Clostridium difficile Toxin A Alters In Vitro–Adherent Neutrophil Morphology and Function

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The effects of purified toxin A in vitro on the shape and function of polymorphonuclear leukocytes (PMNL) were examined. Toxin A induced changes in adherent PMNL shape from a compact spherical or pyramidal shape to a thin and ropelike shape. This change in shape was accompanied by rearrangement of the F-actin cytoskeleton into aggregates. Toxin A–treated PMNL exhibited increased adherence and expressed less L-selectin and more Mac-1, compared with nontreated PMNL. In contrast to these proinflammatory actions, toxin A impaired both directed and nondirected PMNL migration in response to N-formylmethionylleucylphenylalanine. In addition, toxin A decreased the oxidative activity of adherent PMNL stimulated by recombinant human tumor necrosis factor–α. These effects could be explained by toxin A–induced glucosylation of the signaling small-size guanine 5′-triphosphate–binding proteins of the Rho family in human PMNL.

Clostridium difficile, an anaerobic gram-positive bacterium, causes 20%–30% of cases of antibiotic-associated diarrhea, 50%–75% of cases of antibiotic-associated colitis, and 90% of pseudomembranous colitis. The latter is a severe disease characterized by adherent intestinal plaques (i.e., pseudomembranes) composed of neutrophils, fibrin, mucus, and cell debris localized at areas of intestinal tissue destruction [1–6].

The virulence of C. difficile has been attributed principally to the release of the exotoxin toxin A (308 kDa), the primary enterotoxigenic product of C. difficile. purified toxin A causes intestinal secretion, destruction of the intestinal epithelium, and hemorrhagic colitis when introduced in vivo to the intestinal lumen [7–12]. Strains of C. difficile that lack toxin A production are nonvirulent [13].

Toxin A has been reported to be a polymorphonuclear leukocyte (PMNL) chemoattractant [14], and the presence of numerous PMNL both within pseudomembranes and between the pseudomembranes and the intestinal mucosa is a prominent aspect of antibiotic-induced diarrhea and colitis caused by infection with C. difficile [11]. In addition, toxin A stimulates the release of endogenous mediators of inflammation, including tumor necrosis factor (TNF)–α, interleukin (IL)–1, IL-8, platelet-activating factor, and leukotriene B4 [15–19]. Inhibition of PMNL adherence with antibodies to β2-integrins or P-selectin decreases the PMNL inflammatory response and intestinal damage in toxin A–exposed animals [20, 21], suggesting that adherent PMNL may play a role in C. difficile–induced disease.

C. difficile toxin A monoglucosylates RhoA, Rac1, and Cdc42Hs at threonine-37 in a large variety of cells [22], a modification that is believed to inactivate these proteins. However, to our knowledge, toxin A–induced glucosylation of these proteins has not been demonstrated in intact human neutrophils. The Rho class of small GTP-binding proteins (including RhoA, Rac1, and Cdc42) directs cytoskeletal-dependent PMNL activities, including cell adhesion, cell shape, exocytosis, motility, chemotaxis, and the adherent oxidative burst [23]. In addition, Rac2, a protein that is 92% homologous to Rac1, is essential for the assembly of the PMNL NADPH oxidase [23]. Therefore, toxin A–mediated alteration of RhoA, Rac1, and Cdc42 (and possibly also Rac2) has the potential to disrupt several functions of PMNL. This study shows the effects of toxin A on PMNL morphology; on organization of F-actin, t-selectin, and Mac-1 expression, adherence, and superoxide production; and on glucosylation of small proteins of the Rho family in intact human neutrophils.

