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The *Bacteroides fragilis* Toxin Fragilysin Disrupts the Paracellular Barrier of Epithelial Cells

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Bacteroides fragilis **is a member of the normal colonic microflora of most mammals and is the most commonly isolated anaerobe from human clinical specimens. Some strains produce a toxin (fragilysin, a zinc-metalloproteinase) implicated as a cause of diarrheal disease in farm animals and humans. Studies in our laboratory confirm that the proteolytic activity of this toxin is responsible for the fluid secretion and tissue damage observed in vivo. In this study, we investigated the effects of fragilysin on the paracellular barrier of epithelial cells. Researchers suggest that, since the toxin rapidly intoxicates HT-29 cells, it may be internalized. However, we could not prevent cell rounding by using inhibitors of receptor-mediated endocytosis, which indicates that the toxin may act outside the cell. Based on these observations, we studied the effects of the highly purified *B. fragilis* fragilysin on the barrier function of cultured epithelial cells. Fragilysin rapidly increased the permeability of the paracellular barrier of epithelial cells to ions (decrease in electrical resistance across monolayers) and to larger molecules (increase in mannitol flux across monolayers). We tested a human colon cell line and cell lines from the lung and the kidney; the human colon cell line was most sensitive, but all three were affected in the same manner. Our studies show that *B. fragilis* fragilysin alters the barrier function of the epithelial lining, possibly by degrading the tight junction proteins, such as ZO-1. The proteolytic activity is required to cause this effect. The toxin's action has been assumed to be limited to the intestine; however, our studies show that fragilysin could also contribute to the pathogenesis of *B. fragilis* in extraintestinal infections.**

Bacteroides fragilis is an important clinical pathogen and is the most common anaerobe isolated from human clinical specimens (30). It is associated with abscesses, soft tissue infections, and bacteremias (47). In the mid-1980s, Myers et al. reported that some strains of *B. fragilis* produced an enterotoxic factor that could be detected in lamb ileal loops (38). Since then, these enterotoxigenic *B. fragilis* strains have been implicated as the cause of diarrhea in calves (5, 37), piglets (8, 34), foals (35), rabbits (31), and, more recently, humans (28, 36, 40, 41, 43, 44, 46). Several animal models, including neonatal rabbits and neonatal germfree pigs, have been established to study the pathogenicity of enterotoxigenic strains (10, 32, 33, 39). Together, the results from these studies support the role of enterotoxigenic *B. fragilis* in gastrointestinal disease in humans and animals. Weikel et al. showed that culture filtrates from the enterotoxigenic strains caused rapid morphological changes on human colon carcinoma cell lines, particularly HT-29 (50). These effects are characterized by rounding of the cells and reorganization of F-actin. Furthermore, Donelli et al. confirmed the toxin's effect by showing that there was reorganization of the F-actin structure and membrane blebbing on HT-29 cells (9).

By using the HT-29 assay to monitor the toxin's activity, our laboratory purified the toxin from culture supernatants and characterized it as a single polypeptide (M_r, ~20,600) (49). We showed that the toxin gene codes for a zinc-containing metalloproteinase belonging to the metzincin family (4, 11, 48) and later confirmed that fragilysin was a proteinase (29). The purified metalloproteinase also caused fluid secretion and exfoliation of intestinal epithelial cells in vivo, which could be prevented with specific inhibitors of metalloproteinases (39). Recently, in collaboration with Wells et al., we showed that fragilysin increases the epithelial permeability of HT-29 enterocytes, which allows increased internalization of bacteria (51). Together, these data suggest that the mechanism of fragilysin's action may be to disrupt the epithelial paracellular barrier through proteolytic degradation, leading to the tissue damage and fluid secretion.

The epithelial barrier plays an active role in ion and water transport (6). Distinct structural and functional intercellular regions of the epithelial cell membrane help to maintain this barrier between biological compartments (18). These intercellular complexes are composed of specific proteins that regulate the passage of substrates and communication between cells (25). Damage to the epithelial barrier by bacterial toxins or proteolytic enzymes can alter these junctional complexes and promote bacterial translocation, inflammation, and infection (14). This damage will also affect the transport and function of the epithelium (7).

Our study was undertaken to answer the following questions. First, does *B. fragilis* fragilysin enter the cell, or does it cause its effect from outside the cell like other metalloproteinases? Second, does the toxin affect the paracellular barrier (the tight junctions) of cells? Third, is the proteolytic activity responsible for these actions? And fourth, is this action confined to the intestinal epithelium, or are other epithelial junctional complexes affected?

To answer these questions, we used tissue-cultured cells from the rat lung epithelium (type II alveolar cells), the canine kidney epithelium (MDCK; ATCC CRL 6253), and the human intestine (HT-29; ATCC HTB 38) to determine if fragilysin disrupts the barrier function of these epithelial cells. We also

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used immunocytochemistry to determine if the tight junctions were being altered. Our results support the hypothesis that fragilysin disrupts the paracellular barrier and that the proteolytic activity is responsible for this effect. The toxin's action on the tight junction complexes may contribute to the pathogenesis of *B. fragilis* in both intestinal and extraintestinal infections in humans and animals.

MATERIALS AND METHODS

Reagents. *Pseudomonas aeruginosa* elastase was supplied by U.S. Biochemical (Amersham Biochemical, Arlington Heights, Ill.). *Clostridium difficile* toxin A was a gift from J. Scott Moncrief (TechLab, Inc., Blacksburg, Va.). All other reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise stated.

Preparation of purified *B. fragilis* fragilysin. *B. fragilis* VPI 13784 was grown anaerobically according to standard methods described in the Virginia Polytechnic Institute *Anaerobe Laboratory Manual* (17). Fourteen-liter batches of brain heart infusion broth supplemented with hemin (4.96 mg/liter), vitamin K (0.5 μl/liter), and sodium bicarbonate (2 g/liter) were inoculated (0.5 ml) with an 8-h turbid culture of *B. fragilis* VPI 13784 and incubated at 37°C for 14 to 16 h as previously described (49). The culture was then passed through a Pelicon filter unit with a 0.45-μm-pore-size membrane (Millipore, Bedford, Mass.). Fragilysin was purified as previously described (49). Briefly, fragilysin 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 toxin was monitored by its characteristic rounding of HT-29 cells and hydrolysis of rabbit skeletal muscle actin by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (29). Fragilysin was homogeneous, as shown by SDS-PAGE. Purified toxin was frozen at −30°C immediately after elution from the Mono-Q column to assure maximal biological activity.

