IL-8 release and neutrophil activation by Clostridium difficile toxin-exposed human monocytes

Joanne K. Linevsky, Charalabos Pothoulakis, Sarah Keates, Michel Warny, Andrew C. Keates, J. Thomas Lamont, and Ciarán P. Kelly. IL-8 release and neutrophil activation by Clostridium difficile toxin-exposed human monocytes. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G1333–G1340, 1997.—Neutrophil infiltration is central to the pathogenesis of Clostridium difficile toxin A-induced enterocolitis. This study examines whether monocyte activation by C. difficile toxins is instrumental in initiating neutrophil activation and recruitment. Human monocytes were exposed to low concentrations of highly purified C. difficile toxins, and the conditioned media were harvested for cytokine and functional assays. Monocytes exposed to C. difficile toxin A (10⁻¹⁰ M) or toxin B (10⁻¹² M) released 100 and 20 times basal levels, respectively, of the neutrophil chemoattractant interleukin-8 (IL-8). Reverse transcriptase-polymerase chain reaction demonstrated a marked increase in IL-8 mRNA expression by monocytes 3 h after toxin exposure. Conditioned media from toxin A- and toxin B-treated monocytes stimulated neutrophil migration (324 and 245% of control, respectively). This effect was completely blocked by IL-8 antiserum. These media also upregulated neutrophil CD11b/CD18 and endothelial cell intercellular adhesion molecule-1 expression. C. difficile toxins, at low concentrations, potentiate activation of monocytes to release factors, including IL-8, that facilitate neutrophil extravasation and tissue infiltration. Our findings indicate a major role for toxin-mediated monocyte and macrophage activation in C. difficile colitis.

Pseudomembranous colitis; intestinal inflammation; cytokines; adhesion molecules; interleukin-8

Our current understanding of the pathogenesis of Clostridium difficile colitis is that antibiotic therapy disturbs the normal colonic microflora to allow colonization by toxigenic C. difficile (17). The organism releases two toxins, A and B. Toxin A, a 308-kDa protein, is a potent inflammatory enterotoxin that, when injected into rabbit ileal loops, elicits fluid secretion, increased mucosal permeability, and a marked destructive inflammatory response (14, 17, 22, 24, 34). Toxin B, a 269-kDa protein, is a potent cytoxin in vitro but does not produce intestinal inflammation or alter intestinal permeability in the rabbit ileal loop model (23, 33). However, more recent studies indicate that toxin B may be even more harmful to human colonic tissue than toxin A (27). The intracellular mechanism of action of both toxin A and toxin B has recently been described. Both toxins prevent ADP-ribosylation of the low-molecular-weight guanosine triphosphatase Rho, resulting in actin depolymerization and cell death (8, 9, 13).

Neutrophil recruitment appears to be an essential step in the pathogenesis of C. difficile toxin-induced intestinal injury. Biopsy specimens from patients with C. difficile colitis show striking vascular congestion, neutrophil infiltration of the lamina propria, and inflammatory pseudomembrane formation (22, 25). Systemic polymorphonuclear activation is evidenced by the common finding of an elevated peripheral blood neutrophil count with toxic granulations and band forms, as well as the occasional finding of a “leukemoid” reaction (20). Furthermore, we have previously shown that inhibition of neutrophil recruitment using a blocking antibody to the CD18 leukocyte adhesion molecule results in a marked reduction in fluid secretion, epithelial injury, and mucosal inflammation in toxin A-exposed rabbit intestinal loops (14). The mechanism by which neutrophils are activated in C. difficile colitis has yet to be elucidated. Toxin A directly stimulates human neutrophils as evidenced by a rise in neutrophil cytosolic Ca²⁺ levels and stimulation of neutrophil chemotaxis (14, 24). However, the concentrations of toxin A needed to achieve these effects are relatively high (10⁻⁷ M). In addition, toxin B has no direct stimulatory effect on neutrophils (24). We therefore sought an alternative mechanism by which C. difficile toxins, at low concentration, can effect neutrophil activation and tissue infiltration. The monocyte/macrophage was a clear candidate for an important role in this process.