Materials and Methods

Materials

Reagents were purchased as follows. Hanks’s balanced salt solution (HBSS) was obtained from Whittaker M. A. Bioproducts; human serum albumin from Baxter Healthcare; heparin from Lyphomed Fujisawa USA; and cytochrome c (type VI from horse

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human (rh) TNF-α (specific activity, 600 pg/U) was a gift from D. Lyerly (TechLab). Recombinant human TNF-α (specific activity, 600 pg/U) was a gift from D. Lyerly (TechLab). Recombinant human (rh) TNF-α (specific activity, 600 pg/U) was a gift from D. Lyerly (TechLab). Recombinant human (rh) TNF-α (specific activity, 600 pg/U) was a gift from D. Lyerly (TechLab). Recombinant human (rh) TNF-α (specific activity, 600 pg/U) was a gift from D. Lyerly (TechLab). Recombinant human (rh) TNF-α (specific activity, 600 pg/U) was a gift from D. Lyerly (TechLab). Recombinant human (rh) TNF-α (specific activity, 600 pg/U) was a gift from D. Lyerly (TechLab). Recombinant human (rh) TNF-α (specific activity, 600 pg/U) was a gift from D. Lyerly (TechLab). Recombinant human (rh) TNF-α (specific activity, 600 pg/U) was a gift from D. Lyerly (TechLab).

PMNL Preparation

Purified PMNL (~98% PMNL; > 95% viable, as determined by trypan blue exclusion) containing < 50 pg/mL lipopolysaccharide (as determined by limulus amebocyte lysate assay) were obtained from normal, heparinized (10 U/mL) venous human blood by a 1-step ficoll-hypaque separation procedure [24]. The PMNL were washed 3 times with HBSS. Residual erythrocytes were removed by hypotonic lysis. PMNL experiments were conducted in HBSS containing 0.1% human serum albumin, unless otherwise stated. PMNL viability after 2 h of incubation with toxin A (3–15 μg/mL) was determined by trypan blue exclusion.

PMNL Morphology

PMNL migration under agarose. PMNL (5 × 10^6 leukocytes/mL) were incubated with or without toxin A (3 μg/mL) for 2 h, after which PMNL shape was examined microscopically after migration under agarose to FMLP (100 nM), fixation, and staining with Giemsa stain [25]. Under undisclosed codes, 200 leukocytes/field were counted to determine the percentage of cells with morphologic changes, and the mean ± SE of 4 different fields was calculated.

Scanning electron microscopy. Washed round (12-mm-diameter) glass coverslips were coated with membrane-filtered (0.45-μm pore) fibrinogen (0.5 mL; 5 mg/mL) dissolved in 1.5% NaHCO₃ in 24-well flat-bottomed tissue culture plates and were incubated at 37°C overnight. The wells were emptied, and each coverslip was washed 2 times with 1 mL of normal saline.

PMNL (4 × 10^6 leukocytes/mL) were incubated at 37°C for 2 h in culture tubes with or without toxin A (3 μg/mL for 2 h), cytochalasin B (5 μg/mL for the last 5 min), and rhTNF-α (10 U/mL for the last 15 min). Five percent autologous serum was added to the samples, and the PMNL suspensions were transferred to the tissue culture wells containing the fibrinogen-coated coverslips. The PMNL on the coverslips were incubated for 30 min at 37°C in 5% CO₂. The samples were washed with PBS and then were fixed with HBSS containing 2% glutaraldehyde and were held overnight at 19°C. The fixed samples were dehydrated with ethanol, critical point-dried, and sputter-coated with gold/palladium for viewing with a scanning electron microscope (JEOL 35-C) in the University of Virginia Central Electron Microscopy Facility.

PMNL Cytoskeleton

The coverslips were coated with fibrinogen, and PMNL were incubated as stated above for the scanning electron microscopy samples. The adherent PMNL monolayers were then washed with PBS and fixed with 3.2% paraformaldehyde in PBS and stored overnight at 19°C. The samples were permeabilized with 1-α-lysophosphatidylcholine (0.3 mg/0.5 mL for 60 min) and then were stained with rhodamine-phalloidin (5 U/0.5 mL for 60 min) at room temperature. The stained monolayers were washed 2 times with PBS and once with distilled water and then were mounted with Vectashield (Vector Laboratories), and confocal images were obtained with a laser scanning microscope (Zeiss 410) equipped with a rhodamine filter set. The PMNL were scanned, and then images focused at the level of cell contact with the fibrinogen-coated substrate were recorded.

