an alternative and cheaper model to study the histology, pathology, and mechanism of action of the purified enterotoxin in vivo. Thus, the rat intestinal loop model may facilitate future studies of the role of this enterotoxin in increased mucosal turnover and bowel physiology.

Collins et al. (4) and Duimstra et al. (8) examined the pa-

thology associated with the enterotoxigenic strains of *B. fragilis* in the colons of piglets. Their observations show that only enterotoxigenic strains caused crypt hyperplasia and exfoliation of epithelial cells with the accumulation of neutrophils and inflammation of the villus. Furthermore, Myers et al. (20, 24, 25) examined the pathology of enterotoxigenic strains and culture filtrates from enterotoxigenic strains of *B. fragilis* injected into lambs and rabbits. They observed ultrastructural lesions, accumulation of fluid subsequent to the detachment and rounding of epithelial cells, and glandular crypt elongation with a mild efflux of neutrophils into the lamina propria. Our results showed that the purified enterotoxin caused extensive tissue damage similar to that caused by pure cultures and culture filtrates from enterotoxigenic strains. This damage included extensive exfoliation of individual surface epithelial cells, crypt elongation, and hyperplasia, with an attenuated intestinal mucosa. This type of damage was observed in all species in ileal as well as colonic loops. There were significant levels of albumin in the fluid responses in the lamb and rabbit, both in the ileum and colon. We believe this increase in protein was most likely due to leakage of serum proteins into the intestine and to proteins from the sloughed epithelial cells. In an initial time course experiment with lambs (data not shown), there was very little fluid secreted into the loop at up to 10 h; however, some characteristic tissue damage was evident. These data are consistent with the results of Myers et al. (20), who showed that the fluid response was secondary to the sloughing of epithelial cells caused by enterotoxigenic strains. Collectively, these findings support the idea that the fluid response resulted from the tissue damage and not active secretion.

Our next series of studies were designed to determine if factors that inactivate the protease activity of the enterotoxin also inactivate the enterotoxic and cytotoxic activity. We showed in previous studies that the protease activity is inactivated by incubation with the chelating agents EDTA and 1,10-phenanthroline. In the present study, when we injected enterotoxin treated with EDTA or 1,10-phenanthroline into loops, the enterotoxin was no longer enterotoxic and no hemorrhage was observed. This was true for all species, both in the ileum and colon. The cell-rounding activity was also inhibited by EDTA and 1,10-phenanthroline. When we added back zinc to the chelated enterotoxin, both the cell-rounding and entero-

toxic activities were restored to some extent. These findings support the idea that the protease activity is responsible for both the cell-rounding and enterotoxin activity.

The results of our studies raise the interesting question of how a metalloprotease acts as an enterotoxin. Other metalloproteases that have a similar zinc motif, such as those included in our study, do not cause this type of response in the intestine. This may be due to different substrate specificities. The enterotoxin may have a lower K_m for a particular substrate, or the environment of the intestine may be optimal for this enzyme and not for the other metalloproteases; alternatively, this protease may gain access to the cytosol of intestinal cells. Our laboratory is currently investigating why this metalloprotease is so potent in the intestine and how it differs from other metalloproteases. We are identifying the proteins cleaved by the enterotoxin on the intestinal mucosa and determining whether or not the enterotoxin is being internalized by the intestinal epithelial cells. We are also using tissue culture cells in situ to determine how the proteolytic activity alters tight junctions and if there is an internal effect on the cytoskeleton resulting in cell rounding. These types of studies will give us new information on the mechanisms of this metalloprotease and how it alters the barrier function of the intestinal epithelium.

ACKNOWLEDGMENTS

We thank Geoff Saunders and Amelia Walton for their assistance in the histology staining and interpretations. Also, we thank Eddy Carter and David Moore for their assistance in the loop assays and Stephanie Gaulding for maintaining the HT-29 cells.

