Shiga Toxin Induces Superoxide Production in Polymorphonuclear Cells with Subsequent Impairment of Phagocytosis and Responsiveness to Phorbol Esters

Andrew J. King, Sumuk Sundaram, Miguel Cendoroglo, David W. K. Acheson, and Gerald T. Keusch

Divisions of Nephrology and of Geographic Medicine and Infectious Diseases, Tupper Research Institute, New England Medical Center and Tufts University School of Medicine, Boston, Massachusetts

The role of inflammatory cells in the pathogenesis of hemolytic-uremic syndrome induced by Shiga toxin (Stx)—producing Escherichia coli (STEC). The pathogenesis of this form of thrombotic microangiopathy remains unclear, but a role for the Shiga toxin (Stx) family of bacterial toxins has been firmly established [1]. The parent toxin, Stx itself, is produced by Shigella dysenteriae type 1 and consists of an enzymatically active A subunit associated with a pentamer of non-covalently linked B subunits that bind to a cell surface glycolipid, globotriaosylceramide (Gb3) (reviewed in [2]). Following binding of Stx to target cells, the toxin irreversibly inhibits protein elongation. Stx1 and Stx2 derived from E. coli are structurally similar and share the same enzymatic and receptor specificity. STEC induce a spectrum of intestinal disease from watery diarrhea to overt hemorrhagic colitis and systemic complications, such as HUS. The consensus regarding the pathogenesis of HUS suggests that toxin traverses the intestine, circulates, and then binds to target cells, mainly the glomerular and cerebral endothelial cells, leading to cell injury.

The role of inflammatory cells in the pathogenesis of HUS remains unclear. Human colonic biopsies of patients with STEC often have infiltrates of monocytes and polymorphonuclear leukocytes (PMN) [3]. In rabbits infected with E. coli O157:H7, infusion of antibodies directed toward the PMN adhesion molecule, CD18, prevent intestinal infiltration of PMN and render histologic and functional protection [4]. Taken together, these data support a role for PMN in the intestinal phase of the disease. Fever and leukocytosis are frequently observed upon presentation with STEC-associated HUS. Leukocytosis can be extreme and is a positive predictor of acute mortality and residual nephropathy [5]. There is evidence of degranulation and enhanced adhesion of PMN to endothelial cells from patients with HUS [5, 6]. Finally, HUS is associated with increased circulating levels of various chemokines and proinflammatory cytokines, including interleukin (IL)-8, IL-6, and tumor necrosis factor-α [5]. The importance of these inflammatory mediators has yet to be confirmed, although they can increase expression of Gb3 in certain endothelial cells in vitro and, thus, might increase vulnerability to toxin. Peripheral blood mononuclear cells (PBMC) express low concentrations of Gb3, produce increased amounts of cytokines in response to toxins, and are not easily killed by Stx [7]. Little attention has been directed on the effects of Stx on PMN viability or function, prompting the current studies.

Methods

Isolation of neutrophils and PBMC. PMN and PBMC were isolated from heparinized blood (10 U/mL) obtained from healthy donors. PBMC were harvested by ficoll-hypaque separation and suspended in ultrafiltered tissue culture medium (RPMI 1640, pH 7.4; Sigma, St. Louis), containing 10 mmol/L L-glutamine, 24 mmol/L NaHCO3 (Mallinckrodt, Paris, KY), 10 mmol/L HEPES (Sigma), 100 U/mL penicillin (Irvine Scientific, Santa Ana, CA), and 100 mg/mL streptomycin (Sigma). PBMC were counted with a standard hemocytometer, and 0.5 mL of a suspension of 5 × 10⁶ PBMC/mL was aliquoted into four polypropylene tubes. PMN were isolated in high purity by density-gradient centrifugation, as previously described [8]. After harvest of PMN, the PBMC, plasma, and ficoll-hypaque were removed. The pellet, con-
taining red blood cells (RBC) and PMN, was resuspended in 50 mL of PBS with 1.5% dextran. After RBC were allowed to settle, the supernatant was centrifuged for 4 min at 400 g, and the pellet was resuspended in 1 mL of saline. Hypotonic saline (0.24%) was added to lyse contaminating RBC, and cells were then centrifuged at 900 g for 4 min. The pellet was resuspended in 1 mL of Hank’s buffered salt solution (HBSS). Cells were checked for viability using trypan blue and resuspended in HBSS at a concentration of 10 × 10^6 cells/mL.