PMNL Expression of Adhesion Molecules (t.-Selectin [CD62L] and Mac-1 [CD11b])

PMNL (10^6 leukocytes/mL) were incubated with or without toxin A (10 μg/mL) or rhTNF-α (10 U/mL) for 30 min or 120 min at 37°C and then were stained for 30 min at 4°C with FITC-conjugated anti-CD62L and RPE-conjugated anti-CD11b for 30 min at 4°C or an FITC or RPE isotype-matched control antibody. The samples were washed, and PMNL 2-directional fluorescence (FITC/RPE) was measured with a FACSscan fluorescence-activated cell sorter (Becton Dickinson) at an excitation wavelength of 488 nm. The emission wavelengths were wavelength/band pass of 530/30 for FITC and wavelength/band pass of 585/42 for RPE.

PMNL Adherence

PMNL adherence to a fibrinogen-coated surface was measured as follows (adapted from [26]): 24-well flat-bottomed tissue culture plates were incubated at 37°C overnight with 0.5 mL of fibrinogen (5 mg/mL) dissolved in 1.5% NaHCO₃. The plates were emptied, and each well was washed 2 times with 1 mL of normal saline. The wells were filled with PMNL (10^6 leukocytes/mL). The PMNL within the coated wells were incubated for 120 min at 37°C with 5% CO₂ with or without toxin A (1–10 μg/mL). In some wells, with or without toxin A, R15.7 (50 μg/mL), an antibody to CD18, was added. After incubation, the supernatant was pooled with 2 normal saline washes (0.5 mL each) of the monolayer, and this supernatant with washes was centrifuged (200 g for 10 min), the cell-free supernatant was retained, and the pelleted PMNL were lysed with 0.1% Triton X. The adherent monolayer of PMNL was lysed with 0.1% Triton X, and 3 fractions were ob-
tained (cell-free supernatant, lysed nonadherent PMNL, and lysed PMNL from the adherent monolayer). The amount of lactate dehydrogenase in each fraction was assayed (lactate dehydrogenase kit; Sigma) and was compared with a standard curve relating the lactate dehydrogenase content to PMNL numbers. The results were shown as the percentage of PMNL that adhered, based on the amount of lactate dehydrogenase.

PMNL Migration

Directed and nondirected migration under agarose was quantified according to the method of Nelson et al. [25]. Purified PMNL (5×10^6 leukocytes/mL) were incubated in HBSS containing 0.1% human serum albumin (1 mL), with or without toxin A (1–10 μg/mL for 0–2 h) treatment. The leukocytes were centrifuged (150 g for 5 min), and nondirected and directed migration to FMLP (100 nM) was quantified. For some experiments, toxin A (15 μg/mL) or PMNL (5×10^6 leukocytes/mL), incubated at 37°C for 2 h in the presence or absence of toxin A (15 μg/mL) and then concentrated 10 times, was used as a chemotaxogen. The results were reported as the distance from the edge of the well of the leading front of PMNL after 2 h of incubation at 37°C or as the chemotactic differential (directed migration to the chemoattractant minus nondirected migration to control medium).

Superoxide Release by Adherent PMNL

Twenty-four-well flat-bottomed tissue culture plates were coated with fibrinogen (see “PMNL Adherence”). The wells were then filled with PMNL (10^6 leukocytes/mL) that either were nonintoxicated or had been intoxicated with toxin A (3 μg/mL) for 2 h at 37°C, cytochrome c (1.44 mg/mL), and catalase (0.07 mg/mL), with or without rhTNF-α (1 U/mL) and toxin A (3 μg/mL). Matching wells were prepared the same way with the addition of superoxide dismutase (50 μg/mL). The plates were incubated for 120 min at 37°C with 5% CO₂. After incubation, each sample was removed from the well, iced, and centrifuged (2000 g for 10 min). The optical density of each supernatant was read at 550 nm against a paired sample containing superoxide dismutase, to determine the superoxide dismutase–inhibitable change in optical density. Superoxide release (in nanomoles) was calculated [27].

