Molecular Basis for Up-Regulation by Inflammatory Cytokines of Shiga Toxin 1 Cytotoxicity and Globotriaosylceramide Expression

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Mortality in postdiarrheal hemolytic-uremic syndrome (HUS) is associated with brain injury. Normally, brain cells are resistant to Shiga toxin (Stx), the putative pathogenic toxin in HUS. However, exposure of human brain endothelial cells (HBECs) to tumor necrosis factor (TNF) and/or interleukin (IL)–1 markedly up-regulates Stx receptor (globotriaosylceramide; Gb3) expression and cytotoxicity. To investigate how Gb3 is augmented, ceramide glucosyltransferase (CGT), lactosylceramide synthase (GalT2), Gb3 synthase (GalT6), and α-galactosidase were studied in HBECs exposed to TNF and IL-1. TNF, both alone and in combination with IL-1, increased Stx-1 toxicity, Gb3 content, and Stx-1 binding. TNF in combination with IL-1 increased CGT, GalT2, and GalT6 but did not change α-galactosidase activities or mRNA levels. Cytokine treatment did not change CGT, GalT2, or GalT6 mRNA half-lives. Thus, inflammatory cytokine up-regulation of the sensitivity of HBECs to Stx-1 is the result of up-regulation, most likely via transcription, of the activities of 3 enzymes involved in Gb3 synthesis.

Hemolytic-uremic syndrome (HUS) is the leading cause of acute renal failure in children; the disorder derives its name from the classic presentation: acute renal injury, microangiopathic hemolytic anemia, and thrombocytopenia [1, 2]. HUS mainly causes kidney damage; most patients recover completely, but a small percentage have residual renal insufficiency or hypertension [3]. Approximately 5% of patients with HUS die during the acute phase or shortly thereafter [4]. Importantly, these deaths are usually not the result of renal disease but of extrarenal involvement. Several other organ systems may be affected in HUS, including the lungs, heart, central nervous system (CNS), pancreas, liver, muscle, skin, parotid gland, and retinas [1, 2, 5]. Of these, it is now evident that, when death occurs in HUS, it is most commonly caused by damage to the brain [6].

HUS is associated with enteric infection by Shiga toxin (Stx)–producing organisms, predominantly *Escherichia coli* O157:H7 [1, 2]. Several isoforms of Stx, including Stx-1, Stx-2, and Stx-2v, for the most part bind the same receptor and have similar biologic actions [2]. No clear association between Stx isoform and CNS manifestations in patients with HUS due to *E. coli* O157:H7 infection has been established. Stx binds to cell-surface glycosphingolipids, terminating in galactose-α1,4-galactose, whereupon the complex is internalized, with resultant inhibition of peptide elongation [2, 7]. The major glycosphingolipid that binds Stx is galactose-α1,4-galactose-β1,4-glucoseceramide (globotriaosylceramide; Gb3) [7]. Several studies support the idea that Stx is important in the pathogenesis of brain injury in HUS. Rabbits given intravenous Stx-2 developed severe CNS injury associated with lesions suggestive of ischemic damage [8]. Piglets injected with Stx-2–producing strains of *E. coli* O157:H7 developed arteriolar necrosis in the CNS [9]. Another group studying piglets observed that inoculation with *E. coli* O157:H7 caused convulsions and severe encephalopathy; examination of the brains of the piglets revealed pyknosis and karyorrhexis of endothelial cells [10]. Taken together, these studies suggest that Stx causes CNS dysfunction and that this damage is associated with endothelial cell injury.

