

***Clostridium difficile* Toxin A–induced Microvascular Dysfunction**

Role of Histamine

Iwao Kurose, C. Pothoulakis,* J. Thomas LaMont,* Donald C. Anderson,† James C. Paulson,§ Masayuki Miyasaka,|| Robert Wolf, and D. Neil Granger

Departments of Physiology and Medicine, Center of Excellence in Arthritis and Rheumatism, Louisiana State University Medical Center, Shreveport, Louisiana 71130; *Section of Gastroenterology, University Hospital, Boston University School of Medicine, Boston, Massachusetts 02118; †Discovery Research, UpJohn Laboratories, Kalamazoo, Michigan 49001; §Cytel Corporation, San Diego, California 92121; and ||Department of Immunology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

Abstract

Clostridium difficile toxin A (Tx-A) mediates secretion and inflammation in experimental enterocolitis. Intravital video microscopy was used to define the mechanisms that underlie the inflammatory reactions elicited by direct exposure of the microvasculature to Tx-A. Leukocyte adherence and emigration, leukocyte-platelet aggregation, and extravasation of FITC-albumin were monitored in rat mesenteric venules exposed to Tx-A. Significant increases in leukocyte adherence and emigration (LAE) and albumin leakage were noted within 15–30 min of Tx-A exposure. These responses were accompanied by mast cell degranulation and the formation of platelet-leukocyte aggregates. The Tx-A–induced increases in LAE and albumin leakage were significantly attenuated by pretreatment with either monoclonal antibodies (mAbs) directed against the leukocyte adhesion glycoproteins, CD11/CD18, intercellular adhesion molecule-1, and P-selectin (but not E-selectin) or with sialyl Lewis x, a counter-receptor for P-selectin. The mast cell stabilizer, lodoxamide, an H₁- (but not an H₂-) receptor antagonist, and diamine oxidase (histaminase) were also effective in reducing the LAE and albumin leakage elicited by Tx-A. The platelet-leukocyte aggregation response was blunted by an mAb against P-selectin, sialyl Lewis x, and the H₁-receptor antagonist. These observations indicate that Tx-A induces a leukocyte-dependent leakage of albumin from postcapillary venules. Mast cell–derived histamine appears to mediate at least part of the leukocyte-endothelial cell adhesion and platelet-leukocyte aggregation by engaging H₁-receptors on endothelial cells and platelets to increase the expression of P-selectin. The adhesion glycoproteins CD11/CD18 and intercellular adhesion molecule-1 also contribute to the inflammatory responses elicited by toxin A. (*J. Clin. Invest.*

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Introduction

Clostridium difficile, a gram-positive anaerobic bacillus, is the principal etiologic agent responsible for antibiotic-associated pseudomembranous colitis in humans (1) and experimental animals (2). *C. difficile* produces two protein exotoxins: toxin A (Tx-A)¹ and toxin B, which have different physicochemical, biologic, and antigenic properties (3). Several studies indicate that Tx-A mediates the inflammatory responses and transport alterations (fluid secretion) observed in experimental *C. difficile* enterocolitis (4–7). Injection of Tx-A into rabbit ileal loops elicits an influx of neutrophils into the mucosa that is accompanied by fluid secretion, increased mucosal clearance of mannitol, and release of prostaglandin E₂, leukotriene B₄ (LTB₄), and platelet activating factor (PAF) (8, 9).

Both mast cells and leukocytes have been implicated in the pathobiology of Tx-A–induced mucosal dysfunction. Ketotifen, an inhibitor of inflammatory cells, including mast cells, attenuates the production/release of PAF and LTB₄, inflammatory cell infiltration, increased epithelial permeability, and tissue necrosis normally observed in the intestinal mucosa exposed to Tx-A (9). Recent experiments also indicate that mast cell degranulation is an early (15 min) event in rat ileal loops exposed to Tx-A (10). A role for leukocytes is suggested by observations that Tx-A is directly chemotactic for human and rabbit neutrophils (11) and that administration of a monoclonal antibody directed against the leukocyte adhesion glycoprotein CD11/CD18 significantly inhibited the secretion and inflammation observed in ileal loops exposed to Tx-A (12). Taken together, these observations suggest that the recruitment of circulating neutrophils into intestinal segments infected with *C. difficile* may result from the activation and/or degranulation of mucosal mast cells and that the adhesion of neutrophils to microvascular endothelium is a rate-limiting step in Tx-A–mediated mucosal dysfunction.

Intravital videomicroscopy has been used to examine the influence of different inflammatory stimuli on vascular permeability and its relation to leukocyte-endothelial cell adhesion in the microcirculation (13). While several inflammatory conditions are known to elicit both a rise in vascular permeability and an enhanced adherence and emigration of leukocytes in postcapillary venules (14), the dependence of the albumin leakage response on leukocyte adhesion appears to vary among inflammatory stimuli (13, 15). While it is well established that *C. difficile* Tx-A promotes the recruitment of neutrophils in the gut, the interaction between leukocytes and endothelial cell

Address correspondence to D. Neil Granger, Ph.D., Department of Physiology and Biophysics, LSU Medical Center in Shreveport, 1501 Kings Highway, P.O. Box 33932, Shreveport, LA 71130-3932.

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1. Abbreviations used in this paper: BBS, bicarbonate-buffered saline; DAO, diamine oxidase; D_v, venular diameter; ICAM-1, intercellular adhesion molecule-1; PAF, platelet activating factor; SLE^x, sialyl Lewis x; SLN, sialyl lactosamine; Tx-A, toxin A; V_{RBC}, red blood cell velocity.

adhesion molecules that are elicited by Tx-A as well as the impact of such interactions on the barrier function of microvascular endothelium remain unclear.