In the normal colon, intestinal macrophages lie in close proximity to the surface epithelial cells. These macrophages, which are members of the mononuclear phagocytic system, are derived from monocytes in the bone marrow. After entering the systemic circulation, monocytes gain access to the intestinal tissue where they mature into tissue macrophages. Both monocytes and macrophages have similar functions including antigen presentation and phagocytosis (2). In addition, both are key sources for an array of proinflammatory cytokines. Our laboratory previously reported that C. difficile toxin A at a concentration of 10⁻⁹ M activates mouse peritoneal macrophages to secrete interleukin (IL)-1 (21). In another study, both toxins A and B stimulated human monocytes to release IL-1B, tumor necrosis factor-α (TNF-α), and IL-6 (6). Monocytes and macrophages are also a major source of IL-8, a low-molecular-mass (10 kDa) protein that is a member of the chemokine family of chemotactic cytokines (1). In addition to its chemoattractant properties, IL-8 regulates neutrophil adhesion molecule expression and directs neutrophil adhesion to the vascular endothel-
lrium, neutrophil diapedesis, and tissue infiltration (12, 30).

In this study we demonstrate that human monocytes are activated by C. difficile toxins A and B to release IL-8 in addition to the proinflammatory cytokines IL-1β and TNF-α. Much lower concentrations of toxin A (10^{-10} M) are needed to elicit neutrophil migration via monocyte-derived IL-8 as compared with those needed for direct toxin-mediated neutrophil stimulation (10^{-7} M). Toxin B, which has no apparent direct effect on the neutrophil (24), stimulates monocyte IL-8 release at even lower concentrations (10^{-12} M). Both toxin A and toxin B also activate IL-8 production by macrophages. Cytokines released by C. difficile toxin-exposed monocytes upregulate the expression of neutrophil and endothelial cell adhesion receptors, thereby facilitating neutrophil extravasation. These findings support our hypothesis that macrophage activation is an important step in the pathogenesis of C. difficile toxin-induced colitis.

METHODS

Toxin preparation. Toxins A and B were purified, as previously described, from broth culture supernatants of C. difficile strain 10463 (14, 23, 31, 33). The enterotoxicity of purified toxin A was confirmed in the ligated rabbit ileal loop assay (18, 14, 33), and the cytotoxicity of both toxins was determined by cell rounding of IMR-90 fibroblasts (18, 23). With the use of the E-toxate (Sigma) assay system for lipopolysaccharide (LPS), the toxin preparations were tested for and found to be free of LPS contamination.

Peripheral blood monocyte isolation. Whole blood was obtained from healthy volunteers and peripheral blood mononuclear cells isolated using lymphocyte separation media (Organon Teknika, Durham, NC) as previously described (3, 16). The peripheral blood mononuclear cells were washed three times with Hanks’ balanced salt solution (Cellog, Herndon, VA) and plated on 24-well plastic culture dishes at a density of 3 x 10^5 cells/well in 0.6 ml of R10 (RPMI 1640 medium (Cellog) supplemented with heat-inactivated 10% fetal calf serum, 2 mM glutamine, 5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 100 U/ml penicillin, and 100 µg/ml streptomycin, all supplied by Sigma, St. Louis, MO). After 90 min incubation, the nonadherent cells were removed, and the adherent monocytes were treated with hIL-1β (10^{-9} M) for 3 or 6 h. Unstimulated monocytes were used as control. The plates were then washed with Hanks’ balanced salt solution, and monocyte RNA was extracted using the single-step method of Chomczynski and Sacchi (4; see also Refs. 10, 11). Cells were homogenized with Tri Reagent LS (phenol and guanidine thiocyanate in a mono-phase solution, Molecular Research Center, Cincinnati, OH). Phase separation was performed using chloroform and centrifugation at 10,000 × g for 25 min. The aqueous phase was removed, and RNA was precipitated with isopropanol.

