Shiga Toxin 2 and Lipopolysaccharide Induce Human Microvascular Endothelial Cells To Release Chemokines and Factors That Stimulate Platelet Function

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Received 2 July 2005/Returned for modification 17 August 2005/Accepted 7 September 2005

Hemolytic-uremic syndrome (HUS) is an infectious disease affecting mainly children, characterized by hemolytic anemia, thrombocytopenia, and acute renal failure (15, 76). HUS is primarily caused by Shiga toxins 1 and 2 (Stx1 and Stx2) produced by Shigella dysenteriae type 1 and enterohemorrhagic Escherichia coli (9). Both toxins have similar functions but different antigenicities due to minor differences in amino acid sequences. These differences are reflected in the different host-cell binding specificities that determine the different tissue sites of these toxins (22). For example, E. coli O157:H7-derived Stx2 and lipopolysaccharide (LPS) release chemokines and other factors such as cytokines and/or chemokines that may be involved in the pathogenesis of HUS (22). The toxins cause endothelial cells to acquire a prothrombotic phenotype with lesions confined to microvessels mostly in renal glomeruli. Furthermore, the presence of kidney microthrombi composed of fibrin and platelets (50) and the presence of high concentrations of beta-thromboglobulin, platelet factor 4 (5), and serotonin (87) in HUS patient plasma samples suggest widespread platelet activation even though the mechanisms underlying this activation remain unclear. Work carried out in our laboratory has shown that Stx2 alone does not directly activate platelets under low- or high-shear forces (29, 86). Therefore, we hypothesized that factors other than Stx2, such as cytokines and/or chemokines, may be involved in the pathogenesis of HUS.

The inflammation associated with HUS is marked by the release of cytokines/chemokines, and the levels of interleukin-8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) are significantly increased in urine samples collected from HUS patients (85). Similarly, several authors have reported that treatment of intestinal epithelial cell types with Stxs leads to increased mRNA levels as well as protein expression of chemokines (79), mainly CXC chemokines, which are known to be involved in the chemoattraction and activation of neutrophils (18). Evidence for
expression of inflammatory cytokines, such as IL-6 and IL-8, in macrophages in response to Shiga toxin and LPS has also been reported (78); evidence for expression of inflammatory cytokines has also been reported for THP-1 monocytic cells (33) in response to tumor necrosis factor alpha (TNF-\(\alpha\)) and IL-1\(\beta\). Endothelial cells exposed to Stx2 have recently been shown to release inflammatory chemokines, such as IL-8 and MCP-1, which stimulated adhesion and transmigration of leukocytes for HUVEC (88). This phenomenon apparently involved the activation of NF-\(\kappa\)B. More recently, Harrison et al. reported that Stx1 and/or lipopolysaccharide (LPS) induced chemokine expression of macrophage inflammatory protein 1\(\alpha\) (MIP-1\(\alpha\)), MIP-1\(\beta\), and growth-regulated oncogene \(\beta\) in differentiated THP-1 cells (32).

Development of HUS may require not only the presence of Stx (40) but also additional proinflammatory stimuli, such as LPS, TNF-\(\alpha\), or IL-1\(\beta\) (82). Thus, it has been well documented that these factors potentiate the effect of Stx on HUVEC in vitro (50, 51, 82). IL-1\(\beta\) and TNF-\(\alpha\), released by monocytes/macrophages in response to Stx, are also known to increase the enzymatic activities as well as the mRNA levels of all three enzymes involved in the Gb3 synthesis pathway in human brain endothelial cells (72). These cytokines are able to increase vascular sensitivity to the toxins by up-regulating the endothelial Gb3 receptor (82). On the other hand, since LPS is present during the infection (shigellosis) before the development of HUS (43, 44), it is likely that the presence of LPS in association with the toxin is directly involved in the evolution and severity of the disease.

Subsequently, LPS triggers a wide variety of cellular responses, including production of cytokines and chemokines, release of arachidonic acid metabolites, and generation of reactive oxygen and nitrogen intermediates that can be respon-
sible for pathophysiological reactions (54, 68, 84). Among the candidate chemokines, IL-8 (CXCL8), RANTES (regulated on activation, normal T cell expressed and secreted) (CCL5), and stromal cell-derived factors 1α and 1β (SDF-1α and SDF-1β) (CXCL12) are capable of direct platelet coactivation through specific receptors (27, 69, 74). However, the roles of IL-8, RANTES, SDF-1α, and SDF-1β in platelet-endothelial cell interactions in the pathogenesis of HUS remain to be defined. A recent report describes increased chemokine expression of IL-8, MIP-1α, MIP-1β, and growth-regulated oncogene β in phorbol myristate-acetate-differentiated THP-1 monocytic cells exposed to Stx1 and LPS due to enhanced mRNA stability (32).