The overall objective of this study was to characterize the changes in leukocyte-endothelial cell adhesion and albumin extravasation observed in postcapillary venules exposed to Tx-A and to define the mechanisms responsible for these changes. Monoclonal antibodies directed against different leukocyte (CD11/CD18) and endothelial cell (intercellular adhesion molecule-1 [ICAM-1], P-selectin, and E-selectin) adhesion molecules were used to define the molecular determinants of Tx-A-induced leukocyte adherence and emigration and to determine whether Tx-A-induced microvascular dysfunction is a leukocyte-dependent process. The contribution of mast cell-derived histamine to Tx-A-induced microvascular alterations was assessed using mast cell stabilizers, histamine receptor antagonists, and diamine oxidase (DAO).

Methods

Surgical procedure. Male Sprague-Dawley rats (200–250 g) were maintained on a purified laboratory diet and fasted for 24 h before each experiment. The animals were initially anesthetized with pentobarbital (65 mg/kg body wt), then a tracheotomy was performed to facilitate breathing during the experiment. The right carotid artery was cannulated and systemic arterial pressure was measured with a pressure transducer (model P23A; Statham, Oxnard, CA). Systemic blood pressure and heart rate were continuously recorded with a physiologic recorder (Grass Instruments Co., Quincy, MA). The left jugular vein was also cannulated for drug administration.

Intravital microscopy. Rats were placed in a supine position on an adjustable Plexiglas microscope stage and the mesentery was prepared for microscopic observation as described previously (16, 17). Briefly, the mesentery was draped over a nonfluorescent coverslip that allowed for observation of a 2-cm² segment of tissue. The exposed bowel wall was covered with Saran Wrap (The Dow Chemical Co., Indianapolis, IN), and then the mesentery was superfused (bathed at a constant rate) with bicarbonate-buffered saline (BBS) (37°C, pH 7.4) that was bubbled with a mixture of 5% CO₂, 90% N₂.

An inverted microscope (TMD-2S, Diaphoto; Nikon, Tokyo, Japan) with a $\times 40$ objective lens (Fluor; Nikon) was used to observe the mesenteric microcirculation. The mesentery was transilluminated with a 12-V 100-W direct current-stabilized light source. A video camera (model VK-C150; Hitachi, Tokyo, Japan) mounted on the microscope projected the image onto a color monitor (model PVM-2030; Sony, Japan), and the images were recorded using a video cassette recorder (model NV8950; Panasonic, Tokyo, Japan). A video time-date generator (model WJ810; Panasonic) projected the time, date, and stopwatch functions onto the monitor.

Single unbranched venules with diameters ranging between 25 and 35 μm and length $> 150 \mu\text{m}$ were selected for study. Venular diameter (D_v) was measured either on- or off-line using a video image-shearing monitor (IPM, Inc., La Mesa, CA). The number of adherent leukocytes was determined off-line during playback of videotaped images. A leukocyte was considered to be adherent to venular endothelium if it remained stationary for a period ≥ 30 s (16). Adherent cells were expressed as the number per 100- μm length of venule. The number of emigrated leukocytes was also determined off-line during playback of videotaped images. Any interstitial leukocytes present in the mesentery at the onset of the experiment were subtracted from the total number of leukocytes that accumulated during the course of the experiment. Leukocyte emigration was expressed as the number per field of view surrounding the venule. Platelet-leukocyte aggregates which were visible within postcapillary venules were quantified and expressed as the number of aggregates crossing a fixed point within the venule over a 2-min period (13, 18, 19). Mast cells were visualized by staining of mesentery with 0.1%

toluidine blue upon completion of the intravital microscopy measurements. The percentage of degranulated mast cells surrounding the postcapillary venule was calculated as described previously (19).

Centerline red blood cell velocity (V_{RBC}) was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, TX) that was calibrated against a rotating glass disk coated with rat erythrocytes. Venular blood flow was calculated from the product of mean RBC velocity (V_{mean} = centerline velocity $\div 1.6$) (20) and microvascular cross-sectional area, assuming cylindrical geometry. Venular wall shear rate was calculated based on the Newtonian definition: $\dot{\gamma} = 8 (V_{\text{mean}}/D_v)$ (21).

To quantify albumin leakage across mesenteric venules, 50 mg/kg of FITC-labeled bovine albumin (Sigma Immunochemicals, St. Louis, MO) was administered (intravenously) to the animals 10 min before each experiment. Fluorescence intensity (excitation wavelength 420–490 nm emission wavelength 520 nm) was detected using a silicon-intensified target camera (model C-2400-08; Hamamatsu Photonics, Shizuoka, Japan). The fluorescence intensity of FITC-albumin within three segments of the venule under study (Iv) and in three contiguous areas of perivenular interstitium (Ii) area was measured at various times after administration of FITC-albumin using a computer-assisted digital imaging processor (NIH Image 1.35 on a Macintosh computer). An index of vascular albumin leakage was determined from the ratio of Ii-to-Iv at 60 min after administration of Tx-A, i.e., 90 min after injection of FITC-albumin (13, 18, 19).