Reverse transcription-polymerase chain reaction. Total monocyte RNA (3 µg) was diluted to 10.5 µl with sterile water, heated at 65°C for 10 min, and placed on ice for 5 min. After centrifugation at 12,000 × g for 5 s, 4 µl of 5× avian myeloblastosis virus (AMV) reverse transcriptase buffer (Promega, Madison, WI), 2 µl of a 10 mM mixture of deoxyribonucleoside triphosphates (Pharmacia Biotech, Piscataway, NJ), 20 U RNasin ribonuclease inhibitor (Promega), 400 mM oligo(dT) primer (Promega), and 20 U AMV reverse transcriptase (Promega) were added. The mixture was incubated at 37°C for 15 min, and the resulting complementary DNA (cDNA) was diluted to 200 µl with buffer containing 10 mM tris(hydroxymethyl)aminomethane hydrochloride and 1 mM EDTA (pH 6.5). Preparations of cDNA were stored until use at –20°C.

For subsequent amplification by polymerase chain reaction (PCR), 5 µl monocyte cDNA were added to 45 µl of PCR mixture containing 5 µl 10× Taq polymerase buffer (Promega), 200 µM deoxyribonucleoside triphosphates, 1.5 mM MgCl2, 5 pmol human β-actin specific primers (Clontech, Palo Alto, CA) or 50 pmol human IL-8 specific primers (Stratagene, La Jolla, CA), and 1.25 U Taq DNA polymerase (Promega) (II). Samples were amplified by initial denaturation at 95°C for 3 min, then subjected to 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1.5 min, followed by a final extension at 72°C for 5 min. PCR products (10 µl) were analyzed by electrophoresis through 1% agarose gels containing 1 µg/ml ethidium bromide, and DNA bands were visualized using an ultraviolet transilluminator (Fisher Biotech, Pittsburgh, PA) at 312 nm.

Human neutrophil isolation. Human neutrophils were isolated from heparinized blood as previously described (24) using Ficoll-diatizolate density gradient centrifugation (LSM, Organon Teknika), followed by dextran sedimentation (Phar-
Neutrophil suspensions prepared in this manner contained >98% granulocytes and were >97% viable as determined by trypan blue exclusion.

Neutrophil migration assay. The ability of conditioned medium from both toxin A- and toxin B-exposed monocytes to induce neutrophil migration was measured using a conventional migration assay (15, 24). Conditioned media from toxin A- and toxin B-stimulated human monocytes were placed in the lower chambers of a multiwell chemotaxis assembly (Neuro Probe, Cabin John, MD) (2, 15, 24). Human neutrophils suspended in Dulbecco’s PBS (Cellgro) with 0.2% BSA at a concentration of 5 × 10⁶ cells/ml were placed in the upper chambers. The two chambers were separated by a 3-µm nitrocellulose filter (Sartorius, Cherry Hill, NJ). After a 1-h incubation at 37°C, the filters were removed, placed on glass slides, fixed with ethanol, and stained with hematoxylin.

Neutrophil migration was quantified by counting the number of neutrophils migrating a fixed distance into the filter (mean of 3 high-power fields in each of duplicate filters). This distance was set at a point to which 5–10 neutrophils per high-power field migrated in response to R10 medium. Results were expressed as a mean percentage of basal migration. In some experiments, the conditioned media were incubated with a blocking antibody to IL-8 (10 µg/ml, R & D Systems) before performing the migration assay.

Flow cytometric analysis of neutrophil adhesion receptor expression. Human neutrophils were exposed for 30 min at 37°C to R10 medium, R10 conditioned medium from untreated monocytes, and conditioned medium from toxin A-treated monocytes (prepared as described above). The neutrophils were then washed, suspended in PBS with 0.2% BSA and 0.1% sodium azide, and incubated at 4°C for 25 min with one of the following mouse monoclonal antibodies at a concentration of 5 µg/ml: 1) R15.7 (anti-CD18), 2) TS1.18 (anti-CD11b) (both of these antibodies were provided by Dr. R. Rothlein (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT) and Dr. T. A. Springer (Center for Blood Research, Harvard Medical School, Boston, MA)), 3) DREG56 (anti-L-selectin; AMAC, Westbrook, ME), or 4) DAK-GO5, a mouse immortal mononuclear G (IgG) negative control (Dako, Carpinteria, CA). The cells were washed, labeled with fluorescein isothiocyanate-conjugated F(ab’)2 fragment of goat anti-mouse IgG (Dako), fixed in Formalin, and analyzed using a FACScan flow cytometer (Becton Dickinson, Sanj ose, CA) as previously described (15).