Fragilysin activity. To ensure equivalent levels of biological activity, purified fragilysin preparations were diluted to 1 μg/ml and assayed by the cell rounding assay as described elsewhere (39).

Removal of zinc and reconstitution studies. Purified fragilysin (5 μM, based on an estimated molecular weight of 20,600) was incubated with 10 mM EDTA for 1 h at 37°C in 10 mM phosphate-buffered saline (PBS), pH 7.4. The solution was then dialyzed for 48 h at 4°C against PBS. For reconstitution experiments, the chelated toxin was incubated with 100 μM zinc chloride for 1 h at 37°C followed by dialysis for 24 h at 4°C against PBS. Chelated and reconstituted toxin preparations were stored at −30°C.

Protein assay. Protein concentrations were determined with the Pierce Coomassie Protein Assay Kit (Pierce, Rockford, Ill.) as recommended by the manufacturer. Bovine serum albumin (BSA) was a standard to determine protein concentrations. Absorbance values were measured at 595 nm on a Beckman DU-70 spectrophotometer (Beckman, Fullerton, Calif.).

Cell culture. Unless otherwise noted, HT-29 cells were grown in Dulbecco's minimal essential medium base, supplemented with 4.5 g of D-galactose per liter, 0.11 g of sodium pyruvate per liter, 0.016 g of phenol red per liter, 3.7 g of sodium bicarbonate per liter, and 10% fetal bovine serum. In the absence of glucose in the culture medium, HT-29 cells are highly differentiated and the cells form highly polarized monolayers (23). MDCK cells were grown in Dulbecco's minimal essential medium containing 4.5 g of D-glucose per liter and supplemented with 10% fetal bovine serum. Rat lung type II cells were grown in minimal essential medium supplemented with 10% calf serum for 48 h and then changed to Ham's F-12 medium supplemented with 10% calf serum (1). All cells were incubated in a humidified incubator at 37°C with 5% CO_2.

Internalization studies. Confluent monolayers of HT-29 cells were grown as described above to determine if the toxin was entering the cells by endocytosis. Confluent HT-29 cells were washed three times with sterile PBS, before addition of either fresh medium or medium supplemented with chloroquine (0.1, 0.5, or 1 mM) or ammonium chloride (0.1, 0.5, or 1 mM). The cells were incubated in a humidified chamber at 37°C for 4 h. The *B. fragilis* toxin was added (0.01, 0.1, 1, or 10 μg/ml) in serial 1:2 and 1:4 dilutions. The highest concentrations of chloroquine and ammonium chloride used in this study were determined by titration and represented the maximum doses that did not cause any detectable effect on the cell. Because the *B. fragilis* toxin causes rapid cell rounding on HT-29 cells, the assay was read at 1, 2, 4, 6, and 8 h after addition of toxin. Control experimental mixtures with *C. difficile* toxin A were added to HT-29 cells in 10-fold dilutions under the same conditions as above. HT-29 cells treated with toxin were then incubated at 37°C for 4 h, and the cell rounding units were determined as previously described (39).

Cell injury assay. Confluent monolayers of MDCK and HT-29 cells were grown on porous cell culture membrane inserts (12-mm diameter, 0.4-μm pores) (Costar, Cambridge, Mass.) and labeled with sodium chromate (^{51}Cr, 2 μCi per insert; ICN, Irvine, Calif.) to assess cell injury by *B. fragilis* fragilysin. Cells were labeled overnight at 37°C, and after being washed three times with fresh medium to remove unbound radioactivity, they were treated either with *B. fragilis* fragilysin (10 ng/ml, 100 ng/ml, 1 μg/ml, or 10 μg/ml) or with the same concentration of *P. aeruginosa* elastase, or medium alone, and were incubated in a humidified incubator at 37°C for up to 12 h. Samples of culture supernatant in 100 μl were taken from each well at 1, 2, 4, 8, and 12 h, without disturbing the monolayers; radioactivity (in counts per minute) was measured in a gamma counter (Beckman Instruments). Total ^{51}Cr release was determined by lysing the cells with a 2% SDS solution, which served as an internal control. The percent ^{51}Cr release was calculated according to the following equation: percent release = (counts per minute of supernatant/counts per minute of total release) × 100. The amount released by the treatments was compared with that released by the control, PBS alone. Trypan blue dye exclusion was also used to determine whether the toxin had cytotoxic effects on MDCK cells. Control and treated monolayers on the porous cell culture membrane inserts (12-mm diameter, 0.4-μm pores) were incubated with 0.5% trypan blue for 5 min and washed with PBS, and the average numbers of stained (injured) cells in five separate microscopic fields were determined.

Electrical resistance measurements. MDCK, HT-29, and rat lung cell monolayers were grown on porous cell culture membrane inserts (12-mm diameter, 0.4-μm pores), and their electrical resistance was measured with the EVOM electrical resistance system (World Precision Instruments, New Haven, Conn.). Inserts with no cell monolayers served as blanks to determine the baseline resistance, and those inserts with confluent cell monolayers treated with PBS served as controls. *B. fragilis* fragilysin or *P. aeruginosa* elastase was added at concentrations ranging from 1.0 ng/ml to 10 μg/ml. The transepithelial electrical resistance (TER) (ohms × square centimeter) was calculated from the following equation: $(TER_{sample} - TER_{blank}) \times$ surface area.

Mannitol flux assay. HT-29, MDCK, and rat lung cells were grown to confluency on membrane inserts, and their electrical resistance was measured as described above. Tritiated mannitol (ICN) was added at a final concentration of 0.5 μCi/ml to the apical medium. Then, *B. fragilis* fragilysin or *P. aeruginosa* elastase was added to the apical or basolateral side of the membrane inserts at concentrations ranging from 10 ng/ml to 10 μg/ml. The membrane inserts were then incubated at 37°C for up to 24 h in a humidifed incubator. Medium (50 μl) from the apical or basolateral compartments was captured with a filtered pipette tip, and the radioactivity was recorded for each compartment in an LS 8100 liquid scintillation counter (Beckman Instruments). For each well, the mannitol permeability coefficient (i.e., mannitol flux) was calculated by the following equation: $P_m = F_{ab} \times (\text{mann})_a^{-1} \times S^{-1}$, where P_m is the mannitol permeability (square centimeters per second), F_{ab} is in counts per minute in the basolateral or apical medium tested, $(\text{mann})_a$ is the total count recovered from medium per milliliter, and S is the surface area of the insert filter. The percent flux was determined by dividing the experimental values by the PBS control values times 100.

