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Differentiation-Associated Toxin Receptor Modulation, Cytokine Production, and Sensitivity to Shiga-Like Toxins in Human Monocytes and Monocytic Cell Lines

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Infections with Shiga toxin-producing *Shigella dysenteriae* type 1 or Shiga-like toxin (SLT)-producing *Escherichia coli* cause bloody diarrhea and are associated with an increased risk of acute renal failure and severe neurological complications. Histopathological examination of human and animal tissues suggests that the target cells for toxin action are vascular endothelial cells. Proinflammatory cytokines regulate endothelial cell membrane expression of the glycolipid globotriaosylceramide (Gb_3) which serves as the toxin receptor, suggesting that the host response to the toxins or other bacterial products may contribute to pathogenesis by regulating target cell sensitivity to the toxins. We examined the effects of purified SLTs on human peripheral blood monocytes (PBMn) and two monocytic cell lines. Undifferentiated THP-1 cells were sensitive to SLTs. Treatment of the cells with a number of differentiation factors resulted in increased toxin resistance which was associated with decreased toxin receptor expression. U-937 cells, irrespective of maturation state, and PBMn were resistant to the toxins. U-937 cells expressed low levels of Gb_3, and toxin receptor expression was not altered during differentiation. Treatment of monocytic cells with tumor necrosis factor alpha (TNF-α) did not markedly increase sensitivity or alter toxin receptor expression. Undifferentiated monocytic cells failed to synthesize TNF and interleukin 1β when treated with sublethal concentrations of SLT type I (SLT-I), whereas cells treated with 12-*O*-tetradecanoylphorbol-13-acetate acquired the ability to produce cytokines when stimulated with SLT-I. When stimulated with SLT-I, U-937 cells produced lower levels of TNF than PBMn and THP-1 cells did.

The Shiga toxin family is composed of bacterial proteins that (i) share an AB_5 holotoxin molecular structure, (ii) act as 28S rRNA *N*-glycosidases to inhibit eukaryotic protein synthesis, and (iii) utilize the neutral glycolipid globotriaosylceramide (Gb_3) as the primary functional toxin receptor (for reviews, see references 1, 20, and 22). The prototype toxin of the family, Shiga toxin, is produced by *Shigella dysenteriae* type 1. A limited number of serotypes of *Escherichia coli*, collectively referred to as enterohemorrhagic *E. coli*, synthesize one or more toxins designated Shiga-like toxins (SLTs) or verocytotoxins. SLTs are categorized on the basis of antigenic similarity to Shiga toxin, with the cytotoxic activity of SLT classified as type I (SLT-I) being neutralized by polyclonal antibodies to Shiga toxin, while Shiga-like toxin type II (SLT-II) activity is not neutralized by the antisera (30). Subsequently, SLT-I was shown to be essentially identical to Shiga toxin, while a number of SLT-II toxin variants possess only ~56% homology compared with Shiga toxin at the deduced amino acid sequence level (14).

Despite efforts to improve sanitary hygiene, bacillary dysentery and postdiarrheal acute renal failure (hemolytic-uremic syndrome) continue to cause excessive morbidity and mortality in many developing nations. In 1993, the potential of enterohemorrhagic *E. coli* to cause widespread outbreaks of bloody diarrhea with progression to life-threatening sequelae was dramatically highlighted in the western United States, when over 700 people became ill following the ingestion of undercooked contaminated hamburgers. Approximately 27% of those with bloody diarrhea were hospitalized, 8% developed systemic complications and four children died (8). A common histopathological finding in patients with postdiarrheal sequelae is the destruction of endothelial cells lining small blood vessels in the colon, kidneys, and central nervous system (26). In vivo studies using a number of animal models demonstrated that purified Shiga toxin and SLTs cause vascular damage in many of the same target organs (for a review, see reference 9). In vitro studies utilizing human vascular endothelial cells (HVEC) from a number of sites demonstrated a direct cytopathic effect mediated by the toxins, although in some cases, the toxin concentrations necessary to manifest cytotoxicity were orders of magnitude greater than that estimated to be circulating in the bloodstream of infected patients (23, 33). However, when HVEC were cultured in the presence of the toxins and the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin 1β (IL-1β), cytotoxic doses were markedly reduced, suggesting that the cytokines sensitized the target cells to the toxic action of SLTs (19, 33). Subsequently, van der Kar et al. (37) showed that TNF-α and IL-1β upregulate the expression of Gb_3 on HVEC. Obrig et al. (24) also showed that basal levels of Gb_3 expression on HVEC cultured from different sources are variable and that small vessels in target organs may express higher levels of Gb_3. Collectively, these data suggest that modulation of toxin receptor expression may be a critical determinant in disease progression. In order to manifest the profound vascular damage characteristic of the hemolytic-uremic syndrome, two signals may be needed:

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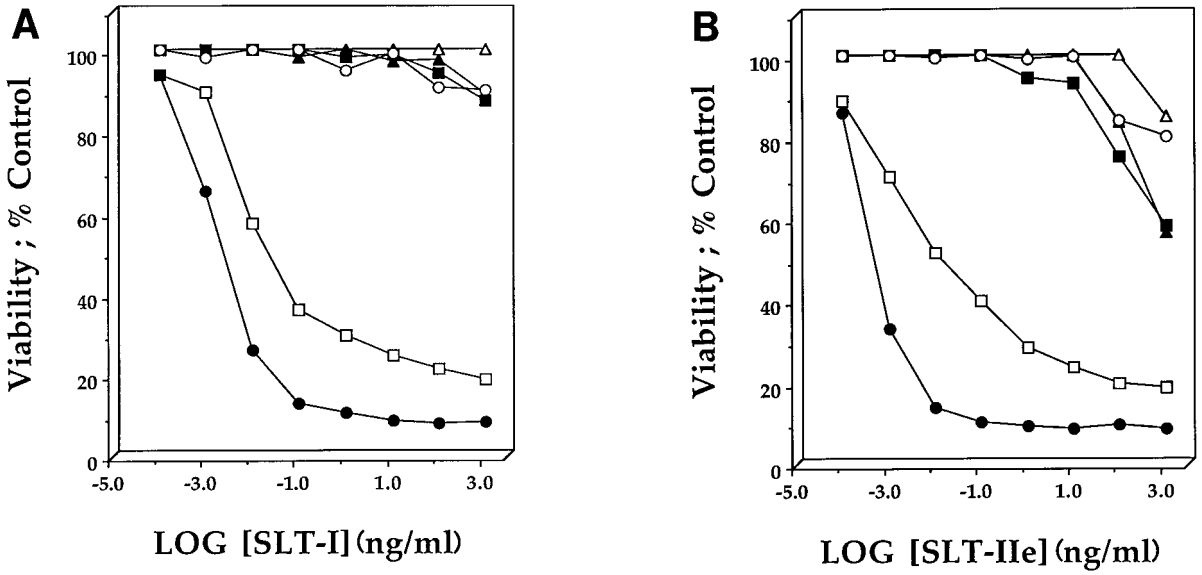

FIG. 1. Comparison of cytotoxicities of purified SLTs for human PBMn and monocytic cell lines. Cells were incubated with 10-fold dilutions of purified SLT-I (A) or SLT-IIe (B) in six replicate wells for 48 h at 37°C. Viability was determined with the dye MTT. Control cells incubated in medium alone were used to calculate percent viability. The data shown in panels A and B are derived from eight and two independent experiments, respectively. Symbols: △, undifferentiated U-937 cells; ▲, differentiated U-937 cells; □, undifferentiated THP-1 cells; ■, differentiated THP-1 cells; ○, human PBMn; ●, Vero cells.

circulating SLTs and elevated levels of toxin receptor-modulating cytokines.

