**Clostridium difficile** Toxins May Augment Bacterial Penetration of Intestinal Epithelium

**Background:** *Clostridium difficile* can be recovered from many high-risk hospitalized patients receiving broad-spectrum antibiotic therapy. *Clostridium difficile* toxins A and B have been associated with increased intestinal permeability in vitro and there is growing evidence that increased intestinal permeability may be a common mechanism whereby enteric bacteria penetrate the intestinal epithelium.

**Hypothesis:** *Clostridium difficile*-induced alterations in the intestinal barrier facilitate microbial penetration of the intestinal epithelium, which in turn facilitates the translocation of intestinal bacteria.

**Design:** Mature Caco-2 enterocytes were pretreated with varying concentrations of toxin A or toxin B followed by 1 hour of incubation with pure cultures of either *Salmonella typhimurium*, *Escherichia coli*, or *Proteus mirabilis*. The effects of toxins A and B on enterocyte viability, cytoskeletal actin, and ultrastructural topography were assessed using vital dyes, fluorescein-labeled phalloidin, and scanning electron microscopy, respectively. The toxins' effects on bacterial adherence and bacterial internalization by cultured enterocytes were assessed using enzyme-linked immunosorbent assay and quantitative culture, respectively. Epithelial permeability was assessed by changes in transepithelial electrical resistance and by quantifying paracellular bacterial movement through Caco-2 enterocytes cultivated on permeable supports.

**Results:** Neither toxin A nor toxin B had a measurable effect on the numbers of enteric bacteria internalized by Caco-2 enterocytes; however, both toxins were associated with alterations in enterocyte actin, decreased transepithelial electrical resistance, and increased bacterial adherence and paracellular transmigration.

**Conclusion:** *Clostridium difficile* toxins A or B may facilitate bacterial adherence and penetration of the intestinal epithelial barrier.

**Arch Surg.** 1999;134:1235-1242

**From the Departments of Surgery (Drs Feltis and Wells and Mr Kim), Laboratory Medicine and Pathology (Dr Wells and Ms Kinneberg), and Genetics, Cell Biology and Development (Dr Erlandsen), University of Minnesota, Minneapolis; and the Department of Biochemistry and Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg (Drs Lyerly and Wilkins). Drs Wilkins and Lyerly are co-owners of TechLab Inc, Blacksburg, Va. This company manufactures and sells intestinal diagnostic assays. One of these products is an enzyme-linked immunosorbent assay that detects *Clostridium difficile* toxins A and B in stool samples.
MATERIALS AND METHODS

BACTERIA, TOXINS, AND CULTURED ENTEROCYTES

Salmonella typhimurium ATCC 14028 was obtained from the American Type Culture Collection, Rockville, Md. Proteus mirabilis M13 and E coli M21 are rodent isolates. These species of enteric bacteria have been used in several in vivo studies of oral infectivity in rodents27 as well as in vitro studies of bacterial internalization by cultured enterocytes.28

Clostridium difficile toxin B (0.24 mg/mL) and toxin A (1 mg/mL) were purified as described,29,30 maintained in sterile normal saline at 4°C, and diluted in tissue culture medium for use in experiments. Toxin activity was confirmed by observation of characteristic cytopathic effect in human foreskin fibroblasts.30 After a 16-hour incubation, C difficile toxins A and B exhibited a cytopathic effect at 10 and 1 ng/mL, respectively.

Caco-2 cells, which are derived from a human colon cancer, have many features of differentiated enterocytes including apical microvilli, tight junctions, structural polarity, ion conductance, and permeability. Caco-2 cells are spontaneously differentiated and in vitro interactions have been shown to have in vivo relevance.31-35 Caco-2 cells, obtained from the American Type Culture Collection, were cultivated in the absence of antibiotics in 24-well plastic dishes (Falcon; Becton Dickinson Co, Lincoln Park, NJ), seeded at 2 × 10⁵ cells per well, and incubated at 37°C in 9.5% carbon dioxide for 15 to 18 days, at which time these enterocytes are considered polarized and differentiated.28,31 Viability was determined by the vital dyes trypan blue (0.36%) and propidium iodide (20 mg/mL).

