Participation of reactive oxygen metabolites in Clostridium difficile toxin A-induced enteritis in rats

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Participation of reactive oxygen metabolites in Clostridium difficile toxin A-induced enteritis in rats. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G485–G490, 1999.—Reactive oxygen metabolites (ROMs) contribute to the pathophysiology of intestinal inflammation. Our aim was to ascertain the involvement of ROMs in experimental ileitis in rats produced by toxin A of Clostridium difficile. Intraluminal toxin A caused a significant increase in hydroxyl radical and hydrogen peroxide production by ileal microsomes starting 1 h following toxin exposure and peaking at 2–3 h, and this was inhibited by pretreatment with DMSO, a ROM scavenger, or superoxide dismutase (SOD), which inactivates ROMs. In contrast, mucosal xanthine oxidase increased only slightly after toxin A exposure, and allopurinol, an inhibitor of xanthine oxidase, had no effect on toxin A-associated intestinal responses. Induction of neutropenia resulted in reduction of toxin-mediated free radical formation, fluid secretion, and permeability. The enterotoxic effects of C. difficile toxin A were associated with increased ROM release in ileal tissues, and the ROM inhibitors DMSO and SOD inhibited these effects. This suggests that ROMs released during toxin A enteritis are released primarily from neutrophils invading the inflamed bowel segment.

superoxide radicals; hydroxyl radicals; intestinal inflammation; intestinal secretion; enterotoxins

THE CELLULAR AND MOLECULAR pathophysiology of Clostridium difficile toxin A-mediated enteritis has been elucidated in considerable detail over the past decade. In experimental animals toxin A binds to brush-border enterocyte receptors (20) and is internalized into the cytoplasm, where it inactivates Rho, a GTP-binding protein of the Ras superfamily (5, 10). Inactivation of Rho leads to disaggregation of filamentous actin and dysfunction of tight junctions in T84 colonicocyte monolayers, as evidenced by increased paracellular permeability and loss of electrical resistance (9). Toxin A enteritis in rats and rabbits is characterized by a marked increase in fluid and electrolyte secretion, comparable to that seen with equimolar concentrations of Vibrio cholerae enterotoxin (23). In contrast to cholera toxin, toxin A elicits a profound neutrophilic infiltrate that commences 2 h after intraluminal toxin administration and at 4 h is associated with widespread enterocyte necrosis and a florid acute inflammatory response in the lamina propria as well as the intestinal lumen (23). Antibodies directed against the neutrophil adhesion molecule CD11-CD18 markedly inhibit fluid secretion and tissue damage (11), and pharmacological blockade of substance P or mast cell degranulation also reduces neutrophil infiltration and tissue damage (18, 20). These findings indicate that neutrophilic invasion and subsequent mediator release in the affected bowel segment are critical components of toxin A pathogenesis.

Reactive oxygen metabolites (ROMs) have been implicated in the pathogenesis of experimental colitis in animal models and in idiopathic inflammatory bowel disease of humans (14, 24). ROMs or free radicals such as superoxide, hydroxyl ion, and hypochlorite radical are thought to directly mediate damage of proteins and lipids in target tissues. Lipid peroxidation of cellular membranes and oxidation of thiol groups in proteins are specific chemical reactions observed in ROM-associated injury. The two main sources of ROMs in experimental enteritis are xanthine oxidase within epithelial cells and NADPH oxidase in phagocytic leukocytes (resident macrophages, eosinophils, and invading neutrophils). The purpose of this study was to ascertain if ROMs were involved in toxin A enteritis and, if so, to determine their source.

MATERIALS AND METHODS

General. Male Wistar rats, weighing 200–250 g (Charles River Breeding Laboratories, Wilmington, MA), were housed at 22°C and 65–70% humidity. Rats were fasted 24 h before experimentation but allowed free access to water. Toxin A was purified to homogeneity from culture supernatants of C. difficile strain 10.463 as described previously by us (19).