Cells of the monocyte/macrophage lineage have been shown to be crucial in orchestrating the immune response to microbes and microbial products (for a review, see reference 36). This cellular control is mediated by both the induction of membrane-associated signaling molecules and the synthesis and secretion of soluble cytokines. Murine macrophages produced TNF-α and IL-1 when stimulated in vitro with purified (endotoxin-free) SLTs, suggesting that macrophages may indeed play a central role in providing the cytokines necessary for target cell sensitization (3, 32). However, these studies have been limited to the use of thioglycolate-elicited peritoneal macrophages, i.e., cells isolated in an activated state. Barrett et al. (4) reported that the administration of bacterial endotoxin after purified SLT-II enhanced lethality, while the pretreatment of rabbits with endotoxin 20 h before SLT challenge afforded significant protection. These experiments suggest that the state of macrophage differentiation or activation is important in determining the cellular response to SLTs. In experiments reported here, we extended our examination of the cytotoxicity and cytokine induction capacity of SLTs by using human peripheral blood monocytes (PBMn) and two monocytic cell lines. The use of monocytic cell lines affords us the opportunity to measure differences in cell response to the toxins as a function of cellular differentiation.

MATERIALS AND METHODS

Toxins. SLT-I was purified as previously described (32). Purified SLT-IIe (pig edema toxin) was the gift of James E. Samuel, Texas A&M University Health Science Center. Prior to use, toxin preparations were passed through ActiClean Etox columns (Sterogene Bioseparations, Arcadia, Calif.) to remove residual endotoxin contaminants. Toxin preparations contained <0.1 ng of endotoxin per ml as determined by *Limulus* amoebocyte lysate assay. Radioiodinated SLT-I was prepared with $Na^{125}I$ and Iodogen beads (Pierce Chemical Co., Rockford, Ill.). Purified lipopolysaccharides (LPS) derived from *E. coli* O111:B4 were purchased from Sigma Chemical Co., St. Louis, Mo.

Cells. Human PBMn were derived from blood collected by venipuncture from healthy adult volunteers. Mononuclear cells were separated by Histopaque 1077 (Sigma) gradient centrifugation, and plastic-nonadherent cells were removed after 1 h of incubation at 37°C. Human myelogenous leukemia cell lines THP-1 (35) and U-937 (31) were purchased from American Type Culture Collection, Rockville, Md., and maintained in RPMI 1640 (GibcoBRL, Grand Island, N.Y.) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (2 μg/ml), and 10% fetal bovine serum (FBS; Intergen, Purchase, N.Y.) at 37°C in 5% CO_2 in a humidified incubator. Vero cells were cultured in medium 199 (Celox, Hopkins, Mich.) with the supplements listed above.

Macrophage differentiation. To induce the human monocytic cell lines to a mature macrophage-like state, THP-1 and U-937 cells (10^6 cells per ml) were treated for 48 h with 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) at 50 ng/ml for THP-1 cells or 100 ng/ml for U-937 cells. Differentiated adherent cells were washed twice with cold Dulbecco's phosphate-buffered saline (PBS; GibcoBRL) and incubated with fresh medium lacking TPA for 48 h prior to use in assays. TPA treatment resulted in an approximately 50% reduction in intracellular protein kinase C levels, as assessed by a colorimetric protein kinase C assay kit (Pierce). In some experiments, cells were stimulated for 3 to 5 days (with daily changes of the medium) with supplemented RPMI 1640 containing 50 ng of 1α,25-dihydroxyvitamin D_3 (Sigma) per ml, 40 ng of recombinant human granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, Minn.) per ml, 500 U of recombinant human gamma interferon (GibcoBRL) per ml, or 0.25 mM *n*-butyric acid (Sigma). The cells were washed with PBS prior to use. Several techniques were used to assess monocyte differentiation. (i) Percent adherence of cells to plastic was determined by hemacytometer counts of cells before and after stimulation with differentiation factors. (ii) Phagocytic index was determined by internalization of fluorescein-conjugated latex beads. (iii) Changes in membrane CD14 expression were measured by flow cytometry with a fluorescein-conjugated murine monoclonal immunoglobulin G2a (IgG2a) directed against human CD14 (UCHM-1; Research Diagnostics, Flanders, N.J.).

Cytotoxicity assays. Vero cells were plated at ~10^4 cells per well, and human monocytic cells were plated at ~10^5 cells per well in 96-well microtiter plates. Purified toxin preparations were serially diluted (1.0 μg/ml to 0.1 pg/ml) in medium 199 or RPMI 1640 and 100-μl portions of each dilution were transferred to the cells in six replicate wells. All cells were incubated with the toxins for 48 h at 37°C in 5% CO_2. Twenty-five microliters of a 5-mg/ml stock solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added to the cells, and incubation was continued for 2 h. The cells were lysed, and the insoluble formazan dye was extracted for 4 h at 37°C with 50% *N*,*N*-dimethylformamide and 20% sodium dodecyl sulfate. A_{570} was measured (Dynatech MR5000; Dynatech Laboratories, Chantilly, Va.), and percent viability was calculated as follows: $[(A_{570}$ of toxin-treated cells $- A_{570}$ background$)/(A_{570}$ of untreated cells $- A_{570}$ background$)] \times 100$. The 50% cytotoxic dose (CD_{50}) is the amount of toxin necessary to kill 50% of the cells in a well.

Cytokine assays. TNF bioactivity in supernatants collected from untreated cells and cells treated with SLT-I or LPS for 18 h was determined by the lysis of actinomycin D (Act D)-treated L929 murine fibroblasts (32). Briefly, L929 cells were cultured in 96-well microtiter plates at a density of 2×10^5 cells per ml in Iscove's modified Dulbecco's medium (Mediatech, Washington, D.C.) supplemented with 5% FBS at 37°C in 5% CO_2 in a humidified incubator. Dilutions of