BACTERIA-ENTEROCYTE INTERACTIONS

To study the effect of C difficile toxins A and B on bacterial internalization, enterocytes were pretreated for 16 hours with varying toxin concentrations. Bacteria were then added and bacterial internalization was assayed as described,28 with C difficile toxin present throughout the assay. Briefly, overnight tryptic soy broth (Difco Laboratories, Detroit, Mich) cultures of individual bacterial strains were washed twice and diluted in sterile isotonic sodium chloride solution, and 100 µl containing 10⁹ viable bacteria was added to each tissue culture well. Bacterial concentrations were determined by densitometry and confirmed by serial dilution, followed by viable plate counts on appropriate agar media. Bacteria were incubated with enterocytes for 1 hour at 37°C. Enterocytes were then washed 5 times with Hanks balanced salt solution (HBSS). Tissue culture medium containing 50 µg/mL of gentamicin sulfate was added to kill residual viable extracellular bacteria. After 2.5 hours, enterocytes were washed 5 times with balanced salt solution and lysed for 5 minutes with 1% Triton X-100 (Sigma-Aldrich Corp, St Louis, Mo). Viable intracellular bacteria were quantified following serial dilution and incubation on appropriate agar media.28

Experiments were designed to test the ability of C difficile toxins to modulate bacterial transmigration between Caco-2 cells. Using 3.0-µm membrane filter inserts with a 0.3-cm² surface area (Becton Dickinson Co), Caco-2 enterocytes were seeded at 3 × 10⁴ per membrane and cultivated as described earlier. Caco-2 cultures had varying concentrations of toxin A or B added to the apical chamber 16 hours prior to the migration assay. Bacteria (10⁶ per well) were then apically introduced, and enterocytes were incubated for 1 hour. The basal chamber was then sampled, serially diluted, and incubated on appropriate agar media.28

Transepithelial electrical resistance (TEER) was used to monitor changes (ohms per square centimeter) in epithelial associated enteric disease.19 Both toxins induce release of several proinflammatory monokines from cultured human monocytes.20 Toxins A and B are similar in size (308 kd and 269 kd, respectively), with 63% amino acid homology. Both toxins contain 3 recognizable domains, namely the amino terminus containing the active site, the carboxy terminus with repeating units responsible for binding, and a hydrophobic domain important for translocating the toxin across the vesicular membrane into the cytoplasm after internalization.21 There is evidence that the intracellular mechanism of action is enzymatic,21,22 with both toxins acting as glucosyltransferases to glycosylate the small guanosine 5C-triphosphatases (GTPases) Rho, Rac, and Cdc42.23-25 Glucosylation inactivates these GTPases, which are intimately associated with maintenance of the cellular cytoskeleton. Inactivation leads to cytoskeletal alterations26 and the resultant cytopathic effect characteristic of these toxins on cultured cells. Although much is known about the epidemiology and activity of C difficile toxins, the effects on bacteria-enterocyte interactions (adherence, internalization, and paracellular transmigration) are unstudied.

Because C difficile can be recovered from many high-risk hospitalized patients receiving broad-spectrum antibiotic therapy,17 because C difficile toxins A and B have
been associated with increased intestinal permeability in vitro, and because there is growing evidence that increased intestinal permeability may be a common mechanism whereby enteric bacteria penetrate the intestinal epithelium, we formulated the following hypothesis. *C. difficile*-induced alterations in the intestinal barrier facilitate microbial penetration of the intestinal epithelium, which in turn facilitates the translocation of intestinal bacteria, a likely initial event in the pathogenesis of systemic infection in many high-risk patients. Herein we report effects of *C. difficile* toxins A and B on bacterial interactions with cultured enterocytes. The data indicate that intestinal colonization with *C. difficile* may play a heretofore unrecognized role in facilitating bacterial transmigration across the intestinal epithelial barrier.

**RESULTS**

**BACTERIAL INTERNALIZATION, TEER, AND BACTERIAL MIGRATION THROUGH CONFLUENT Caco-2 ENTEROCYTES**

The effects of varying concentrations of *C. difficile* toxins A or B on Caco-2 internalization of *S. typhimurium*, *P. mirabilis*, and *E. coli* are presented in Figure 1. For internalization experiments, the maximum toxin A concentration was 10 ng/mL rather than 100 ng/mL, because the higher concentration resulted in enterocyte sloughing, likely facilitated by the multiple washes required by this protocol. Neither toxin had a noticeable...
Effect of Clostridium difficile toxins A (A) and B (B) on adherence of Salmonella typhimurium, Proteus mirabilis, and Escherichia coli to Caco-2 enterocytes as measured by enzyme-linked immunosorbent assay (described in the "Materials and Methods" section). Asterisks indicate significance at P < .05. Values represent mean ± SE of at least 10 tissue culture wells.

