Effects of Toxin A from Clostridium difficile on Mast Cell Activation and Survival

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Toxins A and B from Clostridium difficile are the main cause of antibiotic-associated diarrhea and pseudomembranous colitis. They cause fluid accumulation, necrosis, and a strong inflammatory response when inoculated in intestinal loops. Since mast cells are a rich source of inflammatory mediators, abundant in the gut, and known to be involved in C. difficile-induced enteritis, we studied the in vitro effect of toxin A on isolated mast cells. Normal rats sensitized by infection with Nippostrongylus brasiliensis were used to isolate peritoneal mast cells (PMC). PMC from naive rats were stimulated with calcium ionophore A23187 as a model of antigen-independent activation, and PMC from sensitized rats were stimulated with N. brasiliensis antigens to study immunoglobulin E-dependent mast cell activation. After 4 h, toxin A did not induce release of nitric oxide or histamine in naive PMC. However, 10 ng of toxin per ml caused a significant release of tumor necrosis factor alpha (TNF-α). In contrast, 1 μg of toxin per ml inhibited antigen or A23187-induced histamine release by PMC. Toxin A at 1 μg/ml for 4 h caused disruption of actin which aggregated in the cytoplasm and around the nucleus. After 24 h, chromatid condensation, cytoplasmic blebbing, and apoptotic-like vesicles were observed; DNA fragmentation was documented also. These results suggest that mast cells may participate in the initial inflammatory response to C. difficile infection by releasing TNF-α upon interaction with toxin A. However, longer exposure to toxin A affects the release of inflammatory mediators, perhaps because of the alteration of the cytoskeleton and induction of apoptosis. The impaired functions and survival of mast cells by C. difficile toxin A could hamper the capacity of these cells to counteract the infection, thus prolonging the pathogenic effects of C. difficile toxins.

Clostridium difficile is the etiologic agent of antibiotic-associated diarrhea and pseudomembranous colitis (1). Antibiotics and cytotoxic drugs disturb colonic flora, allowing overgrowth of C. difficile and production of toxins A and B. Toxin A elicits an acute inflammatory response, congestion, and necrosis when inoculated in the gut (1, 14, 36, 44). It is chemotactic and induces the release of inflammatory mediators by macrophages and neutrophils (9, 24, 31). Some studies suggested that mast cells also play an important role in the pathophysiology of toxin A (25). Thus, toxin A administered into ileal loops of rats elicited the release of inflammatory mediators such as leukotriene B4, platelet-activating factor, and rat mucosal mast cell protease II (RMCPII) (6, 26, 35). Moreover, treatment of animals with the antiallergic and antiinflammatory agent ketotifen, with the H1 histamine antagonist iodoxamide, or with histaminase reduced the inflammation and secretory responses caused by toxin A (12, 25, 34). It has been proposed that toxin A induces the secretion of inflammatory mediators from mast cells either directly or indirectly through the release of substance P, a known activator of mucosal mast cells (7, 19, 29).

Mast cells are widely distributed in the intestinal mucosa, in skin and around blood and lymphatic vessels, and in many other tissues and organs. They can be activated to release inflammatory mediators via immunoglobulin E (IgE)-dependent and IgE-independent mechanisms (16). In IgE-independent mechanisms, mast cells can be activated by substances such as calcium ionophore, compound 48/80, substance P, and microbial products (11, 16). They can release potent mediators of inflammation and recently have been shown to play a pivotal role in host defense against bacterial infection (11, 28). The defenses in sepsis are dependent on mast cells that produce tumor necrosis factor alpha (TNF-α), which in turn attracts and activates neutrophils to the site of infection (28). However, in all these studies, direct evidence of C. difficile toxin A effect on mast cells has not been described.

Thus, to investigate whether toxin A has direct effects on mast cells, we analyzed the influence of toxin A on the secretion of histamine, TNF-α, and nitric oxide (NO) in vitro. We found that toxin A did not induce the release of histamine and NO, although it induced the release of small amounts of TNF-α. Moreover, exposure to large doses of toxin A inhibited mast cell activation induced by IgE-dependent and IgE-independent mechanisms and also altered the mast cell cytoskeleton and induced cell death by apoptosis.

