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

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1Section of Gastroenterology, Department of Veterans Affairs Medical Center, Boston 02130; 2Evans Memorial Department of Clinical Research, Boston University School of Medicine, Boston 02118; and 3Gastroenterology Division, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215

Linevsky, Joanne K., 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, potently activate 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.

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 cytotoxin 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 Ca^{2+} 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-1β, 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 chemotactant properties, IL-8 regulates neutrophil adhesion molecule expression and directs neutrophil adhesion to the vascular endothel-
IL-8 levels were also measured in the monocyte media using commercially available ELSIs (R & D Systems).

Monocyte total RNA extraction. Peripheral blood mononuclear cells were isolated as above and plated on 150-cm² coated polystyrene culture dishes at a density of 1 × 10⁹ cells/ml in RPMI media. After a 90-min incubation, the nonadherent cells were removed, and the adherent monocytes were treated with either toxin A or toxin B. Bacterial endotoxin (10 ng/ml, Escherichia coli 0111:B4) was added, and the mixture was incubated for 3 h and washed, fresh medium was added, and the conditioned media were harvested after an additional 21 h.

IL-8 measurement. IL-8 protein levels in monocyte conditioned media were measured using a double-ligand enzyme-linked immunosorbent assay (ELISA) (10, 15, 29). Briefly, the wells of a 96-well Immulon II plate (Dynatech, Chantilly, VA) were coated with goat anti-human IL-8 (R & D Systems, Minneapolis, MN) at a concentration of 5 µg/ml in carbonate coating buffer (pH 9.6) overnight and washed with phosphate-buffered saline (PBS)-0.05% Tween 20, pH 7.5. Nonspecific binding was blocked with 2% bovine serum albumin (BSA) in PBS-Tween 20 for 2 h at room temperature. Plates were washed twice, and 100 µl of the recombinant human (rh) IL-8 standard (R & D Systems) or samples were added to the wells and incubated for 1 h at room temperature. Goat anti-human IL-8 antiserum (R & D Systems), biotinylated using ImmunoPure (NHS-LC-Biotin; Pierce) according to the manufacturer's directions, was used as the secondary antibody. The plates were washed again, the biotinylated goat anti-IL-8 was added, and the plates were incubated for 1 h at room temperature. The plates were washed, and biotinylated peroxidase streptavidin complex (Amersham, Arlington Heights, IL) was added and incubated for 30 min at room temperature. After careful washing with PBS, 100 µl tetramethylbenzidine substrate solution (Kirkegaard and Perry Labs, Gaithersburg, MD) were added to each well, and the reaction was stopped after 5 min with 100 µl of 1 M o-phosphoric acid. The optical density (OD) at 450 nm was then read using an automated microplate photometer (Dynatech), and concentrations of IL-8 were determined by comparison with the standard curve. IL-1β and TNF-α levels were also measured in the monocyte media using a commercially available ELISA kit (R & D Systems).

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 both toxins was determined by cell rounding of IMR-90 fibroblasts (18, 23). With the use of the E-toxase (Sigma) assay system for lipopolysaccharide (LPS), the toxins 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 (Cellgro, Herndon, VA) and plated on 24-well plastic culture dishes at a density of 3 × 10⁵ cells/well in 0.6 ml of RPMI 1640 medium (Cellgro) 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, nonadherent lymphocytes were removed by washing. Monocyte purity, determined by nonspecific esterase positivity, was >90%. After resting overnight, the adherent monocytes were treated with varying doses of either toxin A or toxin B. Bacterial endotoxin (10 ng/ml, Escherichia coli 055:B5 LPS, Sigma) was used as a positive control. Unless otherwise stated, monocytes were treated for 3 h and washed, fresh medium was added, and the conditioned media were harvested after an additional 21 h.

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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 (Pharmacia Biotech, Piscataway, NJ), 20 U RNasin ribonuclease inhibitor (Promega), 100 pmol oligo(dT) primer (Promega), and 20 U Taq DNA polymerase (Promega) were added. The mixture was incubated at 37°C for 90 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-diatrizoate density gradient centrifugation (LSM, Organon Teknika, followed by dextran sedimentation (Phar-
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 to R10 medium alone. rhIL-8 (100 ng/ml) was used as a positive control. 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) TS11.18 (anti-CD11b) both of these antibodies were provided by Dr. R. Rothlein (Boehringer Ingleheim Pharmaceuticals, Ridgefield, CT) and Dr. T.A. Springer (Center for Blood Research, Harvard Medical School, Boston, MA); 3) DREG56 (anti-ICAM-1), DAK-G05, a mouse IgG (Dako), fixed in Formalin, and analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, 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. After the cells were repeatedly washed, the primary antibody RR11, a mouse IgG1 monodonal directed against human ICAM-1 (provided by Dr. R. Rothlein) in a 1:200 dilution in 2% BSA was added and incubated for 1 h at 37°C. The plates were washed, and 100 μl horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL) in a 1:20,000 dilution in 2% BSA were added to each well and incubated for 1 h at 37°C. After the cells were carefully washed with PBS, 100 μl of 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 in 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 10⁻¹¹ 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 24 h. Conditioned media from toxin A- and toxin B-exposed 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 10⁻¹¹ 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 24 h. Conditioned media from toxin A- and toxin B-exposed 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 10⁻¹¹ 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 24 h. Conditioned media from toxin A- and toxin B-exposed 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 10⁻¹¹ 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 24 h. Conditioned media from toxin A- and toxin B-exposed 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 10⁻¹¹ 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 24 h.
24 h (Fig. 2). This time course of IL-8 release paralleled that of LPS-stimulated monocytes.

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.

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^{-10} 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 inhibited toxin-stimulated neutrophil migration.

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

<table>
<thead>
<tr>
<th>Condition</th>
<th>IL-1β (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
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<tr>
<td>Untreated monocytes</td>
<td>4.0 ± 0.4</td>
<td>10 ± 2.4</td>
</tr>
<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 three 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.
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 10⁻⁹ M toxin A (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).

Fig. 5. A neutralizing antibody to IL-8 inhibits neutrophil migration induced by C. difficile toxin-exposed monocytes. Human monocytes were exposed to C. difficile toxin A (10⁻⁹ M) or toxin B (10⁻¹² M) for 2 h. Cells were washed, and fresh medium was added, which was harvested after overnight incubation. Monocyte conditioned media (MCM) were then incubated with a neutralizing antibody to IL-8 (solid bar) before neutrophil migration assay was performed. Recombinant human IL-8 (rhIL-8; 100 ng/ml) was used as a positive control. Data are expressed as a percentage of neutrophil migration to medium alone (% basal migration) and presented as means ± SE; n ≥ 4.

Fig. 6. C. difficile toxin-exposed monocytes regulate neutrophil adhesion molecule expression. Human neutrophils were incubated for 30 min with R10 medium (control), with conditioned medium from unstimulated monocytes (untreated MCM), or with conditioned medium from monocytes that had previously been 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 monocytic 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 ($10^{-11}$ 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 mucosal injury 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...
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 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-α, 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-1β as well as other monocyte-derived cytokines such as TNF-α. 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 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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