Effect of Clostridium difficile toxins A (A) and B (B) on internalization of any of the bacterial species tested (Figure 1).

Both toxin A and toxin B caused a dose-dependent decrease in TEER (r = 0.9 and 0.8, respectively) (Figure 2), indicating an opening of enterocyte tight junctions. Correspondingly, toxins A and B were also associated with dose-dependent increases in bacterial transmigration through mature Caco-2 enterocyte cultures (Figure 3).

BACTERIAL ADHERENCE TO Caco-2 ENTEROCYTES

To determine if increased bacterial transmigration (Figure 3) was related to increased bacterial adherence, enterocytes were pretreated with either 100 ng/mL of toxin B or 10 ng/mL of toxin A and bacterial adherence was assayed by enzyme-linked immunosorbent assay. Both toxins were associated with increased bacterial adherence and statistical significance was achieved with Proteus mirabilis and Escherichia coli (P < .05) (Figure 4). Wright-Giemsa stains of adherent bacteria (viewed by light microscopy) also confirmed that both toxins were associated with increased bacterial adherence.

ENTEROCYTE VIABILITY, MORPHOLOGY, AND FILAMENTOUS ACTIN

Pretreatment of Caco-2 cultures with 0, 1, 10, or 100 ng/mL of either toxin A or B had no apparent effect on viability, and enterocyte cultures were consistently 95% viable or higher. Wright-Giemsa staining showed noticeable separation of individual enterocytes, an effect that increased with increasing toxin concentrations (not shown). Ultrastructural observations of untreated (control) Caco-2 cultures showed confluent enterocytes with tightly apposed borders (Figure 5A). Although ultrastructural alterations appeared more pronounced with toxin A, both toxins caused distortions in apical microvilli, enterocyte rounding, and individual cells separated from each other (Figure 5B). Bacteria appeared preferentially adherent to distorted apical microvilli (Figure 5C and D). Following incubation of enterocytes with toxin A or toxin B, staining with fluorescein-labeled phalloidin revealed alterations in distribution of filamentous actin (Figure 6), and these cytoskeletal alterations appeared more prominent with toxin A than toxin B.

COMMENT

Although exact mechanisms whereby C difficile toxins A and B damage intestinal epithelium are not completely understood, there is evidence that both toxins have direct and indirect effects in vivo. Direct effects, manifested as cytoskeletal damage and increased paracellular permeability, likely result from toxin binding, internalization, and intracellular enzymatic activity.21-26 Directly, toxins A and B have been shown to damage human colonic explants20 as well as cultured enterocytes.11,12 Indirectly, studies involving ligated ileal loops in experimental animals have revealed that toxin A can activate lamina propria neuroimmune cells29 and cause infiltration of the submucosa by neutrophils.3 Chaves-Olarte et
al recently suggested that the initial event in *C. difficile* pathophysiology may be toxin A–induced activation of the enteric nervous system. This hypothesis assumes that toxin A binding to the intestinal mucosa results in signal transduction, resulting in secretion of proteins that signal neuroimmune cells to become effectors of inflammatory damage. Intracellular enzyme-mediated (glucosyltransferase) cytotoxicity then becomes important as a secondary mediator of damage.

Whether by a direct or indirect effect, both toxins increase intestinal permeability in explanted colonic tissue and in cultured enterocytes, and toxin A increases permeability in ligated intestinal loops. Hecht et al have shown that toxins A and B can increase permeability of cultured intestinal epithelium (T84 enterocytes) in the absence of inflammatory cells or their products. Using dual mannitol/sodium flux studies, this group also suggested that the toxin-induced permeability defect may be mediated by tight junctional disruption. Defects in this barrier potentially enhance transepithelial movement of peptides or macromolecules, including bacterial cell wall fragments and toxins that are known to induce intestinal inflammation.