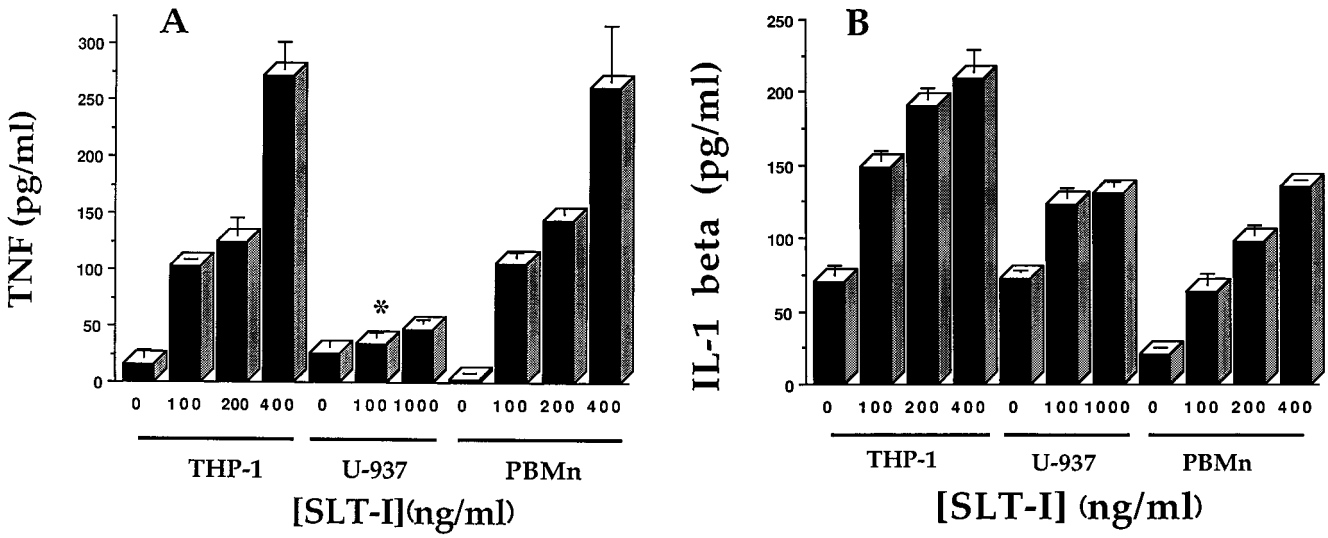

FIG. 2. Cytokine production by human PBMn and monocytic cell lines stimulated with purified SLT-I. PBMn and TPA-differentiated THP-1 and U-937 cells were incubated with media containing the indicated concentrations of purified SLT-I for 18 h (A) or 24 h (B) at 37°C. Cell-free culture supernatants were collected, and TNF bioactivity (A) was determined by the L929 lysis assay. IL-1β levels (B) were determined by ELISA. The data shown are the means ± standard deviations of 10 determinations from two independent experiments. The asterisk denotes nonsignificance by Student's paired *t* test. All other test values differ significantly compared with the control value ($P < 0.05$).

macrophage supernatants or recombinant human TNF-α (R&D Systems) were made in medium containing 1 μg of Act D per ml (Sigma). Each dilution was added to five replicate wells of L929 cells and incubated for 18 h. MTT was added to each well, and after a 2-h incubation, the cells were lysed and the formazan dye was extracted from the cells. A_{570} was measured, and L929 survival values were calculated as follows: (A_{570} of L929 cells treated with supernatants plus ActD)/(A_{570} of L929 cells treated with ActD only). TNF bioactivity in each sample was calculated by substituting the corresponding survival value in the regression equation generated by the recombinant human TNF-α standard curve. Treatment of L929 cells with Act D and SLT-I consistently resulted in <10% cytotoxicity compared with untreated cells. Levels of immunoreactive IL-1β in supernatants collected from cells exposed to stimulants for 24 h were measured by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems). Statistical analyses using Student's paired *t* test were performed with Microsoft Excel version 4.0 software (Microsoft Corp., Redmond, Wash.).

Toxin receptor expression. Relative levels of cell surface toxin receptor expression were evaluated by indirect immunofluorescence (33). Briefly, 10^6 cells per ml were mixed with a receptor-saturating concentration of SLT-I (~1 mg/ml) at 4°C for 1 h with constant agitation. Unbound toxin was removed by three washes with ice-cold phenol red-free Earle's balanced salt solution (EBSS; GibcoBRL) containing 10% FBS and 0.1% NaN_3. The cells were then suspended in 500 μl of a 1:100 dilution of MAb 13C4, a murine monoclonal IgG1 directed against SLT-I B subunits (29), and agitated for 45 min at 4°C. Unbound primary antibody was removed by washes with cold EBSS, and the cells were treated with a 1:100 dilution of fluorescein isothiocyanate-conjugated goat $F(ab')_2$ anti-mouse IgG (Organon-Teknika, Durham, N.C.) for 45 min at 4°C. Cells were washed with EBSS lacking FBS and placed in a flow cytometer (EPICS V; Coulter Electronics, Hialeah, Fla.) equipped with an argon laser tuned to 488 nm. Fluorescence intensity of 10^4 events was recorded. Integration analyses were performed with the Coulter Easy 88 system. Controls included cells in which primary or secondary antibodies had been excluded and cells treated with an isotype-matched primary monoclonal antibody directed against an irrelevant antigen.

Toxin internalization studies. Toxin internalization was measured by the temperature-dependent loss of cell surface fluorescence as described by Cohen et al. (5). Briefly, purified SLT-I (7 μg/ml) was added to 10^6 cells per ml at 4°C for 1 h. The cells were extensively washed with EBSS. Some cells were incubated at 37°C in 5% CO_2 for 1 or 3 h. Control cells were maintained at 4°C. All the cells were then treated with MAb 13C4 and fluoresceinated goat anti-mouse IgG as outlined above. Fluorescence intensity was measured by flow cytometry. Percent internalization was calculated as [(MFI at 4°C − MFI at 37°C)/MFI at 4°C] × 100, where MFI is mean fluorescence intensity expressed as channel number ± standard error of the mean. Radioiodinated SLT-I was also used to examine toxin binding and internalization. Briefly, 4×10^6 cpm of ^{125}I-SLT-I (specific activity, 1.6×10^6 cpm/μg of protein) was added to 5×10^6 cells per ml and incubated at 4°C for 1 h to allow toxin binding and prevent internalization. All cells were washed three times with cold RPMI 1640, and some cells were then shifted to 37°C for 1 h, while others were maintained at 4°C. Cells were removed from the culture dishes by scraping, and the radioactivity associated with cell pellets was determined by scintillation counting. In some experiments, cells were treated with trypsin (50 μg/ml) at room temperature for 15 min and extensively washed prior to scintillation counting. The trypsinization procedure did not decrease trypan blue exclusion or alter cell morphology by light microscopy.

Toxin sensitization experiments. TPA-differentiated THP-1 cells (10^6 cells per ml) were cultured in RPMI 1640 supplemented with 0.25 mM *n*-butyric acid or 8-bromoadenosine 3′:5′-cyclic monophosphate (br-cAMP; Sigma) for 24 h. The medium was removed, and fresh medium containing the sensitizing agents was added for an additional 24 h. Cells were treated with serial dilutions of purified SLT-I for 48 h. MTT was used to measure cytotoxicity as outlined above.

RESULTS

Comparative cytotoxicity of purified SLTs for human monocytes and monocytic cell lines. The cytotoxic effects of purified SLT-I and SLT-IIe for human PBMn and the monocytic cell lines THP-1 and U-937 was assessed by incubation of the cells with 10-fold dilutions of the toxins for 48 h. As shown in Fig. 1, undifferentiated THP-1 cells were sensitive to SLT-I (CD_{50}, ~18 pg/ml) and SLT-IIe (CD_{50}, ~10 pg/ml). TPA treatment of THP-1 cells resulted in increased adherence of the cells to plastic, increased phagocytic activity, and a dramatic increase in resistance to SLTs (CD_{50}, >1.0 μg/ml). Primary PBMn and undifferentiated U-937 cells were refractory to the cytotoxic effects of SLT-I and SLT-IIe, with CD_{50}s at least 5 log units greater than that characterized for undifferentiated THP-1 cells. Differentiation-dependent changes in toxin sensitivity with U-937 cells were not noted. Vero cells were used as a toxin-sensitive control cell (CD_{50}, ~1.0 pg/ml).

