Microbes and Microbial Toxins: Paradigms for Microbial-Mucosal Interactions
II. The integrated response of the intestine to Clostridium difficile toxins

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Pothoulakis, Charalabos, and J. Thomas LaMont. Microbes and Microbial Toxins: Paradigms for Microbial-Mucosal Interactions. II. The integrated response of the intestine to Clostridium difficile toxins. Am J Physiol Gastrointest Liver Physiol 280: G178–G183, 2001.—Clostridium difficile, the major etiologic factor of antibiotic-associated diarrhea and colitis, mediates its effects by releasing two large protein exotoxins, toxins A and B. A major toxin effect is related to the disassembly of actin microfilaments, leading to impairment of tight junctions in human colonocytes. The mechanism of actin disaggregation involves monoglucosylation of the signaling proteins Rho A, Rac, and Cdc 42, which control stress fiber formation directly by toxins A and B. An important aspect of C. difficile infection is the acute necroinflammatory changes seen in patients with pseudomembranous colitis. The early mechanism of toxin-mediated inflammation involves toxin effects on cellular mitochondria, release of reactive oxygen species, and activation of mitogen-activated protein kinases and the transcription factor nuclear factor-κB. Injection of toxin A into animal intestine triggers secretion of fluid and intestinal inflammation characterized by epithelial cell destruction and neutrophil activation. A critical feature of C. difficile enterotoxicity is communication between enterocytes and lamina propria nerves, macrophages, and mast cells mediated via release of neuropeptides and proinflammatory cytokines.

intestinal inflammation; neuropeptides; Rho proteins; mast cells; neutrophils

Since its identification 20 years ago as the major cause of antibiotic-associated pseudomembranous colitis, Clostridium difficile has now emerged as the leading cause of nosocomial enteric infections, with an estimated annual incidence of more than 3 million cases in the United States. C. difficile produces colitis entirely by the action of two potent exotoxins, toxin A (308 kDa) and toxin B (270 kDa). C. difficile toxins can trigger profound intestinal inflammation (colitis), in contrast to cholera toxin and Escherichia coli enterotoxin, which elicit secretion (diarrhea) without an acute inflammatory component. The bacterium itself is not invasive, and all of the pathological manifestations are related to an integrated hierarchy of proinflammatory signals triggered by binding of toxins to their receptors located in the apical (luminal) surface of enterocytes. The mechanisms of C. difficile toxins can be divided into two broad categories: direct actions on enterocytes and indirect actions on lamina propria cells triggered by cytokines, neuropeptides, and other neuroimmune mediators. Our review focuses on how these two mechanisms produce a complex pathophysiological response involving specific neuroimmune and inflammatory pathways.

STRUCTURE OF C. DIFFICILE TOXINS

Toxins A and B are encoded by two very large genes (Fig. 1) present in all toxigenic strains of C. difficile (17). The toxin A gene (8,130 nucleotides) encodes one of the largest bacterial toxins known (2,710 amino acids, mol. mass 308 kDa). Toxin A, often referred to as the “enterotoxin,” produces fluid secretion and a necro-inflammatory response in intestinal loops of experimental animals. The toxin B gene (7,098 bp) encodes a 2,366-amino acid protein (mol. mass 270 kDa) that possesses potent cytotoxic activity against cells in culture but no enterotoxic effects in rodent or rabbit models. Recent experiments in human intestinal explants exposed in vitro to pure toxins indicate that both toxins possess enterotoxic activity in human colon, thus making the designations “enterotoxin” and “cytotoxin” rather nonspecific.

The two toxins are closely related to each other, with 49% amino acid identity and similar overall structure. They are members of a family of related toxins from C.
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sordelli and C. novyi, which share considerable sequence homology (23). The enzymatic (catalytic) domain containing the critical glucosyltransferase activity is expressed in the first 550 NH₂-terminal amino acids of both toxins, and the receptor binding domain resides in the COOH-terminal one-third of toxin A and probably also in toxin B, although this has not yet been proven (11, 12). Deletion mutants of toxin B lacking the COOH-terminal repeats have a 10-fold reduction in cytotoxicity compared with holotoxin, whereas removal of the repeat region plus the conserved cysteine at position 1625 causes complete loss of activity. The receptor binding domain in toxin A consists of repeating units of 20–30 amino acids that function as a multivalent lectin with high binding specificity for the trisaccharide Galα1–3Galβ1–4GlcNAc (15). This structure is thought to bind toxin A to its intestinal receptor in rodents and other animals but not in humans, who lack the α-galactosyltransferase required for assembly of this trisaccharide. This implies that binding of toxin A to human enterocytes involves a different oligosaccharide or possibly a protein-protein interaction.