Figure 5. Scanning electron micrographs of Caco-2 enterocytes showing ultrastructural effects of 16-hour incubation with 100 ng of *Clostridium difficile* toxin A. A. Untreated enterocytes with dense, relatively uniform apical microvilli and with individual enterocytes tightly apposed to each other. B. Rounding of toxin-treated enterocytes, with some adjacent enterocytes pulling apart from each other (arrows); microvilli appear elongated and distorted, with denuded enterocytes also evident. C. Preferential association of *Escherichia coli* with apical microvilli of toxin-treated enterocytes, with higher magnification (D) revealing *E. coli* flagella (arrow) intimately entwined amongst microvilli. Scale bars: A and B, 10 µm; C, 5 µm; and D, 2 µm.
Sepsis), intercellular tight junctions are a likely target for injury. We have speculated that in these high-risk patients, intestinal bacterial toxins may affect tight junctional integrity, facilitating bacterial translocation.28

We have previously shown that exposure of the lateral membranes of cultured enterocytes (using low extracellular calcium,43 Bacteroides fragilis enterotoxin,28 or cytochalasin44) promotes preferential bacterial adherence to the exposed lateral enterocyte membranes, and also augments bacterial internalization. These observations have led us to speculate that, in compromised patients, translocation of enteroinvasive bacteria may occur via a similar mechanism. However, in the present study, toxin A– or toxin B–induced exposure of the enterocyte lateral membrane was associated with increased bacterial adherence but not increased internalization, although bacterial paracellular (between enterocytes) migration was augmented in a dose-dependent fashion.

Our working hypothesis links C difficile intestinal overgrowth with compromised intestinal epithelial barrier function and with bacterial penetration of the intestinal epithelium. Herein we report that C difficile toxins A and B caused confluent Caco-2 enterocytes to pull apart, resulting in decreased TEER and increased transmigration of enteric bacteria. This cytopathic effect was accompanied by disruptions in enterocyte filamentous actin. Others have also noted similar toxin-induced actin disruptions in cultured enterocytes, namely T84 cells.11,12 However, our study is the first to correlate these effects of C difficile toxins with bacteria-enterocyte interactions. If the present in vitro results can be extrapolated to the in vivo situation, the data presented herein suggest that C difficile toxins could act in vivo to compromise the intestinal epithelial barrier and facilitate bacterial penetration into extraintestinal tissues.


This work was supported in part by Public Health Service grant AI23484 from the National Institutes of Health, Bethesda, Md.

Reprints: Brad Allen Feltis, MD, Department of Surgery, University of Minnesota, Box 195 UMHC, 420 Delaware St SE, Minneapolis, MN 55455 (e-mail: felti001@tc.umn.edu).

REFERENCES

DISCUSSION

Donald Fry, MD, Albuquerque, NM: Thank you very much for the opportunity to discuss this well-presented paper. I find an analogy in this proposed mechanism of increased gut permeability as being quite analogous to what one sees with the increased vascular permeability of the changes that endothelial cells undergo: specifically, endothelial cells undergo cytoskeletal changes and some degree of global formation as vascular permeability is increased by any of a number of different specific chemical signals. So the changes of the enterocyte that permit between-cell or paracellular movement of bacteria has a lot more appeal to me than the traditional Dr Wells’ explanation of transcellular movement of bacteria. So I find this intellectually a little more satisfying as well.

I guess the question that comes up at this point is about the remodeling of the cytokates. Is the change of permeability a specific response to the toxin, or is this a nonspecific response of the cell to any of a host of different noxious stimuli? Have you studied 2 very specific toxins? I would like to begin the question of whether you have looked at other potentially noxious stimuli to your study model here? Is it specific to the toxin or is it potentially a nonspecific response?

One of the other interesting things that you actually allude to in your manuscript is that, in addition to direct effects of the toxin, there are potentially indirect effects. The release of histamine by mucosa-associated mast cells is probably one of the best-studied in vivo models of changes in gut permeability. So I am curious as to whether you have looked at agents such as histamine in terms of whether it potentially changes the cytoskeletal structure of the enterocyte as yet another potential indirect mechanism that might speak to cytoskeletal changes of the enterocyte cells?

As you may lastly see,朱朱 what these cytoskeletal changes are permanent. Is the cell that has been attacked by the specific toxin irreversibly changed into the global configuration, or can the cell really recover its normal native morphology and function?