Cytokine production by human monocytes and monocytic cell lines stimulated with purified SLT-I or LPS. PBMn and TPA-treated THP-1 and U-937 cells were incubated with SLT-I or LPS for 18 or 24 h, and TNF bioactivity and immunoreactive IL-1β levels in cell-free culture supernatants were determined by the L929 cell lysis assay or ELISA, respectively. PBMn and THP-1 cells responded to SLT-I stimulation by synthesizing TNF and IL-1β in a dose-dependent manner (Fig. 2). In contrast, U-937 cells were less responsive to SLT-I, producing significantly elevated TNF activity only at the highest toxin dose tested ($P < 0.05$ by Student's paired *t* test). All the cells used in these experiments produced TNF and IL-1β

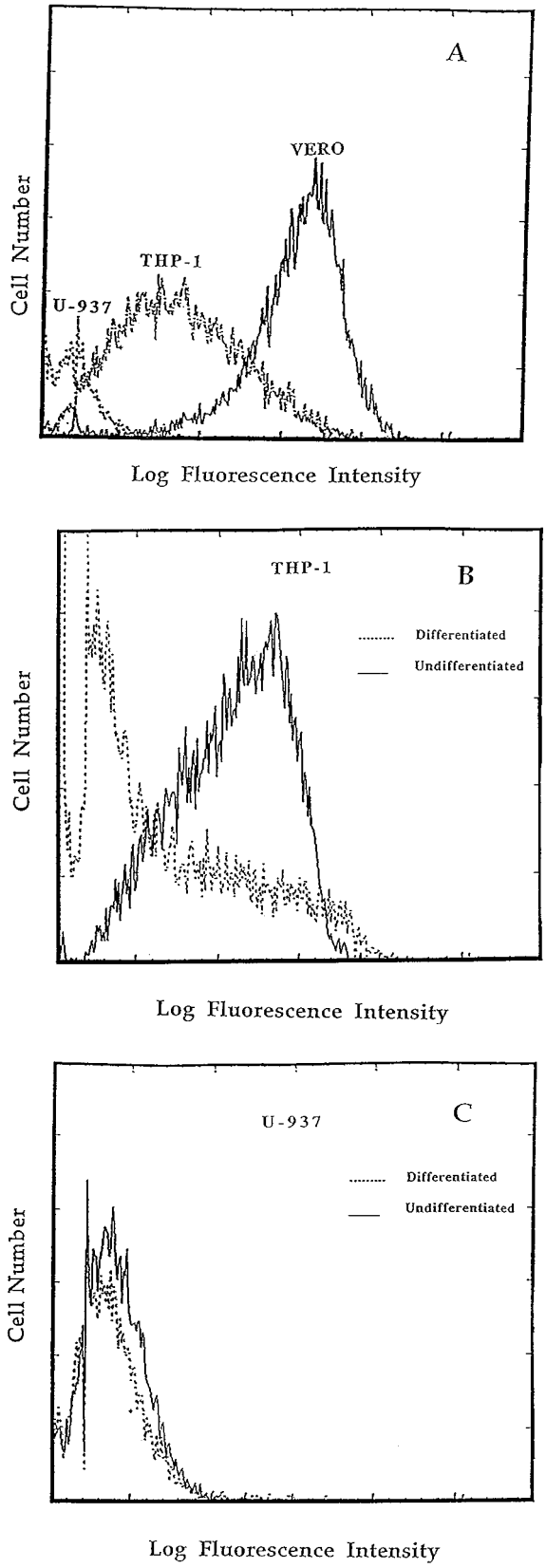

FIG. 3. Toxin receptor profiles of THP-1, U-937, and Vero cells monitored by indirect immunofluorescence and flow cytometry. Cell suspensions (10^6 cells per ml) were treated with 1 mg of SLT-I per ml at 4°C. Bound toxin was then detected with a murine anti-SLT-I monoclonal antibody and a fluorescein-conjugated secondary antibody. The cells were extensively washed and placed in a flow cytometer. Fluorescence emitted by 10^4 events was recorded. The data shown are representative of five independent analyses. (A) Comparison of Gb_3 expression on undifferentiated cells. (B) Comparison of Gb_3 expression on undifferentiated versus differentiated THP-1 cells. (C) Comparison of Gb_3 expression on undifferentiated versus differentiated U-937 cells.

when stimulated with LPS at doses as low as 5.0 ng/ml (data not shown). We detected slightly elevated levels of IL-1β in supernatants from untreated control monocytic cells, probably caused by residual IL-1β production after TPA treatment (7). Treatment of undifferentiated U-937 cells with SLT-I or LPS and treatment of undifferentiated THP-1 cells with LPS did not evoke significantly elevated TNF or IL-1β production (data not shown). Detection of soluble cytokines produced by SLT-treated undifferentiated THP-1 cells was complicated by the extreme sensitivity of the cells to the toxins. However, when undifferentiated THP-1 cells were treated with sublytic doses of SLT-I (up to 10 pg/ml), we did not detect elevated cytokine production (data not shown). Thus, optimal cytokine production by human monocytic cell lines stimulated with SLT-I or LPS required some degree of cellular differentiation. Furthermore, as THP-1 cells acquired differentiation-associated resistance to SLTs, the cells responded to the toxins by elaborating TNF and IL-1β.

Analysis of toxin receptor expression on human monocytic cell lines. Several studies have demonstrated a correlation between the sensitivity of some cell lines to SLTs and membrane expression of Gb_3 (6, 11, 38). To evaluate whether the differential toxin sensitivity we observed with THP-1 and U-937 cells was related to toxin receptor expression, we monitored Gb_3 levels by indirect immunofluorescence and flow cytometry. Mean fluorescence intensity (MFI) was used as a relative measure of Gb_3 expression. In accordance with earlier studies (16), toxin-sensitive undifferentiated THP-1 cells possessed relatively high levels of toxin receptors (Fig. 3A; MFI = 140.7 ± 12.7). Consistent with their relative resistance to SLTs, undifferentiated U-937 cells displayed an approximately fivefold reduction in SLT-I binding (MFI = 29.3 ± 2.7) from that of THP-1 cells. Vero cells, the toxin-sensitive control cell line used in this study, exhibited Gb_3 levels approximately 30% higher (MFI = 182.6 ± 11.3) than undifferentiated THP-1 cells. These data suggest that levels of toxin receptor expression may explain, at least in part, the observed differences in toxin sensitivity between undifferentiated THP-1 and U-937 cells.

Monocyte maturation is accompanied by the gain or loss of a number of enzymatic activities or cell surface markers (2, 10, 17). Therefore, we evaluated whether the differentiation-associated reduction in toxin sensitivity we observed with THP-1 cells was related to changes in toxin receptor expression. TPA treatment of THP-1 cells resulted in an approximately 50% reduction in cell surface fluorescence (Fig. 3B; MFI of untreated THP-1 cells = 140.7 ± 12.7 versus MFI of TPA-treated THP-1 cells = 74.5 ± 17.6). When ^{125}I-SLT-I was used to examine toxin binding, we detected a >2-log-unit decrease in binding in TPA-treated THP-1 cells from that of undifferentiated THP-1 cells (data not shown). TPA-treated U-937 cells showed only a slight reduction in Gb_3 expression in comparison to the undifferentiated counterpart (Fig. 3C; MFI of untreated U-937 cells = 29.3 ± 2.7 versus MFI of TPA-treated U-937 cells = 26.5 ± 3.4). These data show that the differentiation-associated resistance to SLTs we observed with THP-1 cells was associated with a decrease in membrane toxin receptor expression.