The goal of the present study was to evaluate whether mediators released from HMEC-1 exposed to Stx2, LPS, and the Stx2-LPS combination could stimulate human platelet function. Our overall research is directed to understanding the mechanisms responsible for the involvement of platelets in the inflammation and thrombotic events associated with HUS (55, 57, 76). We used human microvascular endothelial cells and assessed the presence of four chemokines (IL-8, SDF-1α, SDF-1β, and RANTES) in supernatants of endothelial cells incubated with Stx2, LPS, and the Stx2-LPS combination. We now report that factors released from endothelial cells stimulated with the combination of Stx2 and LPS were able to induce platelet aggregation, serotonin secretion, and formation of platelet-monocyte aggregates. We also show that the combination of the RANTES chemokine with SDF-1α or other chemokines and very low levels of primary agonists, such as ADP, was effective at inducing platelet aggregation.

MATERIALS AND METHODS

Reagents. Eagle’s medium was obtained from Gibco BRL (Rockville, MD) and other chemicals used for platelet experiments, and purified lipopolysaccharide derived from E. coli O55:B5 was purchased from Sigma Chemicals (St. Louis, MO). All reagents and chemokines used for enzyme-linked immunosorbent assays were from R & D Systems (Minneapolis, MN).

Toxins. Stx2 was purified by an affinity purification procedure with anti-Stx2 antibodies (11E10), using an E. coli lysate provided by Allison O’Brien (Uniformed Services University of the Health Sciences, Bethesda, MD). Following treatment of the sample with Detoxi-Gel (Pierce Chemical, Rockford, IL), LPS was undetectable in the Stx2 preparation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis revealed the presence of only Stx2A subunit (32-kDa) and Stx2B subunit (7.7-kDa) proteins. The 50% lethal dose of this purified Stx2 was 0.1 nM as determined in a Vero cell cytotoxic assay. After purification, the Stx2 was stored at −80°C until use.

Cell lines and culture conditions. HMEC-1 cells were obtained from the CDC (Atlanta, Ga.). They were originally derived by transfection of human microvascular endothelial cells with a pB322-based plasmid containing the coding region for the simian virus 40A gene product, large T antigen (2). The HMEC-1 cells exhibit typical cornerstone morphology when grown in monolayer culture and exhibit cell surface markers typical of normal endothelial cells. The HMEC-1 cells were maintained in MCDB 131 medium (Gibco BRL, Grand Island, N.Y.) containing 1 μg/ml hydrocortisone and 10 ng/ml epidermal growth factor supplemented with 15% fetal bovine serum (Intergen, Purchase, N.Y.). Undifferentiated human leukemia THP-1 cells were purchased from American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 (Gibco BRL, Grand Island, N.Y.), supplemented with 10% fetal bovine serum. Both cell lines were maintained at a density of 1 × 10^6 cells/ml at 37°C in 5% CO2 in a humidified incubator in the absence of penicillin, streptomycin, or amphotericin B. The media were routinely checked for contamination, and none was detected.

Chemokine immunoassays. HMEC-1 (10^6 cells/ml) were incubated with 0.1 nM Stx2, 5 ng of LPS per ml (64), or with the Stx2-LPS combination at the same concentrations from 0 up to 24 h. At each time point, aliquots of the cell suspensions were collected and centrifuged at 1,500 rpm, and the resultant supernatants were stored at −80°C until needed. The supernatants were assayed for the chemokines IL-8, SDF-1α, SDF-1β, and RANTES. For each chemokine, the microwell plate-wells were coated overnight with anti-human or mouse antibodies in sterile phosphate-buffered saline (PBS). Subsequent steps included adding biotinylated polyclonal human anti-human chemokine, horseradish peroxidase-conjugated streptavidin, and a mixture of H2O2 and tetramethylbenzidine as the substrate.