One of the things that is evident to me is that this particular research effort is opening up another Pandora’s box of a large number of signals that may be responsible for changing the cytoskeletal structure of the enterocyte. I somehow suspect this will increase indirect cost recovery at the University of Minnesota over the next few years.

Dr Feleti: In answer to your first question about whether or not C difficile toxigenic activity is a specific or nonspecific response, although toxin interactions with enterocytes have not been rigorously studied, some data are available. Previously, we have studied the effects of Bacteroides fragilis enterotoxin on enterocytes and we noted morphologic changes similar to what we have observed with C difficile toxins, notably, rounding up, and pulling apart of adjacent cells. This argues that these effects may be seen with other intraluminal toxins as well.

Your second question concerns indirect effects of C difficile toxins. As you point out, a similar phenomenon of increased permeability due to tight junctional disruption is observed in vascular endothelium in response to inflammatory cytokines. One current hypothesis on the mechanism of C difficile


ficile toxin activity describes the initial event as toxin A–induced activation on the enteric nervous system. This hypothesis assumes that toxin A binding to the intestinal mucosa results in signal transduction, followed by secretion of proteins that signal neuroimmune cells to become effectors of inflammatory damage. A secondary effect of the toxin may be the direct effect on disruption of the actin cytoskeleton. Therefore, similar to inflammatory changes in vascular endothelium, there may be a direct effect on the enterocytes by cytokines from neuroimmune inflammatory cells.

To answer your last question, we do not yet know if the changes in the cell cytoskeleton are reversible. We do know that in cultured fibroblasts, if you remove the toxin stimulus, the cells return to their normal morphologic state after a few hours.

Ed Deitch, MD, Newark, NJ: I would like to compliment the authors on a very nice study. As you know, we have been working with this model of Caco cells since about 1990 and have had a lot of fun with this model. Now, one of the problems with studying the gut in a jar is that you miss the entire unstirred layer. In vivo there is an unstirred layer on top of the enterocytes that’s made up of mucous and a number of other factors that prevents many of the things that might injure the enterocyte from reaching it. So, clearly, the gut is wearing a bulletproof vest under normal circumstances and what you have done in this study is make it naked.

So my question is, have you done any studies to look and see whether the same sort of injury would occur if you add mucous back? We have done that and it can be done relatively easily to see if the gut is protected from these toxins by the addition of low levels of virus.

Second, what happens if you put a mixed flora in or try to recreate the competitive environment that resides in the gut? There are a lot of good things that bacteria do as well as bad.

You have made a very important observation. You have dissected out in a reductionist mode a very interesting phe- nomenon. Have you put the pieces back together to see how it fits when we have a whole picture?

Dr Feltis: The short answer is that we aren’t sure what effect an unstirred mucous layer might have on bacteria-enterocyte interactions. Preliminary results from similar experiments in our lab with mucous-secreting HT-29 enterocytes indicate that in vitro the presence of mucous does not alter the morphologic effects of C difficile toxin.

Henri Ford, MD, Pittsburgh, Pa: Very interesting study. We have been also interested in trying to figure out the preferred route of bacterial migration across the epithelium. When we take the isolated piece of colonic mucosa and mount it in the Ussing chamber, we are able to see that the mere presence of bacteria is able to induce similar changes with regards to the TEER. It is clearly associated with a paracellular defect; however, we are not able to demonstrate any increase in bacterial migration across that mucosa, even though when you look at the mannitol flux, it is clearly increased. So clearly, there must be some divergent properties here.

I was wondering if, number one, you looked at mannitol flux within your system and is there any evidence besides what you have surmised that this is indeed a paracellular defect that is allowing the bacteria to get across? Second, have you been able to demonstrate that by either transmission electron microscopy or by scanning electron microscopy that the bacteria are indeed going through the defect that you presume is there?

Dr Feltis: No, we have not performed ion flux studies in our system. We relied on electrical resistance measurements to represent disruption of the enterocyte tight junctions caused by C difficile toxins. Interestingly, in regard to colonic explant models, if the primary activity of the toxins is induction of the gut neuroimmune system via transcellular signaling, having an intact myenteric plexus might be important to elicit the full effect. This is another reason that an in vivo model is essential for further study.