MATERIALS AND METHODS

Rat PMC. Peritoneal mast cells (PMC) were from 250- to 300-g male Sprague-Dawley rats (Charles River, Canada Inc.), maintained under standard laboratory conditions with food and water ad libitum. PMC were obtained by lavage of the peritoneal cavity with HEPES-buffered Tyrode’s solution (HTBS) containing 0.1% bovine serum albumin and isolated in a discontinuous gradient of sterile Percoll (Pharmacia Ltd., Uppsala, Sweden). Purity of isolated cells was checked by staining with toluidine blue (3) and observed under light microscopy. Mast cells used in all experiments were 97 to 99% pure, with viability of >96%. To study the IgE-dependent activation of mast cells, rats were infected with 3,000 third-stage larvae of Nippostrongylus brasiliensis 5 to 6 weeks before PMC isolation (2). To evaluate activation by calcium ionophore A23187 or toxin A, mast cells were obtained from naive rats. All protocols used for mast cell isolation
from animals were approved by Animal Ethics Committees from University of Alberta (Canada), as well as from the Mexican Institute of Social Security. Reagents. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulfanilamide, naphthylethylenediamine dihydrochloride, HEPEs (sodium salt), H-PL, PBS, Bovine Serum Albumin (BSA), Calcein-AM and EthD-1 (Invitrogen, Carlsbad, Calif.), 2-mercaptoethanol, N-acetyl-L-cysteine and L-lysine hydrochloride, Luminas amebocyte lysate, for detection of endotoxin) were from Sigma Chemical Co. (St. Louis, Mo). C. difficile toxin A free of endotoxin, as tested with the E-toxate test, was produced and purified as previously described (45). N. brasiliensis antigen (NbAg) was obtained according to the procedure of White and Pearce (47). RPMI 1640 medium was from Gibco-BRL (Grand Island, N.Y.). Fetal bovine serum was purchased from HyClone (Logan, Utah). Monoclonal antibodies to actin were produced and purified as described by Diaz-Barriga et al. (40). Polyclonal antibodies to toxin A were produced by immunization of rabbits with purified toxin A as described previously (46).

TNF-α bioassay. For the TNF-α assay, 2.5 ×10⁵ naïve PMC were mixed with different concentrations of toxin A (0.001, 0.01, 0.1, and 1 µg/ml) in RPMI 1640 supplemented with 10% FBS (RPMI-FBS) and incubated for 4 h at 37°C under a 5% CO₂ atmosphere. Unbound toxin A was removed by centrifugation at 600 × g, and PMC were suspended in fresh RPMI-FBS. The cell suspension was further incubated for 6 h at 37°C. The cells were then centrifuged at 900 × g, and the supernatant was tested for TNF-α cytotoxicity on WEHI 164.13 cells (13). As a control, the direct effect of toxin A (1 to 100 ng/ml) on WEHI 164.13 cells was tested and shown not to be toxic; by contrast, with 1 µg of toxin A per ml, we observed significant cytotoxicity. This alteration was enhanced with rabbit anti-toxin A antibodies (1:50 dilution). Under these conditions, TNF-α-mediated toxicity was not modified by toxin A. Antibody to toxin A did not alter the TNF-α-mediated toxicity of mast cells, given that antibody to TNF-α fully blocked the toxicity against WEHI 164.13 cells induced by supernatants from mast cells pretreated with TNF-α (Fig. 1A). A recombinant TNF-α (Genzyme Co., Cambridge, Mass.) was used as a standard. Test samples were assayed with concentrations of TNF-α from 0.8 to 100 µg/ml. WEHI 164.13 cells were incubated in 50 µl of RPMI-FBS with 50 µM 2-mercaptoethanol for 20 h at 37°C in 5% CO₂ atmosphere. Viability of cells was measured 3 h following addition of 10 µl of MTT (5 mg/ml) per well. Isopropyl-nitro-HCl (150 µl) was added to dissolve the purple formazan precipitates. The plate was read on a Vmax kinetic microplate reader (Molecular Devices Co., Menlo Park, Calif.) at 340 nm.