Platelet preparation. Blood was collected in acid-citrate-dextrose anticoagulant (11.5 mM), and washed platelets were prepared from platelet-rich plasma (PRP) by procedures developed in our laboratory (26). The final platelet pellet was resuspended in Eagle’s medium containing HEPES supplemented with 0.5 mg/ml fibrinogen (26). The average final platelet count was 2 × 10^10/ml.

Platelet stimulation by HMEC-1 supernatants by aggregation assays. (i) Particle counting. To assess platelet aggregation, 20-μl samples of washed platelets were preincubated at 37°C for 10 min with saline (static control) or 20-μl samples of medium from HMEC-1 cultures incubated with ADP (0.5 μM) alone. To test the potential cyclooxygenase involvement in platelet aggregation induced by medium samples from HMEC-1 cultures, platelets were also preincubated with indomethacin (2.5 μg/ml) for 10 min at 37°C without stirring. All samples were then stirred for 30 s (1,200 rpm) at 37°C, under orbital shaking conditions developed in our laboratory to reproduce in vitro, low-shear stresses (74). Reactions were quenched with glutaraldehyde (1%) in 0.15 M NaCl, and platelet aggregation was then evaluated by the loss of platelet singlets (26).

(ii) Optical aggregometry. Aggregation of washed platelets was assessed under low-shear stress of about 1 dyn/cm² (1,000 rpm) in an optical aggregometer (Chronolog, Havertown, PA). Two hundred fifty microliters of the platelet suspension (2 × 10^10 cells/ml) was incubated with 250 μl of the supernatant collected
Platelet secretion assay of serotonin. Platelets were labeled by incubating PRP with 0.2 μCi [3H]serotonin for 30 min at 37°C, followed by the procedures used to prepare washed preparations. The labeled platelets were then incubated in the test tube stirring system with 250 μl of supernatant collected from HMEC-1 culture flasks containing Stx2, LPS, or the Stx2-LPS combination. ADP (2.5 μM) was used again as a platelet agonist, and when needed, indomethacin (2.5 μg/ml) was used to prevent TxA2 synthesis. After 3 min, the reaction was quenched with glutaraldehyde (1%), and platelets were pelleted in a microcentrifuge. Aliquots of the supernatants were then mixed with a scintillation cocktail for analysis (Beckman LS 6500), and secretion was determined by the method of Jones et al. (38).

P-selectin exposure. Platelets were resuspended in HEPES buffer without fibrinogen (1 × 10^7 platelets/ml) and then incubated with equal volumes of the supernatants collected at different times after incubation of HMEC-1 with Stx2, LPS, or the Stx2-LPS combination. Along with supernatants, platelets were incubated with ADP (0.5 μM) alone or with indomethacin (2.5 μg/ml) for 10 min at 37°C. Control supernatants were obtained from the cell cultures which had not been exposed to any stimulus (LPS and/or Stx2). Phycocerythrin (PE)-conjugated anti-CD41 and fluorescein isothiocyanate (FITC)-conjugated anti-CD62P were used for dual-color analysis by flow cytometry. Platelets (100 μl) were incubated at 37°C for 30 min in the dark with PE-conjugated anti-CD41 or FITC-conjugated anti-CD62P. Out of the total platelet population, the percentages of platelets that were positive for P-selectin exposure are shown in Fig. 5. The percentage of platelets binding nonspecific IgG1 conjugated to FITC was set as zero percent (i.e., negative control). All samples were analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using Cell Quest software, and experiments were repeated with three different platelet donors.