The NH₂-terminal glucosyltransferase domain is also critical for toxin action. As discussed below, the molecular targets of this enzyme activity are low-molecular-weight GTPases of the Rho family (Rho ABC, Rac, and CDC 42), key regulators of cellular actin (9). A 63-kDa fragment comprising the first 546 amino acids of toxin B contains the glucosyltransferase activity and causes cell rounding when microinjected into target cells. By itself, this fragment is nontoxic because it is unable to bind the receptor and enter the cell. The middle portion of these toxins, between the NH₂-terminal one-third carrying the catalytic function and the COOH-terminal repeats, may be involved with cellular uptake and processing. Mutant toxins deleted of the middle third but containing intact catalytic and binding domains have sharply reduced cytotoxicity. Site-directed mutagenesis and deletion mutations should allow more precise assignment of structure-function relationships of C. difficile toxins.

**RECEPTOR BINDING AND INTERNALIZATION**

The specific cell surface receptors for toxins A and B have not yet been identified, although some information on receptor structure and function is available. The observations that nearly all cultured cells as well as freshly isolated neutrophils, lymphocytes, and macrophages are sensitive to these toxins suggests that their receptors are ubiquitous on mammalian cells. As noted above, the trisaccharide Galα1–3Galβ1–4GlcNAc, expressed in all mammals and some nonhuman primates, is required in some cells for toxin A binding. It is likely that the mammalian cell receptor is a glycoprotein, whereas on rabbit erythrocytes toxin A binds to a cell surface glycolipid. Rabbit intestinal sucrase-isomaltase binds toxin even when expressed in nonintestinal cells, and antibody to this digestive enzyme blocks toxin A fluid secretion in rabbit ileal loops (19). However, this enzyme is not expressed in adult colon and therefore is not likely to be a colonocyte receptor. The carbohydrate antigens I, X, and Y, all of which are expressed on human colonocytes, bind toxin A, but the functional role of such binding is unknown.

Once toxin A binds to its plasma membrane receptor, internalization begins within minutes. Toxin-receptor binding is irreversible within a few minutes, after which time rescue of cells with antitoxins or by washing is ineffective. After 5–10 min toxin is localized to mitochondria, and this binding is accompanied by a precipitous fall in intracellular ATP concentrations, release of mitochondrial cytochrome c, and generation of reactive oxygen species (10). Mitochondrial dysfunction has been observed in toxin A-exposed Chinese hamster ovary cells and human colonic cell lines and occurs well before the onset of Rho glucosylation at 15–30 min. Toxin A also appears to localize to a lysosomal compartment, because prior treatment of cells with ammonium chloride or chloroquine partially prevents cell rounding. It is not yet known whether internalized toxins require processing in acid compartments or by activating proteases or if they preferentially modify Rho proteins segregated in a specific organelle or in the cytoplasm.

**RHO GLUCOSYLATION AND CYTOSKELETAL DAMAGE**

C. difficile toxins catalyze the transfer of glucose from UDP-glucose to threonine-37 in Rho A and threonine-35 in Rac and Cdc 42, rendering these signaling proteins nonfunctional (1). Rho proteins are critical regulators of a large and growing number of cell functions including the formation of actin-containing stress fibers, cell-cell contacts, regulation of tight junctions,
and membrane trafficking. Impairment of Rho function by *C. difficile* toxins leads to disaggregation of actin-containing stress fibers, loss of adhesion, and cell rounding of tissue culture cells. In human colonic epithelial cell lines and human colonic explants, both toxins dose-dependently impair tight junction function with increased paracellular permeability, loss of the perijunctional actinomyosin ring, and diminished electrical resistance (21). Increased blood-to-lumen permeability is also observed in vivo in rat and rabbit intestine exposed to toxin A. Subsequent events in intoxicated cells include diminished protein synthesis, reduced cell division, and apoptosis or necrosis.