**Experimental procedure.** Immediately following isolation, PMN were incubated for 30 min at 37°C in the presence of test substances. A stock Stx1 solution (isolated as previously described [9]; 100 µg/mL) was diluted using RPMI to 0.01, 1, and 100 ng/mL. A portion of the stock solution was boiled for 1 h in a water bath to heat-inactivate the toxin, a procedure known to abrogate the effects of Stx1 on protein synthesis. Stx1 B subunit was purified as previously described [10]. To remove contaminating lipopolysaccharide, 1 mL each of stock solution of Stx1 and Stx1 B subunit (100 µg/mL) was treated with End-X (Associates of Cape Cod, Falmouth, MA) overnight per the manufacturer’s instructions. After treatment, the material was tested for endotoxin using the limulus amebocyte assay (Associates of Cape Cod) and for cytotoxicity on Vero cells. Endotoxin levels of the stock solutions were <300 pg/mL, and there was no difference in the biologic activity between the End-X–treated Stx1 and the starting stock solution.

**Superoxide production.** Superoxide production by PMN was determined by measuring the capacity of cells to reduce ferricytochrome C to ferrocytochrome C [8]. For each of the test conditions, resting and stimulated tubes were set up after the 30-min incubation with test substances (see above). Unstimulated tubes contained 125 µL of cells (initial concentration, 10 × 10^6 cells/mL), 25 µL of ferricytochrome C (12.3 mg/mL) with or without superoxide dismutase (SOD; 1 mg/mL), and buffer (HBSS) to a final volume of 250 µL. Stimulated cells were prepared in a similar fashion with the addition of stimuli (5 µM solution of 4-β phorbol 12-β myristate 13-α-acetate, PMA; Sigma) and brought to a final volume of 250 µL. After a 10-min incubation at 37°C with rotation, tubes were centrifuged for 5 min at 2000 g at 4°C, and the absorbance at dual wavelength (550-nm test filter with a reference wavelength of 570 nm) of the supernatants was measured. Results are the difference between with SOD and without SOD and are expressed as nanomoles of cytochrome C reduced per 5 × 10^6 PMN per 10 min, based on an extinction coefficient for a 1-cm light path (ferrous-ferri) of 21.1.

**Phagocytosis.** Phagocytosis of opsonized bacterial particles was determined by measuring the uptake of 111 In-labeled heat-killed Staphylococcus aureus, as previously described [8]. Phagocytosis is expressed as an index (ratio of cell-associated counts to total counts added).

**Apoptosis and cell surface markers.** To 0.5 mL of cell suspension (5 × 10^6 cells/mL in RPMI) in triplicate 12 × 75 mm polystyrene tubes, an equal volume of the following test solutions was added to individual tubes: medium (RPMI), untreated or End-X–treated Stx1 (100 ng/mL), untreated or End-X–treated B subunit (100 ng/mL), endotoxin (10 ng/mL, Sigma), or cycloheximide (10 µg/mL; Aldrich Chemical, Milwaukee). Cells were then incubated for 20 h at 37°C and washed in PBS. In one set of tubes, cells were resuspended in 2 mL of PBS, bis-benzimide dye (5 µg/mL; Sigma) was added, and the cells were incubated at 37°C for 30 min. To prevent further uptake of the dye, cells were rapidly cooled to 4°C. Then propidium iodide (PI; final concentration, 5 µg/mL; Sigma) was added, and the cells were taken immediately to flow cytometry analysis on a FACStar plus (Becton Dickinson, Mansfield, MA) dual-laser flow cytometer (flow rate = 1000 cells/s). Cells were excited with optics of UV at 352 nm for bis-benzimide and at 488 nm for PI; 10,000 cells were counted. For each assay, PMN exposed to RPMI medium were analyzed first, in order to determine the gates delineating high and low bis-benzimide and PI uptake. Cells with high PI uptake were considered nonviable. Among the viable cells, those with low bis-benzimide uptake were considered apoptotic, according to definitions previously described [11].