Platelet–THP-1 cell interactions. Washed platelets (100 μl, 1 × 10^5 cells/ml) and THP-1 cells (100 μl, 1 × 10^5 cells/ml) were mixed together and incubated for 10 min at 37°C with 100-μl aliquots of medium samples from the HMEC-1 cultures. For a positive control, THP-1 monocytic cells were incubated with thrombin-activated platelets. These platelets were stimulated with 1 U/ml thrombin for 10 s, when the reaction was stopped by adding 10 U/ml hirudin and the THP-1 cells were then added to the activated platelets. Subsequently, the following antibodies were added. The THP-1 cell population was identified by FITC-conjugated anti-CD11b (2.5 μl) and PE-conjugated anti-CD14 (2.5 μl). To label platelets and to determine nonspecific binding, FITC-conjugated anti-CD41a antibody (2.5 μl) and isotype-matched FITC-conjugated IgG1 (2.5 μl) were used, respectively. Platelets and THP-1 cells were incubated with appropriate antibodies for 20 min at 37°C, and the reaction was stopped by adding 2% paraformaldehyde in PBS. Samples were centrifuged for 10 min at 620 × g and resuspended in 600 μl PBS. Platelet–THP-1 cell aggregate formation was measured by a FACSCalibur flow cytometer (Becton Dickinson), and the data represent 15,000 monocyte events. Monocytes were distinguished from platelets by their size and granularity as well as by their PE-conjugated anti-CD14 and FITC-conjugated anti-CD11b fluorescence. The percentage of platelet–THP-1 cell conjugates was measured in single-parameter histograms of FITC-conjugated anti-CD41a fluorescence displaying events from the THP-1 cell gate. The positive analysis region was determined using the FITC-conjugated IgG1 isotype control. Data are expressed as the percentages of THP-1 cell-platelet aggregates detected out of the total THP-1 cell population.

Statistical analysis. Analysis of variance was performed, and data are expressed as means ± standard errors. Tests for potential significance between control and treated samples were carried out with the Student’s t test (unpaired test) and analysis of variance (Bonferroni test). Probability values were considered significant when P was < 0.05.

RESULTS

Chemokine release by HMEC-1 upon stimulation by Stx2 and/or LPS. HMEC-1 were responsive to all stimuli in a time-dependent manner with significant increases in IL-8, RANTES, SDF-1α, and SDF-1β reached after 24 h of incubation (Fig. 1A to D). IL-8 was released in the greatest amount (Fig. 1A), especially when the Stx2-LPS combination was employed. The data in Fig. 1B show that more SDF-1α was released when the cells were stimulated for 24 h with Stx2 alone (783 ± 42 pg/ml) than with LPS alone or the Stx2-LPS combination. Unlike IL-8,
release of RANTES was much slower initially (more than 8 h), but after 24 h of incubation, substantial release occurred (Fig. 1C), which ranged from 86.7 ± 7.7 pg/ml (no stimulus) to 1,058 ± 363 pg/ml with the Stx2-LPS combination. Figure 1D shows that the release of SDF-1 by HMEC-1 was also stimulated with Stx2, LPS, and the Stx2-LPS combination in a time-dependent manner; however, 24 h of incubation with LPS proved to be a stronger stimulus than incubation with the Stx2-LPS combination (\(P < 0.05\)).

Platelet aggregation by supernatants. (i) Light scattering. The results displayed in Fig. 2A show that the supernatant collected after 8 h of incubation of HMEC-1 with Stx2-LPS did not induce any platelet aggregation, but when combined with a low dose of a primary agonist, 2.5 \(\mu\)M ADP, the supernatant then caused irreversible aggregation during the 150 s of observation. This concentration of ADP usually stimulates about 20% of aggregation, which is reversible within 2 min. In addition, Fig. 2A also shows that indomethacin prevented the potentiation of platelet aggregation caused by the supernatants from the HMEC-1 culture incubated with the Stx2 and LPS combination at 8 and 24 h. In addition, indomethacin restored the reversibility of platelet aggregation. The same pattern of platelet aggregation was observed when platelets were stimulated with the supernatant collected after 24 h of stimulation of HMEC-1 by the Stx2-LPS combination (Fig. 2B).

(ii) Single-particle counting. Results shown in Fig. 3 reveal that media collected at the beginning (0 h) of the incubation induced 21% ± 2% of platelet aggregation. Similar aggregation was observed after stimulation with a low dose of ADP (0.5 \(\mu\)M) and the ADP plus indomethacin combination. Loss of singlets was significantly increased (\(P < 0.05\)) to 42% ± 2% when the platelets were exposed to supernatants collected after 8 h of incubation of HMEC-1 with Stx2-LPS, and even more strongly after 24 h (46% ± 1%). These singlet losses at 8 and 24 h due to aggregation were prevented by platelet preexposure to indomethacin. Supernatants from HMEC-1 cells exposed only to Stx2 or LPS did not enhance platelet aggregation (data not shown).