Intercellular adhesion molecule-1 cell ELISA. Surface expression of intercellular adhesion molecule-1 (ICAM-1) by human endothelial cells (EndoPack-UV, Clonetics, San Diego, CA) was measured by cell ELISA (16). Endothelial cells were grown to confluence on a 96-well culture plate. Toxin-treated monocyte conditioned media were mixed in equal parts with endothelial cell medium and incubated with the endothelial cell monolayers for 24 h. The monolayers were then fixed with 1% paraformaldehyde, washed with PBS, and nonspecific binding blocked with 2% BSA in PBS for 1 h at 37°C. The cells were washed, and 100 µl horseradish peroxidase-conjugated goat anti-mouse IgGs (Pierce, Rockford, IL) in a 1:200 dilution in 2% BSA were added and incubated for 1 h at 37°C. The plates were washed, and 100 µl tetramethylbenzidine substrate solution (Kirkegaard and Perry) were added to each well, and the reaction was stopped after 5 min with 100 µl of 1 M o-phosphoric acid. Results are expressed as an ELISA index (EI) derived as (OD 450 nm test – OD 450 nm background)/(OD 450 nm basal – OD 450 nm background), where background refers to blank wells with no endothelial cells and basal refers to unstimulated endothelial cells.

Monocyte and macrophage differentiated THP-1 cells. THP-1 cells, a human monocyte cell line (American Type Culture Collection, Rockville, MD), were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 5 mM HEPES, 50 mM β-mercaptoethanol, 50 U/ml penicillin G, and 50 µg/ml streptomycin ( Gibco, Grand Island, NY). For differentiation into macrophages, THP-1 cells (500,000/ml) were seeded into 96-well plates (Primaria, Falcon, Lincoln Park, NJ) and stimulated with 10 ng/ml phorbol 12-myristate 13-acetate (Sigma) for 48 h. They were then further cultured in fresh medium for 4 days (5, 26, 32). Monocyte and macrophage differentiated THP-1 cells were stimulated for 3 h with varying doses of toxin A, toxin B, or LPS. The conditioned media were then harvested, and IL-8 levels were measured by ELISA.

Statistical analyses. Statistical analyses were performed using SigmaStat (Jandel Scientific Software, San Rafael, CA). Analysis of variance followed by the Student-Newman-Keuls method were used for intergroup comparisons.

RESULTS

IL-8 release from C. difficile toxin-exposed monocytes. Conditioned media from unstimulated monocytes contained low concentrations of IL-8 (150 ± 13 pg/ml). As expected, monocytes treated with 10 ng/ml LPS produced greater amounts of IL-8 (2,820 ± 290 pg/ml). Conditioned media from monocytes exposed to toxin A at concentrations of 10⁻¹⁻ to 10⁻⁸ M also contained significantly greater amounts of IL-8 as compared with control (Fig. 1). Toxin B at low concentrations (10⁻¹² to 10⁻¹⁴ M) stimulated monocyte IL-8 release, but at higher concentrations (10⁻¹¹ and 10⁻¹⁰ M) caused a reduction in IL-8 release (Fig. 1). Stimulation of monocyte IL-8 release by toxin A was evident within 2 h of toxin exposure, peaked at 6 h, and persisted for at least 10 h (P < 0.01 vs. control).
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Fig. 2. Time course of IL-8 release from C. difficile toxin-exposed human monocytes. Human monocytes were exposed to C. difficile toxin A at 10^{-9} M. Conditioned media were removed, and fresh medium was added after 2, 4, 6, 8, and 24 h. IL-8 levels in monocyte conditioned media were measured by ELISA. Unstimulated monocytes (control), toxin A-stimulated monocytes, and monocytes stimulated by lipopolysaccharide (LPS) were studied. Results are expressed as means ± SE; n = 4. At all time points, values for toxin A- and LPS-treated monocytes were significantly higher than control (P < 0.01).