Glucosyltransferase Assay

The quantitative assay for measuring Rho glucosylation is based on filter-trapping assays for measuring GTP binding to Rho small GTPases. PMNL were purified and were incubated with or without toxin A at 3 or 10 μg/mL for 2 h, as described above. The cells were pelleted and were resuspended in a lysis buffer containing 150 mM KCl, 3.0 mM NaCl, 3.5 mM MgCl₂, 10 mM piperazine-N,N’-bis(2-ethanesulfonic acid; pH 7.3), 1 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, and 30 μg/mL leupeptin at 4°C. The cells were lysed by sonication 3 times, and the debris was pelleted by centrifugation at 12,000 g for 10 min at 4°C. The protein concentration of the lysates was determined by use of a bicinchoninic acid protein assay (Pierce). The lysates were then incubated with UDP-[14C]glucose (40 μM) at 37°C for 1 h in the presence or absence of toxin A (200 μg/mL). The samples were loaded immediately on Millipore HA 0.45-μm filters set in a Millipore vacuum manifold, which had been prerinsed with water. The vacuum was applied, and the membranes were washed 4 times with 3 mL of water. The membranes were dried and were placed in Ready Gel scintillation fluid (Beckman), and the radioactivity was determined by scintillation counting. Rho family protein glucosylation was calculated as counts per minute per milligram of total protein of lysate.

Statistics

Significance (P < .05) was determined by paired Student’s t test.

Results

PMNL morphology. After migration to FMLP under agarose, the toxin A–treated PMNL displayed an exaggerated elon-

![Figure 1](https://academic.oup.com/jid/article-abstract/185/9/1297/937282)

**Figure 1.** Effect of *Clostridium difficile* toxin A on shape of polymorphonuclear leukocytes (PMNL) migrating to FMLP under agarose. A, Control in absence of toxin A; B, PMNL incubated with toxin A (3 μg/mL) for 2 h before chemotaxis assay. Bar, 10 μm. Shown are representative data from 5 separate experiments.
gated shape, compared with untreated PMNL migrating to FMLP (figure 1). Scanning electron microscopy showed PMNL without toxin A that adhered to a fibrinogen-coated surface to be spherical in shape (figure 2A). In contrast, toxin A–exposed PMNL displayed an elongated or stretched shape (37% ± 4.4% had an altered shape), sometimes showing multiple extensions, or a “multiarmed” shape (figure 2B and 2C). The appearance of toxin A–treated PMNL was quite different from that of rhTNF-α–exposed PMNL. Stimulation with TNF-α caused the PMNL to have a pancake-like spread appearance (figure 2D). Treatment with both toxin A and rhTNF-α resulted in PMNL with an intermediate shape. These PMNL were spread out more than were toxin A–exposed cells but also contained elongated arms extending away from the cell, which were not often seen in cells treated with rhTNF-α alone (figure 2E).

Because there is evidence from studies of other cell types that toxin A causes an aberration in cytoskeletal assembly [28], we compared the effect on PMNL shape of cytochalasin B (which prevents F-actin polymerization) with that of toxin A. Cytochalasin B–treated cells were quite different in shape from toxin A–exposed PMNL; they were spherical or teardrop-shaped and did not extend pseudopodia (figure 2F).

**PMNL cytoskeleton.** We visualized F-actin within fibrinogen-adherent PMNL by viewing rhodamine-conjugated phalloidin–stained cells, using confocal microscopy focused at the level of attachment to the substrate. The F-actin distribution in adherent unstimulated PMNL was relatively homogenous, with a rim of bright staining (figure 3A). Within toxin A–treated PMNL, large, bright areas of concentrated F-actin were seen, especially within elongated PMNL (figure 3B and 3C). PMNL

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**Figure 2.** Effect of *Clostridium difficile* toxin A vs. recombinant human tumor necrosis factor (rhTNF)-α and cytochalasin B on the shape of adherent polymorphonuclear leukocytes (PMNL), as viewed by scanning electron microscopy. PMNL were treated as follows: A, No treatment; B and C, toxin A (3 μg/mL for 120 min); D, rhTNF-α (10 U/mL for 15 min); E, toxin A (3 μg/mL for 105 min) followed by rhTNF-α (10 U/mL for 15 min); and F, cytochalasin B (5 μg/mL for 5 min). Bar, 10 μm. Shown are representative data from 3 separate experiments.