Platelet secretion of serotonin. Figure 4 shows that the supernatants collected from HMEC-1 culture media at the beginning of HMEC-1 incubation (0 h) induced minimal secretion of serotonin from platelets, even when combined with ADP. However, a significant increase occurred when platelets were exposed to a low level of ADP (2.5 \(\mu\)M) plus the Stx2-LPS combination for 8 and 24 h; S (0 h), supernatant obtained at the beginning of incubation. Data are means ± standard errors (error bars) of three different experiments. Values that were significantly different from the S (0 h) value are indicated by asterisks (*, \(P < 0.05\); **, \(P < 0.02\)).

Platelet activation by HMEC-1 supernatants: P-selectin exposure. Only minimal exposure of CD62P (P-selectin) was detected on the surfaces of platelets exposed to the HMEC-1 culture supernatants obtained at the beginning of incubation (0 h) with Stx2-LPS (data not shown). Significant exposure of P-selectin occurred during platelet stimulation with supernatants derived after 8 h of incubation of the HMEC-1 with Stx2 alone or the Stx2-LPS combination, especially in the presence of low-level ADP (0.5 \(\mu\)M) (Fig. 5), as well as after 24 h (data not shown). Thrombin (1 U/ml) was used as a positive control (data not shown).
Combination of chemokines activate platelet aggregation.
Earlier work from our laboratory and others has clearly shown that chemokines, such as SDF-1α/β, and macrophage-derived chemokine (MDC), are more effective at inducing platelet aggregation when combined with low levels of primary platelet agonists, such as ADP and thrombin (1, 14, 28, 45). However, chemokines like RANTES and IL-8, which may be produced in substantial amounts during inflammatory situations, are very weak inducers of platelet function, even in the presence of ADP (28). We therefore tested whether combinations of chemokines might activate platelet aggregation, especially since our data on Stx2-LPS revealed strong production of IL-8, RANTES, and SDF-1α and -1β (Fig. 1).

The data presented in Fig. 6 reveal that combining the chemokine RANTES (CCL5) with low levels of ADP or thrombin did not stimulate platelet aggregation. However, the inclusion of an additional chemokine, such as SDF-1α (CXCL12), MDC (CCL22), or thymus and activation-regulated chemokine (TARC) (CCL17), induced significantly more aggregation as detected in our sensitive single-particle counting assay. An exception was that RANTES did not enhance SDF-1α-induced aggregation when thrombin was a coagonist, in contrast to the significant enhancement seen when ADP was present.

Platelet–THP-1 cell heterotypic aggregate formation. Brief stimulation of platelets with thrombin induced a marked increase in the formation of platelet–THP-1 monocytic cell conjugates, with 56% of platelets bound to the THP-1 cells (Table 1). We observed little interaction between platelets and THP-1 cells when platelets were stimulated with medium control supernatants derived from HMEC-1 culture after 24 h of incubation (14.2% ± 3.6%). In contrast, supernatants derived after 8 or 24 h of stimulation of HMEC-1 with LPS, Stx2, and the Stx2-LPS combination caused significant increases in platelet–THP-1 cell heterotypic aggregate formation. Combination of supernatants with a low level of ADP (0.5 μM) enhanced the observed increases in platelet interaction with the THP-1 cells.

DISCUSSION

In the present study we have shown that Stx2 caused HMEC-1 to release proinflammatory chemokines, such as IL-8, RANTES, SDF-1α, and SDF-1β, establishing a possible link between platelet activation and the presence of Stx2 and/or LPS in the circulation systems of patients with HUS (Fig. 1). Such chemokine release may promote the development of HUS, since when combined with low doses of platelet primary agonists (74), chemokines can induce platelet aggregation, an important feature of HUS (67). However, there is little evidence that Stx2 interacts with platelets and initiates thrombosis in the kidney. Previous studies have shown that Stx2 does not bind to quiescent platelets (29), suggesting that Stx2 may initially interact with other vascular wall cells to release proinflammatory mediators likely to be involved in subsequent platelet aggregation. Indeed, the levels of these mediators, including cytokines/chemokines, growth factors, soluble adhesion molecules, and interleukins, are significantly increased in patients with HUS (85).