As discussed in detail below, toxin A produces a severe acute necroinflammatory reaction in the mammalian small and large intestine that involves activation of mast cells, nerves, vascular endothelium, and immune cells. Because the toxins bind to apical receptors facing the lumen and are too large to be transported intact into the plasma, it is presumed that this integrated inflammatory response results from cytokines or other mediators released by enterocytes, but the details of this linkage are still not complete. It is likewise not clear whether Rho inactivation is essential for cytokine release or whether other Rho-independent effects of toxins (for example, mitochondrial dysfunction) may activate epithelial and other cell types.

An important early event in *C. difficile* pathogenesis is activation of nuclear factor-κB (NF-κB) with subsequent release of neutrophil chemoattractants. Macrophage inflammatory protein 2 (MIP-2), a potent neutrophil chemoattractant in rodents, is secreted by rat macrophages and epithelial cells after exposure to inflammatory stimuli. MIP-2 mRNA and protein are elevated in rat ileal epithelial cells 30 min after intraluminal exposure to toxin A (4). Pretreatment of rats with an antibody to MIP-2 significantly blocked the enterotoxicity of toxin A (4), suggesting that enterocyte release of MIP-2 is a major early signal in the enterotoxic cascade. However, these results would not explain the observation that rat intestinal mast cells and substance P (SP)-containing neurons are activated before MIP-2 release.

Several observations support the evolving hypothesis that some important cellular responses to *C. difficile* toxins may occur before (and independently of) Rho glucosylation. For example, mitochondrial damage from toxins A and B occurs before Rho glucosylation and may be an important mechanism of interleukin (IL)-8 release in enterocytes. We recently reported that p38 mitogen-activated protein (MAP) kinase activation after toxin A exposure is independent of Rho and is the major signal transduction pathway for IL-8 release (24). Thus the classic teaching that the enzymatic effect of *C. difficile* toxins is required for toxicity may not be entirely correct, but further exploration of these potentially important non-Rho pathways is required to allow a more definitive statement.

**TOXIN A IS A PROINFLAMMATORY ENTEROTOXIN**

One of the primary features of *C. difficile* colitis in humans is the presence of an inflammatory infiltrate in the colonic mucosa characterized by epithelial cell necrosis and presence of neutrophils in the colonic lumen. Experiments in intact animals demonstrated that injection of toxin A into ileal or colonic loops stimulates secretion of fluid accompanied by increased mucosal permeability, epithelial cell destruction, and neutrophil infiltration (4, 5, 18). This is in contrast to cholera toxin and *E. coli* enterotoxins, which trigger intestinal secretion without intestinal inflammation. The mechanism of toxin A-mediated intestinal secretion and inflammation involves activation of neurons and immune and inflammatory cells in the intestinal mucosa, leading to release of proinflammatory mediators and recruitment of neutrophils (Fig. 2). Several mediators are released in the intestinal mucosa in response to toxin A, including leukotriene (LT)B4, PGE2, and tumor necrosis factor (TNF)-α in animal intestine (3, 6, 22) and IL-8 in human colon (2). Furthermore, administration of drugs that reduce cytokine synthesis results in a reduction of the intestinal effects of toxin A (22), indicating that proinflammatory cytokines mediate part of the in vivo responses to this toxin. However, toxin A is also able to directly activate monocytes to release IL-1β and IL-6 (8) and increase neutrophil migration in vitro (14). These responses may be important in the pathogenesis of toxin A-induced enterocolitis after the inflammation is established, allowing toxin A to cross the epithelial cell barrier and activate cells of the lamina propria.

**Role of neutrophils and mast cells.** Several studies indicate that neutrophil activation and transmigration are important determinants in the pathophysiology of toxin A enterocolitis. Neutrophil-derived proinflammatory mediators act on epithelial cells, causing destruction and necrosis of enterocytes and colonocytes (22). The pathophysiological importance of neutrophils in the in vivo mechanism of toxin A is underscored by the significant inhibition of fluid secretion and inflammation when neutrophil infiltration is inhibited by administration of antibodies directed against the leukocyte adhesion molecule CD18 or against the potent chemotactic MIP-2 (4, 14). Induction of neutropenia in rats resulted in a significant inhibition of toxin A-mediated ileal secretory and inflammatory changes (20).