The other two sets of tubes were used for staining PMN and PBMC with fluorescein isothiocyanate (FITC)–conjugated antibodies. PMN (2.5 × 10^6 cells) were incubated with 25 µL of FITC-conjugated anti-CD11b (Sigma) or with 25 µL of FITC-labeled isotype control (IgG1; Sigma). PBMC (2.5 × 10^6 cells) were incubated with 25 µL of FITC-conjugated anti-CD14 (Sigma) or 25 µL of FITC-labeled isotype control (IgG2a). After incubation for 20 min at 37°C in the dark, cells were washed with PBS, fixed in 2 mL of 1% paraformaldehyde, and placed on ice. Flow cytometry was performed as before; 10,000 cells were counted. The mean FITC-channel fluorescence intensity was measured using 4-decade logarithmic amplification for CD14 or CD11b/CD18 and the respective isotype control staining, and the differences in fluorescence values were calculated.

**Statistics.** Analysis was performed using SYSTAT software (SYSTAT, Evanston, IL). Univariate analysis of variance was used to compare the different test conditions. Dose-response curves were analyzed using analysis of variance with repeated measures. Data are expressed as mean ± SE, with significance ascribed when P < .05.

**Results**

To characterize toxin-mediated effects on PMN oxidative burst, a dose-response curve was determined using 6 healthy donors (figure 1A). Toxin increased superoxide formation in a dose-dependent fashion, with a significant rise at 10 pg/mL (P < .001 vs. medium) and near maximal effect at 1 ng/mL. The dose-response curve was not significantly affected by pretreatment of Stxs for endotoxin removal (figure 1A). Addition of PMA to medium-treated cells led to a robust increase in superoxide production, whereas Stx-treated cells failed to show augmented oxygen radical formation with a dose as low as 10 pg/mL. Stx1 (figure 1B). The impaired superoxide response to PMA was mirrored by a dose-dependent reduction of phagocytic ability (figure 1C). PMN exposed to as little as 10 pg/mL Stx1 had a 50% reduction in bacterial phagocytosis (P < .001). B subunit was not different from medium control, but heat-inactivated Stx1 partially impaired phagocytosis with values between those of medium and Stx1 (data not shown).
Figure 1. Dose-dependent effect of Stx on superoxide production of unstimulated (A) and phorbol myristate acetate–stimulated (B) polymorphonuclear PMN and phagocytosis (C), comparing untreated Stx1 with End-X–treated Stx1. Stx1 stimulation of oxidative burst is dose-dependent, with near maximal effects at 1 ng/mL, and is not affected by End-X treatment, whereas substantial blunting of PMA–induced superoxide production and phagocytosis occurs in the presence of Stx1.

Because heat inactivation markedly lessened the effects of toxin on PMN oxidative burst and phagocytosis but did not abrogate the response, we compared in the same experiments the effects of active Stx, heat-inactivated Stx, and purified B subunit (all at 100 ng/mL) (table 1). Heat inactivation did not completely abolish the effect of toxin on the pattern of superoxide response. The effect of purified B subunit and heat-inactivated toxin were very similar.

To assess the effects of Stx on cell viability and on the induction of apoptosis, PMN and PBMC were treated overnight with test substances, stained with PI and bis-benzimide, and analyzed by flow cytometry (n = 5). Stx and B subunit had no more effect on cell necrosis and apoptosis of PMN than medium or endotoxin (10 ng/mL). Necrosis and apoptosis were both enhanced when the cells were treated with cycloheximide, a known inducer of apoptosis and necrosis of PMN used as a positive control in these experiments (table 1). To assess for changes in adhesion molecule expression, immunofluorescent studies were performed using antibodies for CD11b/CD18. Neither Stx, nor endotoxin, nor B subunit controls altered expression of CD11b/CD18 on PMN (data not shown). Stx (100 ng/mL) also had no effect on necrosis or apoptosis in PBMC compared with vehicle, endotoxin (10 ng/mL), or B subunit (100 ng/mL, data not shown). Furthermore, cell-surface CD14 on PBMC was not different from vehicle or endotoxin-treated cells (data not shown).

Discussion

In these studies, Stx1 induced a rapid dose-dependent increase in oxygen free radical production by PMN, whereas the response was blunted when heat-inactivated toxin was used. Nonetheless, toxin exposure rendered the cells dysfunctional by at least two measures: There was reduced responsiveness to PMA stimulation, and there was reduced phagocytic ability. These effects were noted to occur at doses as low as 10 pg/mL Stx1, a concentration of toxin considered to occur in the stool of patients with hemorrhagic colitis. Toxin-induced PMN death does not account for the dysfunctional changes, and even at concentrations of 100 ng/mL, toxin caused no more apoptosis and necrosis than did medium control. These findings are also unlikely to be due to low-dose contamination with endotoxin. First, Stx-induced effects on PMN were identical in untreated Stx compared with Stx subjected to treatment for endotoxin removal. Second, the effects of Stx were largely blocked by heat inactivation, a procedure that is known to abrogate the effects of Stx on protein elongation in sensitive cells (e.g., Vero cells) and yet has no effect on endotoxin activity. While heat inactivation did not completely block the effects on PMN oxidative burst or function, the isolated B subunit behaved similarly. We have previously found that heat-inactivated Stx1 does retain some binding properties (unpublished observations). Indeed, isolated B subunit has been shown to trigger apoptosis in Burkitt’s lymphoma cells, suggesting that binding to Gb3 via the B subunit might...
induce cellular responses independent of the action of the A subunit [12].