Ileal loop experiments. Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (55 mg/kg). At laparotomy the renal pedicles were tied, and two closed ileal loops were formed in each animal as described (3, 4, 18). Immediately before toxin A administration, 10 µCi of [3H]mannitol (New England Nuclear) was injected into the inferior vena cava. Toxin (5 µg) was administered by intraluminal injection in 0.4 ml of 50 mM Tris buffer (pH 7.4). The abdominal incision was then closed, and at 4 h the animals were killed. Ileal loops were removed, and their weights and lengths were measured. Mucosal permeability to mannitol (dpm/cm of loop) and fluid secretion (loop weight (mg)/length (cm)) were then measured as described (3, 4, 18).

In some animals intravenous cannulas were implanted to deliver superoxide dismutase (SOD), an inhibitor of ROM formation. Polyethylene catheters (1.27 mm in diameter) filled with heparinized saline (500 IU/300 ml) to prevent clotting were implanted in the right jugular vein under...
aseptic conditions and subcutaneously exteriorized in the interscapular region. Three days after surgery animals were injected intravenously with either 0.9% NaCl (saline) or SOD solutions in a volume of 0.6 ml, according to the protocol to be described.

Intestinal mucosal xanthine oxidase and SOD. Xanthine oxidase activity in ileal mucosa was determined spectrophotometrically (21). Ileal mucosa was removed with a glass slide and homogenized in ice-cold phosphate buffer. After centrifugation for 20 min at 15,000 rpm, the supernatants were passed over a buffer-equilibrated G25 Sephadex (Pharmacia Biotechnology) column at 4°C to remove low-molecular-weight inhibitors. Xanthine oxidase activity was determined by measuring uric acid formation in the presence of xanthine and ambient oxygen. The absorbance of the solutions was measured using ultraviolet light with a wavelength of 295 nm.

SOD activity was determined by the nitro blue tetrazolium (NBT) reactions (21). Each cuvette contained 0.1 mM xanthine, 25 µM NBT, 0.1 mM EDTA, 50 mM NaCO₃ 0.1 ml sample, and 6 mM xanthine oxidase in a total volume of 3 ml at 25°C and a final pH of 10.2. The reaction was stopped by adding CuCl₂ at 25°C 3 min later. Inhibition of NBT reduction in the samples was monitored spectrophotometrically at 560 nm.

Hydroxyl radical and hydrogen peroxide formation. After toxin A administration, the ileal loops were removed and the mucosa was scraped off with a glass slide. Microsomal fractions were prepared from a 10% homogenate of the mucosa in 0.9% NaCl (pH 7.4). The homogenate was sequentially centrifuged at 1,000 g for 10 min and 12,000 g for 20 min. The microsomal fraction was suspended in 0.9% NaCl (pH 7.4) at a concentration of 20 mg protein/ml. Microsomes from control and toxin A-treated loops were tested for generation of hydroxyl or hydroxyl-like species by assaying formaldehyde production from DMSO as described (15). To verify the hydroxyl radical assay we incubated microsomal fractions from mucosal scrapings of control rats (n = 8/group) with catalase (20 mg/ml) or the nonchelator deferoxamine (0.1 mM) before measuring hydroxyl or hydroxyl-like species (15). Previous studies showed that both catalase and deferoxamine effectively prevent hydroxyl radical formation (25, 26). Our results showed that both catalase and deferoxamine effectively inhibited (by 88.3 and 90%, respectively, P < 0.01 for both) hydroxyl radical formation, confirming the specificity of the assay used in our experiments.

Hydrogen peroxide formation was measured with the ferricinium sulfate potassium thiocyanate method in the presence of NaN₃ to block peroxidase activity (1). The incubation mixture of 1.5 ml contained 1 mg protein, 50 mM Tris-chloride buffer (pH 7.4), and 0.75 µmol NADPH. The microsomes were incubated 20 min at 30°C under continuous shaking. Controls included incubation mixtures to which TCA was added before the microsomal suspension.