Experimental protocols. After all parameters measured on-line (arterial pressure, V_{RBC} , and D_v) were in a steady state, the mesentery was superfused (2 ml/min) with BBS alone for a period of 30 min, with video recordings and measurements of all parameters made at the onset and at 30 min into the superfusion period. For the following 10 min, the mesentery was superfused with either BBS containing Tx-A (2 $\mu\text{g}/\text{ml}$, 6 mM) or BBS alone (controls). Then, the mesentery was superfused for an additional 50 min with BBS alone. All data shown in figures 2–8 represent values obtained at the final 50 min of Tx-A. In some mesenteric preparations, either hydroxyzine (H₁-receptor antagonist, 10 μM ; Sigma Immunochemicals), cimetidine (H₂-receptor antagonist, 10 μM ; Sigma Immunochemicals), or lodoxamide (mast cell stabilizer, 100 μM ; The Upjohn Co., Kalamazoo, MI) was added to the superfusate 5 min before exposure to Tx-A. In other experiments, animals were pretreated with ketotifen (mast cell stabilizer, 1.0 mg/kg., p.o., twice a day for 2 d before experiments, p.o.) or DAO (2.1 U/kg, 10 min before Tx-A exposure). Previous studies have shown that the effect of Tx-A on tissue damage or secretion in *in vivo* rabbit ileal loops is present even if Tx-A is removed after a 5-min exposure. A dose of 2 $\mu\text{g}/\text{ml}$ was used because previous studies indicated that injection of 1–5 μg of Tx-A resulted in a substantial enterotoxic effect (9, 10, 12). Ketotifen was used in this study because prefeeding rats with the drug dramatically inhibited the intestinal effects of Tx-A and this inhibition was accompanied by a substantial reduction of mast cell mediator release (9). The dose of DAO used in this study effectively abolishes the recruitment of rolling leukocytes elicited by superfusion of the rat mesentery with histamine 10^{-5} M (our unpublished observation).

In another series of experiments, a monoclonal antibody (mAb) directed against either the β -subunit (CD18) of CD11/CD18 (mAb CL26, 100 $\mu\text{g}/\text{rat}$), ICAM-1 (mAb 1A29, 2.0 mg/kg), P-selectin (mAb PB1.3, 2.0 mg/kg), E-selectin (mAb CL3, 1.5 mg/kg), or a nonbinding antibody (PNB1.6, 2.0 mg/kg) was administered intravenously at 30 min before commencing the superfusion with Tx-A and before obtaining the baseline measurements. To evaluate the involvement of sialyl Lewis X [SLe^x; NeuAc- α -2,3Gal β 1,4(Fuca α 1,3)GlcNAc β 1,3Gal β -O(CH₂)₅COOCH₃], a counterreceptor for selectins or a control oligosaccharide sialyl lactosamine [SLN; NeuAc α 2,3Gal β 1,4GlcNAc β 1,3Gal β -O(CH₂)₅COOCH₃] was administered (i.v., 10 mg/kg) 10 min before the experiment (15). The concentration of mAbs used in this study was based upon experiments which determined the minimal amount of mAb needed to maximally reduce the leukocyte adherence and emigration in rat mesenteric venules induced by either LTB₄ or PAF (22). mAbs CL26 (23), CL3 (24), and 1A29 (25) were produced by The Upjohn Company,

while PNB1.6 and PB1.3 (26) were provided by the Cytel Corporation (San Diego, CA).

Purification of Tx-A. Toxin-A was purified to homogeneity from culture supernatants of *C. difficile* strain 10,463 as described previously by us (12). Enterotoxicity of Tx-A was tested in the ligated rat ileal loop assay (9, 10) and cytotoxicity was determined by cell rounding of IMR-90 fibroblasts (27).

Statistics. The data obtained in this study were expressed as mean \pm SE, with six rats per group. The data were analyzed using standard statistical analyses, i.e., analysis of variance with Scheffe's post-hoc test. Statistical significance was set at $P < 0.05$.

Results

In untreated (control) rats, the V_{RBC} and wall shear rate in mesenteric venules were 3.5 ± 0.2 (mm/s) and 553 ± 19 s^{-1} , respectively, under baseline conditions. 60 min after superfusion with Tx-A, V_{RBC} (3.1 ± 0.1 mm/s) and wall shear rate (536 ± 30 s^{-1}) showed no significant change. None of the agents which were used in this study (hydroxyzine, cimetidine, DAO, lodoxamide, ketotifen, mAbs against CD18, ICAM-1, P-selectin, and E-selectin, the nonbinding mAb, SLe^x, or SLN) altered V_{RBC} or wall shear rate, either under control conditions or after Tx-A exposure.

Fig. 1 illustrates the time course of changes in the number of adherent (A) and emigrated (B) leukocytes and the albumin leakage (C) elicited by 10 min of exposure of the mesentery to Tx-A. In mesenteric preparations superfused with BBS alone (controls), leukocyte adherence was 1.3 ± 0.3 per 100 μm , with 1.2 ± 0.5 emigrated leukocytes per field and an albumin leakage index of $7.0 \pm 2.2\%$ after 60 min of superfusion. The number of adherent leukocytes was significantly elevated within 15 min after Tx-A exposure, while the increase in emigrated leukocytes became significant at 30 min. The number of adherent and emigrated leukocytes further increased to 8.8 ± 0.4 (cells/100 μm) and 6.8 ± 0.6 (cells/field), respectively, at 60 min. Albumin leakage from venules was significantly increased at 10 min and was further elevated to $43.0 \pm 3.5\%$ at 60 min. The albumin leakage observed at 60 min after Tx-A exposure was found to be highly correlated with both leukocyte adherence ($r = 0.69$, $P < 0.05$) and emigration ($r = 0.80$, $P < 0.05$). Albumin leakage was greater in regions of the venule which exhibited a high level of leukocyte adherence/emigration.