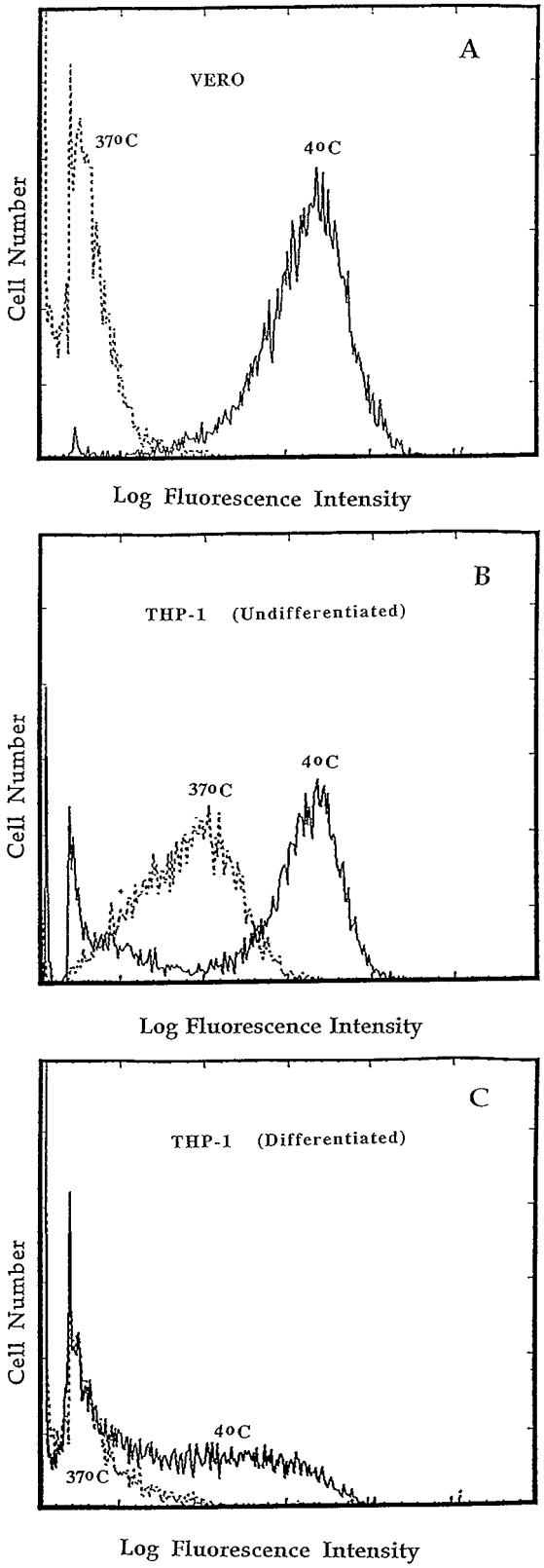

FIG. 4. Internalization of SLT-I by THP-1 and Vero cells. Cells (10^6 cells per ml) were incubated with purified SLT-I for 1 h at 4°C. After three washes with ice-cold buffer, some cells were incubated at 37°C to allow internalization and other cells were maintained at 4°C. Cell-bound toxin was detected by flow cytometry as described in Materials and Methods. The graphs shown are representative of five independent experiments.

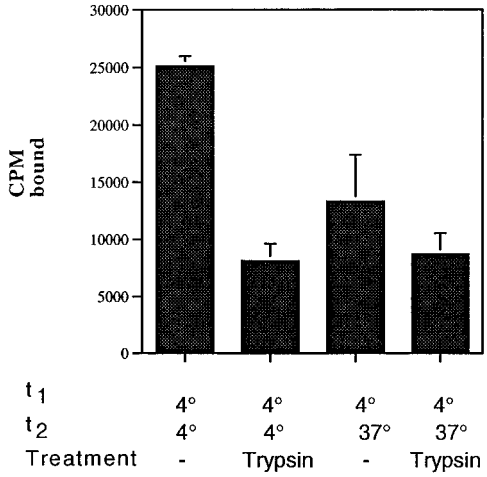

FIG. 5. Binding and internalization of ^{125}I-SLT-I by undifferentiated THP-1 cells. Radioiodinated SLT-I was incubated with 5×10^6 cells per ml at 4°C for 1 h (t_1). Unbound toxin was removed by washing, and the cells were either maintained at 4°C or shifted to 37°C for 1 h (t_2). Cells were treated with trypsin for 15 min at room temperature or not treated with trypsin (−). Binding of ^{125}I-SLT-I to cells was quantitated by scintillation counting.

Internalization of SLT-I by THP-1 and Vero cells. Our flow cytometric analyses of THP-1 cells demonstrated a differentiation-dependent reduction in SLT-I binding. However, as estimated from the MFIs, the amount of toxin binding to resistant TPA-treated THP-1 cells was roughly 2.5 times that binding to toxin-resistant U-937 cells. We therefore questioned whether the toxin resistance observed for TPA-treated THP-1 cells was due to an inability to internalize membrane-bound toxin. Temperature-sensitive toxin internalization was monitored by flow cytometry (Fig. 4), and MFIs were used to calculate percent internalization as described in Materials and Methods. After 1 h of incubation at 37°C, toxin-sensitive Vero cells and undifferentiated THP-1 cells showed 77.5 and 47.2% reduction of surface fluorescence, respectively. Although differentiated THP-1 cells showed decreased toxin binding, the percent loss of surface fluorescence (51.5%) was comparable to that observed for undifferentiated THP-1 cells. To ensure that the loss of surface fluorescence correlated with toxin internalization, we monitored the localization of ^{125}I-SLT-I after binding to cells at 4°C and a temperature shift to 37°C (Fig. 5). When THP-1 cells were maintained at 4°C, approximately 75% of the cell-associated counts were removed by brief trypsinization, suggesting that the toxin was not extensively internalized. Incubation for 1 h at 37°C resulted in ~56% reduction in cell-associated radioactivity from that of cells kept at 4°C, and trypsin treatment did not result in a statistically significant loss of radiolabel from the cells, suggesting that after the temperature shift, the toxin was present in a trypsin-inaccessible (i.e., intracellular) form.

Modulation of toxin receptor expression on THP-1 and U-937 cells by recombinant human TNF-α. Earlier studies demonstrated that the treatment of HVEC with TNF-α resulted in the upregulation of cell surface Gb_3 expression and increased toxin sensitivity (24, 37). To investigate whether a similar phenomenon is operative in monocytes, we treated cells with TNF-α (200 U/ml) for 24 h prior to SLT-I treatment and measurement of toxin receptor expression by flow cytometry. TNF pretreatment of undifferentiated THP-1 cells resulted in a modest increase in toxin binding (MFI of untreated cells = 144.9 ± 37.8 versus MFI of TNF-treated cells = 155.3 ± 36.8