Histamine release assay. PMC (2.5 ×10⁵) from N. brasiliensis-infected rats were suspended in 100 µl of HBTS containing 0.1% bovine serum albumin and incubated with the different concentrations of toxin A (0.001, 0.01, 0.1, and 1 µg/ml) for 30 min or 4 h at 37°C in a 5% CO₂ atmosphere. After incubation, cells were washed and resuspended in HBTS and challenged with 10 worm equivalents of NbAg per ml for 20 min. Isolated PMC from infected rats were also treated with 2.5 µg/ml of C. difficile (25 µm) or medium alone under the same conditions. The supernatant and cell pellets were then separated by centrifugation at 3,000 × g. Histamine levels were measured in both supernatant and pellet fractions with a fluorometric assay (43) using a CytoFluor 2350 fluorescence spectrometer (Milipore, Billerica, Mass.). Histamine was detected using a 1:30 dilution of 0.8% E-toxate (1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride, 25% H₃PO₄, NaNO₂, calcium ionophore A23187 (Calon), 2-mercaptoethanol for 20 h at 37°C in 5% CO₂ atmosphere. Viability of cells was tested and shown not to be toxic; by contrast, with 1 µg of toxin A per ml, we observed evidence of toxicity. This alteration was abolished with rabbit anti-toxin A antibodies (1:50 dilution). Under these conditions, TNF-α-mediated toxicity was not modified by toxin A. Antibody to toxin A did not alter the TNF-α-mediated toxicity of mast cells, given that antibody to TNF-α fully blocked the toxicity against WEHI 164.13 cells induced by supernatants from mast cells pretreated with TNF-α (Fig. 1A).

Statistical analysis. The two-tailed paired Student's t test was used for statistical evaluation of TNF-α and NO studies. Results were considered significantly different when P was <0.05. For histamine release, a three-way interaction analysis was used. Results were considered significantly different when P was <0.01 with the Scheffe test.

RESULTS

The viability of PMC from both N. brasiliensis-infected and uninjected rats was not affected by exposure of up to 4 h with doses of toxin A from 0.001 to 1 µg/ml. However, exposure to 1 µg/ml for longer periods of time (24, 48, and 72 h) decreased the viability of mast cells (17, 30, and 60%, respectively), whereas exposure to 10 µg of toxin A per ml for 4 and 24 h reduced cell viability to 12 and 26%, respectively. Accordingly, for studies of mediator secretion, toxin A doses of up to 1 µg/ml and exposure for up to 4 h were used.

Effect of C. difficile toxin A on TNF-α and NO production. To determine the effects of toxin A on TNF-α production, PMC from uninfected rats were incubated with different concentrations of toxin A (0.001 to 1 µg/ml) for 4 h. TNF-α in cell-free supernatants was assessed on TNF-α-sensitive WEHI cells. Treatment of PMC with 0.01 or 0.1 µg of toxin A per ml stimulated (P < 0.05) release of TNF-α (Fig. 1A), from 14.4 pg/10⁶ in sham-treated PMC to 36.5 or 46 pg/10⁶ cells, respectively.

To investigate the effects of toxin A on NO production by mast cells from infected rats, we exposed PMC to different concentrations of toxin A. Toxin A did not induce NO production in PMC (Fig. 1B) at any of the concentrations tested. Constitutive NO production by PMC in absence of toxin A was not affected (Fig. 1B).

Effect of toxin A on histamine release from PMC stimulated by IgE- and non-IgE-dependent pathways. To investigate the effects of toxin A on IgE-dependent histamine release, PMC from N. brasiliensis-infected and uninjected rats were incubated with different concentrations of toxin A (Fig. 2). Toxin A (4-h incubation, doses from 0.001 to 1 µg/ml) did not stimulate histamine secretion from normal or N. brasiliensis-sensitized mast cells above the level of spontaneous release (2.6% ± 0.5%, mean ± standard error [SE]). However, exposure to 1 µg of toxin A per ml significantly inhibited histamine release (77%, P < 0.01) from sensitized mast cells stimulated with NbAg. To analyze the inhibitory effect of toxin A on IgE-independent histamine release, naïve PMC were incubated with toxin A and then treated with A23187 (2.5 µM) for 20 min. A significant inhibition of histamine release (41%, P < 0.01) was observed when PMC were treated with 1 µg of toxin A per ml for 4 h. However, treatment of PMC with up to 1 µg of toxin A per ml for 30 min did not inhibit IgE-independent histamine release (data not shown).