The effects of immunoneutralization of different adhesion glycoproteins on Tx-A-induced leukocyte adherence and emigration (at 60 min) are summarized in Figs. 2 and 3. mAbs directed against either CD18 (66%), ICAM-1 (58%), or P-selectin (57%) significantly reduced the Tx-A-induced increase in adherent leukocytes, while the E-selectin and nonbinding mAbs showed no effect. SLe^x (52%), but not the control sugar (SLN), also significantly attenuated the leukocyte adherence observed at 60 min after Tx-A exposure. A similar pattern of effectiveness in reducing leukocyte emigration was observed with the different antibodies, i.e., mAbs directed against CD18, ICAM-1, and P-selectin reduced the number of emigrated leukocytes by 74, 71, and 41%, while the E-selectin and nonbinding mAbs had no effect. Neither SLe^x nor SLN altered Tx-A-induced leukocyte emigration.

Fig. 4 summarizes the influence of the mAbs against different adhesion molecules on the albumin leakage response elicited by Tx-A. mAbs directed against CD18, ICAM-1, and P-selectin reduced Tx-A-induced albumin leakage by 78, 64, and 55%, respectively, while the E-selectin-specific and nonbinding

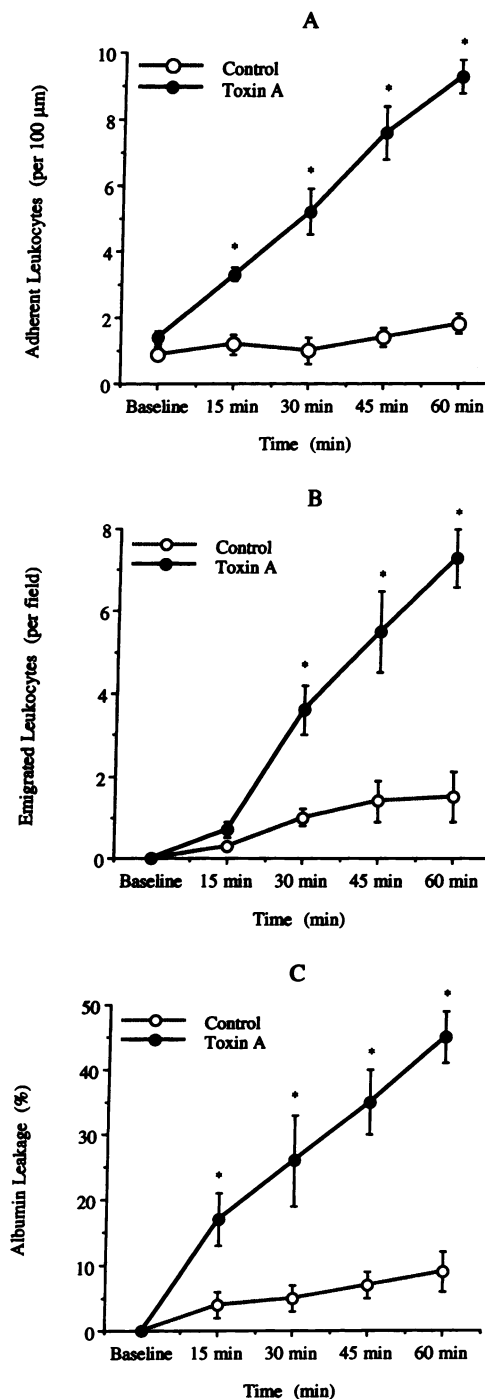


Figure 1. Time course of changes in leukocyte adherence (A) and emigration (B) and albumin leakage (C) in mesenteric venules exposed to *C. difficile* Tx-A. Tx-A. * $P < 0.05$ relative to control value ($n = 6$ in each group).

mAbs had no effect. SLe^x (92%) also prevented the albumin leakage observed in mesenteric venules exposed to Tx-A, while the control sugar (SLN) did not significantly influence this parameter.

A significant increase in the number of degranulated mast cells surrounding mesenteric venules ($31.1 \pm 5.1\%$) was also noted 60 min after Tx-A exposure, while superfusion with BBS

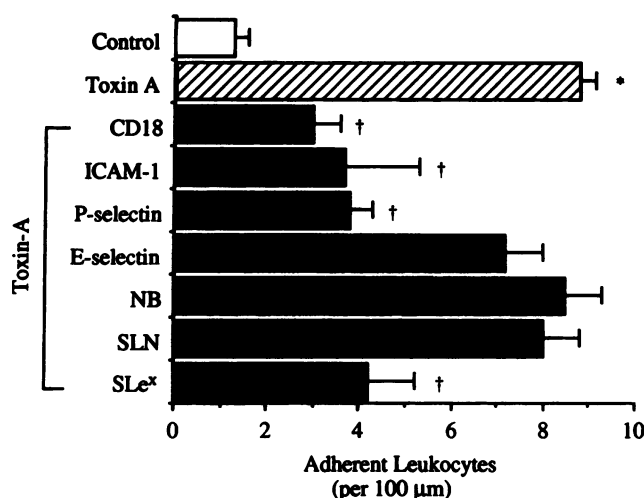


Figure 2. Effects of monoclonal antibodies directed against CD18, ICAM-1, P-selectin, E-selectin, SLe^x, and SLN on the increased leukocyte adherence induced by Tx-A. **P* < 0.05 relative to control value; †*P* < 0.05 relative to Tx-A alone. *n* = 6 in each group.