Tight junction resistance recovery assay. To test the effects of *B. fragilis* fragilysin on the recovery of the TER, we used the methods of Gumbinar and Simons (12) with the following modifications. Monolayers of MDCK cells were grown on membrane inserts (12-mm diameter, 0.4-μm pores) until they reached confluency and expressed high electrical resistance values (250 to 300 $\Omega \times cm^2$ for HT-29 cells, 1,400 to 2,200 $\Omega \times cm^2$ for MDCK and rat lung cells). Calcium-free medium was then added, and at the baseline electrical resistance, *B. fragilis* fragilysin was added (10, 50, and 100 ng/ml) with the calcium recovery medium. Electrical resistance measurements were taken at various intervals to detect the toxin's effects on the recovery of the tight junctions.

Immunohistochemistry of ZO-1. We used immunohistochemical techniques to assess the effect of fragilysin on a specific junctional protein, ZO-1. Replicate MDCK cell monolayers were grown on glass slides and treated with toxin (100, 500, or 1,000 ng/ml), elastase (10, 100, or 500 ng/ml), or PBS for 4 h at 37°C. Cells were washed three times for 2 min in a PBS–1 mM EDTA solution, simultaneously fixed and made permeable with chilled (−20°C) methanol for 10 min, washed three times with PBS, and incubated with a 1% BSA-PBS solution for 30 min. The cells were incubated with monoclonal antibody against the ZO-1 protein (Chemicon, Temecula, Calif.) in a 1:200 dilution, incubated at 37°C for 1 h, washed with BSA-PBS, and developed with a 1:1,000 dilution of fluorescein-conjugated rabbit anti-rat immunoglobulin. After 1 h at 37°C and three additional washes with BSA-PBS, the glass slides were mounted with a coverslip and examined by epifluorescence with a Zeiss IM-35 microscope with a 63×, 1.4 numerical aperture objective and narrow pass fluorescein filter set (Garden City, N.Y.). The samples were photographed with a charge-coupled device Cohu 4915 camera under the control of IPLab Spectrum software (Signal Analytics, Vienna, Va.) with capture to a Macintosh computer with a Scion LG-3 frame grabber board (Scion, Gaithersburg, Md.).

RESULTS

***B. fragilis* fragilysin—purity and activity.** The purified toxin yielded a single band on SDS-PAGE at a molecular weight of ~20,600. We monitored the biological activity of the purified preparations (diluted to 1 μg) immediately before performing assays to ensure consistency between sample preparations and purification batches. Cell rounding titers (in cell rounding units

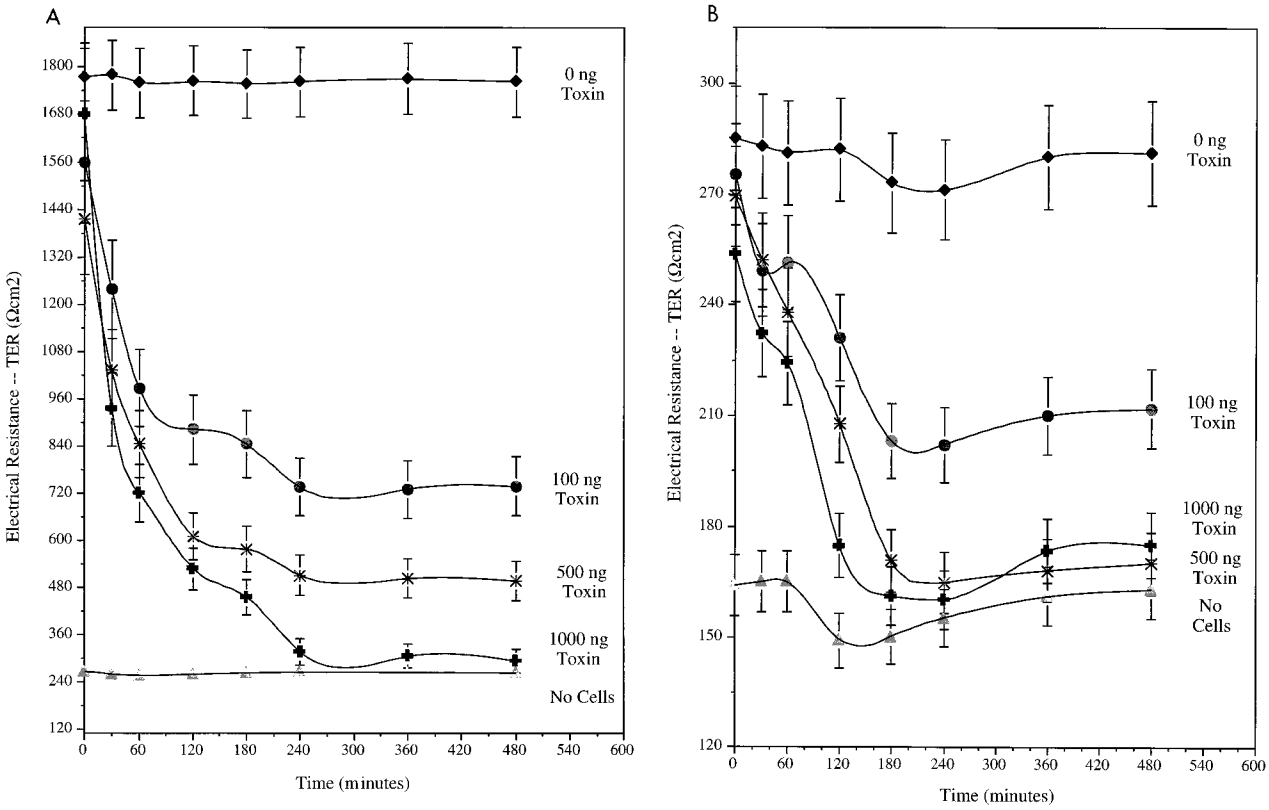


FIG. 1. Fragilysin decreases the electrical resistance across HT-29 (human colon epithelium) (B) and MDCK (canine kidney epithelium) (A) cell monolayers. The responses are time and dose dependent. The data points represent the means and standard deviations for triplicate samples from three experiments.