Cytoskeletal changes induced with the early treatment on PMC. There were cytoskeletal changes in rat PMC after incubation with 1 µg of toxin A per ml for 4 h (Fig. 3). Shamtreated PMC cultured for 4 h showed a normal distribution of actin filaments (Fig. 3A). In contrast, PMC treated with 1 µg of toxin A per ml for 4 h showed cytoplasm retraction and cy-
toskeletal rearrangements with actin-containing dense deposits in the cytoplasm (Fig. 3B).

Morphological changes in PMC induced by C. difficile toxin A. TEM and SEM were used to examine naive PMC treated with 1 μg of toxin A per ml for 4 h (Fig. 4). Naive cells showed a regular distribution of granules with well-dispersed chromatin in the nuclei. The plasma membrane was normal, and granule contents were clearly visible (Fig. 4A). However, PMC treated with toxin A (Fig. 4C) showed a decreased number of membrane microvilli, changes in electron-dense granules in the cytoplasm, and pyknotic nuclei with condensation of nuclear chromatin, all features characteristic of apoptosis. Furthermore, PMC treated with toxin A and observed with SEM (Fig. 4D) showed morphological changes and evidence of enhanced granule release compared with untreated cells (Fig. 4B).

DNA fragmentation. DNA from PMC treated with 1 μg of toxin A per ml showed a characteristic ladder pattern after agarose electrophoresis (Fig. 5, lane C). DNA from sham-treated PMC (Fig. 5, lane B) did not show DNA fragmentation.

DISCUSSION

In experimental enterocolitis, toxin A causes inflammation and necrosis of the intestinal wall (24, 35, 44). Both mast cells and other leukocytes have been implicated in the pathophysiology of toxin A-induced mucosal damage. To understand the mechanisms involved, several in vivo and in vitro approaches have been used. For example, the in vitro interaction of toxin A with rat peritoneal macrophages induces release of interleukin-1β and TNF-α, which attract and activate neutrophils (40). Toxin A also causes release of RMCPII when injected in ileal loops of rats (6). This effect can be inhibited with a specific antagonist of substance P (34), suggesting that toxin A activates mast cells via substance P released from adjacent sensory neurons (30). Thus, macrophages, other leukocytes, and mast cells may play a role in the inflammation and necrosis associated with toxin A.

Although mast cells are an important source of several inflammatory mediators and have been implicated in toxin A-induced enterocolitis, no studies about the direct action of toxin A on isolated mast cells have been published. Thus, our study showed that low doses of toxin A induce the secretion of TNF-α from freshly isolated mast cells in vitro. This is to our knowledge, the first direct report showing that a bacterial enterotoxin causes release of TNF-α from mast cells. The ability of toxin A to induce release of TNF-α is not limited to mast cells, as both toxins A and B cause release of TNF-α by human monocytes (15).

In contrast to macrophages, mast cells store TNF-α in granules and can therefore release it immediately after toxin A activation and participate in the early phase of the inflammatory reaction. TNF-α is also known to cause necrosis of epithelial cells of the intestine, as well as inhibition of gastric motility (42). Thus, all the above effects suggest that mast cell-derived TNF-α may play a role in the mucosal damage and fluid accumulation observed in C. difficile-associated colitis.

Although the in vitro ability of toxin A to activate inflammatory cells such as neutrophils, macrophages, and mast cells (this study) has been demonstrated, the in vivo access of the toxin to these cells is in dispute because of the large size of the toxin. However, toxin A can cause detachment and apoptosis of enterocytes (27). In this disrupted epithelium, toxin A might diffuse and interact with inflammatory cells in the lamina propria, including mast cells.