The results of the present studies suggest that PMN oxygen free radical formation, induced by toxin, might play a role in intestinal tissue injury early in the course of infection with STEC. PMN migrating to the gut may also be rendered dysfunctional by Stx and potentially impair the host response to the bacterial infection. Several lines of evidence suggest that invasion of the bowel wall by PMN and monocytes might contribute to the pathogenesis of the disease. Histologic examination of human colon biopsies reveals infiltrates of PMN in ~45% of patients; about half of these patients had crypt abscesses [3], and ~70% of patients with O157:H7 infection had fecal leukocytes [13]. In rabbit models of STEC, PMN infiltration into the lamina propria and epithelium occurs as early as 2 days into the infection [14], and αCD18 blocks experimental O157:H7 colitis in this model [4].

We have recently demonstrated increased IL-8 production by intestinal epithelial cells treated with Stx, a cytokine with chemotactic properties for PMN (unpublished data). In the present studies, there was a rapid increase in superoxide formation by PMN upon exposure to low-dose toxin. Others have reported evidence of enhanced production of reactive oxygen species and decreased antioxidant mechanisms in patients with HUS, including increased levels of malonyldialdehyde and markers of lipid peroxidation [15]. Enhanced bowel wall reactive oxygen intermediates (ROIs) might have several important effects on the epithelium and endothelium favoring absorption of Stx and promoting an inflammatory response. ROIs induce secretion of von Willebrand factor from human vascular endothelial cells, thereby increasing surface expression of P-selectin [16]. Further, these oxygen species can rapidly increase endothelial intercellular adhesion molecule-1 expression, favoring binding of the PMN [17].

Over the time course studied, we were unable to discern a difference in CD11b/CD18 expression of the PMN treated with Stx, although binding of PMN from patients with HUS to reporter normal human umbilical vein endothelial cells (HUVEC) is enhanced, perhaps suggesting an increase in other adhesion molecules [18]. This binding of PMN to HUVEC resulted in the degradation of endothelial cell fibronectin, a form of injury that might have important effects on the passage of toxin into the circulation [18]. Our results support the hypothesis that the pathogenesis of STEC infection and its systemic complications may be partly mediated by direct effects of Shiga toxins on PMN.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (ng/mL)</th>
<th>Superoxide production (μM O₂⁻/10⁶ PMN/10 min)</th>
<th>No PMA</th>
<th>PMA</th>
<th>Apoptosis %</th>
<th>Necrosis %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium, RPMI</td>
<td>—</td>
<td>14.3 ± 0.9</td>
<td>41.5 ± 1.2</td>
<td>14.8 ± 3.4</td>
<td>28.8 ± 8.6</td>
<td></td>
</tr>
<tr>
<td>Stx1</td>
<td>100</td>
<td>28.4 ± 1.2</td>
<td>15 ± 1.2</td>
<td>14.9 ± 2.0</td>
<td>32.8 ± 7.9</td>
<td></td>
</tr>
<tr>
<td>HI/Stx1</td>
<td>100</td>
<td>18.3 ± 0.6</td>
<td>30.2 ± 2</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>B subunit</td>
<td>100</td>
<td>18.5 ± 0.5</td>
<td>32.9 ± 1.3</td>
<td>14.4 ± 2.7</td>
<td>35.0 ± 11.1</td>
<td></td>
</tr>
<tr>
<td>Endotoxin</td>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>10.1 ± 2.0</td>
<td>29.1 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>Cyclohexamide</td>
<td>10 µg/mL</td>
<td>ND</td>
<td>45.3 ± 5.3</td>
<td>58.8 ± 14.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: PMA, phorbol myristate acetate; HI, heat-inactivated; ND, not done.

a P < .05 vs. medium.

References