Supernatants derived from the cultures of HMEC-1 exposed to Stx2-LPS were able, in the presence of low levels of ADP, to stimulate platelet aggregation (Fig. 2 and 3), consistent with microthrombus formation associated with HUS. In addition, our results demonstrating that HMEC-1 supernatants trig-
RANTES and SDF-1α (Fig. 1), with the release of IL-8 reaching near maximum levels at 8 h of incubation with the Stx2-LPS combination. IL-8 is a known proinflammatory chemokine that is expressed in macrophage-rich areas of human coronary atherosmas (70), and increased plasma levels of IL-8 have been detected in children with HUS (49). Thus, Stx2 and LPS through increased production and secretion of chemokines (IL-8, RANTES, and SDF-1α/β) from activated endothelial cells could lead to subsequent chemokine accumulation in the vascular extracellular matrix of the kidney, enhancing the thrombogenicity of kidney microvessels and contributing to the progression of kidney failure in HUS.

The development of HUS is also closely linked to the presence of LPS in the circulation system (42). LPS is a potent activator of both immune and nonimmune cells, such as endothelial cells. In response to LPS, endothelial cells undergo activation with enhanced expression of cell adhesion molecules (20), release of cytokines/chemokines, and increased leukocyte-binding activity (7). Our results build on earlier findings showing that Stx2 and LPS induced release of cytokines, such as IL-1β and TNF-α, from monocytes (65), thereby promoting leukocyte adhesion (88) and development of thrombotic changes in the kidney.

Our findings that stimulation of platelet aggregation and secretion upon exposure to HMEC-1 supernatants were strongly inhibited by indomethacin suggest cyclooxygenase involvement. Since TxA2 has a very short half-life, it most likely originates from the stimulated platelets rather than being present in the HMEC-1 supernatants. Such platelet stimulation can occur via activation of platelet G-protein-coupled receptors (12) targeted by chemokines, ADP, and other po-

TABLE 1. Supernatants collected from HMEC-1 exposed to Stx2, LPS, and the Stx2-LPS combination stimulate platelet–THP-1 cell aggregate formation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Platelet–THP-1 cell aggregates after incubation for:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Medium + saline (medium control)</td>
<td>7.4 ± 1.9</td>
</tr>
<tr>
<td>Stx2 supernatant</td>
<td>6.5 ± 1.6</td>
</tr>
<tr>
<td>Stx2-LPS supernatant</td>
<td>6.7 ± 1.6</td>
</tr>
<tr>
<td>Medium + ADP (0.5 μM)</td>
<td>12.0 ± 3.0</td>
</tr>
<tr>
<td>LPS supernatant + ADP (0.5 μM)</td>
<td>23.5 ± 5.9</td>
</tr>
<tr>
<td>Stx2 supernatant + ADP (0.5 μM)</td>
<td>12.9 ± 3.2</td>
</tr>
<tr>
<td>Stx2-LPS supernatant + ADP (0.5 μM)</td>
<td>29.1 ± 4.4</td>
</tr>
</tbody>
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* The presence of platelet-monocyte conjugates induced by factors released from HMEC-1 cultures treated with Stx2 (0.1 nM), LPS (5 ng/ml), and the Stx2-LPS combination was analyzed by flow cytometry. Platelets were incubated with HMEC-1 supernatants for 10 min at 37°C with gentle agitation, followed by 30 s of stirring at 1,200 rpm.

* Data are expressed as percentages of THP-1 cell-platelet aggregates out of the total THP-1 cell population. Results are from a representative experiment of three different experiments. Values that were significantly different from the 0-h values (at the beginning of incubation) (P < 0.05) are shown in bold type.

* Medium control is supernatant obtained from HMEC-1 cultured in the absence of any stimulation.
Potential factors present in the supernatants tested in our experiments. Previous data support TxA2 involvement, since Stx1 and/or LPS induces arachidonate release and eicosanoid formation by human glomerular epithelial cells (68). Thus, substantial amounts of stable metabolites of TxA2 were found in plasma samples from patients with HUS (81), while renal levels of the anti-thrombotic factor prostacyclin were decreased (58). Vascular inflammation and thrombosis are often accompanied by platelet-monocyte aggregate formation (21, 37). The presence of these heterotypic conjugates in microcirculation may then promote formation of clotting complexes as well as the production and release of more cytokines/chemokines and other proinflammatory factors by platelets, endothelial cells, and other blood cells (25). Furthermore, activated monocytes, a rich source of tissue factor, could amplify this process (10).

