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-cytoskeletal actin, and ultrastructural morphology. Translocation of intestinal bacteria was 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.

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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.

Annually in the United States, an estimated 10% of hospitalized patients develop nosocomial infections involving more than 2 million people (with 58,000 deaths), and costing more than $4.5 billion. Many nosocomial infections have an undefined focus and seem to be caused by translocating enteric bacteria. These infections, which include gram-negative bacteremia (primarily Escherichia coli and other Enterobacteriaceae), are often life-threatening. Septicemia, often acquired in the nosocomial setting, is the 13th leading cause of death in the United States, and its incidence is increasing. Despite appropriate antimicrobial therapy, the mortality associated with gram-negative bacteremia is 20% to 50%.

Clostridium difficile is an anaerobic, gram-positive, spore-forming bacillus found in the normal intestinal flora of approximately 3% of healthy adults, 15% to 30% of hospitalized patients, and up to 50% of neonates. The incidence of C. difficile–associated diarrhea is increasing and is highly correlated with the use of broad-spectrum antibiotics, particularly third-generation cephalosporins. C. difficile produces tissue-degrading enzymes (proteases, collagenase, hyaluronidase, heparinase, and chondroitin-4-sulfatase) as well as 2 toxins, A and B, the widely recognized etiologic agents of antibiotic-associated diseases ranging from diarrhea to pseudo-membranous colitis. These toxins, recovered from 90% to 100% of patients with C. difficile–associated diarrhea, are known to alter the integrity of confluent cultured intestinal epithelial cells.

A growing literature documents that intestinal barrier function is compromised in the diverse clinical conditions associated with translocation of intestinal bacteria; these conditions include enteric bacterial overgrowth, surgery, burn wounds, and other trauma. It may not
toxins into rodent ligated intestinal loops, investigating the toxin across the vesicular membrane into the cytoplasm after internalization. There is evidence that the intracellular mechanism of action is enzymatic, with both toxins acting as glucosyltransferases to glucosylate the small guanosine 5′-triphosphatases (GTPases) Rho, Rac, and Cdc42. Glucosylation inactivates these GTPases, which are intimately associated with maintenance of the cellular cytoskeleton. Inactivation leads to cytoskeletal alterations 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, because *C. difficile* toxins A and B have associated enteric disease. Both toxins induce release of several proinflammatory monokines from cultured human monocytes. 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. There is evidence that the intracellular mechanism of action is enzymatic, with both toxins acting as glucosyltransferases to glucosylate the small guanosine 5′-triphosphatases (GTPases) Rho, Rac, and Cdc42. Glucosylation inactivates these GTPases, which are intimately associated with maintenance of the cellular cytoskeleton. Inactivation leads to cytoskeletal alterations 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.

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cell culture integrity, presumably caused by loosening of the tight junctions; decreasing values reflect increasing epithelial permeability. 

The TEER of Caco-2 enterocytes was studied with the Millicell Electrical Resistance System (Millipore Corp, Bedford, Mass). All electrical resistance readings were recorded after subtracting the average resistance of 2 membranes in the absence of enterocytes; ie, membranes equilibrated overnight in tissue culture medium. Because TEER values often vary among individual enterocyte cultures, the electrical resistance value was recorded for each membrane before and after experimental treatment, and the percentage decrease from baseline was calculated for each membrane.

Bacterial adherence to cultured enterocytes was assayed as previously described. Briefly, 10^6 bacteria (prepared as described above) were incubated with 10^6 confluent Caco-2 cells. Washed enterocytes (with adherent bacteria) were lightly fixed with 0.01% paraformaldehyde, incubated with primary rabbit antibody (diluted in 5% goat serum), washed, incubated with horse radish peroxidase–conjugated goat anti-rabbit IgG (Organotechnika-Cappel, West Chester, Pa), washed, the blue color developed with tetramethylbenzidine-hydrogen peroxide color development reagent (Sigma-Aldrich Corp), the reaction stopped with sulfuric acid, and the color intensity read at 450 nm in an enzyme-linked immunosorbent assay plate reader. Resulting data are optical density units, a measure of relative adherence. This method does not determine absolute numbers of adherent bacteria, but the goal is to compare differences in bacterial adherence between untreated and toxin-treated enterocytes. Wright-Giemsa stains were also used to qualitatively assess relative bacterial adherence.

Statistical analyses were performed using StatView 4.5 (Abacus Concepts Inc, Berkeley, Calif). Experiments were repeated at least twice on separate days to verify reproducibility. On a given day, for bacterial internalization, assays were performed in triplicate tissue culture wells and the average was considered 1 assay value. For bacterial migration and adherence assays, each well was considered an assay value. Bacterial numbers were log_{10} transformed and analyzed by analysis of variance followed by the Fisher test for significant difference. Dose responses were analyzed by correlation coefficient (r). Statistical significance was P < .05.