This work was supported by Public Health Service grant AI 322940 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

- Booth, B. A., M. Boesman-Finkelstein, and R. A. Finkelstein. 1984. *Vibrio cholerae* hemagglutinin/protease nicks cholera enterotoxin. *Infect. Immun.* **45**:558–560.
- Border, M. M., B. D. Firehammer, D. S. Shoop, and L. L. Myers. 1985. Isolation of *Bacteroides fragilis* from the feces of diarrheic calves and lambs. *J. Clin. Microbiol.* **21**:472–473.
- Chowdhury, M. A. R., S. L. Miyoshi, and S. Shinoda. 1990. Role of *Vibrio mimicus* protease in enterotoxigenicity. *J. Diarrhoeal Dis. Res.* **9**:322–334.
- Collins, J. E., M. E. Bergeland, L. L. Myers, and D. S. Shoop. 1989. Exfoliating colitis associated with enterotoxigenic *Bacteroides fragilis* in a piglet. *J. Vet. Diagn. Invest.* **1**:349–351.
- Cury, J. D., E. J. Campell, C. J. Lazarus, R. J. Albin, and H. G. Welgus. 1988. Neutrophil activation. *J. Immunol.* **141**:4306–4312.
- DasGupta, B. R., and W. Tepp. 1993. Protease activity of botulinum neurotoxin type E and its light chain: cleavage of actin. *Biochem. Biophys. Res. Commun.* **190**:470–474.
- Dowling, J. N., A. K. Saha, and R. H. Glew. 1992. Virulence factors of the family *Legionellaceae*. *Microbiol. Rev.* **56**:32–60.
- Duimstra, J. R., L. L. Myers, J. E. Collins, D. A. Benfield, D. S. Shoop, and W. C. Bradbury. 1991. Enterovirulence of enterotoxigenic *Bacteroides fragilis* in gnotobiotic pigs. *Vet. Pathol.* **28**:514–518.
- Galloway, D. R. 1991. *Pseudomonas aeruginosa* elastase and elastolysis revisited: recent developments. *Mol. Microbiol.* **5**:2315–2321.
- Hase, C. C., and R. A. Finkelstein. 1993. Bacterial extracellular zinc-containing proteases. *Microbiol. Rev.* **57**:823–837.
- Holdeman, L. V., E. P. Cato, and W. E. C. Moore. 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg, Va.
- Kamata, R., K. Matsumoto, R. Okamura, T. Yamamoto, and H. Maeda. 1985. The serratal 56K protease as a major pathogenic factor in serratal keratitis: clinical and experimental study. *Ophthalmology* **92**:1452–1459.
- Kapur, R., and X. Shiriniwas. 1987. Effect of *Pseudomonas aeruginosa* protease and elastase on ligated rabbit ileal loops. *Indian J. Med. Res.* **86**:295–297.
- Lima, A. A. M., D. M. Lyerly, T. D. Wilkins, D. J. Innes, and R. L. Guerrant. 1988. Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestine in vivo and on cultured cells in vitro. *Infect. Immun.* **56**:582–588.
- Lyerly, D. M., L. Gray, and A. Kreger. 1981. Characterization of rabbit corneal damage produced by serratal keratitis and by a serratal protease. *Infect. Immun.* **33**:927–932.
- Lyerly, D. M., M. D. Roberts, C. D. Phelps, and T. D. Wilkins. 1986. Purification and properties of toxins A and B of *Clostridium difficile*. *FEMS Microbiol. Lett.* **33**:31–35.
- Moncrief, J. S., R. J. Obiso, Jr., L. A. Barroso, J. J. Kling, R. L. Wright, R. L. Van Tassel, D. M. Lyerly, and T. D. Wilkins. 1995. The enterotoxin of *Bacteroides fragilis* is a metalloprotease. *Infect. Immun.* **63**:175–181.
- Moon, H. W., and S. C. Whipp. 1971. Systems for testing the enteropathogenicity of *E. coli*. *Ann. N. Y. Acad. Sci.* **176**:197–211.
- Moore, W. E. C., E. P. Cato, and L. V. Holdeman. Some current concepts in intestinal bacteriology. *Am. J. Clin. Nutr.* **31**:S33–S42.
