

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

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**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*

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**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, collagenases, hyaluronidase, heparinase, and chondroitin-4 sulfatase) as well as 2 toxins, A and B, the widely recognized etiologic agents of antibiotic-associated disease ranging from diarrhea to pseudomembranous 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
be coincidental that patients at highest risk for systemic infection caused by enteric flora are the same patient populations with a high incidence of *C. difficile* intestinal overgrowth. In vivo, the pathologic effect of the *C. difficile* toxins can include disruption of enterocyte brush border membranes followed by extensive mucosal damage, leukocytic infiltration into the lamina propria, toxic megacolon, and intestinal perforation. Formation of pseudomembranes (consisting of fibrin, mucus, and leukocytes) can occur from 2 to 10 days after first use of antibiotics to 1 to 2 weeks after cessation of antibiotics. Resultant mortality may approach 10%.8

Following initial experiments with injecting *C. difficile* toxins into rodent ligated intestinal loops, investigators concluded that toxin A alone was responsible for pathogenic activity because it causes a florid inflammatory response including hemorrhagic fluid secretion, tissue necrosis, and mucosal inflammation as well as increased intestinal permeability.19 Toxin B was termed a cytoxin, because it caused a cytopathic effect in tissue culture while having no detectable activity in ligated intestinal loops.18 However, data from experiments using human colonic explants indicated that toxin B was more potent than A in causing mucosal damage, suggesting that both toxins play a role in pathogenesis of *C. difficile*-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 membrane binding, and a hydrophobic domain important for translocation 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 glucosylate the small guanosine 5C-triphosphatases (GTPases) Rho, Rac, and Cdc42.23 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.\textsuperscript{11,12} 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. \textit{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 \textit{C difficile} toxins A and B on bacterial interactions with cultured enterocytes. The data indicate that intestinal colonization with \textit{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 \textit{C difficile} toxins A or B on Caco-2 internalization of \textit{S typhimurium}, \textit{P mirabilis}, and \textit{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

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Effect of \textit{Clostridium difficile} toxins A (A) and B (B) on internalization of \textit{Salmonella typhimurium}, \textit{Proteus mirabilis}, and \textit{Escherichia coli} by Caco-2 enterocytes. Lower limit of assay detection was 1.7 log\textsubscript{10}. Values represent mean ± SE of at least 4 assays.
Effect of Clostridium difficile toxins A (A) and B (B) on transepithelial electrical resistance (TEER) of cultured Caco-2 enterocytes. The TEER is presented as percent change from baseline. Values represent mean ± SE of at least 12 tissue culture wells. Asterisks indicate significance at P < .05. Values represent mean ± SE of at least 10 tissue culture wells.

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 mirabilis and E 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 5 A). 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 5 B). Bacteria appeared preferentially adherent to distorted apical microvilli (Figure 5 C and D). Following incubation of enterocytes with toxin A or 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. Indirectly, toxins A and B have been shown to damage human colonic explants as well as cultured enterocytes. Directly, toxins A and B have been shown to damage human colonic explants as well as cultured enterocytes. 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. 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.

Multiple mediators of inflammation, including vasoactive 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. 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 *Clostridium 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.

**Statement of Clinical Relevance**

Surgical patients are considered high risk for both intestinal colonization with *Clostridium 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.


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 also been 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.