[CRU]) were consistently between 2,084 and 2,187 CRU/μg at 4 h for native toxin samples. Toxin exposed to 65°C for 30 min was inactive and produced no cell rounding. Toxin treatment with EDTA reduced the titers to approximately 128 to 196 CRU/μg at 4 h. Chelated toxin that was partially reconstituted with zinc had titers of 512 CRU/μg at 4 h.

Internalization studies. Chloroquine and ammonium chloride were used to ascertain if these inhibitors could prevent the rounding of HT-29 cells caused by the toxin. The titer of the toxin was determined at concentrations of 0.01, 0.1, 1, or 10 μg/ml in tissue culture wells with and without the inhibitors. There was no decrease in cell rounding titer at any time point when the inhibitor chloroquine or ammonium chloride was used. *C. difficile* toxin A, an exotoxin internalized by receptor-mediated endocytosis, was used as a control. We observed a 99% inhibition of the cytotoxic effect with these inhibitors under the same conditions. All toxin internalization assays were done in duplicate in two separate trials and gave similar results. Our data agree with those of Saidi and Sears (45), who reported that the *B. fragilis* toxin (fragilysin) does not intoxicate HT-29 cells by pH-sensitive endocytosis.

Electrical resistance measurements. Changes in the TER of MDCK, HT-29, and rat lung type II cells exposed to the *B. fragilis* toxin are shown in Fig. 1. The *B. fragilis* toxin decreased the electrical resistance across previously tight monolayers of all cell lines tested. These effects were time and dose dependent. In 6 h, apical treatment of a low concentration of toxin (100 ng/ml) decreased the TER of the monolayers by 40% in MDCK cells (Fig. 1A), 18% in HT-29 cells (Fig. 1B), and 28% in rat lung cells (see Fig. 3); at a higher concentration of toxin (1 μg/ml), the toxin reduced the electrical resistance by 87% in MDCK cells (Fig. 1A), 40% in HT-29 cells (Fig. 1B), and 62% in rat lung cells (see Fig. 3). Heat-inactivated toxin did not alter the permeability. Also, toxin treated with metal chelators prevented the decrease in electrical resistance by 75 to 85%, depending on the cell line used. *Pseudomonas* elastase, used as a positive control, decreased the electrical resistance to approximately the same extent as the *B. fragilis* toxin in the HT-29 and MDCK monolayers.

Interestingly, there was a more acute electrical resistance drop (within the first hour of toxin exposure) when toxin was added to the basolateral compartment compared to when toxin was added to the apical compartment. This suggests that the toxin has easier access to the extracellular matrix proteins and junctional complex components through the basolateral side. After the second hour of toxin exposure, this basolateral effect diminished and the drop was equal to that for toxin added to the apical compartment at the same concentration (data not shown).

In MDCK and rat lung cells, the general appearance of the cells did not change during the experiment, suggesting that the toxin's effects were subtle; furthermore, the cells did not become round or detach from the surface. With HT-29 cells, rounding of cells was obvious by the second hour, indicating that these cells are more sensitive to the toxin. There could be differences in sensitivity to the toxin between different species of cells, especially with regard to the amounts and types of extracellular matrix proteins expressed.

^{51}Cr release assay. Cells were labeled with ^{51}Cr prior to addition of *B. fragilis* toxin or *P. aeruginosa* elastase to determine whether the decreased resistance of the monolayers was caused by injury to the cell membrane. Parallel cultures of

TABLE 1. Effects of *B. fragilis* toxin on ^{51}Cr release from MDCK and HT-29 cells in relation to TER[a]

Treatment (ng/ml)	MDCK		HT-29	
	% ^{51}Cr release (± SD)	% Decrease in TER (± SD)	% ^{51}Cr release (± SD)	% Decrease in TER (± SD)
PBS	4.8 ± 3.5	None	3.9 ± 2.7	None
P. aeruginosa elastase				
500	4.5 ± 0.5	51 ± 4.1	3.6 ± 1.1	48 ± 5.3
1,000	4.7 ± 0.6	62 ± 3.8	5.2 ± 2.3	61 ± 3.6
B. fragilis toxin				
500	3.1 ± 0.3	37 ± 4.6	4.1 ± 0.7	43 ± 3.7
1,000	4.3 ± 0.7	55 ± 6.3	5.4 ± 1.8	52 ± 1.7

[a] Data are triplicate measurements from two separate experiments. Cells were labeled with 2 μCi of ^{51}Cr sodium chromate 24 h prior to treatment. Then the cells were washed extensively with PBS before sample addition. Aliquots were taken every 2 h for 12 h. Data reported are at 12 h.

labeled and unlabeled cells were incubated with *B. fragilis* toxin (0.01, 0.1, 1, and 10 μg/ml) or *P. aeruginosa* elastase (0.01, 0.1, 1, and 10 μg/ml). We measured the TER of the unlabeled monolayers and the release of radioactivity from these labeled cells. Although electrical resistance dropped in MDCK and HT-29 cells, there was no increase in the release of ^{51}Cr. Table 1 shows that there was no significant ^{51}Cr released from cells treated with the toxin or elastase. Furthermore, greater than 95% of the *B. fragilis* toxin- and *P. aeruginosa* elastase-treated cells remained intact and metabolically viable during the experiment, as shown by trypan blue exclusion. These data indicate that the toxin does not injure or damage the membranes of cells and that they are not killed by the toxin. The reduction of the electrical resistance across the monolayers in *B. fragilis*-fragilysin or *P. aeruginosa* elastase-treated cells cannot be attributed to nonspecific membrane injury.