Intestinal mast cells are critically involved in neutrophil activation in toxin A-mediated inflammation. Mucosal mast cells are activated after administration of toxin A into the intestinal lumen, as evidenced by the increased mucosal and circulating levels of the specific mucosal mast cell enzyme rat mast cell protease II (RMCP II) and by electron microscopy studies demonstrating significant degranulation of mucosal mast cells 15–30 min after toxin A exposure (5). Administration of mast cell inhibitors not only reduced the intestinal levels of mast cell-derived mediators but
also attenuated toxin A-induced neutrophil activation and fluid secretion. Using intravital microscopy in toxin A-exposed rat mesenteric venules, Kurose et al. (16) showed substantially increased leukocyte adherence and emigration at 15–30 min of toxin A exposure that were associated with mast cell degranulation and albumin leakage. In addition, toxin A-induced increased albumin leakage was significantly reduced by pretreatment with the mast cell blocker lodoxamide, the histaminase diamine oxidase, or the histamine receptor subtype 1 (H1) antagonist hydroxyzine (16). Thus histamine is responsible, at least in part, for the leukocyte-endothelial cell adhesion in response to toxin A by interacting with H1 receptors on endothelial cells.

Experiments using mice genetically deficient in mast cells provide evidence for the critical involvement of mast cells in the secretory and inflammatory effects of toxin A. Previous studies demonstrated that these animals do not contain mast cells in the periphery, including the gastrointestinal tract. Compared with normal mice, mast cell-deficient mice had significantly attenuated fluid secretion and neutrophil infiltration after toxin A administration (25). Reconstitution of mast cell-deficient mice with mast cells obtained from normal mice restored toxin A-associated responses (25). These results strongly suggest that mast cell activation represents a major step in the expression of toxin A-mediated enteritis and that neutrophil recruitment in response to this enterotoxin is strongly mast cell dependent.

**Nitric oxide as a mediator of the toxin A inflammatory response.** Nitric oxide (NO) is an important modulator of intestinal injury and inflammation. Because NO is involved in mast cell degranulation, leukocyte adherence, and mucosal permeability, it seemed likely that NO may be involved in toxin A-associated changes in rat ileum. We found that the toxin A-induced increases in mannitol permeability and secretion of fluid were enhanced in animals treated with either the NO synthase inhibitor nitro-L-arginine methyl ester (L-NAME) or the neuronal NO synthase inhibitor 7-nitroindazole, whereas pretreatment of rats with the NO donor S-nitroso-N-acetyl-L-cysteine (SNAC) substantially inhibited these toxin A responses (20). Mast cell degranulation and neutrophil infiltration occurring after ileal injection of toxin A were also blocked by the NO donor SNAC (20). These results suggest that NO, probably of neuronal origin, protects the intestinal mucosa from the effects of toxin A by reducing mast cell degranulation and neutrophil infiltration activated by this toxin.

**Role of neuropeptides and nerve-immune cell interactions.** One of the highlights of C. difficile toxin A pathophysiology is the dependence of the toxin A-associated intestinal responses on activation of intestinal nerves and neuropeptides. Early experiments using anesthetized animals demonstrated that either local application of the anesthetic lidocaine or systemic administration of the ganglionic blocker hexamethonium dramatically inhibited toxin A-induced ileal fluid secretion and mucosal permeability and inflammation (5). Interestingly, chronic administration of capsaicin, a neurotoxin that desensitizes primary sensory neurons, almost completely normalized the intestinal effects of toxin A, including degranulation of mucosal mast cells (5). These results suggested a major role for primary sensory neurons in the induction of toxin A-mediated enteritis. Because SP and calcitonin gene-related peptide (CGRP) are known to be released from sensory nerves in response to inflammatory stimuli, we investigated the role of CGRP in the enterotoxic response to toxin A. We found that topical application of CGRP to the rat ileum significantly increased fluid secretion and mucosal permeability, whereas pretreatment with the CGRP antagonist BIBN4096BS attenuated these responses (20). These results suggest that CGRP, probably of neuronal origin, plays a significant role in the enterotoxic response to toxin A by activating sensory nerves and mediating the release of other inflammatory mediators.