**Figure 3.** Effect of *Clostridium difficile* toxin A vs. recombinant human tumor necrosis factor (rhTNF)-α and cytochalasin B on F-actin distribution in adherent polymorphonuclear leukocytes (PMNL), as viewed by fluorescein isothiocyanate–conjugated phalloidin staining and confocal microscopy. PMNL were treated as follows: A, No treatment; B and C, toxin A (3 μg/mL for 120 min); D, rhTNF-α (10 U/mL for 15 min); E, toxin A (3 μg/mL for 105 min) followed by rhTNF-α (10 U/mL for 15 min); and F, cytochalasin B (5 μg/mL for 5 min). Shown are representative data from 3 separate experiments.
treated with rhTNF-α had numerous small foci of F-actin evenly spread across the cell surface in contact with the substrate (figure 3D). In contrast, when PMNL were exposed to both toxin A and rhTNF-α, the foci accumulated into distinct regions, usually within thin “necks” of cytoplasm adjacent to the main body of the cell (figure 3E). In the small, round cytochalasin B–exposed PMNL, the distribution of F-actin was punctate, especially along the cell periphery (figure 3F).

**PMNL expression of adhesion molecules.** Incubation with toxin A (10 μg/mL for 2 h) resulted in a decrease in PMNL L-selectin expression, compared with that expressed after incubation for 2 h in the absence of toxin A (P < .001, compared with 2-h control; figure 4D and 4B, respectively). This was not observed after a 30-min incubation with toxin A (P = .310, compared with 30-min control; figure 4C and 4A, respectively). rhTNF-α treatment of PMNL resulted in L-selectin disappearance at both 30 min and 2 h (figure 4E and 4F, respectively; P < .001, compared with 30-min and 2-h controls).

A preliminary dose-response experiment indicated that 3 μg/mL toxin A resulted in a partial response (data not shown), and 10 μg/mL stimulated most of the PMNL to express increased Mac-1 at 2 h. Thus, 10 μg/mL was used for further studies. Toxin A (10 μg/mL) caused an increase in PMNL Mac-1 expression in 2 h (P < .001, compared with 2-h control; figure 4D and 4B, respectively) but not in 30 min (P = .775, compared with 30-min control; figure 4C and 4A, respectively). PMNL exposed to toxin A for 2 h displayed 2 populations of cells, one that had lost expression of L-selectin but did not express more Mac-1 than did 2-h control cells (figure 4D, lower left quadrant) and another that had lost L-selectin and now expressed Mac-1 (figure 4D, upper left quadrant).

**PMNL adherence.** Adherence was quantitated by relating the amount of lactate dehydrogenase recovered from adherent PMNL monolayers to a standard curve of PMNL lactate dehydrogenase content. Because this assay of PMNL adherence is based on the assumption that the PMNL do not change lactate dehydrogenase content with treatment, the amounts of lactate dehydrogenase in the monolayer, suspended cells, and cell-free supernatant were monitored to detect possible lactate dehydrogenase leakage from treated cells. Two-hour exposure to toxin A

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**Figure 4.** Effect of *Clostridium difficile* toxin A vs. recombinant human tumor necrosis factor (rhTNF)–α on polymorphonuclear leukocyte (PMNL) L-selectin and Mac-1 expression. Shown are representative fluorescence-activated cell sorting contour plots from 4–13 separate experiments, performed as follows: A, C, and E, 30-min treatment; B, D, and F, 2-h treatment. A and B, Untreated PMNL; C and D, PMNL treated with toxin A (10 μg/mL); E and F, PMNL treated with rhTNF-α (10 U/mL). Percentages shown are mean percentage of PMNL in each quadrant.
A (1–10 µg/mL) increased PMNL adherence (figure 5) without stimulating the release of lactate dehydrogenase to the surrounding medium (data not shown).

We found that, although treatment of human PMNL with the neutralizing antibody to human CD18, R15.7 (50 µg/mL), had no effect on adherence of unstimulated PMNL (from 13% of PMNL adhered without R15.7 to 12% adhered with R15.7; \( P = .112 \)), toxin A (10 µg/mL)–stimulated adherence to a fibrinogen-coated surface was blocked by this antibody (from 21% of PMNL adhered without R15.7 to 14% adhered with R15.7; \( P = .005 \)), suggesting that toxin A–induced changes in human PMNL adherence are \( \beta_2 \)-integrin dependent.