Addition of neutrophils to rats. To examine the role of neutrophils in toxin A-induced ileitis, neutropenia was induced by the intraperitoneal injection of a specific adsorbed polygonal rabbit anti-rat neutrophil antiserum as described previously by von Ritter et al. (24). Preliminary studies demonstrated that intraperitoneal injection of a 1:10 dilution of antibody (Al-ADS1140; Accurate Chemical and Scientific Corporation, Westbury, NY) at a dose of 2 ml/100 g body weight produced a marked neutropenia, reaching a nadir ~8 h following injection and being sustained for the following 48 h. Consequently, rats received antibody 1 day before the experiments to achieve a 90% or greater reduction in peripheral blood neutrophil counts at the time of toxin injection. Neutrophil counts were determined on venous blood samples from a lateral tail vein using a hemocytometer.

Tissue MPO activity. Tissue myeloperoxidase (MPO) activity was measured to estimate tissue polymorphonuclear cell accumulation during toxin A enteritis. This enzyme has previously been shown to correlate significantly with numbers of polymorphonuclear cells determined histologically in colonic tissues (11, 12). Tissue MPO activity was determined by the method of Krawisz et al. (12). Approximately 0.2-g samples of mucosa were homogenized in 0.05 M potassium phosphate buffer, containing hexadecyltrimethylammonium bromide (Sigma). The mucosal homogenate was then centrifuged at 20,000 g for 15 min. An aliquot (0.1 ml) of the supernatant was added to 3.0 ml o-dianisidine (Aldrich Chemical, Milwaukee, WI), and the change in absorbance at 450 nm was determined. One unit of MPO activity was equivalent to the catalysis of 1 nmol hydrogen peroxide per minute at 25°C.

In some experiments the effects of DMSO and SOD on toxin A-induced increased tissue MPO activity were examined. Rats (n = 6/group) were pretreated with 12,000 U/kg of SOD or with 1% of DMSO, as described below, before injection of either buffer or 5 µg of toxin A into ileal loops. After 4 h animals were killed and tissue MPO activity was measured as previously described.

Drug administration. All drugs were freshly prepared within 1 h of use. SOD dissolved in 0.9% NaCl solution was injected intravenously 15 min before operation, and three additional injections with the same dose of SOD were given at 1-h intervals after toxin A administration to achieve a steady-state serum concentration. The doses of SOD tested were 4,000, 8,000, 12,000, and 16,000 U/kg. DMSO at concentrations of 0.1, 0.5, 1, and 5% was added to drinking water for 7 days before surgery. Allopurinol at 0.01 or 0.1 g/kg body weight (17) was administered by gavage 24 and 0.5 h before toxin A administration.

Statistical analysis. Results are expressed as means ± SE. Data were analyzed by the two-tailed unpaired Student’s t-test. Differences between groups exposed to multiple factors in the same experimental conditions were examined by post hoc test following one-way or mixed ANOVA significance.

RESULTS

ROM generation following toxin A exposure. The production of hydroxyl radicals (Fig. 1A) and hydrogen peroxide (Fig. 1B) in intestinal microsomes was increased significantly above baseline at 2, 3, and 4 h following toxin A administration. These increases were temporally correlated with alterations of intestinal permeability, blood-to-lumen mannitol clearance, and histological damage (Table 1). The major effects of 5 µg toxin A on most parameters studied were statistically increased or altered after 2 h of toxin exposure, and this correlated with increases in hydroxyl radicals and hydrogen peroxide at 2 h post-toxin exposure. At 4 h post-toxin exposure parameters of enterotoxicity were maximal (Table 1), whereas ROMs were declining slightly from their peak values at 3 h.