Reverse transcriptase PCR analysis of IL-8 mRNA in human monocytes. RNA from untreated monocytes (0 h) and monocytes treated with toxin A for 3 and 6 h was reverse transcribed, and the IL-8 gene was amplified by PCR. The results are shown in Fig. 3. We observed a substantial increase in IL-8 mRNA expression by monocytes exposed to toxin A for 3 h as compared with untreated monocytes. Monocyte IL-8 mRNA expression returned to baseline after 6 h of toxin A exposure. Southern hybridization with an IL-8 cDNA probe confirmed the identity of the IL-8 mRNA reverse transcriptase PCR product.

Table 1. Clostridium difficile toxin-exposed monocytes release IL-1β and TNF-α

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<tr>
<th>Condition</th>
<th>IL-1β (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
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<tbody>
<tr>
<td>Untreated monocytes</td>
<td>4.0 ± 0.4</td>
<td>10 ± 2.4</td>
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<tr>
<td>Toxin A-treated monocytes</td>
<td>165 ± 27*</td>
<td>377 ± 26*</td>
</tr>
<tr>
<td>LPS-treated monocytes</td>
<td>55 ± 6*</td>
<td>117 ± 9.4*</td>
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Values are means ± SE of 4 or more experiments. Human monocytes were exposed to Clostridium difficile toxin A at a concentration of 10^{-9} M or lipopolysaccharide (LPS; 10 ng/ml) with untreated monocytes serving as control. After 3 h, toxin was removed, cells were washed 3 times, and fresh medium was added. Monocyte conditioned media were harvested after an additional 21 h, and interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) levels were measured by enzyme-linked immunosorbent assay. * P < 0.01 vs. untreated monocytes.

IL-1β and TNF-α release from C. difficile toxin-exposed monocytes. Monocytes treated with toxin A produced significantly higher levels of IL-1β and TNF-α as compared with untreated monocytes (Table 1). Peak levels of IL-1β and TNF-α were obtained after exposure to toxin A at a concentration of 10^{-9} M for IL-1β release and 10^{-12} M for TNF-α.

C. difficile toxin-exposed monocytes stimulate neutrophil migration. Conditioned media from control monocytes increased neutrophil migration only marginally (135% of basal migration, Fig. 4). Conditioned media from monocytes exposed to toxin A or to toxin B caused a dramatic increase in neutrophil migration (Fig. 4). The greatest effect was seen after exposure of monocytes to toxin A at a concentration of 10^{-9} M (438% of basal migration) or to toxin B at a concentration of 10^{-12} M (330% of basal migration). Thus the concentrations of toxin A and toxin B that resulted in maximal stimulation of neutrophil migration were the same as those that induced maximal monocyte IL-8 release (Fig. 1). The addition of a human IL-8 blocking antibody to the monocyte conditioned media completely suppressed neutrophil migration.

Fig. 3. C. difficile toxins stimulate increased IL-8 mRNA levels in human monocytes. RNA from monocytes exposed to C. difficile toxin A (10^{-9} M) for 0, 3, or 6 h was extracted, and cDNA was prepared by reverse transcription. Amplification by polymerase chain reaction (PCR) was performed using oligonucleotide primers for human IL-8 and human β-actin. PCR products were electrophoresed through a 1% agarose gel and visualized with ethidium bromide.

Fig. 4. C. difficile toxin-exposed monocytes stimulate human neutrophil migration. Human monocytes were exposed to C. difficile toxins A or B at varying concentrations, and their conditioned media were harvested after 24 h. Ability of monocyte conditioned media to stimulate human neutrophil migration was then determined using a multi-well chemotaxis assembly. Data are expressed as a percentage of neutrophil migration to medium alone (% basal migration) and are presented as means ± SE; n = 4. * P < 0.05, ** P < 0.01 vs. control.
inhibited their ability to stimulate neutrophil migration (Fig. 5).

C. difficile toxin-exposed monocytes regulate neutrophil adhesion molecule expression. Exposure of neutrophils to conditioned media from resting monocytes had little effect on neutrophil surface expression of L-selectin, CD11b, or CD18 as compared with control medium (Fig. 6, A–C). However, exposure to conditioned medium from monocytes treated with toxin A ($10^{-9}$ M) resulted in marked shedding of neutrophil L-selectin and increased expression of CD11b/CD18 adhesion receptors. These effects were similar to those observed after exposure of neutrophils to rhIL-8 (positive control). To be certain that neutrophil stimulation was not due to residual toxin A in the conditioned media, we examined adhesion molecule expression by neutrophils exposed directly to $10^{-9}$ M toxin A (the same dose used to stimulate the monocytes in these and earlier experiments). Toxin A at this dose has no direct effect on neutrophil adhesion molecule expression as compared with control (data not shown).