In earlier reports, there is strong evidence that histamine is involved in the C. difficile toxin A experimental colitis in rats (25, 35). However, our results in vitro indicate that toxin A was
not able to directly induce the secretion of histamine or NO from mast cells. This contrasts to the in vivo degranulation observed after injection of toxin A in intestinal loops (25), suggesting that histamine is released in vivo due to indirect activation of mast cells, such as with substance P released by enteric nerves (6, 30, 34).

Our results further document that in mast cells, the mechanisms for TNF-α release are different from those involved in the release of histamine. Furthermore, we found that toxin A inhibited antigen-dependent and -independent release of histamine by mast cells. To release histamine, vesicles must move and fuse with the cell membrane, a process that requires the participation of the cytoskeleton. We found that in mast cells, like in other cell types, toxin A disrupts the actin microfilaments; this alteration may reduce the capacity of mast cells to release histamine contained in granules. It was recently reported that *C. difficile* toxins A and B alter cytoskeleton by monoglycosylation of GTP-binding proteins Rho and Rac (17, 22, 23). These proteins have been shown to regulate the role of the cytoskeleton during secretion of inflammatory mediators in mast cells (33, 37, 38). However, the concentration of toxin used to alter the PMC cytoskeleton was high, and it is probably not achieved in vivo. Once again, degranulation of mast cells may occur in vivo by activation of neuronal circuits.

NO is also an effector molecule produced by mast cells that could be involved in the pathophysiology of enterocolitis (41). Interestingly, NO is able to potentiate mast cell cytotoxicity mediated by TNF-α (5). However, toxin A did not stimulate NO synthesis by mast cells at any of the concentrations of toxin used. Thus, direct interaction of toxin A with mast cells in vitro may not induce NO production, although this does not preclude synthesis of NO by mast cells or other cells induced by indirect mechanisms such as neuropeptides. Recently, Qiu et al. (39) demonstrated that NO inhibits the enteroc-
toxicity of toxin A on rat small intestine; if NO acts as a mechanism of defense, this might come from cells other than mast cells.

We found that prolonged exposure to toxin A caused apoptosis of PMC. This is the first report on induction of apoptosis in mast cells caused by a bacterial toxin. The induction of apoptosis by bacterial products has been documented in other cell types (8, 21, 32). In fact, toxin A caused apoptosis in human intestinal epithelial cells (27). The ability of toxin A to cause apoptosis raises the interesting possibility that Rho and Rac proteins may be involved in the mechanisms regulating program cell death. We are currently studying the mechanisms involved in toxin A-induced apoptosis in mast cells.

During the acute phase of inflammation and necrosis in the intestinal wall caused by C. difficile toxins, mast cells are active and releasing mediators such as RMCPII, platelet-activating factor, and leukotriene B4 (6, 26, 35). This finding suggests that toxin A-induced apoptosis may not occur at this stage. However, it could be that in severe cases of pseudomembranous colitis, mast cells in the lamina propria may be exposed to toxin A at doses or times that induce apoptosis; macrophages, monocytes, and neutrophils may also be affected (14, 15, 40). This would impair the ability of these cells to counteract infection and may explain in part why severe cases are so refractory to treatment and prone to recrudescence.

Our results suggest that mast cells may participate in the initial inflammatory reaction by releasing TNF-α in response to toxin A. In contrast, histamine and NO may not participate when toxin A acts directly on mast cells. The disruption of the cytoskeleton caused by the toxin A may alter the ability of mast cells to secrete histamine and NO after antigen-dependent or -independent activation. In opposition to the necrotic action of toxin A in other cell types, it induces apoptosis in mast cells. Prolonged exposure to toxin A decreases function and viability of intestinal inflammatory cells, hindering their ability to counteract infection; this would probably favor severe and long-lasting cases of pseudomembranous colitis.
Toxin A for 24 h. Lane A, molecular weight marker profile from a 123-bp ladder from Gibco-BRL; lane B, DNA from PMC incubated in RPMI 1640–5% FBS for 24 h; lane C, fragmented genomic DNA from PMC treated with toxin A for 24 h.

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