Mannitol flux assay. *B. fragilis* fragilysin increased the mannitol flux across previously tight monolayers in all cell lines. Low concentrations of toxin (100 ng/ml) increased the mannitol flux of the monolayers 3-fold in MDCK cells (Fig. 2A), 5.5-fold in HT-29 cells (Fig. 2B), and 1.5-fold in rat lung cells (Fig. 3). At a 10-fold higher concentration of toxin, mannitol flux increased by 12-fold in MDCK cells (Fig. 2A), 10-fold in HT-29 cells (Fig. 2B), and 3.5-fold in rat lung cells (Fig. 3). *B. fragilis* fragilysin treated with metal chelators inhibited the mannitol flux of previously tight junctions by approximately

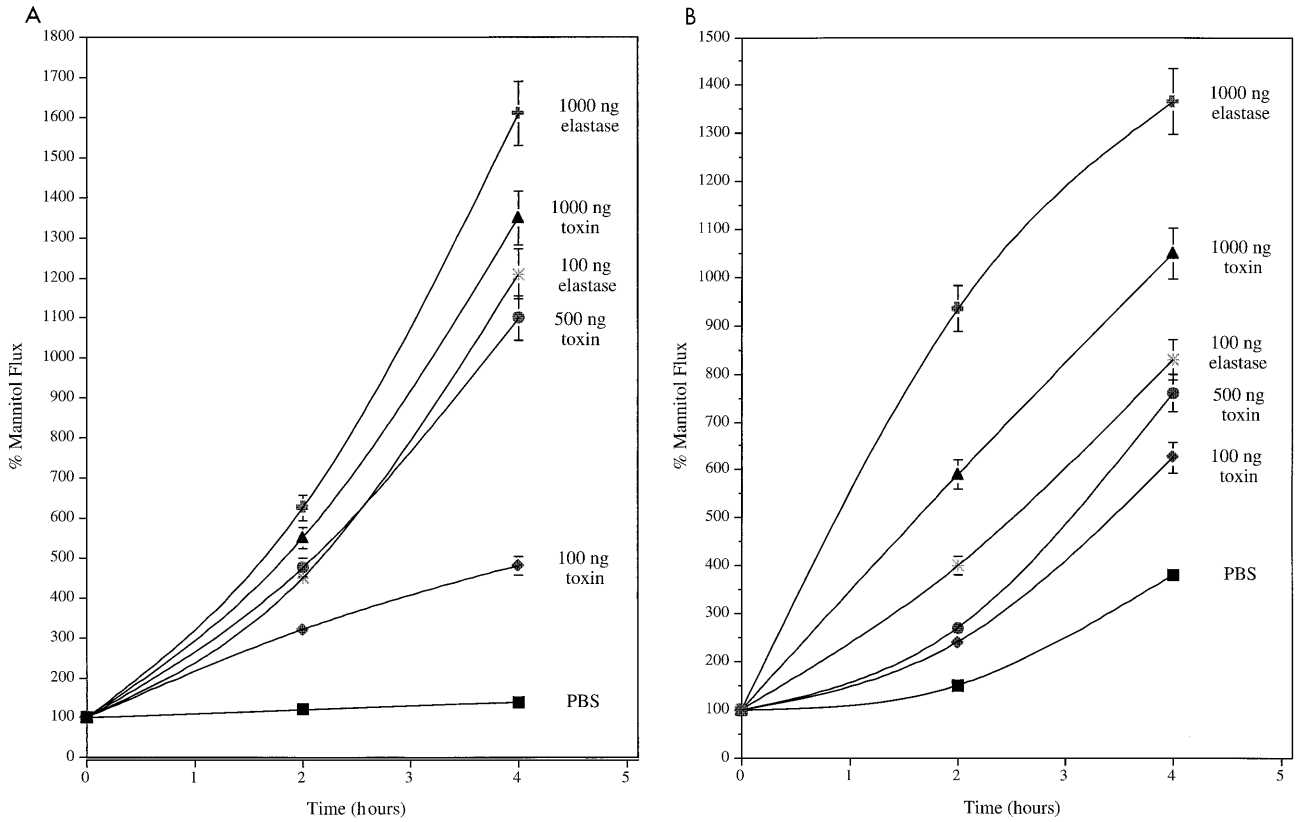


FIG. 2. Fragilysin increases the mannitol permeability (mannitol flux) across MDCK (canine kidney epithelium) (A) and HT-29 (human colon epithelium) (B) cell monolayers. The responses are time and dose dependent. The data points represent the means and standard deviations for triplicate samples from three experiments.

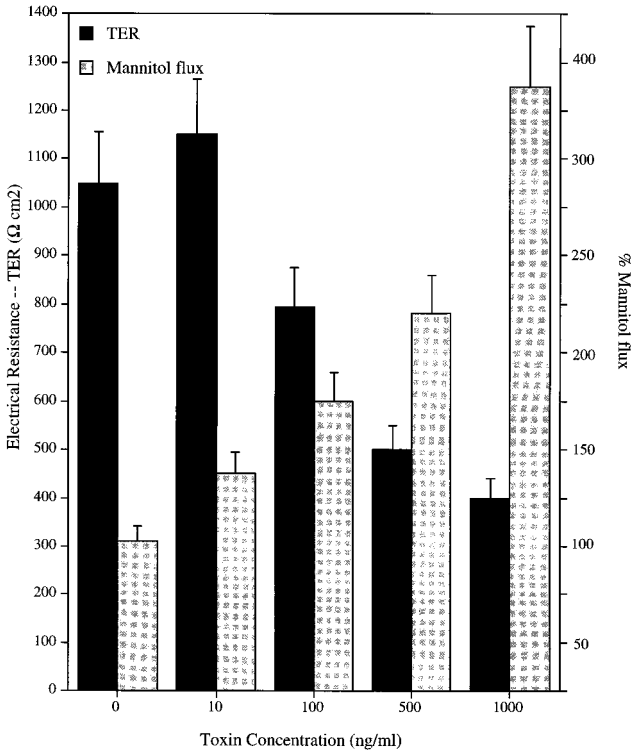


FIG. 3. Fragilysin decreases the electrical resistance with a concomitant increase in the mannitol permeability across rat type II (alveolar epithelial cell) cell monolayers. The responses are time and dose dependent. The data points represent the means and average standard deviations for triplicate samples from three experiments. Toxin was incubated at the indicated concentrations for 6 h at 37°C.