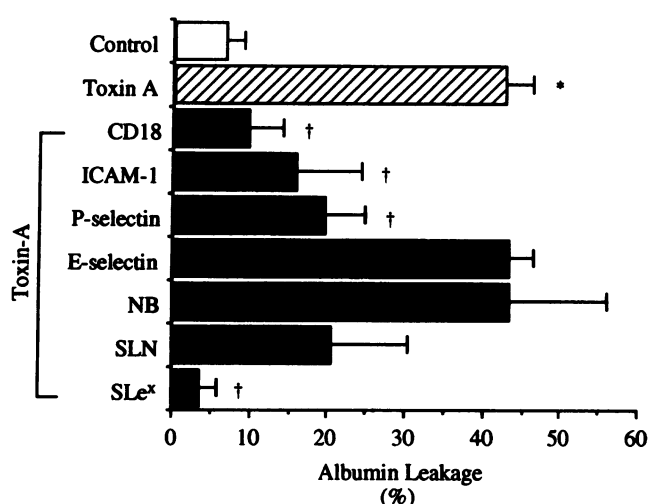


Figure 4. Effects of monoclonal antibodies directed against CD18, ICAM-1, P-selectin, E-selectin, SLe^x, and SLN on the increased albumin leakage induced by Tx-A. **P* < 0.05 relative to control value; †*P* < 0.05 relative to Tx-A alone. *n* = 6 in each group.

alone (control) resulted in only $3.9 \pm 1.6\%$ mast cell degranulation (Fig. 5). Both mast cell stabilizers, lodoxamide ($6.1 \pm 2.2\%$, 80% inhibition) and ketotifen ($15.0 \pm 4.4\%$, 52% inhibition), reduced the percentage of degranulated mast cells, however, lodoxamide was more effective in attenuating the mast cell degranulation. Hydroxyzine, cimetidine, and DAO did not affect Tx-A-induced mast cell degranulation. None of the antibodies or oligosaccharides used in this study attenuated the Tx-A-induced mast cell degranulation.

In view of the mast cell degranulation elicited by Tx-A, we next examined the influence of mast cell stabilizers, histamine antagonists, and DAO (histaminase) on Tx-A-induced leukocyte adherence (Fig. 6) and emigration (Fig. 7), and albumin leakage (Fig. 8) in mesenteric venules. Hydroxyzine (H₁-receptor antagonist), DAO, and lodoxamide (mast cell stabilizer)

significantly attenuated the Tx-A-induced leukocyte adherence by 66, 63, and 58%, while cimetidine (H₂-receptor antagonist) and ketotifen (mast cell stabilizer) had no effect. A similar pattern of effectiveness in reducing leukocyte emigration was also observed with the different agents, i.e., hydroxyzine, DAO, and lodoxamide reduced the number of emigrated leukocytes by 56, 49, and 46%, while cimetidine and ketotifen had no effect. Hydroxyzine (72%), DAO (52%), and lodoxamide (69%) also significantly reduced the albumin leakage elicited by Tx-A. Cimetidine and ketotifen did not alter the Tx-A-induced albumin leakage.

Exposure of the rat mesentery to Tx-A was often associated with the appearance of large platelet-leukocyte aggregates which filled the venule lumen and rapidly coursed through the vessel with flowing blood. These aggregates have been pre-

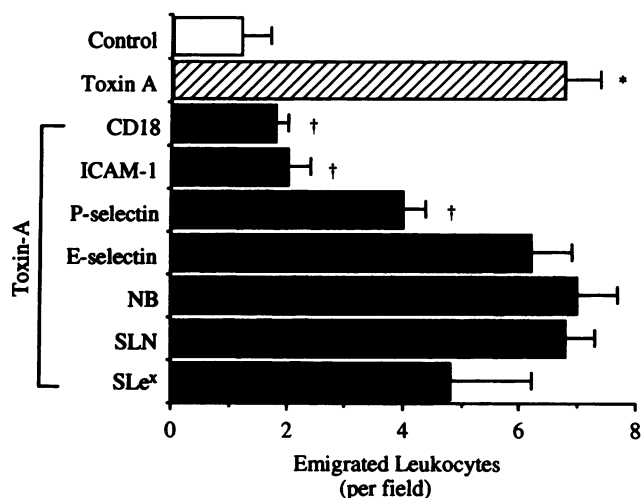


Figure 3. Effects of monoclonal antibodies directed against CD18, ICAM-1, P-selectin, E-selectin, SLe^x, and SLN on the increased leukocyte emigration induced by Tx-A. **P* < 0.05 relative to control value; †*P* < 0.05 relative to Tx-A alone. *n* = 6 in each group.

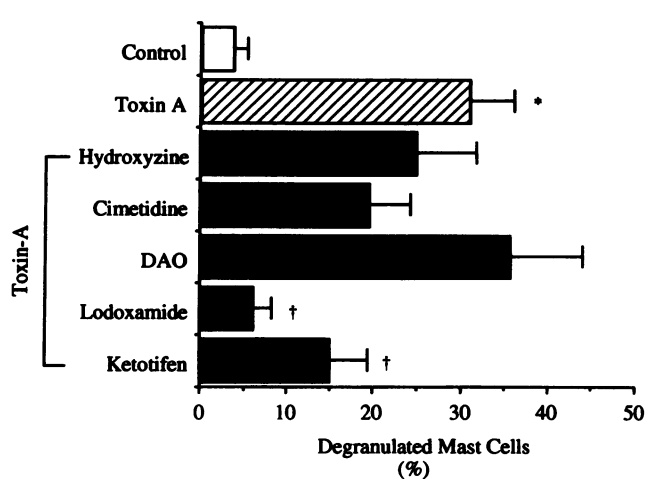


Figure 5. Effects of H₁- (hydroxyzine) and H₂- (cimetidine) histamine receptor antagonists, DAO, and mast cell stabilizers (lodoxamide and ketotifen) on the Tx-A-induced mast cell degranulation. **P* < 0.05 relative to control value; †*P* < 0.05 relative to Tx-A alone. *n* = 6 in each group.