C. difficile toxin-exposed monocytes upregulate endothelial cell ICAM-1 expression. Human endothelial cells exposed to toxin-treated monocyte conditioned media demonstrated increased ICAM-1 expression. Upregulation of endothelial ICAM-1 was dose dependent, and maximal effect was again seen with media from monocytes exposed to $10^{-9}$ M toxin A (5.05 ± 1.93 EI) or $10^{-12}$ M toxin B (3.46 ± 1.72 EI). Conditioned media from unstimulated monocytes had little effect on endothelial cell ICAM-1 expression (1.24 ± 0.47 EI). Because IL-1 is known to upregulate ICAM-1 expression on endothelial cells, we preincubated endothelial cells with a receptor antagonist to IL-1 (rhIL-1ra, 25 µg/ml; R & D Systems) before exposing them to the monocyte conditioned media. rhIL-1ra pretreatment resulted in a 72% reduction in ICAM-1 upregulation using conditioned media from monocytes exposed to C. difficile toxin A at $10^{-9}$ M (toxin A MCM). Flow cytometric analysis was then performed to examine neutrophil surface expression of adhesion molecules CD18 (A), CD11b (B), and L-selectin (C).
IL-8 release from C. difficile toxin-exposed macrophages. C. difficile toxin A, toxin B, and LPS each stimulated IL-8 release from macrophage-differentiated THP-1 cells (Fig. 8). Macrophages showed greater sensitivity to each stimulus than monocyctic THP-1 cells. Macrophages were ~100-fold more sensitive to toxin A (Fig. 8A) and toxin B (Fig. 8B) and ~10-fold more sensitive to LPS (Fig. 8C). The response of macrophages was similar to that observed in peripheral blood monocytes (Fig. 1) in a number of respects: toxin B stimulated IL-8 production at lower doses than toxin A; the highest doses of toxin B were associated with lesser IL-8 stimulation, and the greatest IL-8 production was seen with high levels of toxin A (Fig. 1). The amount of IL-8 produced by macrophages after maximal toxin stimulation was similar in degree to maximal production in response to the potent macrophage activator LPS (Fig. 8).

DISCUSSION

The main finding of this study is that C. difficile toxins A and B potently stimulate monocytes to release the neutrophil chemotactic factor IL-8 with maximal effects at toxin concentrations of 10^{-10} M for toxin A and 10^{-12} M for toxin B. Significant monocyte stimulation continued to be evident at even lower toxin concentrations (10^{-13} M for toxin A and 10^{-14} M for toxin B). Toxin B at concentrations higher than 10^{-12} M reduced IL-8 release to levels below those seen in control monocytes. One possible explanation is that this reflects monocyte toxicity and death secondary to the cytotoxic effects of higher doses of toxin B, as previously reported (28). The concentration of toxin B needed to stimulate IL-8 release was 100-fold less than that of toxin A. This difference in potency is similar to that reported in a recent study which found that toxin B was 10 times more potent than toxin A in producing mucosal damage in human colonic explants (27). Increased IL-8 mRNA levels and IL-8 protein release by monocytes occurred within 3 h of toxin exposure.

In C. difficile colitis, peripheral blood monocytes are unlikely to be exposed directly to substantial amounts of toxin A or toxin B. However, tissue macrophages may well contact these toxins especially when colonic microulceration has developed (25, 27). This led us to examine activation of macrophage-differentiated THP-1 cells (5, 26, 32). Our studies confirm that macrophages, like monocytes, are activated by both toxin A and toxin B. Macrophage differentiation of THP-1 cells results in
monocyte stimulation by C. difficile toxins

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heightened sensitivity to C. difficile toxins. However, the absolute concentrations of toxin required to activate THP-1 macrophages are somewhat higher than for nontransformed human monocytes.