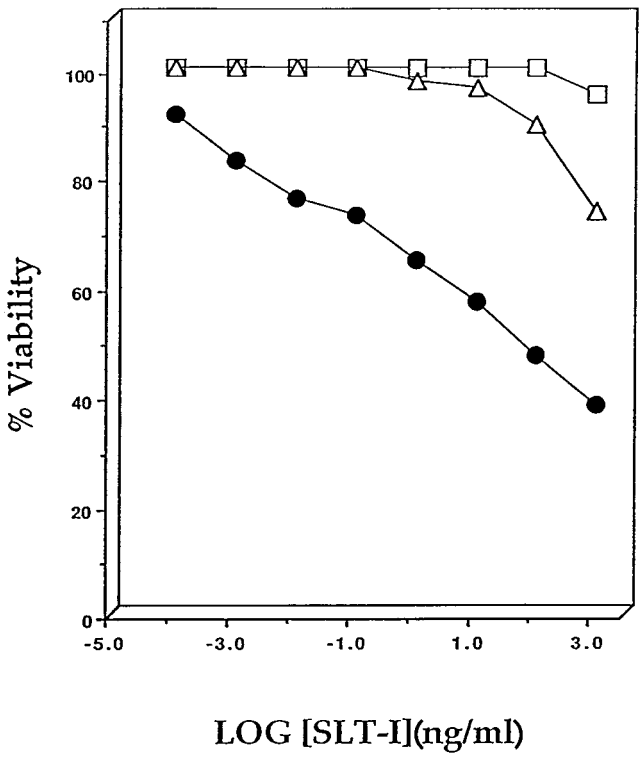

FIG. 6. Toxin sensitization of differentiated THP-1 cells. TPA-treated THP-1 cells were incubated with 0.25 mM *n*-butyric acid or br-cAMP for 24 h at 37°C. The medium was removed, and the cells were treated with the stimulants for an additional 24 h. Cells were then incubated with serial dilutions of SLT-I or medium for 48 h at 37°C. Cell viability was determined with the dye MTT. The data shown were derived from five independent experiments. Symbols: □, untreated cells; △, *n*-butyric acid-treated cells; ●, br-cAMP-treated cells.

[$P < 0.05$ by Student's paired *t* test]). In contrast, TPA-treated THP-1 cells did not respond to TNF-α pretreatment by significantly increasing membrane immunofluorescence (MFI of untreated cells = 57.8 ± 17.6 versus MFI of TNF-treated cells = 60.5 ± 14.6). Thus, during the course of maturation, THP-1 cells not only downregulate toxin receptor levels but also remain unresponsive to TNF-mediated increases in toxin receptor expression. Neither undifferentiated nor TPA-treated U-937 cells displayed statistically significant elevations in cell surface Gb_3 expression following exposure to TNF compared with untreated cells (data not shown).

Sensitization of differentiated THP-1 cells to SLT-I toxicity. Although TPA-treated THP-1 cells are refractory to the toxic action of SLT-I, they bind and internalize the toxin. Using A431 cells, a human epidermoid carcinoma cell line, Sandvig et al. (27) noted a similar response to Shiga toxin. Furthermore, treatment of A431 cells with the differentiation agent butyric acid or 8-br-cAMP sensitized the cells to Shiga toxin via a mechanism involving, in part, increased intracellular transport of the toxin to the endoplasmic reticulum. To determine if a similar sensitization mechanism was operating in human monocytic cells, we treated differentiated THP-1 cells with *n*-butyric acid or 8-br-cAMP prior to exposure to 10-fold dilutions of SLT-I (Fig. 6). Butyrate treatment produced only a modest increase in toxin sensitivity at the highest toxin dose tested. However, treatment of TPA-differentiated THP-1 cells with the cell-soluble cAMP analog sensitized the cells to the cytotoxic action of SLT-I (CD_{50}, ~50 ng/ml) but did not restore toxin sensitivity to the values characteristic of undifferentiated THP-1 cells.

Effects of various differentiation factors on toxin receptor expression and SLT-I cytotoxicity. We measured the effects on toxin sensitivity and Gb_3 expression of THP-1 cells using differentiation factors which may be physiologically more relevant than the pharmacologic agent TPA. Differentiation was monitored by flow cytometric detection of the CD14 surface antigen and estimation of percent adherence to plastic. All the differentiation agents tested diminished the expression of membrane toxin receptor from that of untreated cells (Table 1). Receptor reduction was as high as 46% of control cells in TPA-treated cells, while butyrate was the least-effective modulator of toxin receptor expression. All the differentiation factors induced toxin resistance, although the extent of resistance differed from an approximate 10-fold increase in CD_{50} following butyrate treatment to a $>10^5$-fold increase in resistance mediated by TPA or gamma interferon treatment. These data suggest that THP-1 cell differentiation mediated by a variety of agents is associated with decreased toxin receptor expression and resistance to SLTs.

DISCUSSION

Although our understanding of the roles of Shiga toxin and SLTs in the pathogenesis of bloody diarrhea and hemolytic-uremic syndrome are at an inchoate stage, one concept that has emerged from numerous studies is that HVEC are target cells of toxin action. Toxin receptor expression on HVEC may be upregulated by endogenous cytokine mediators, including TNF-α and IL-1β (37), and exogenous modulators, such as butyrate and vitamin D_3 (18). Thus, regulation of enzymes involved in target cell glycolipid biosynthesis and degradation may be an important determinant in the development of the vascular lesions characteristic of infections with SLT-producing bacteria. Macrophages may participate in pathogenesis by producing cytokines which alter glycolipid biosynthesis and sensitize HVEC to the toxins. The data presented here show that plastic-adherent human PBMn are essentially resistant to the cytotoxic action of SLTs. Two homogeneous human monocytic leukemia cell lines display a differential maturation-associated susceptibility to SLTs. Undifferentiated THP-1 cells, but not U-937 cells, are sensitive to the cytopathic effects of SLTs. Incubation of THP-1 and U-937 cells with phorbol diesters results in reduced cellular replication, increased adherence to plastic surfaces, increased phagocytic activity, and reduced protein kinase C-dependent phosphorylation of selective endogenous substrates (21, 34, 39). We show here that TPA treatment of THP-1 cells also induces resistance to SLTs as characterized by a CD_{50} of >1.0 μg/ml. In contrast, U-937 cells are resistant to SLTs, irrespective of degree of cellular differentiation. The reason for the differential response to SLTs may be related to the developmental stage in which the cell line is transformed. Unlike the polyploid U-937 cell line, THP-1 cells possess a normal complement of chromosomes and are considered more representative of primary monocyte-derived macrophages in terms of inducible functions (2, 17). Interestingly, Jacewicz et al. (13) have noted the differential maturation-associated response of human intestinal epithelial cell lines to SLT-I. Thus, the differentiation stage of epithelial cells and monocytes may be important in the development of bloody diarrhea and systemic complications, respectively.

PBMn and TPA-treated THP-1 and U-937 cells synthesized and secreted TNF and IL-1β when incubated with purified SLT-I, although the dose-response studies suggested that THP-1 cells were more responsive to TNF induction than were

TABLE 1. Effects of differentiation factors on THP-1 cell Gb_3 expression and SLT-I sensitivity

Treatment[a]	CD14[b]	% Adherence[c]	% Fluorescence[d]	CD_{50}	Fold increase in toxin resistance
TPA	17.4 ± 3.5	71.3	56.4	>1.0 μg/ml	$>10^5$
IFN-γ	25.4 ± 5.5	48.5	65.4	>1.0 μg/ml	$>10^5$
GM-CSF	19.6 ± 1.0	55.8	66.3	~75 ng/ml	$\sim10^4$
Vit D_3	15.5 ± 0.6	10.1	69.7	1.0 ng/ml	10^2
n-Butyric acid	5.5 ± 1.1	5.0	84.2	0.1 ng/ml	10
None	3.7 ± 0.4	0	100	14 pg/ml	

[a] Abbreviations: IFN-γ, gamma interferon; GM-CSF, granulocyte-macrophage colony-stimulating factor; Vit D_3, vitamin D_3.
[b] Data shown are MFI (channel number ± standard error of the mean).
[c] Calculated from hemacytometer counts before and after treatment.
[d] Percent Gb_3 expression relative to undifferentiated THP-1 cells as assessed by flow cytometry.