90% compared to native toxin at the same concentration; heat-inactivated toxin had no effect. Elastase also increased the mannitol flux to approximately the same extent in the HT-29 and MDCK cells; however, elastase increased the mannitol flux slightly better than the toxin in rat lung cells.

Recovery of tight junctions. Once we had evidence that the tight junctions of these cultured epithelial cells were being affected, we examined the effect of the toxin on the recovery of the tight junction through calcium flux. This ion is continuously needed to maintain a sealed junction; calcium also affects the opening and closing within the tight junction. In this experiment, we used MDCK cells that had reached confluency and had maintained tight junctions. The results are shown in Fig. 4. When calcium-free medium was added, the electrical resistance fell to near baseline in 60 min. We then added *B. fragilis* fragilysin at 0, 10, 50, and 100 ng/ml in calcium recovery medium. Control cells recovered within 8 h. The toxin had greatly increased the recovery time of the tight junction. At 10 ng of toxin per ml, the cells required three times as long to recover their tight junctions, and this recovery was only partial (650 $\Omega \times cm^2$) at 16 h. At 50 and 100 ng/ml, the cells would not recover unless they were washed several times to remove the proteinase. Then, after the normal recovery time, the electrical resistance of the tight junctions increased to previous levels. MDCK cells treated with chelated toxin did not recover fully within 12 h, but the recovery was nearly complete after 16 h. This indicates that the toxin has a direct effect on the tight junction.

Localization of the junctional protein ZO-1 by immunofluorescence. Figure 5 shows the results of experiments in which MDCK cells were treated either with heat-inactivated toxin or with 1 μg of *B. fragilis* fragilysin for 2 h (Fig. 5A and B, respectively) and 4 h (Fig. 5C). These figures show confluent monolayers in untreated samples; in the fragilysin-treated samples, only slight changes at the cell-to-cell contacts occurred. Figure 5 also shows the results for the MDCK cells when treated either with heat-inactivated toxin or with 1 μg of the *B.*

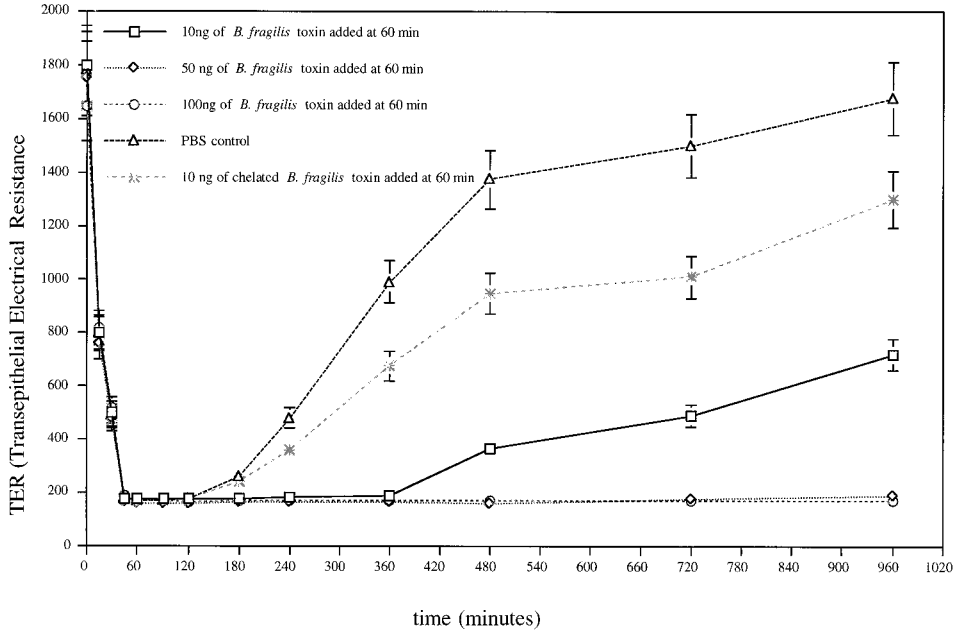
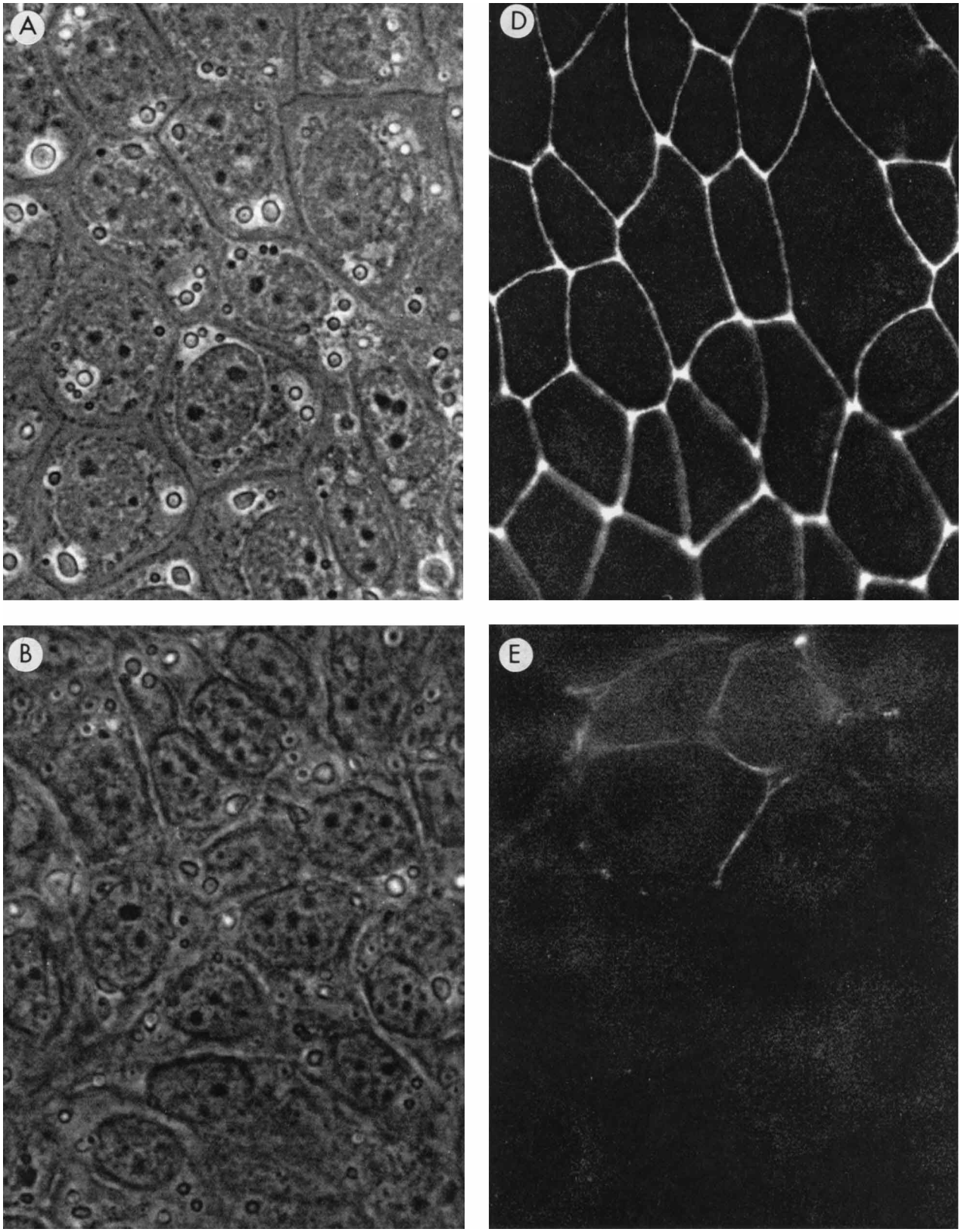