We also examined the functional importance of monocyte/macrophage activation by demonstrating that both C. difficile toxins A and B activate human monocytes to produce factors that promote both neutrophil migration and neutrophil adhesion to the vascular endothelium. Conditioned media from monocytes exposed to C. difficile toxin A or toxin B potently stimulated neutrophil migration in comparison with conditioned media from unstimulated monocytes. This effect is due primarily to IL-8 release as evidenced by complete inhibition of migration using a neutralizing antibody to IL-8. These data suggest that IL-8 released by C. difficile toxin-exposed macrophages in the lamina propria creates a chemotactic gradient that induces neutrophil migration to the site of mucosal inflammation in C. difficile colitis.

In addition to promoting neutrophil migration, the conditioned media of C. difficile toxin-exposed monocytes caused shedding of neutrophil L-selectin and increased the surface expression of neutrophil CD11b/CD18. A variety of monocyte-derived factors, including IL-8 and TNF-\(\alpha\), may be responsible for these changes in neutrophil adhesion molecule expression. L-selectin mediates neutrophil rolling along the vascular wall while CD11b/CD18 (Mac-1) mediates firm adhesion of the neutrophil to the vascular endothelium (12). Shedding of L-selectin and upregulation of CD11b/CD18 marks a critical step in neutrophil recruitment to sites of tissue injury, since CD11b/CD18-dependent firm adhesion is a prerequisite to neutrophil migration across the vascular endothelium. Again, toxin A had no direct effect on neutrophil adhesion molecule expression.

Expression of ICAM-1, the endothelial cell ligand for neutrophil CD11b/CD18, was upregulated on human endothelial cells after exposure to conditioned media from C. difficile toxin-exposed monocytes. ICAM-1 upregulation was largely, but not completely, inhibited in the presence of rhIL-1ra. This is consistent with endothelial stimulation by IL-1\(\beta\) as well as other monocyte-derived cytokines such as TNF-\(\alpha\). Direct exposure of endothelial cells to toxin had no demonstrable effect on ICAM-1 expression (data not shown). Thus C. difficile toxins, acting through the monocyte/macrophage, may activate both neutrophil and endothelial cell adhesion receptors. We have previously shown that a blocking antibody to CD18 markedly reduced neutrophil infiltration of toxin A-exposed rabbit ileal loops (14). Inhibition of neutrophil recruitment in this model was associated with a substantial reduction in C. difficile toxin-induced intestinal permeability, fluid secretion, and mucosal injury. In another study, intravital video microscopy was used to examine the direct effects of toxin A on the intestinal microvasculature (19). In that model, monoclonal antibodies to CD11/CD18 and to ICAM-1 also inhibited toxin A-induced leukocyte adhesion and extravasation. These studies underscore the importance of neutrophil CD18 and endothelial cell ICAM-1 interaction in the pathogenesis of C. difficile toxin-induced intestinal inflammation.

Monocyte/macrophage stimulation by C. difficile toxins requires these toxins to gain access to the lamina propria of the colon. Both toxins A (308 kDa) and B (270 kDa) are very large molecules that would not easily cross the intact intestinal epithelium. T84 colonic cell monolayers exposed to C. difficile toxins A and B exhibit a marked increase in permeability to mannitol (7). However, the increase in monolayer permeability in this in vitro system is limited to molecules with hydrodynamic radii <5.7 A, significantly smaller than either toxin A or toxin B. A more recent study examined sheets of normal human colonic mucosa that were exposed to toxins A or B. After 5 h, epithelial cell rounding and detachment from the basal membrane were noted. Toxin B was more potent than toxin A in inducing this colonocyte injury (27). Interestingly, undamaged epithelium was observed immediately adjacent to severely damaged areas, consistent with the classical in vivo findings of patchy pseudomembranes in human C. difficile colitis (17, 25, 27). The localized areas of injury and inflammation seen in vitro and in vivo may result from cell rounding causing localized breaches in the colonic epithelium through which tiny amounts of toxins A and B can pass. These small amounts of toxin, although unable to directly activate neutrophils, might activate tissue macrophages to produce IL-8 and other proinflammatory cytokines. Once initiated, this inflammatory cascade may result in a marked acute inflammatory cell infiltration, further mucosal injury, and, ultimately, focal pseudomembrane formation.

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