Pharmacological inhibition of toxin A effects. Two types of inhibitors were used to determine if ROMs contributed to fluid secretion or changes in mannitol permeability caused by toxin A exposure. SOD catalyzes the conversion of superoxide radical to hydrogen peroxide, which is then quickly inactivated by catalase. When administered to animals undergoing oxidative
Again, dose response effects on fluid secretion (Fig. 3) and mucosal permeability to mannitol, and histological severity of enteritis were observed with oral pretreatment with DMSO starting 7 days before toxin exposure. Similar results were observed with water. We also examined the effect of DMSO (1%) and SOD (12,000 U/kg) pretreatment on mucosal MPO activity following toxin A administration. Injection of toxin A into ileal loops of nontreated rats increased mucosal MPO activity by 7.3-fold compared with MPO levels in buffer-injected loops (P < 0.01, n = 6). Pretreatment with DMSO inhibited toxin A-induced increased mucosal MPO activity by 71.5% (P < 0.05, compared with toxin A alone).

Table 1. Effect of toxin A on rat ileal fluid secretion, mucosa permeability to mannitol, and histological severity of enteritis

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.5 h</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal secretion, cm/mg</td>
<td>110 ± 20</td>
<td>125 ± 11</td>
<td>140 ± 4</td>
<td>220 ± 11*</td>
<td>372 ± 11*</td>
</tr>
<tr>
<td>[3H]Mannitol permeability, dpm/cm</td>
<td>1,350 ± 295</td>
<td>1,550 ± 180</td>
<td>1,350 ± 190</td>
<td>4,250 ± 130*</td>
<td>42,500 ± 3,300*</td>
</tr>
<tr>
<td>Epithelial necrosis</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.4</td>
<td>0.8 ± 0.4*</td>
<td>2.3 ± 0.1*</td>
</tr>
<tr>
<td>Mucosal infiltration</td>
<td>0.4 ± 0.1</td>
<td>0.8 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>2.0 ± 0.3*</td>
<td>2.6 ± 0.2*</td>
</tr>
<tr>
<td>Neutrophil infiltration</td>
<td>0.3 ± 0.1</td>
<td>0.8 ± 0.3</td>
<td>1.4 ± 0.2*</td>
<td>1.8 ± 0.1*</td>
<td>2.5 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8–12 loops/group. Rat ileal loops were injected with either 5 µg of toxin A or buffer (control). At indicated time intervals, loops were excised and fluid secretion was assessed by loop weight (mg)-to-length (cm) ratio. Intestinal permeability to mannitol was estimated as blood-to-lumen clearance of [3H]mannitol. Histological severity of enteritis was graded by a score of 0–3 for epithelial cell damage, congestion, and edema of mucosa and neutrophil infiltration. *P < 0.05 vs. control.
Table 2. Effects of neutropenia on toxin A-mediated intestinal secretion, mannitol permeability, MPO, and hydroxyl radical formation

<table>
<thead>
<tr>
<th>Group</th>
<th>Fluid Secretion, mg·cm⁻¹·4 h⁻¹</th>
<th>Permeability, dpm·cm⁻¹·4 h⁻¹</th>
<th>MPO, U/mg protein</th>
<th>Hydroxyl Radical, nmol/mg protein</th>
</tr>
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<tbody>
<tr>
<td>Buffer</td>
<td>128 ± 20</td>
<td>1,896 ± 256</td>
<td>0.31 ± 0.04</td>
<td>4.22 ± 0.71</td>
</tr>
<tr>
<td>Toxin A</td>
<td>335 ± 14*</td>
<td>28,800 ± 4,200†</td>
<td>1.67 ± 0.28†</td>
<td>9.89 ± 0.90†</td>
</tr>
<tr>
<td>Anti-neutrophil antibody</td>
<td>220 ± 12§</td>
<td>17,344 ± 2,198‡</td>
<td>0.42 ± 0.06†</td>
<td>5.91 ± 0.74†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 loops/group. MPO, myeloperoxidase. Anti-neutrophil antibody at a dose of 2 ml/100 g body wt produced marked neutropenia after injection that was sustained for the following 48 h. *P < 0.05, †P < 0.01 when compared with buffer group. §P < 0.05, §P < 0.01 when compared with toxin A group.

n = 6), whereas pretreatment with SOD (n = 6) did not inhibit increased MPO activity in response to toxin A.

