Over the past few years, our understanding of the pathophysiology of enterotoxin actions has evolved from a classic cell-oriented model to an integrated model incorporating neuronal and immune mediators in the lamina propria. According to the classic model, the pathogenesis of cholera toxin-mediated diarrhea requires binding of the toxin to a brush-border receptor and increases in enterocyte adenosine 3’,5’-cyclic monophosphate (cAMP) levels, followed by intestinal chloride and water secretion via the paracellular pathway. An expanded model to explain cholera toxin diarrhea was advanced by Lundgren and associates (10) based on in vivo experiments. These workers showed that diarrhea caused by intraluminal instillation of cholera toxin or Escherichia coli heat-stable toxin in rats or cats could be completely inhibited by pretreating animals with the nerve blockers tetrodotoxin, hexamethonium, or lidocaine (reviewed in Ref. 10). Because these agents had no effect on intracellular levels of cAMP in villus enterocytes, the authors concluded that a rate-limiting step in cholera diarrhea was regulation by intestinal neurons.

Recent studies from several laboratories indicate that a complex neural cascade is involved in the cholera response. The secretory effects of cholera toxin in vivo are mediated by release of 5-hydroxytryptamine and prostaglandins from enterochromaffin-like cells on the villus of small intestine, followed by activation of the enteric nervous system and release of vasoactive intestinal peptide (VIP). VIP then binds to crypt cell receptors, triggering secretion of NaCl and water (reviewed in Ref. 3) (Fig. 1). This new paradigm of toxin-mediated intestinal secretion maintains that the nervous system amplifies signals originating in the lumen when toxins or other noxious agents such as bile salts stimulate villus cell receptors. Our laboratory has incorporated this new model of toxin action into our studies of clostridial enterotoxins, which, like cholera toxin, are capable of stimulating massive intestinal secretion. As outlined below, cholera toxin and clostridial enterotoxins stimulate separate neuronal pathways involving distinct neuropeptides. Not surprisingly, the clinical manifestations of these two toxigenic diarrheas are also quite distinct.

**Clostridium difficile** toxin A is a proinflammatory enterotoxin

Clostridium difficile, a noninvasive toxigenic bacteria, is the principal cause of antibiotic-associated diarrhea and colitis, an important human disease that affects millions of patients each year (reviewed in Ref. 8). Toxigenic strains of *C. difficile* release two large protein exotoxins, toxin A (308 kDa) and toxin B (275 kDa), which mediate the intestinal responses seen in *C. difficile* infection. In contrast to cholera toxin, which causes a watery diarrhea without inflammatory cells, toxin A diarrhea is accompanied by considerable tissue necrosis and an intense acute neutrophilic infiltrate (7). Toxin A initiates this complex signal cascade by binding to brush-border receptors on enterocytes facing the intestinal lumen. Such binding triggers a stereotypical response characterized by fluid secretion, increased epithelial permeability, and acute enteritis with infiltration of neutrophils, mucosal edema, and necrosis (Fig. 2) (7). Although toxin A can directly decrease epithelial cell resistance and increase tight junction permeability in vitro in cell cultures or organ baths (4, 14), these processes cannot explain the acute inflammatory diarrhea that occurs when the toxin is instilled in an ileal or colonic loop of an anesthetized rat or rabbit.
Primary afferent sensory neurons and sensory neuropeptides mediate diarrhea and intestinal inflammation

Ileal loop experiments in intact rats or rabbits suggest that sensory neuronal input is essential for the acute fluid secretion and intestinal inflammation caused by toxin A. In parallel with Lundgren's earlier observations (10), we observed that toxin A-mediated diarrhea and inflammation in rats were abolished by lidocaine and hexamethonium (2). Furthermore, chronic desensitization of primary sensory afferent nerves by capsaicin almost completely normalized toxin A-mediated responses in rat ileum (2). These experiments suggested major input by visceral sensory neurons in the observed toxin A responses (Fig. 2). Because substance P (SP) and calcitonin gene-related peptide (CGRP) are the principal neurotransmitters released from sensory neurons, we next examined whether these peptides were involved in toxin A responses. For these experiments, we used a potent, highly specific SP receptor antagonist to study the involvement of SP. Treatment of rats with CP-96,345, a specific SP receptor antagonist, before toxin A exposure significantly inhibited fluid secretion and mucosal permeability (11). Administration of a CGRP antagonist to rats also significantly reduced the in vivo intestinal responses to toxin A, suggesting that this peptide participates with SP in toxin A effects (6). In contrast to these results with toxin A, neither capsaicin nor the SP antagonist prevented the effects of cholera toxin in rat intestine (2, 11). We conclude that toxin A activates SP- and CGRP-containing primary sensory afferents (Fig. 2), whereas cholera toxin activates a neuronal pathway that utilizes serotonin and VIP as neurotransmitters (Fig. 1).