FIG. 4. Inhibition of recovery of transmonolayer resistance of MDCK cells by *B. fragilis* fragilysin. MDCK cell monolayers were incubated in a Ca^{2+}-free medium for 60 min at 37°C. Then, *B. fragilis* fragilysin was added at 10, 50, and 100 ng/ml with recovery medium for 16 h. MDCK cells treated with 10 ng/ml show recovery. Chelated toxin (10 ng/ml)-treated MDCK monolayers show a dramatic increase in their recovery. Values are the means of duplicate samples from two experiments.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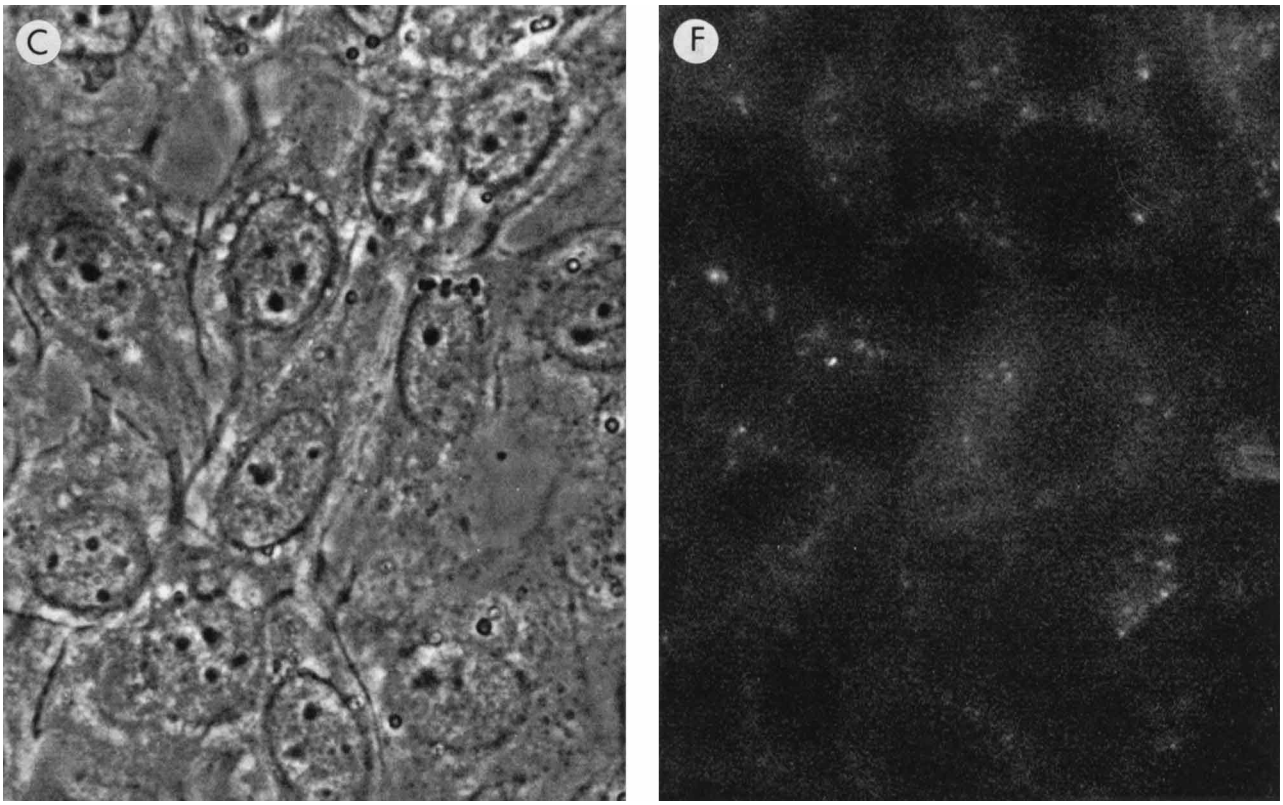

FIG. 5. (A to C) Bright-field micrographs of MDCK (canine kidney) monolayers after treatment with heat-inactivated fragilysin (A) or the *B. fragilis* toxin for 2 h (B) and 4 h (C). Fragilysin shows no obvious morphological changes; however, there is slight detachment at the cell-to-cell contacts in fragilysin-treated cells. (D to F) Micrographs of MDCK (canine kidney) monolayers stained with a monoclonal antibody to ZO-1 after treatment with heat-inactivated fragilysin or the *B. fragilis* fragilysin. (D) Fluorescence micrograph of heat-inactivated fragilysin-treated MDCK monolayers immunolabeled for ZO-1. Note the intact tight junctions which create a border around the cells. (E) Fluorescence micrograph of a 2-h incubation of fragilysin-treated MDCK monolayer immunolabeled for ZO-1. The border staining is diminishing, indicative of an effect on the ZO-1 junctional protein. (F) Fluorescence micrograph of a 4-h incubation of fragilysin-treated MDCK monolayer immunolabeled for ZO-1. The border staining is further diminished, with very few ZO-1 molecules staining.