**PMNL motility and chemotaxis.** PMNL intoxication with enterotoxigenic concentrations of toxin A resulted in impaired nondirected motility and decreased directed migration to the chemoattractant FMLP in a dose- and time-dependent fashion (figure 6A and 6B).

Although toxin A has been reported to be a PMNL chemoattractant and stimulates the release of chemoattractants, we observed that toxin A (15 µg/mL) is not a chemoattractant when assayed under agarose. In contrast, 2-h intoxication of PMNL with toxin A (15 µg/mL) resulted in these PMNL acting as a chemoattractant for nonintoxicated PMNL (figure 6C).

PMNL viability after 2 h of incubation with toxin A (3–15 µg/mL) was >95%, as assessed by trypan blue exclusion.

**Oxidative burst by adherent PMNL.** Because toxin A, like rhTNF-\( \alpha \), stimulated PMNL adherence to a fibrinogen-coated surface, we studied the effects of toxin A on the oxidative response of adherent PMNL. Although toxin A (3 µg/mL) stimulated PMNL adherence in 2 h, it did not stimulate an adherent PMNL oxidative response (\( P = .210 \); figure 7, \( \Rightarrow \) TA). Furthermore, incubation with toxin A (3 µg/mL) for 2 h before the adherent PMNL oxidative assay did not result in an adherent PMNL oxidative burst (\( P = .575 \); figure 7, TA\( \Rightarrow \)).

Because TNF-\( \alpha \) is released in response to toxin A and stimulates an adherent oxidative burst, we examined the effect of coexposure of PMNL to toxin A plus rhTNF-\( \alpha \) on the oxidative burst of adherent PMNL. rhTNF-\( \alpha \) (1 U/mL), in the absence of toxin A, stimulated a 6-fold increase in superoxide release,
Fig 7. Effect of Clostridium difficile toxin A on superoxide production by adherent polymorphonuclear leukocytes (PMNL). Medium, no additions; ➔TA, toxin A (3 μg/mL) added for second 2-h incubation; ➔TNF, recombinant human tumor necrosis factor (rhTNF)-α (1 U/mL) added for second 2-h incubation; ➔TA + TNF, TA (3 μg/mL) + rhTNF-α (1 U/mL) added for second 2-h incubation; TA➔, TA (3 μg/mL) added for first 2-h incubation; TA➔TNF, TA (3 μg/mL) added for first 2-h incubation followed by rhTNF-α (1 U/mL) added for second 2-h incubation. Data are mean ± SE of 5 separate experiments. *Significantly increased superoxide production (P < .05), compared with controls, in absence of TA and rhTNF-α; #significantly decreased superoxide production (P < .05), compared with rhTNF-α alone.

compared with PMNL without rhTNF-α (P = .005; figure 7, ➔TNF). Coexposure of PMNL to toxin A plus rhTNF did not decrease the adherent oxidative response to TNF (figure 7, ➔TA + TNF). In addition, prior (2-h) incubation with toxin A markedly decreased the subsequent adherent oxidative response to rhTNF-α (P = .016; figure 7, TA➔TNF).

Rho glucosylation induced by toxin A in intact human PMNL. The quantitative assay for measuring Rho family protein glucosylation showed that the incubation of PMNL with toxin A at 3 μg/mL for 2 h reduced subsequent incorporation of UDP-[14C]glucose to the cell lysate by 36% and, at 10 μg/mL, reduced incorporation to 45% (P = .0033; toxin A treated, 7241 ± 681 counts/mg of total protein, vs. control, 13,380 ± 703.5 counts/mg of total protein; mean ± SE of 3 separate experiments) of that induced by a higher dose of the same toxin. These data show that a 2-h incubation of intact human PMNL with toxin A results in glucosylation of Rho family proteins.