The pathways by which primary sensory neurons are activated when intestinal epithelial cells are exposed to toxin A have not yet been elucidated. An interesting possibility is that inflammatory cytokines, like macrophage inflammatory protein-2, are released from enterocytes in response to toxin A and activate primary sensory neurons. Neurotransmitter release following toxin exposure occurs in the early phase of the signal cascade. For example, treatment of rats with the SP antagonist 30 min after intraluminal toxin A administration failed to reduce toxin A mediated-intestinal responses (11). Moreover, 30 min after injection of toxin A into rat ileum, SP and CGRP content and SP mRNA are elevated in lumbar dorsal root ganglia, followed 30 min later by increased levels of these peptides in ileal mucosa (1, 6). In contrast, fluid secretion and mannitol permeability in response to toxin A are only evident 2 h after toxin administration (1). Lamina propria macrophages are also activated and release SP during the course of toxin A enteritis (1). These results suggest that binding of the toxin to intestinal epithelial cells transmits...
a signal or series of signals to the underlying lamina propria, activating a sensory afferent reflex via the dorsal root ganglia (Fig. 2). This pathway is reminiscent of experimental neurogenic inflammation in other organs. For example, SP and CGRP levels are increased in the dorsal root ganglia during adjuvant monoarthritis in the rat.

**Nerve-mast cell interactions**

How does release of SP or CGRP from sensory nerves in the lamina propria trigger intestinal secretion and acute inflammation? Although SP and other neuropeptide receptors are expressed in intestinal epithelial cells, we reasoned that SP would more likely activate mast cells, macrophages, and lymphocytes in the intestinal lamina propria. We focused initially on mucosal mast cells as a likely target because previous investigators had shown close physical contact between neurons and mast cells and because mast cells were shown to trigger a number of intestinal allergic and inflammatory conditions.

Recent observations in our laboratory support a major role for mucosal mast cells in the pathogenesis of *C. difficile* toxin A-mediated enteritis. For example, electron microscopy studies showed that significant mast cell degranulation occurred early after administration of toxin A into rat ileal loops (2) and before the onset of fluid secretion and mucosal permeability. These results indicated that mast cell activation, as well as neuronal SP release, was an early event after intestinal exposure to the toxin. Injection of toxin A into rat ileal loops also caused increased release of the mast cell products leukotriene C₄ and platelet-activating factor (PAF) (12). Both compounds are able to stimulate fluid secretion by direct action on intestinal epithelial cells and thus may participate in the resultant diarrhea (Fig. 3). Intraluminal toxin A administration also stimulated the release of rat mast cell protease II (RMCPII) (2, 11, 12), indicating that mucosal mast cells are activated in toxin A enteritis. Furthermore, pretreatment of rats with ketotifen, a stabilizer of mast cells and inflammatory cells, profoundly inhibited toxin A-associated enteritis in vivo and significantly reduced the levels of RMCPII (12). In contrast, injection of cholera toxin into rat ileum was not associated with increases in RMCPII release, indicating that mucosal mast cells may not be involved in cholera diarrhea. Finally, chronic ablation of sensory neurons by capsaicin or pretreatment of rats with the specific SP antagonist, CP-96,345, not only inhibited secretion and intestinal inflammation in response to toxin A but also

**FIGURE 2.** Pathogenesis of inflammatory diarrhea caused by *C. difficile* toxin A. Toxin A binds to its brush-border receptor(s) on intestinal epithelial cells, causing release of cytokine(s) from these cells. This cytokine(s) diffuses into the lamina propria and activates primary sensory afferent neurons whose cell bodies are present in the dorsal root ganglia (DRG). Activation of primary sensory neurons causes early release of substance P (SP) and calcitonin gene-related peptide, which stimulate mucosal mast cells and other resident immune cells, such as macrophages. PMN, polymorphonuclear neutrophils.
almost completely inhibited mast cell degranulation following toxin administration (2, 11). These results indicate that capsaicin-sensitive afferent neurons that release SP functionally and anatomically are linked with intestinal mast cells in toxin A enteritis.

Mucosal mast cells and the neutrophil response

The next question was whether mast cells were involved in neutrophil recruitment and activation, a key feature of intestinal inflammation following exposure to C. difficile toxin A (Fig. 4). Injection of toxin A into rabbit or rat ileal loops resulted in release of leukotriene B4, a potent mediator that directly stimulates intestinal inflammation and ileal fluid secretion. Pretreatment of rabbits with a monoclonal antibody directed against the neutrophil adhesion molecule, CD18, inhibited polymorphonuclear neutrophil recruitment and mucosal damage and markedly attenuated fluid secretion and mucosal permeability in response to toxin A (7). Recent evidence indicates that activation of mast cells is also important in leukocyte transmigration seen in response to toxin A. Using intravital video microscopy, Kurose et al. (9) reported that exposure of rat mesenteric venules to toxin A elicited leukocyte adherence and emigration and caused increased albumin leakage and mast cell degranulation. Interestingly, pretreatment with the mast cell stabilizer, lodoxamide, or the histamine 1 receptor antagonist, hydroxyzine, before toxin A exposure inhibited these responses (9). These data provide indirect evidence for a link between mast cells and neutrophils in the pathogenesis of C. difficile toxin A enteritis (Fig. 4) and are relevant to C. difficile colitis in humans, in whom neutrophil infiltration of the colonic mucosa is a prominent feature.