fragilis fragilysin for 2 h (Fig. 5D and E, respectively) and 4 h (Fig. 5F) and then stained for ZO-1. Fluorescence microscopy showed tightly adherent cells in a compact monolayer, and the microscopic appearance of the cells did not change with any of the toxin treatments. When these cells were immunostained for ZO-1, monolayers of control cells displayed a well-defined pattern of fluorescence in the perijunctional region (Fig. 5D). But, in monolayers treated with toxin, ZO-1 staining decreased over time, showing an alteration or loss of the ZO-1 protein (Fig. 5E and F). Elastase has been shown to degrade the tight junctional proteins and was used as a positive control (2). Elastase degraded ZO-1 as rapidly as *B. fragilis* fragilysin; however, degradation of the ZO-1 by elastase was complete, with no staining of the ZO-1 in 4 h (data not shown).

DISCUSSION

The intestinal epithelium, like all other epithelia in higher organisms, separates two environments by creating an efficient barrier against the penetration of microorganisms, their toxins, and other bacterial products (26). The junctions between these epithelial cells also regulate passage of micronutrients, ions, and phagocytes. Epithelial cells can do this because of two properties: (a) the ability to establish tight junctions and cellular adhesions that seal the intercellular space between the cells and (b) their polarization into an apical and a basolateral domain (7).

Tight junctions (zonula occludens) hold cells to each other and play an integral role in cellular architecture. They are composed of several proteins that act to seal the junctional space and that form an apparent fusion between the lateral membranes, which look like "kisses" on replicas of freeze fractured membranes (26). There seems to be a close relationship between the tight junction and the cytoskeleton at the attachment site on the plasma membrane (18, 24, 26). Several researchers have found that disruption or alteration of one of these components causes the disruption or alteration of the other (24, 26). For example, the *C. difficile* toxins cause the disorganization of the actin cytoskeleton through the UDP-glucosylation of a protein called rho (19); this disruption causes the actin cytoskeleton to rearrange and then the tight junctions between cells to open, which in turn increases the intestinal permeability (15, 16). Tight junctions also can be opened by the direct effect of proteinases; this is the mechanism of action of elastase from *P. aeruginosa* (2). This metalloproteinase degrades the tight junctions and basement membranes of the epithelial cells, which results in tissue damage and fluid accumulation in the lungs of infected patients and animals (2, 3, 42).

B. fragilis fragilysin increases the paracellular permeability of the intestinal epithelium. We have previously shown that fragilysin is a low-molecular-weight zinc-containing metalloproteinase, similar to that of elastase. Therefore, the most obvious mechanism of action would seem to be direct proteolytic degradation of the intestinal tight junctions and cell-to-cell con-

tacts. Although the intestinal tract must be protected from such direct degradation of the tight junctional proteins, our results suggest that this is indeed the mechanism by which this toxin causes diarrhea.

Our studies suggest that fragilysin may not enter the epithelial cells, since we found that inhibitors of receptor-mediated endocytosis do not inhibit the rounding of HT-29 cells. This contrasts with the *C. difficile* toxin, which renders its effects by internal action of the host cells. We also found that fragilysin is not lethal to the cells; trypan blue dye exclusion showed that >90% of the cells remained viable, even at high toxin doses. These data agree with those of Saidi and Sears (45), who reported that the *B. fragilis* toxin (fragilysin) intoxication does not involve pH-sensitive endocytosis.

B. fragilis fragilysin decreases the TER in cultured monolayers of epithelial cell lines from the colon, lung, and kidney in a dose- and time-dependent manner. Changes in the electrical resistance could be caused either by disruption of the cell membrane or by alteration of the tight junctions between cells. The results of our radiolabeling studies using chromium showed that there was no change in the integrity of the cell membranes treated with the *B. fragilis* metalloproteinase. The following findings, however, indicate that the toxin acts on the tight junctions to alter this electrical resistance. Firstly, the change in the electrical resistance is accompanied by an increase in mannitol flux across cell monolayers, which occurs only when intercellular spaces are opened between the cells. Secondly, there is a direct effect on the tight junction, because monolayers that were treated with the toxin resisted recovery of the functional tight junctions through calcium flux. Thirdly, we also observed a dramatic effect on the MDCK tight junction protein ZO-1. By the use of immunofluorescence, we were able to observe a decrease in the amount of this tight junction protein between the cells, and this decrease correlated with the amount of toxin and length of exposure.

We previously showed that the proteinase activity, enterotoxic activity, and cell rounding activity are all dependent on zinc and can be inhibited with metal chelators (29, 39). Metal chelators also prevented the effects of the toxin on the paracellular barrier, indicating that the proteinase activity also is responsible for these results.

The *B. fragilis* toxin is a member of a growing family of proteinases that can act as virulence factors (for recent reviews, see references 13 and 14). As previously mentioned, *P. aeruginosa* produces elastase, a metalloproteinase that increases the permeability of epithelial cells in the lung by degrading the tight junction and basement membrane proteins of cells (2, 3). *Serratia* species produce metalloproteinases that are responsible for tissue damage in the eye (20). *B. fragilis* metalloproteinase is a member of the metzincin family; some metzincins cause hemorrhage by degrading fibrin, fibrinogen, and the complement cascade proteins (27); other members of this family act by degrading host serine-proteinase inhibitors, which results in the activation of host serine proteinases from cells and neutrophils (21, 22). The wide variety of effects caused by metalloproteinases makes us wary of concluding that the enterotoxic effect is due solely to direct degradation of the tight junctions. Indeed, the amount of destruction is so massive in intestinal loops that we believe that some type of cascade phenomenon may be involved. Proteinases can activate cell-bound cytokines, cellular proteinases and enzymes, and other cellular information pathways, which could lead to increased destruction by host neutrophils and phagocytic cells and further exacerbate the destruction. We are concentrating our research on these possibilities. Still unanswered is the intriguing question of how a proteinase can get around the protection that the intestinal epithelium must have against its constant proteolytic environment.

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