Discussion

This study shows that a 2-h incubation of PMNL with C. difficile toxin A causes substantial changes in the shape of these cells when migrating in response to FMLP. It has been reported that the migration of neutrophils is mediated by selectin-carbohydrate interaction, which is responsible for the initial loose attachment to the vessel wall, resulting in PMNL “rolling,” followed by immobilization of the PMNL via integrin CD11b/CD18 (Mac-1) [29]. To enable continued migration, the binding via integrins must be down-regulated to allow new bonds to be formed [30]. On stimulation of PMNL with inflammatory cytokines such as TNF-α or a chemoattractant such as FMLP, L-selectin is shed from the PMNL surface, and β2-integrins, including Mac-1, are expressed and activated on the PMNL surface. A possible explanation of the effect of toxin A on the shape of cells in response to FMLP could be an alteration in the expression and/or activation of these adhesion molecules, leading the cells to remain adhered to the substrate. Therefore, despite the forward motion of leading pseudopodia, the trailing portion of the cell remains adhered, resulting in elongated PMNL. Consistent with this explanation, it was observed in this study that 2 h but not 30 min of incubation of PMNL with toxin A decreased the expression of L-selectin and increased the expression of Mac-1 in most of the cells. A similar effect of rhTNF-α was also found but with a different time course. rhTNF-α induced a more rapid change in adhesion molecule expression, which was observed after a 30-min incubation. In fact, the rhTNF-α–induced expression of Mac-1 showed a tendency to decline after 2 h. Because PMNL migration is dependent on the expression followed by down-regulation of integrins, the late expression of integrins induced by toxin A may cause the neutrophils to remain firmly anchored to the substrate, causing the observed PMNL shape changes, increasing the adherence, and resulting in impeded cell migration.

The demonstration that toxin A increases the adherence of PMNL to a fibrinogen-coated surface and decreases nondirected and direct migration induced by FMLP is in accordance with the previous hypothesis. There is an apparent contradiction between the impairment of the migration shown in this study and the intense neutrophil infiltration found in experimental colitis induced by toxin A. In vivo, toxin A at 3–10 μg/mL, concentrations used in most of our studies, causes intense mucosal damage and PMNL infiltration in ligated rabbit ileal loops [8]. However, it has been shown that toxin A binds to the apical receptor on the intestinal epithelial cells facing the lumen and is too large to be transported intact into the bloodstream [31], thus avoiding early contact with PMNL that could inhibit their initial migration into affected mucosa. Furthermore, it is presumed that the severe inflammatory response present in C. difficile colitis results mainly from toxin-induced release of cytokines or other mediators rather than from a direct effect of the toxin [15, 17, 32]. On arrival in the affected tissue, PMNL are exposed to toxin A, and the changes in PMNL shape and adherence could be a contributing factor to the formation of pseudomembranous plaques typical of C. difficile pseudomembranous colitis. Although toxin A has been reported to be a PMNL chemoattractant [14], we did not find that toxin A (15 μg/mL) was a chemoattractant in an assay under agarose. These different results may be explained by the higher concentrations used in the previous report, which showed significant migration beginning at twice the dose used in this study. Additionally, they used a multiwell chemotaxis assembly in which the increased PMNL adhesiveness to the filter surface induced by toxin A may affect the results by augmenting the number of neutrophils counted.
even if individual migrating ability is impaired [33]. We also found that a 2-h incubation of PMNL with toxin A resulted in these PMNL acting as a chemoattractant for nonintoxicated PMNL. Hence, once at the site of the inflammation, toxin A–activated PMNL may be a source of potential chemoattractants, such as IL-8, platelet-activating factor, or leukotriene B4 [15, 18], that can further aggravate the inflammatory process.

PMNL adherence is a result of both expression and activation of the β2-integrin Mac-1 (reviewed by Rosales and Juliano [34]). It has been observed that pretreatment of rabbits with the neutralizing antibody R15.7 to the β2 portion of Mac-1 (CD18) lessens neutrophil migration into toxin A–exposed ileal loops and prevents damage to these loops [20]. We found that, although treatment of human PMNL with R15.7, an antibody to CD18, had no effect on unstimulated PMNL adherence, it did block toxin A–stimulated adherence to a fibrinogen-coated surface, suggesting that toxin A–induced changes in human PMNL adherence are β2-integrin dependent.