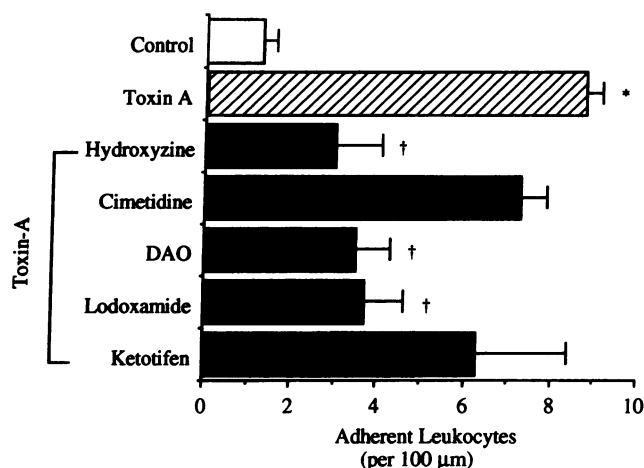


Figure 6. Effects of H₁- (hydroxyzine) and H₂- (cimetidine) histamine receptor antagonists, DAO, and mast cell stabilizers (lodoxamide and ketotifen) on the Tx-A-induced adherent leukocytes. **P* < 0.05 relative to control value; †*P* < 0.05 relative to Tx-A alone. *n* = 6 in each group.

viously observed in mesenteric venules exposed either to the nitric oxide synthase inhibitor, L-NAME (13), or to ischemia/reperfusion (18, 19). Although such aggregates were never observed under control conditions, 6.7 ± 0.9 aggregates per 5 min were observed in venules exposed to 10 min of Tx-A superfusion. mAbs directed against P-selectin reduced the formation of platelet-leukocyte aggregates (2.5 ± 1.7 aggregates/5 min), while the other antibodies used in this study had no effect. SLe^x (2.0 ± 1.6 aggregates/5 min) reduced the Tx-A-induced platelet-leukocyte aggregation. The control oligosaccharide SLN slightly reduced the rate of aggregate formation (3.3 ± 0.8 aggregates/5 min), however, this effect was not statistically significant. Hydroxyzine also reduced the formation of platelet-leukocyte aggregates in venules exposed to Tx-A (1.7 ± 1.1 aggregates/5 min, 75% inhibition). DAO (3.3 ± 1.1 aggregates/5 min)

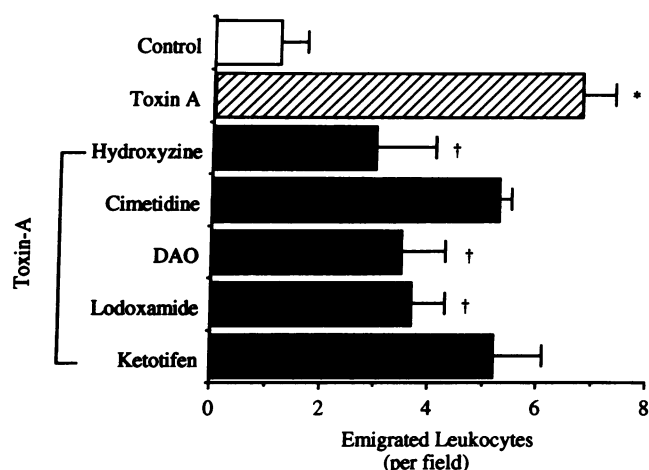


Figure 7. Effects of H₁- (hydroxyzine) and H₂- (cimetidine) histamine receptor antagonists, DAO, and mast cell stabilizers (lodoxamide and ketotifen) on the Tx-A-induced emigrated leukocytes. **P* < 0.05 relative to control value; †*P* < 0.05 relative to Tx-A alone. *n* = 6 in each group.

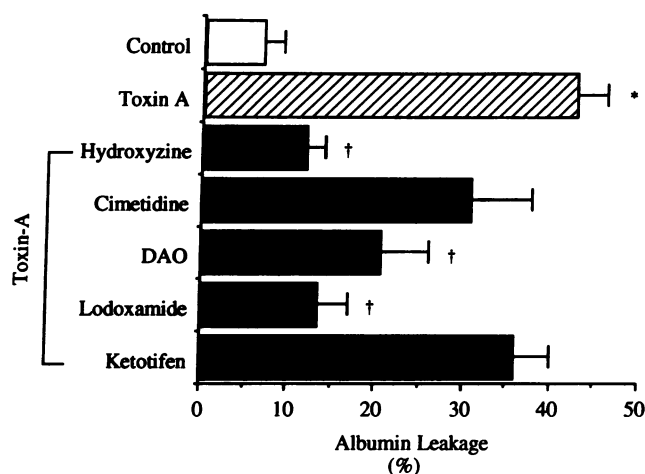


Figure 8. Effects of H₁- (hydroxyzine) and H₂- (cimetidine) histamine receptor antagonists, DAO, and mast cell stabilizers (lodoxamide and ketotifen) on the Tx-A-induced albumin leakage. **P* < 0.05 relative to control value; †*P* < 0.05 relative to Tx-A alone. *n* = 6 in each group.

and lodoxamide (3.7 ± 1.6 aggregates/5 min) tended to reduce the number of aggregates, however, these reductions were not statistically significant.