ENTEROCYTE ACTIN AND ULTRASTRUCTURE

Phalloidin is a phallotoxin that preferentially binds actin filaments as opposed to actin monomers. The distribution of filamentous actin in the epithelial cell cytoskeleton was observed using the method of Howard and Meyer, with minor modifications. Epithelial cultures were incubated at 37°C for 30 minutes with 0.8-μmol/L fluorescein-labeled phallolidin (Sigma-Aldrich) suspended in 3% buffered formalin containing 0.1 mg of lysophosphatidylcholine (Sigma-Aldrich) per milliliter, then washed 3 times with HBSS, mounted in phosphate-buffered saline and glycerin (1:9) containing 0.1% p-phenylenediamine (Sigma-Aldrich) at pH 8, and viewed by epifluorescence microscopy.

High-resolution, low-voltage scanning electron microscopy was used to observe the effect of C difficile toxins A and B on confluent Caco-2 cells. Bacterial surface interactions with toxin-treated enterocytes were also observed. Caco-2 cells were cultivated in wells containing a 12-mm-diameter glass coverslip. Caco-2 cells were pretreated with either toxin A or B, then incubated with or without bacteria as described earlier for bacterial internalization. Enterocytes were then washed, fixed, and processed as described. Fixed samples were dehydrated in ethanol, critical point dried with carbon dioxide, and sputter-coated with a 1-nm discontinuous layer of platinum. A modified YAG crystal scintillator was used for backscatter electron imaging at 3 or 4 kV in a Hitachi S-900 (Hitachi, Tokyo, Japan) field emission scanning electron microscope.

Figure 1. Effect of Clostridium difficile toxins A (A) and B (B) on internalization of Salmonella typhimurium, Proteus mirabilis, and Escherichia coli by Caco-2 enterocytes. Lower limit of assay detection was 1.7 log_{10}. Values represent mean ± SE of at least 4 assays.

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 on enterocyte integrity as assessed by TEER. The effects of varying concentrations of C difficile toxins A or B on Caco-2 internalization of S typhimurium, P
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 *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.

**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.11 Indirectly, studies involving ligated ileal loops in experimental animals have revealed that toxin A can activate lamina propria neuroimmune cells and cause infiltration of the submucosa by neutrophils.5 Chaves-Olarte et
al40 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.23,24

Whether by a direct or indirect effect, both toxins increase intestinal permeability in explanted colonic tissue19 and in cultured enterocytes,11,12 and toxin A increases permeability in ligated intestinal loops.6 Hecht et al11,12 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.12 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.41

Multiple mediators of inflammation, including vasocative amines and kinins, are known to increase the permeability of vascular endothelium directly, or indirectly by recruiting neutrophils and monocytes, the products of which can contribute to disruption of tight junctions.42 Similarly, in clinical conditions associated with increased intestinal permeability (eg, shock, trauma, and

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**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.
Surgical patients are considered high risk for both intestinal colonization with *C difficile* and for systemic infection caused by normal enteric flora. Data from this in vitro study (with enteric bacteria and cultured enterocytes) indicate that the intestinal toxins produced by *C difficile* may facilitate bacterial penetration of the intestinal epithelial barrier. Thus, intestinal colonization with *C difficile* may contribute to a heretofore unrecognized increased risk of septic complications in this patient population.


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**REFERENCES**


**Statement of Clinical Relevance**

Surgical patients are considered high risk for both intestinal colonization with *C difficile* and for systemic infection caused by normal enteric flora. Data from this in vitro study (with enteric bacteria and cultured enterocytes) indicate that the intestinal toxins produced by *C difficile* may facilitate bacterial penetration of the intestinal epithelial barrier. Thus, intestinal colonization with *C difficile* may contribute to a heretofore unrecognized increased risk of septic complications in this patient population.

We have previously shown that exposure of the lateral membranes of cultured enterocytes (using low extracellular calcium, or cytochalasin) 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. 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.

Figure 6. Effect of 16-hour pretreatment with Clostridium difficile toxins on distribution of filamentous actin in Caco-2 cells, visualized by staining with fluorescein-labeled phalloidin. A, Untreated (control) Caco-2 cells showing relatively smooth distribution of actin within enterocytes, as well as in perijunctional areas (arrowheads). B and C, Enterocytes treated with either 10 ng/mL of toxin A (B) or 100 ng/mL of toxin B (C) revealing disruptions in actin distribution evident as focal accumulations and alterations in distribution of perijunctional (arrowheads).

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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 globular 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 cytokines. 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? You have studied 2 very specific toxins. I would like to beg 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?

Your second question concerns indirect effects of Clostridium difficile involved in adhesion to human colonic enterocyte-like Caco-2 and mucus-secretting HT-29 cells in culture. 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 phenomenon. 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.