Neutropenia inhibits toxin A effects. To evaluate the contribution of neutrophils to toxin A effects, we induced profound neutropenia in rats by intraperitoneal injection of a polyclonal anti-neutrophil antibody that reduced peripheral blood neutrophil counts by >90%. Intestinal secretion and mannitol permeability were both significantly diminished in toxin-treated neutropenic rats compared with animals without neutropenia (Table 2). In addition, mucosal MPO activity and hydroxyl radical concentration in response to toxin were both significantly reduced during antibody-associated neutropenia.

Ileal xanthine oxidase activity. Another important source of ROMs in inflammatory or ischemic enteritis is the xanthine oxidase-mediated hypoxanthine-xanthine reaction in intestinal mucosa. Granger and colleagues (8) have proposed that mucosal xanthine oxidase generates ROMs at the time of reperfusion during a cat model of ischemia-reperfusion injury and that these ROMs promote the attraction and activation of circulating granulocytes in the ischemic segment. We measured ileal mucosal xanthine oxidase activity following toxin A exposure. A minor increase (~10%, P > 0.1) in mucosal xanthine oxidase activity was observed in ileal mucosal scrapings from 1 to 4 h following ileal loop exposure to 5 µg purified toxin A (Fig. 4). To assess the possible contribution of xanthine oxidase-derived ROMs in toxin A responses, we pretreated rats with allopurinol at doses of 0.01 and 0.1 mg/kg body weight by gavage 24 and 0.5 h before toxin administration. Similar doses of allopurinol have been shown to inhibit rat small intestinal mucosal xanthine oxidase (7, 13). As shown in Fig. 5, allopurinol pretreatment did not inhibit toxin A-induced fluid secretion or ileal permeability to mannitol at 4 h following toxin exposure.

DISCUSSION

The main finding reported here is an increase in hydroxyl radical and hydrogen peroxide concentrations in ileal mucosal microsomes following intraluminal instillation of 5 µg purified toxin A of C. difficile. The increase in ROMs was temporally correlated with the gradual evolution of typical enterotoxic effects that commence ~2 h after toxin exposure. Importantly, the increase in fluid secretion, mannitol permeability, and inflammatory changes 2 h after toxin A exposure (Table 1) were correlated temporally with a significant increase in hydroxyl radical and hydrogen peroxide at 2 h (Fig. 1). The inhibitory effects of SOD and DMSO on these enterotoxic parameters suggest that ROMs con-
tribute to the mechanism of C. difficile enteritis and diarrhea.

Previous work in our laboratory indicates that toxin A triggers a reproducible sequence of events in the lamina propria that culminates in severe diarrhea and enteritis. The earliest events that we have observed in this model are activation of mucosal mast cells (4) and release of substance P from intestinal sensory afferent neurons (3). Injection of toxin A into ileal loops caused mast cell degranulation after 15 min (4) and increased substance P content in the cell bodies of the dorsal root ganglia at 30 min (3), followed by substance P release in the ileal mucosa at 1 h (3). Mast cell degranulation in this model involves activation of sensory neurons(s) because chronic pretreatment of rats with capsaicin, a drug that desensitizes primary sensory afferents, inhibits mast cell degranulation as well as intestinal secretion and inflammation (4). Leukocyte infiltration begins 1.5 h following intraluminal exposure, and after 2–4 h the lamina propria, epithelial layer, and lumen are heavily invaded by polymorphonuclear leukocytes, mostly neutrophils (3, 11). These greatly amplify the enterotoxic effects, particularly fluid secretion, permeability, and histological changes (11). For example, pretreatment with a specific substance P inhibitor markedly diminishes the toxin A leukocyte response as well as fluid secretion and increased mannitol permeability (18). Similar results were observed in this study in rats made neutropenic by administration of an antibody to neutrophils before toxin exposure (Table 2). Thus neutrophil invasion and activation into toxin-exposed loops amplify the secretory and inflammatory effects of the toxin. We report here that secretion and mannitol permeability are significantly inhibited by an antibody to neutrophils which induces neutropenia in rats (Table 2). In these experiments, both neutrophilic infiltration and hydroxyl radical release were also significantly diminished, suggesting that one likely source of the ROMs was invading neutrophils.