Discussion

This study provides the first direct evidence that *C. difficile* Tx-A promotes leukocyte margination in the microvasculature. Our findings clearly demonstrate that postcapillary venules exposed to Tx-A exhibit an increased level of leukocyte adherence and emigration, enhanced extravasation of albumin, and the formation of platelet-leukocyte aggregates. These intravascular changes are accompanied by the degranulation of mast cells situated in the immediate vicinity of the postcapillary venules. The vascular and extravascular responses to Tx-A occur rapidly, with significant leukocyte adhesion observed within 15 min and leukocyte emigration within 30 min of Tx-A exposure. Neutrophil accumulation in the mucosa of intestinal segments exposed to Tx-A occurs within 1–3 h (9). In the latter situation, access of Tx-A to venules in the lamina propria is significantly impeded by an intact mucosal epithelial barrier. Thus, the rapid inflammatory responses elicited in our studies are more likely to mimic the situation in which Tx-A gains access to the microvasculature via a disrupted mucosal barrier.

All of the microvascular alterations elicited by Tx-A (leukocyte-endothelial cell adhesion, increased albumin permeability, and platelet-leukocyte aggregation) are also observed in rat mesenteric venules exposed to ischemia and reperfusion (18, 19). Despite this similarity, it appears unlikely that the responses observed in Tx-A-treated venules can be attributed to a reduction in blood flow. Our studies indicate that Tx-A does not significantly alter V_{RBC} or wall shear rate in postcapillary venules. Previous reports clearly demonstrate that venular shear rate must be reduced by over 50% before a significant level of leukocyte adhesion is achieved (28).

The rapid onset of Tx-A-induced leukocyte-endothelial cell adhesion suggests that this response is not mediated by factors that act by stimulating the synthesis of proinflammatory factors

or adhesion molecules. Tx-A has been shown to activate macrophages *in vitro* to release IL-1, TNF- α , and IL-6 (29, 30). Endothelial cell monolayers treated with these cytokines can sustain a higher level of neutrophil adhesion (31), however, several hours are needed to upregulate the endothelial cell adhesion molecules that participate in this cytokine-mediated response (32, 33). A more rapid (within minutes) leukocyte adhesion can be elicited by exposing neutrophils and/or endothelial cells to certain inflammatory mediators, such as LTB₄, PAF, or histamine (33–35). These mediators are produced and released by a variety of cells including macrophages and mast cells (36). Consequently, the time course of the Tx-A-induced leukocyte adhesion response in postcapillary venules is consistent with a mechanism that allows for the production and/or release of an inflammatory stimulus that rapidly induces leukocyte adherence.

Our studies provide several lines of evidence that implicate a role for mast cell-derived histamine as one of the mediators of the leukocyte-endothelial cell adhesion elicited by Tx-A. First, we observed that exposure of the mesentery to Tx-A resulted in degranulation of over 30% of the mast cells surrounding postcapillary venules. This observation is consistent with the recent demonstration that Tx-A causes significant release of mast cell-derived protease II from rat ileal explants (9). In the latter study, it was also shown that ketotifen, a mucosal mast cell stabilizer, attenuates the neutrophil infiltration normally observed in Tx-A-treated rat ileal loops. Two mast cell stabilizers, ketotifen and lodoxamide, were used in the present study to assess the contribution of mast cells to the Tx-A-induced leukocyte-endothelial cell adhesion. Although both mast cell stabilizers significantly reduced the mast cell degranulation elicited by Tx-A, lodoxamide (80%) was clearly more effective than ketotifen (52%) in blunting the degranulation response. Our observation that ketotifen had no effect on leukocyte adherence and emigration, while lodoxamide significantly reduced these adhesion responses, suggests that mast cell products may contribute significantly to the inflammatory response elicited by Tx-A. An alternative explanation for these findings is that the inhibitory effects of lodoxamide on leukocyte adhesion are unrelated to its mast cell stabilizing action.

The possibility that mast cell products contribute to Tx-A-induced leukocyte adhesion is further supported by our findings with agents that interfere with the biological actions of histamine, i.e., histamine receptor antagonists and DAO (histaminase). We observed that treatment of the mesenteric microcirculation with either DAO (to prevent extravascular accumulation of histamine) or an H₁-, but not an H₂-, receptor antagonist significantly blunted the leukocyte-endothelial cell adhesion elicited by Tx-A. These observations suggest that Tx-A stimulates mast cells to release histamine, which engages H₁-receptors on endothelial cells and/or leukocytes to cause adhesion. A role for the H₁-receptor in mediating leukocyte-endothelial cell adhesion was demonstrated recently in rat mesenteric venules that were superfused with histamine (15). The increased leukocyte adhesion elicited by histamine was prevented by hydroxyzine but not by cimetidine. A similar role for the H₁-receptor (and mast cell degranulation) has also been demonstrated in a model of lactoferrin-induced leukocyte adherence in rat mesenteric venules (37).