The scheme presented in Fig. 7 outlines a general mechanism by which platelets may become activated as a consequence of Stx2 and LPS stimulation of endothelial cells, leading to platelet aggregation and platelet-monocyte interactions. These events are proposed to contribute to the known thrombotic complications and pathogenesis of HUS (8, 39, 41, 60, 66, 81). We demonstrated (Fig. 1) that the chemokines IL-8, RANTES, SDF-1α, and SDF-1β were released from HMEC-1 cells in significant amounts: 1.1 ng/ml or 0.14 nM for RANTES, assuming a chemokine molecular weight of 8,000, down to 0.3 ng/ml or 0.038 nM for SDF-1β. These concentrations were then lowered by 50% in the platelet function assays (Materials and Methods), since a 1:1 dilution of the HMEC-1 supernatants occurred during the assay. Thus, our chemokine concentrations were about 50- to 500-fold lower than the typical 50% effective concentrations of about 7 to 30 nM for chemokine activation of platelet function (28). The 50% effective concentrations can range from 0.1 up to 200 nM for chemokine cell migration assays and other function tests (3, 31, 63, 64, 80).

We therefore propose that combinations of factors released from HMEC-1 cells exposed to Stx2-LPS, coupled with low levels of released cellular ADP, act together at levels below the individual threshold levels to synergize and stimulate platelet function. This hypothesis is analogous to the ability of epinephrine to enable stable platelet aggregation when combined with low levels of ADP (52, 53, 71). Thus, the combination of low levels of ADP with the supernatants from HMEC-1 cells exposed to Stx2 and LPS consistently induced stable platelet aggregates (Fig. 2 and 3), significant dense-granule serotonin release (41), and endothelial cell activation (10).
secretion, and exposure of α-granule CD62P (Fig. 4 and 5). These outcomes were not observed in the absence of the low level of primary platelet agonist.

Figure 7 also presents a rationale as to why indomethacin can prevent the ability of HMEC-1 supernatants to stimulate platelet aggregation, serotonin secretion, and CD62P exposure. Activation of platelet soluble phospholipase A2 generates arachidonic acid, the precursor of thromboxane A2, an effective platelet agonist (11, 16, 34, 46). Platelet soluble phospholipase A2 may thus be an important component of signaling events stimulated by factors released from endothelial cells exposed to Stx2 and LPS. Indeed, there is strong evidence for the generation of TXA2 during early phases of HUS (68, 81), and severe thrombocytopenia during HUS reflects massive platelet consumption and other indices of hemostasis activation during the progression of the disease (13, 55, 56, 76). Our results showing that indomethacin prevented activation of platelet dense-granule and α-granule secretion (Fig. 4 and 5), as well as aggregation (Fig. 2 and 3) and interaction with THP-1 monocyte cells (Table 1), suggest that use of cyclooxygenase 2 (COX2) inhibitors, such as aspirin, might be indicated during early phases of HUS to help prevent platelet consumption and thrombocytopenia.

Exposure of platelet CD62P (Fig. 5), which can interact with leukocyte P-selectin glycoprotein ligand 1 (6, 16, 47, 83) provides a direct link to our observations of platelet-monocyte aggregate (THP-1 cells) aggregates caused by media from HMEC-1 cells exposed to Stx2, LPS, or to the Stx2-LPS combination with ADP (Table 1). These new data parallel findings of enhanced monocyte-platelet aggregate formation in coronary artery disease (24) and the involvement of chemokines, such as RANTES (36, 37). Monocytes are a rich source of tissue factor, and therefore, the platelet-monocyte interactions, which are a hallmark of HUS-related events in the kidney (22, 23, 62), may stimulate blood coagulation. Other studies also suggest that microvesicles derived from monocytes/macrophages can fuse with activated platelets and thus help initiate coagulation (17) and the thrombotic events associated with HUS (22, 23, 55, 56, 62, 75, 76). A recent related study by our group on the abilities of Stx2 and LPS stimulated platelet function as judged by the criteria set for the human microvascular endothelial cell line. J. Investig. Dermatol. 99:683–690.


ACKNOWLEDGMENTS

This work was supported by Public Health Service grants DK59704 (to A. R. L. Gear) and AI 24431 (to T. Obrig) and by the Carman Trust.

We thank Linda Beggarly for phlebotomy and Joanne Lannigan for technical assistance with the flow cytometry studies.

None of the authors have any commercial or other association that might pose a conflict of interest.