The scanning electron micrographs also showed different patterns of morphologic changes induced by toxin A, cytochalasin, TNF-α, and toxin A plus TNF-α. The toxin A–treated PMNL displayed an elongated or stretched shape, sometimes showing multiple extensions. These changes may also be related to the previously mentioned alteration in adhesion molecule expression and adherence caused by toxin A. Additionally, the effect of toxin A on the cytoskeleton that leads to the disorganization of actin microfilaments is well known. The cytoskeletal arrangement directly correlates with cell shape, migration, oxidative burst, and function of integrins [35–38]. Rates of migration will depend not only on integrin up- and down-regulation but also on cycles of actin polymerization [30]. The mechanism of toxin A’s effects on actin organization involves monoglucosylation of the signaling proteins RhoA, Rac, and Cdc42 [39]. Accordingly, our data strongly suggest that incubation of intact human PMNL with toxin A results in glucosylation of small GTPases of the Rho family. In agreement with the present data, it was demonstrated recently that treatment of PMNL with C3-transferase, an inhibitor of Rho, significantly reduces initial selectin-mediated rolling, reduces migration, and makes neutrophils become stationary and undergo shape change [40]. Thus, the toxin A–induced impairment of PMNL motility or migration observed in this study could be related to its effect on the cytoskeleton via inactivation of the GTP-binding protein Rho. PMNL treated with cytochalasin B, which prevents F-actin polymerization, appeared spherical and did not extend pseudopodia. The differences in the shapes of toxin A– and cytochalasin-treated PMNL could be related not only to the distinct effects on the expression of adhesion molecules and adherence but also to the different effects on F-actin. Cytochalasin B counteracts F-actin polymerization, resulting in less F-actin, whereas toxin A, by disrupting Rho proteins, prevents the normal organization of polymerized F-actin in stimulated cells [39, 41]. It has been shown that cytochalasin-treated neutrophils exhibit a teardrop shape, which is not mediated by β2 integrins [42]. Unlike toxin A, TNF-α is able to activate Rho [43, 44], an event that may lead not only to the organization of the actin cytoskeleton but also to the early expression of integrins. These differences may also be related to the distinct cell shape patterns and distributions of the actin filaments seen in toxin A– and TNF-α–treated neutrophils.

The NADPH superoxide–generating oxidase is an enzymatic complex present in phagocytic leukocytes. Its role is to generate free radicals that are essential for killing microorganisms. Two small G proteins, including Rac, have been shown to be involved in the modulation of oxidase activation [45]. In neutrophils, Rac regulates not only the cytoskeleton but also assembly of NADPH oxidase. This pathway is activated by the interaction of phagocytes with soluble stimuli or opsonized microorganisms [46]. Although adherence is not affected, the oxidative response of adherent PMNL stimulated by inflammatory substances, such as TNF-α, is dependent on F-actin polymerization [47] and the formation of tight focal adhesions where integrins bind to the substrate aggregate. The signal is transduced from stimulated integrins through the formation of complexes composed of the cytoskeleton, signaling proteins, and effector proteins, including the PMNL NADPH oxidase [48]. In contrast to other inflammatory stimulants (e.g., FMLP), toxin A does not excite the oxidative burst of suspended PMNL [14]. This study now adds the observation that toxin A, unlike TNF-α, does not stimulate the oxidative burst of PMNL adhering to a biologic surface. In fact, we observed that toxin A, with a 2-h delay, inhibited the adherent PMNL oxidative burst stimulated by rhTNF-α. The observation that toxin A inhibition of the oxidative burst of adherent PMNL in response to rhTNF-α required a 2-h incubation period suggests that toxin internalization and processing are necessary for its effects on the PMNL oxidase.

This is consistent with the concept that PMNL oxidase assembly is dependent on Rho family proteins. This effect of toxin A may be a part of the microorganism’s strategy to protect itself from the microbicidal effect of oxygen free radicals.

In conclusion, this investigation shows the impact of C. difficile toxin A on the shape and function of human PMNL. We suggest that these effects are a consequence of toxin A–induced glucosylation of the Rho subfamily of small GTP-binding proteins, followed by disorganization of the actin cytoskeleton. These data enable further understanding of the pathogenesis of C. difficile–induced colitis and of the role of the Rho family of small GTPases in human neutrophils. Elucidation of these mechanisms should lead to improved control of the life-threatening colitis and diarrhea induced by C. difficile.

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