The results of recent studies indicate that the leukocyte adhesion in postcapillary venules induced by histamine is related to the ability of the autacoid to increase P-selectin expression

on endothelial cells. The increased leukocyte adhesion elicited by histamine or lactoferrin is largely prevented by prior administration of a monoclonal antibody directed against P-selectin (15, 37). These *in vivo* observations are consistent with experiments on endothelial cell monolayers, which demonstrate rapidly increased surface expression of P-selectin within minutes of exposure to histamine (34, 35), as well as an increased P-selectin-dependent adhesion of neutrophils that is inhibited by a histamine H₁-receptor antagonist (37). The findings of the present study are also consistent with a histamine-induced, P-selectin-mediated leukocyte adhesion response, inasmuch as a P-selectin mAb was as effective in reducing Tx-A-induced leukocyte adherence in mesenteric venules as an H₁-receptor antagonist and DAO. Additional support for a contribution of P-selectin in Tx-A-induced leukocyte adhesion is provided by the observation that a synthetic SLe^x oligosaccharide also attenuates the leukocyte adherence response normally elicited by Tx-A. SLe^x is a fucose-containing oligosaccharide that normally exists on the surface of leukocytes, where it can act as a ligand for P-selectin (38, 39). The SLe^x oligosaccharide, but not a control oligosaccharide lacking fucose, has been shown to inhibit the recruitment of rolling leukocytes in mesenteric venules elicited by either histamine (15) or thrombin-receptor peptide (40).

It is now recognized that P-selectin plays an important role in modulating the low avidity binding of leukocytes to endothelial cells that is manifested *in vivo* as leukocyte rolling (15, 41). Since leukocytes must first roll before they can firmly adhere to, and subsequently emigrate across, microvascular endothelium (42), it is not surprising that P-selectin mAbs are effective in reducing both the rolling and firm adherence of leukocytes elicited by certain inflammatory mediators (13, 15). Nonetheless, it appears that other adhesion glycoproteins participate in the high avidity adhesive interactions between leukocytes and endothelial cells that is manifested *in vivo* as adherence (firm adhesion). The available *in vitro* and *in vivo* data invoke a role for the β_2 -integrins (CD11/CD18) and ICAM-1 in mediating leukocyte adherence and emigration (43). The results of the present study also invoke a role for CD11/CD18–ICAM-1 interactions in mediating the leukocyte adherence and emigration elicited in mesenteric venules exposed to Tx-A. mAbs directed against either the common β -subunit (CD18) of the CD11/CD18 leukocyte adhesion glycoprotein or the endothelial cell adhesion molecule ICAM-1 were as effective as the P-selectin, but not E-selectin, mAb in attenuating Tx-A-induced leukocyte adherence. These observations suggest that the rolling leukocytes recruited by Tx-A are exposed to an inflammatory mediator(s) that elicits an increased expression and/or activation of CD11/CD18 on the leukocyte surface. Since histamine does not exert this effect on neutrophils, it is likely that another mast cell product (e.g., LTB₄, PAF) acts as the yet undefined mediator. The rapid onset of Tx-A-induced leukocyte adherence in mesenteric venules argues against an increased endothelial cell expression of either ICAM-1 or E-selectin in our studies, inasmuch as several hours (due to protein synthesis) are normally required for an increased surface expression of these adhesion molecules on endothelial cells exposed to inflammatory mediators (cytokines) (32, 33, 43). Consequently, it can be assumed that the reduction in leukocyte adherence and emigration observed with the ICAM-1 mAb likely reflects the participation of constitutively expressed ICAM-1 in the Tx-A-induced adhesion response. ICAM-1, but not E- or P-

selectin, is normally present on endothelial cells in postcapillary venules. Although an E-selectin mAb was ineffective in reducing Tx-A-mediated leukocyte-endothelial cell adhesion, it is possible that E-selectin contributes to the leukocyte recruitment observed several hours after exposure of the gut to Tx-A (4, 9). In addition to promoting the adhesion of leukocytes to endothelial cells, Tx-A elicited the formation of platelet-leukocyte aggregates in postcapillary venules. We have demonstrated previously that conditions associated with a reduced production of nitric oxide (NO synthase inhibition or ischemia/reperfusion) are also characterized by the formation of platelet-leukocyte aggregates and that immunoneutralization of P-selectin largely prevents this aggregation process (13, 18). The results of the present study indicate that Tx-A-induced platelet-leukocyte aggregation is also P-selectin dependent and that histamine (via H_1 -receptor engagement) is the likely stimulant of P-selectin expression on platelets. This contention is supported by in vitro studies that have demonstrated histamine-mediated P-selectin expression on platelets as well as endothelial cells (32, 44).

A novel observation of the present study is that *C. difficile* Tx-A causes an increased leakage of albumin across postcapillary venules. This finding is somewhat expected in view of our data implicating histamine, which is well known to increase vascular permeability through engagement of H_1 -receptors (15), in the microvascular responses to Tx-A. Intravital microscopic analyses of histamine-induced albumin leakage across postcapillary venules suggest that this is a leukocyte-independent response (15, 45). In the present study, we found that agents (mAbs, hydroxyzine, DAO, lodoxamide, SLe^x) that are effective in attenuating Tx-A-induced leukocyte-endothelial cell adhesion are also effective in reducing Tx-A-induced albumin leakage. These observations, coupled to the significant positive correlations obtained between albumin leakage and the number of adherent or emigrated leukocytes, suggest that Tx-A-induced albumin leakage in mesenteric venules is dependent, in large part, on histamine-mediated leukocyte-endothelial cell adhesion. Nonetheless, we cannot exclude the possibility that histamine mediates some of the Tx-A-induced albumin leakage through direct stimulation of endothelial cell contraction after engagement of the H_1 -receptors (46, 47). It is also conceivable that products of platelet activation, that are released consequent to aggregation with leukocytes, may contribute to the Tx-A-induced albumin leakage response.

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