U-937 cells. Thus, concomitant with a loss of susceptibility to SLTs, monocytic cells develop the capacity to produce proinflammatory cytokines in response to toxin stimulation. PBMn and TPA-treated THP-1 and U-937 cells produced elevated levels of cytokines when treated with a well-documented cytokine inducer, LPS, at doses as low as 5.0 ng/ml. Undifferentiated THP-1 and U-937 cells failed to produce cytokines when stimulated with LPS or sublytic concentrations of SLT-I.

All Shiga toxin-, SLT-I-, and SLT-II-sensitive cells express the neutral glycolipid Gb_3, and for some cell types, a correlation between receptor expression and toxin sensitivity has been demonstrated (6, 11, 38). Our studies with human monocytic cell lines support this concept; differences in toxin receptor expression between undifferentiated THP-1 and U-937 cells were correlated with differences in sensitivity to the toxins. Furthermore, treatment of THP-1 cells with differentiation agents which reduced toxin receptor expression also rendered the cells resistant to cell killing. As has been previously noted (12), the correlation between toxin receptor expression and toxin sensitivity is not linear. For example, the difference in reduction of surface fluorescence mediated by gamma interferon versus vitamin D_3 treatment of THP-1 cells was approximately 5%, but the difference in toxin sensitivity was approximately 1,000-fold (Table 1). Thus, the proportional change in toxin resistance was greater than the decrease in Gb_3 expression. The precise reason for the nonlinear relationship between toxin receptor expression and cytotoxicity is not known, although one would predict that if stable toxin binding and internalization require cooperative binding between pentameric B subunits and multiple Gb_3 molecules, then minute reductions in Gb_3 expression may manifest marked differences in cytotoxicity. An important caveat in interpreting our data is that quantitative and qualitative differences in Gb_3 may be involved in toxin sensitivity. Modifications in the fatty acid composition of the ceramide moiety of Gb_3 may alter the conformation or surface accessibility of the Gal(α1-4)Gal disaccharide necessary for toxin binding (15, 27). Subtle modifications in toxin receptor structure induced by differentiation agents would not be detected by the methods employed in this study.

We demonstrate here that cells of the human monocyte/macrophage lineage respond differently to SLT-I treatment than cells of epithelial or endothelial origin do. Unlike A431 epithelioid cells, treatment of differentiated THP-1 cells with *n*-butyric acid did not restore, and treatment with br-cAMP only partially restored, the toxin-sensitive phenotype. Unlike HVEC, TNF-α treatment of THP-1 cells did not result in drastic alterations in toxin receptor expression. TPA treatment of THP-1 cells resulted in toxin resistance associated with reduced toxin receptor expression, yet the cells still bound and internalized SLT-I. While the precise intracellular fate of SLTs in undifferentiated versus differentiated THP-1 cells awaits further study, our experiments suggest that SLT binding and internalization per se are not sufficient to cause cell death. While treatments designed to alter the regulation of toxin receptor synthesis and toxin processing or routing may represent effective interventional strategies to inhibit SLT cytotoxicity, the regulation of Gb_3 synthesis and intracellular transport of SLTs may occur by distinct mechanisms in different cell types.

In response to inflammatory stimuli, circulating PBMn adhere tightly to vascular endothelial cells, undergo diapedesis, and migrate to sites of infection or tissue damage (28). In the presence of microbial products and host response factors, a monocyte differentiative program is activated, resulting in the synthesis of products which direct the cells to inflamed tissue, trigger increased major histocompatibility complex class II expression, facilitate increased phagocytosis, and activate the cells to a microbicidal or tumoricidal state. Immunohistochemical studies on rectal biopsies from patients with shigellosis suggest that elevated levels of monocyte-activating cytokines are found in damaged intestinal tissues (25). The abilities of monocytes to undergo differentiation to a microbicidal state and to extravasate to sites of bacterial invasion and tissue damage would seem intuitively advantageous to the host. Our data suggest that as monocytes undergo differentiation, the cells may become increasingly refractory to the cytotoxic action of SLTs while simultaneously acquiring the capacity to produce TNF and IL-1β in response to the toxins. However, excessive localized production of proinflammatory cytokines may exacerbate vascular damage, especially at sites expressing high basal levels of Gb_3 prior to toxin exposure. Studies to further clarify the roles of inflammatory cells and their products in the pathogenesis of hemorrhagic colitis and the hemolytic-uremic syndrome are in progress in our laboratory.

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REFERENCES

1. **Acheson, D. W. K., A. Donohue-Rolfe, and G. T. Keusch.** 1991. The family of Shiga and Shiga-like toxins, p. 415–433. *In* J. E. Alouf and J. H. Freer (ed.), Sourcebook of bacterial protein toxins. Academic Press, London.
2. **Auwerx, J.** 1991. The human leukemia cell line THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. Experientia **47:**22–31.