Our results with DMSO and SOD also support the possibility that ROMs themselves, specifically hydroxyl and superoxide radicals, contribute to toxin A effects in rat ileum. The observation that SOD and DMSO only partially inhibited the enterotoxic effects of toxin A are consistent with the contribution of other mediators in the toxin A response. It is known that, for example, leukotriene B4 and platelet-activating factor, both potent neutrophilic chemotactic factors, are released from the small bowel following exposure to toxin A (20, 23). Clearly, ROMs are only one class of several inflammatory mediators that contribute to this model of inflammation.

Our results are consistent with other observations concerning the contribution of ROMs to intestinal inflammation, particularly in the microvascular injury in the small intestine during ischemia reperfusion (2). In this model injury is associated with granulocyte infiltration of the reperfused ischemic segment and is largely prevented by agents that either inhibit xanthine oxidase or scavenge oxygen radicals. Because inhibition or inactivation of xanthine oxidase inhibits reperfusion injury (15), it has been suggested that mucosal xanthine oxidase is the major source of oxygen radicals in this experimental model and that xanthine oxidase-derived oxidants initiate the leukocyte infiltration that follows reperfusion of the ischemic bowel segment. Direct measurement of oxygen radicals in reperfused cat small intestine revealed three sources for radicals (16). Xanthine oxidase accounted for ~15%, neutrophils 50%, and other sources 35%. In contrast, xanthine oxidase probably does not contribute substantially to ROM formation in our model as evidenced by a lack of effect of allopurinol on toxin A-associated epithelial permeability or fluid secretion (Fig. 4). Although intestinal microsomes alone can generate oxygen radicals (Fig. 1), it is likely that the large increases observed after toxin A exposure are contributed by infiltrating neutrophils. Interestingly, DMSO and SOD pretreatments were equally effective in inhibiting the enterotoxic effects of toxin A, but only DMSO inhibited MPO activity. This suggests that the modulatory effect of SOD may involve a nonneutrophilic mechanism.

Reactive oxygen radicals also appear to contribute to ulcerative colitis and Crohn’s disease in humans, diseases characterized by infiltration of affected tissues with activated monocytes and neutrophils. Reduced concentrations of tissue antioxidant molecules such as ascorbate, glutathione, and ubiquinol have been described in sites of active inflammation in Crohn’s disease and ulcerative colitis. Recently, McKenzie et al. (14) provided direct evidence for in vivo oxidant injury in the inflamed mucosa but not in the normal or healed mucosa in Crohn’s disease patients. Oxidative damage to thiol groups of colonocyte proteins was attributed to reactive oxygen or nitrogen species released from monocytes or neutrophils.

In summary, we report here that the acute enteritis that results when purified toxin A of C. difficile is instilled in the rat ileum is accompanied by local generation of significant amounts of hydroxyl radicals and hydrogen peroxide. ROMs are generated primarily by infiltrating neutrophils, and pharmacological inhibition of radicals with SOD or DMSO diminished the degree of mucosal damage. These results suggest that acute C. difficile toxin-mediated colitis in humans may also depend in part on oxidative injury and that pharmacological inhibition of this pathway might ameliorate this damage.

This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-34583. I. Castagliuolo was supported by a Research Fellowship from the Crohn’s and Colitis Foundation of America.

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Received 25 March 1998; accepted in final form 26 October 1998.

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