Mast cell-deficient mice are less sensitive to toxin A

Direct evidence for participation of mast cells in toxin A-mediated enteritis has been provided by recent experiments in mast cell-deficient mice. Genetically, mast cell-deficient mice have been used extensively as an important tool to study pathophysiology of mast cells. Because of a mutation in a gene that controls mast cell differentiation from a precursor stem cell, these animals lack mast cells in all anatomic sites examined, including the intestine. We showed that mast cell-deficient mice have a 39% reduction in fluid secretion in response to toxin A compared with normal congenic mice (15), thus providing further evidence for a functional role of mast cells in toxin A-mediated enteritis. Neutrophil recruitment in response to intraluminal administration of toxin A was also reduced in mast cell-deficient mice, further supporting the notion of a major link between intestinal mast cells and neutrophils in

FIGURE 3. Pathogenesis of inflammatory diarrhea caused by C. difficile toxin A. After stimulation by substance P (SP) and calcitonin gene-related peptide, significant mucosal mast cell degranulation occurs early after toxin A administration. These cells release several proinflammatory mediators, such as histamine, platelet-activating factor (PAF), leukotriene C4 (LT), and proteases (rat mast cell protease II). Activated intestinal lamina propria macrophages also release potent inflammatory mediators, such as macrophage inflammatory protein-2 (MIP-2), LT, tumor necrosis factor-α (TNF-α), and SP. These mediators directly stimulate fluid secretion from epithelial cells and also upregulate expression of adhesion molecules on endothelial cells and polymorphonuclear neutrophils (PMNs), thus causing entry of PMNs into the tissue.
inflammatory diarrhea mediated by C. difficile toxin A.

The nitric oxide connection

Kanwar et al. (5) have recently performed a series of elegant experiments showing that nitric oxide (NO) is a major regulator of intestinal mast cell function. NO appears to regulate mast cell function by tonically suppressing release of histamine and PAF. Endogenous NO production under normal (i.e., noninflamed) conditions is regulated by constitutive NO synthase (NOS), and inhibition of this enzyme with pharmacological inhibitors causes a dose-dependent increase in mucosal permeability (5). We recently tested whether NO is involved in the intestinal responses to either C. difficile toxin A or cholera toxin. Pretreatment of animals with either the nonspecific NOS inhibitor, nitro-L-arginine methyl ester (L-NAME), or the neuronal NOS inhibitor, 7-nitroindazole, significantly augmented toxin A-mediated fluid secretion and mucosal permeability (13). Conversely, pretreatment of rats with the NO donor, SNAC, dramatically inhibited toxin A-mediated intestinal responses. These results strongly indicate that NO, probably of neuronal origin, protects the gut following exposure to toxin A. This protection involves an NO-mast cell interaction, since pretreatment of rats with the NO donor SNAC also inhibited the release of mucosal mast cell products after toxin A challenge (13). Our experiments also showed a protective effect of NO on tissue neutrophil infiltration in response to intraluminal administration of toxin A, further supporting a neuron-mast cell-neutrophil interaction in toxin A-mediated fluid secretion and intestinal inflammation. In contrast, pretreatment of rats with the NOS inhibitor, L-NAME, had no significant effect on cholera-mediated fluid secretion.

Synopsis

Bacterial enteric toxins are incredibly potent molecules that are capable of disrupting the normal function of the bowel at nanomolar or even lower concentrations. Even a few molecules per cell of certain toxins like diphtheria toxin or C. difficile toxin B are sufficient to “intoxicate” target cells. Despite these potent effects on target cells, recent evidence from a number of laboratories supports a much broader view of enterotoxin action than was previously recognized. Although cholera toxin and clostridial enterotoxins indeed have profound effects on enterocytes, their effects in whole animals are not completely explained by changes in enterocyte second messengers. Rather, toxins initiate a complex signal cascade in the lamina propria that involves neuronal pathways and release of neurotransmitters. Toxin A of C. difficile, in addition, activates mucosal mast cells whose granule contents regulate blood flow and activate adhesion molecules on vascular endothelium that initiate recruitment of circulat-
ing neutrophils to sites of injury. These signal transduction cascades are entirely different for cholera toxin and *Clostridium difficile* toxin, and, perhaps not surprisingly, the diseases they produce in humans are very different. It is likely that other enteric pathogens elicit specific amplification pathways involving neurons, neuroimmune cells, vascular endothelium, and circulating inflammatory cells. Dissecting out these complex pathways for specific pathogens and devising ways to interrupt them may provide new weapons for disease control.

**References**