- Myers, L. L., J. E. Collins, and D. S. Shoop. 1991. Ultrastructural lesions of enterotoxigenic *Bacteroides fragilis* in rabbits. *Vet. Pathol.* **28**:336–338.
- Myers, L. L., B. D. Firehammer, D. S. Shoop, and M. M. Border. 1984. *Bacteroides fragilis*: a possible cause for acute diarrheal disease in newborn lambs. *Infect. Immun.* **44**:241–244.
- Myers, L. L., and D. S. Shoop. 1987. Association of enterotoxigenic *Bacteroides fragilis* with diarrheal disease in young pigs. *Am. J. Vet. Res.* **48**:774–775.
- Myers, L. L., D. S. Shoop, and T. B. Byars. 1987. Diarrhea associated with enterotoxigenic *Bacteroides fragilis* in foals. *Am. J. Vet. Res.* **48**:1565–1567.
- Myers, L. L., D. S. Shoop, and J. E. Collins. 1990. Rabbit model to evaluate enterovirulence of *Bacteroides fragilis*. *J. Clin. Microbiol.* **28**:1658–1660.
- Myers, L. L., D. S. Shoop, J. E. Collins, and W. C. Bradbury. 1989. Diarrheal disease caused by enterotoxigenic *Bacteroides fragilis* in infant rabbits. *J. Clin. Microbiol.* **27**:2025–2030.
- Myers, L. L., D. S. Shoop, B. D. Firehammer, and M. M. Border. 1985. Association of enterotoxigenic *Bacteroides fragilis* with diarrheal disease in calves. *J. Infect. Dis.* **152**:1344–1347.
- Myers, L. L., D. S. Shoop, L. L. Stackhouse, F. S. Newman, R. J. Flaherty, G. W. Letson, and R. B. Sack. 1987. Isolation of enterotoxigenic *Bacteroides fragilis* from humans with diarrhea. *J. Clin. Microbiol.* **25**:2330–2333.
- Sack, B. S., M. J. Albert, K. Alam, P. K. B. Neogi, and M. A. Akbar. 1994. Isolation of enterotoxigenic *Bacteroides fragilis* from Bangladeshi children with diarrhea: a controlled study. *J. Clin. Microbiol.* **32**:960–963.
- Sack, R. B., L. L. Myers, J. Aleido-Hill, D. S. Shoop, W. C. Bradbury, R. Reidand, and M. Santosham. 1992. Enterotoxigenic *Bacteroides fragilis*: epidemiologic studies of its role as a human diarrheal pathogen. *J. Diarrhoeal Dis. Res.* **10**:4–9.
- Schiavo, G., F. Benfenati, B. Poulain, O. Rossette, P. P. de Laureto, B. R. DasGupta, and C. Montecucco. 1992. Tetanus and botulinum B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature (London)* **359**:832–835.
- Seifert, S., and E. Harper. 1971. The collagenases, p. 649–697. *In* P. D. Boyer (ed.), *The enzymes*. Academic Press, Inc., New York.
- Simon, G. L., and S. L. Gorbach. 1984. Intestinal health and disease. *Gastroenterology* **86**:174–193.
- Van Tassel, R. L., D. M. Lyerly, and T. D. Wilkins. 1992. Purification and characterization of an enterotoxin from *Bacteroides fragilis*. *Infect. Immun.* **60**:1343–1350.
- Weikel, C. S., F. D. Greico, and L. L. Myers. 1991. Production and partial purification of a *Bacteroides fragilis* enterotoxin, 25–28. *In* Abstracts of the 27th Joint Conference on Cholera and Related Diarrheal Diseases, Charlottesville, Va.
- Weikel, C. S., F. D. Greico, J. Reuben, L. L. Myers, and R. B. Sack. 1992. Human colonic epithelial cells, HT-29/C₁, treated with crude *Bacteroides fragilis* enterotoxin dramatically alter their morphology. *Infect. Immun.* **60**:321–327.
- Wretling, B., and O. R. Pavlovskis. 1983. *Pseudomonas aeruginosa* elastase and its role in pseudomonas infections. *Rev. Infect. Dis.* **5**(Suppl.):S998–S1004.