3. **Barrett, T. J., M. E. Potter, and N. A. Strockbine.** 1990. Evidence for participation of the macrophage in Shiga-like toxin II-induced lethality in mice. Microb. Pathog. **9:**95–103.
4. **Barrett, T. J., M. E. Potter, and I. K. Wachsmuth.** 1989. Bacterial endotoxin both enhances and inhibits the toxicity of Shiga-like toxin II in rabbits and mice. Infect. Immun. **57:**3434–3437.
5. **Cohen, A., V. Madrid-Marina, Z. Estrov, M. H. Freedman, C. A. Lingwood, and H.-M. Dosch.** 1990. Expression of glycolipid receptors to Shiga-like toxin on human B-lymphocytes: a mechanism for the failure of long-lived antibody response to dysenteric disease. Int. Immunol. **2:**1–8.
6. **Eiklid, K., and S. Olsnes.** 1980. Interaction of *Shigella shigae* cytotoxin with receptor on sensitive and insensitive cells. J. Recept. Res. **1:**199–212.
7. **Fenton, M. J., M. W. Vermeulen, B. D. Clark, A. C. Webb, and P. E. Auron.** 1988. Human proIL-1 beta gene expression in monocytic cells is regulated by two distinct pathways. J. Immunol. **140:**2267–2273.
8. **Griffin, P. M., B. P. Bell, P. R. Cieslak, J. Tuttle, T. J. Barrett, M. P. Doyle, A. M. McNamara, A. M. Shefer, and J. G. Wells.** 1994. Large outbreak of *Escherichia coli* O157:H7 infections in the western United States: the big picture, p. 7–12. *In* M. A. Karmali and A. G. Goglio (ed.), Recent advances in verocytotoxin-producing *Escherichia coli* infections. Elsevier Science, Amsterdam.
9. **Gyles, C. L.** 1994. VT toxemia in animal models, p. 233–240. *In* M. A. Karmali and A. G. Goglio (ed.), Recent advances in verocytotoxin-producing *Escherichia coli* infections. Elsevier Science, Amsterdam.
10. **Harris, P., and P. Ralph.** 1985. Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. J. Leukocyte Biol. **37:**407–422.
11. **Jacewicz, M., H. A. Feldman, A. Donohue-Rolfe, K. A. Balasubramanian, and G. T. Keusch.** 1989. Pathogenesis of *Shigella* diarrhea. XIV. Analysis of Shiga toxin receptors on cloned HeLa cells. J. Infect. Dis. **159:**881–889.
12. **Jacewicz, M., M. Mobassaleh, S. K. Gross, K. A. Balasubramanian, P. F. Daniel, S. Raghavan, R. H. McCluer, and G. T. Keusch.** 1994. Pathogenesis of *Shigella* diarrhea. XVII. A mammalian cell membrane glycolipid, Gb_3, is required but not sufficient to confer sensitivity to Shiga toxin. J. Infect. Dis. **169:**538–546.
13. **Jacewicz, M. S., D. W. K. Acheson, M. Mobassaleh, A. Donohue-Rolfe, K. A. Balasubramanian, and G. T. Keusch.** 1995. Maturational regulation of globotriaosylceramide, the Shiga-like toxin I receptor, in cultured human gut epithelial cells. J. Clin. Invest. **96:**1328–1335.
14. **Jackson, M. P., R. J. Neill, A. D. O'Brien, R. K. Holmes, and J. W. Newland.** 1987. Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933. FEMS Microbiol. Lett. **44:**109–114.
15. **Kiarash, A., B. Boyd, and C. A. Lingwood.** 1994. Glycosphingolipid receptor function is modified by fatty acid content: verotoxin 1 and verotoxin 2c preferentially recognize different globotriaosylceramide fatty acid homologues. J. Biol. Chem. **269:**11138–11146.
16. **Kniep, B., D. A. Monner, U. Schwulera, and P. F. Muhlradt.** 1985. Glycosphingolipids of the globo-series are associated with the monocytic lineage of human myeloid cells. Eur. J. Biochem. **149:**187–191.
17. **Koeffler, H. P.** 1983. Induction of differentiation of human acute myelogenous leukemia cells: therapeutic implications. Blood **62:**709–721.
18. **Louise, C. B., S. A. Kaye, B. Boyd, C. A. Lingwood, and T. G. Obrig.** 1995. Shiga toxin-associated hemolytic-uremic syndrome: effect of sodium butyrate on sensitivity of human umbilical vein endothelial cells to Shiga toxin. Infect. Immun. **63:**2766–2769.
19. **Louise, C. B., and T. G. Obrig.** 1991. Shiga toxin-associated hemolytic-uremic syndrome: combined cytotoxic effects of Shiga toxin, interleukin-1β, and tumor necrosis factor alpha on human vascular endothelial cells in vitro. Infect. Immun. **59:**4173–4179.
20. **Maloney, M., and C. A. Lingwood.** 1993. Interaction of verotoxins with glycosphingolipids. Trends Glycosci. Glycotechnol. **5:**23–31.
21. **Minta, J. O., and L. Pambrum.** 1985. In vitro induction of cytologic and functional differentiation of the immature human monocyte-like cell line U-937 with phorbol myristate acetate. Am. J. Pathol. **119:**111–126.
22. **O'Brien, A. D., V. L. Tesh, A. Donohue-Rolfe, M. P. Jackson, S. Olsnes, K. Sandvig, A. A. Lindberg, and G. T. Keusch.** 1992. Shiga toxin: biochemistry, genetics, mode of action and role in pathogenesis. Curr. Top. Microbiol. Immunol. **180:**67–94.
23. **Obrig, T. G., P. J. DelVecchio, J. E. Brown, T. P. Moran, B. M. Rowland, T. K. Judge, and S. W. Rothman.** 1988. Direct cytotoxic action of Shiga toxin on human vascular endothelial cells. Infect. Immun. **56:**2373–2378.
24. **Obrig, T. G., C. B. Louise, C. A. Lingwood, B. Boyd, L. Barley-Maloney, and T. O. Daniel.** 1993. Endothelial heterogeneity in Shiga toxin receptors and responses. J. Biol. Chem. **268:**15484–15488.
25. **Raqib, R., A. A. Lindberg, B. Wretlind, P. K. Bardhan, U. Andersson, and J. Andersson.** 1995. Persistence of local cytokine production in shigellosis in acute and convalescent stages. Infect. Immun. **63:**289–296.
26. **Richardson, S. E., M. A. Karmali, L. E. Becker, and C. R. Smith.** 1988. The histopathology of the hemolytic uremic syndrome (HUS) associated with verocytotoxin-producing *Escherichia coli* (VTEC) infections. Hum. Pathol. **19:**1102–1108.
27. **Sandvig, K., M. Ryd, Ø. Garred, E. Schweda, P. K. Holm, and B. van Deurs.** 1994. Retrograde transport from the Golgi complex to the ER of both Shiga toxin and the nontoxic Shiga-B-fragment is regulated by butyric acid and cAMP. J. Cell Biol. **126:**53–64.
28. **Springer, T. A.** 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell **76:**301–314.
29. **Strockbine, N. A., L. R. M. Marques, R. K. Holmes, and A. D. O'Brien.** 1985. Characterization of monoclonal antibodies against Shiga-like toxin from *Escherichia coli*. Infect. Immun. **50:**695–700.
30. **Strockbine, N. A., L. R. M. Marques, J. W. Newland, H. W. Smith, R. K. Holmes, and A. D. O'Brien.** 1986. Two toxin-converting phages from *Escherichia coli* O157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. Infect. Immun. **53:**135–140.
31. **Sundström, C., and K. Nilsson.** 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U937). Int. J. Cancer **17:**565–577.
32. **Tesh, V. L., B. Ramegowda, and J. E. Samuel.** 1994. Purified Shiga-like toxins induce expression of proinflammatory cytokines from murine peritoneal macrophages. Infect. Immun. **62:**5085–5094.
33. **Tesh, V. L., J. E. Samuel, L. P. Perera, J. B. Sharefkin, and A. D. O'Brien.** 1991. Evaluation of the role of Shiga and Shiga-like toxins in mediating direct damage to human vascular endothelial cells. J. Infect. Dis. **164:**344–352.
34. **Tsuchiya, S., Y. Kobayashi, Y. Goto, H. Okamura, S. Nakae, T. Konno, and K. Tada.** 1982. Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. Cancer Res. **42:**1530–1534.
35. **Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada.** 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int. J. Cancer **26:**171–176.
36. **Unanue, E. R., and P. M. Allen.** 1987. The basis for the immunoregulatory role of macrophages and other accessory cells. Science **236:**551–557.
37. **Van der Kar, N. C. A. J., L. A. H. Monnens, M. A. Karmali, and V. W. M. van Hinsbergh.** 1992. Tumor necrosis factor and interleukin-1 induce expression of the verocytotoxin receptor globotriaosylceramide on human endothelial cells: implications for the pathogenesis of the hemolytic uremic syndrome. Blood **80:**2755–2764.
38. **Waddell, T., A. Cohen, and C. A. Lingwood.** 1990. Induction of verotoxin sensitivity in receptor-deficient cell lines using the receptor glycolipid globotriaosylceramide. Proc. Natl. Acad. Sci. USA **87:**7898–7901.
39. **Ways, D., R. Riddle, M. Ways, and P. Cook.** 1991. Effect of phorbol esters on cytosolic protein kinase C content and activity in the human monoblastoid U-937 cell. J. Biol. Chem. **262:**1258–1264.

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