**Fig. 2. Mechanism of toxin-mediated inflammation.** NF-kB, nuclear factor-kB; SP, substance P; CGRP, calcitonin gene-related peptide; NT, neurotensin.
related peptide (CGRP) are the major constituents of primary sensory neurons, we tested the possibility that these peptides are involved in the intestinal effects of toxin A. Administration of either nonpeptide SP receptor antagonists or a peptide CGRP antagonist to rats dramatically reduced intestinal secretion, mucosal permeability, and release of proinflammatory cytokines from the intestinal mucosa in response to toxin A (13, 18), suggesting a proinflammatory role for these peptides during toxin A enteritis. Consistent with these observations, administration of toxin A into ileal segments led to an early (30 min) increase of SP and CGRP mRNA and increased content of these peptides in the cell bodies of spinal dorsal root ganglia followed by increased SP and CGRP expression in the intestinal mucosa (3, 13). In addition, lamina propria macrophages, activated during the inflammatory response to toxin A, express high-affinity (neurokinin-1; NK-1) receptors for SP and binding of SP to these receptors leads to release of the potent chemokine TNF-α (3). A major role for SP and its NK-1 receptor in the mediation of the enterotoxic effects of toxin A is further supported by recent results showing that mice lacking NK-1 receptors have significantly attenuated intestinal responses to toxin A (6). Thus activation of primary sensory afferent nerves and binding of the sensory peptides SP and CGRP to receptors on mucosal immune cells represent a major amplification system that controls the in vivo effects of toxin A.

Neurotensin (NT) is a brain and gastrointestinal neuropeptide released in the intestine in response to several stimuli. NT has been implicated in several intestinal functions, including intestinal motility and mast cell activation. Because previous studies suggested a neuronal communication between NT and SP, we investigated whether NT is involved in the pathophysiological mechanism of C. difficile toxin A colitis. Injection of toxin A into colonic loops increased colonic NT expression within 30–60 min, whereas intestinal secretagogues and mucosal permeability changes in response to toxin A were present after 2 h (7). Pretreatment of rats with a specific NT receptor antagonist caused a substantial reduction in all toxin A-associated colonic responses, including activation of mucosal mast cells. Furthermore, NT-mediated degranulation of colonic mast cells in vitro was inhibited by a SP receptor antagonist, suggesting a functional communication between NT and SP during toxin A colitis (7).

One of the curious characteristics of toxin A enterocolitis is the dramatic upregulation of receptors for both NT and SP on intestinal epithelial cells 15–30 min after toxin A administration. Both SP and NT receptors belong to the G protein receptor superfamily with seven transmembrane domains, and the promoter regions of these receptors contain NF-κB binding sites. As noted above, toxin A also stimulates increased NF-κB activity in target cells, which in turn leads to the release of IL-8 and probably other inflammatory cytokines (25). Thus the NF-κB system may represent a major proinflammatory mechanism leading to up-regulation of epithelial SP and NT receptors.

**SUMMARY**

The mechanism of enterotoxin action has been expanded considerably in the past several decades after the pioneering experiments of Lundgren and colleagues, who showed the critical role of the neuroimmune system in the physiological responses to toxins and other secretagogues. Cholera toxin, the prototypical enterotoxin, produces a fluid secretory response that is dependent on the activation of enteric neural pathways mediated by cholinergic and vasoactive intestinal polypeptide (VIP)-dependent nerves. C. difficile toxin A exerts its potent secretory and necroinflammatory responses in mammalian intestine by a separate neural pathway involving the activation of mucosal mast cells and release of SP and CGRP from sensory neurons. The cholera toxin and toxin A neural pathways are distinct and specific with no molecular or junctional overlap. Their function appears to be amplification of initiating signals in enterocytes after binding of small amounts of toxins to enterocyte receptors facing the lumen. It seems likely that parallel but separate neuroimmune pathways exist for other toxins and luminal secretagogues (e.g., bile salts). Elucidation of the elements of these amplification pathways should lead to better control of diarrheal diseases arising from a wide variety of causes.

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