Despite these observations implicating Stx-mediated endothelial cell injury in the pathogenesis of CNS damage in HUS, there is evidence that human brain microvascular endothelial cells are resistant to Stx-1–induced cytotoxicity [11, 12]. However, recent studies indicate that inflammatory cytokines may play a key role in HUS-associated brain injury. Mice injected with *E. coli* O157:H7 develop flaccid paralysis associated with brain edema and microhemorrhage; treatment with tumor necrosis factor (TNF) worsened the pathologic response to *E. coli* O157:H7, whereas a TNF inhibitor markedly reduced brain injury [13]. In addition, TNF enhanced Stx toxicity by ~1000-fold in cultured human brain microvascular endothelial cells (HBECs) [11, 12]. Similarly, interleukin (IL)–1 augmented Stx-1 toxicity in HBECs by ~1000-fold [11, 12]; IL-6 had no significant effect. Importantly, TNF and IL-1 also increased Gb3 expression and Stx binding in HBECs, suggesting that up-regulation of Stx receptors was important in the cytokine response.

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Finally, Stx has been shown to stimulate TNF and IL-1 production by several cell types, including endothelial cells [11, 12, 14–17]. Taken together, these findings suggest that brain endothelial cell injury in HUS may be critically dependent on stimulation by cytokines of the action of Stx, most likely through enhanced expression of Gb3.

Several components of the Gb3 metabolic pathway may be involved in cytokine-stimulated Gb3 expression in HBECs. Gb3 is synthesized from lactosylceramide (LacCer) and UDP-galactose by Gb3 synthase (GalT6) [18] and is metabolized to LacCer by α-galactosidase [19]. LacCer is derived, in turn, from glucosylceramide (GlcCer) and UDP-galactose by LacCer synthase (GalT2) [20], whereas GlcCer is synthesized from ceramide and UDP-glucose by ceramide glucosyltransferase (CGT) [21]. Consequently, increased Gb3 content in HBECs could, conceivably, be due to increased activity of GalT6, GalT2, or CGT, and/or it could be due to decreased activity of α-galactosidase. Currently, there is no information on cytokine regulation of these enzymes in the brain (and very little information on regulation in other organs). The present study was undertaken, therefore, to define how inflammatory cytokines modulate the enzymes that are involved in controlling the levels of Gb3 expression in HBECs.

Materials and Methods

Cell culture. HBECs were obtained at primary culture (Cell Systems) and studied at passages 7–9. Cells were grown to confluence in EGM2-MV medium (Clonetics) and switched to serum-free Maintenance Formula medium (Cell Systems) 24 h before all studies were initiated. In addition to characterizing these cells by culture in Cell Systems medium, we determined that these cells had uniformly positive immunofluorescence for von Willebrand factor and platelet endothelial cell adhesion molecule but were negative for cytokeratin.

Stx-1 purification. Stx-1 was purified from *E. coli* HB101 containing pNAS13, which encodes Stx-1 (gift of Alison D. O'Brian, Uniformed Services University of the Health Sciences, Bethesda, MD), as described elsewhere [22]. The crude toxin preparation from bacterial lysates was dialyzed extensively against 50 m*M* Tris-HCl (pH 8), subjected to CL-6B DEAE-sepharose (Pharmacia) anion-exchange chromatography with the same buffer, and eluted with a 0–0.5 *M* NaCl gradient in the same buffer. Fractions were pooled on the basis of their cytotoxicity to Vero cells and dialyzed against PBS (pH 7.4). Crude toxin was then concentrated and subjected to immunoaffinity chromatography with a monoclonal antibody to the B-subunit of Stx-1 (ATCC 13C4) linked to an AminoLink column (Pierce). Eluted samples were tested for cytotoxicity on Vero cells, and protein was directly visualized by electrophoresis on 15% native and denaturing polyacrylamide gels. Cytotoxic fractions were combined, concentrated, and dialyzed against PBS (pH 7.4). Lipopolysaccharide contamination was determined to be minimal by use of an E-Toxate assay (Sigma). Toxin used for all cell-culture experiments was purified a second time over the immunoaffinity column, concentrated, and dialyzed against PBS. Toxin concentration was based on spectrophotometric optical density and Bradford protein assay.

Cytotoxicity. Cells grown in 96-well plates were analyzed for neutral red stain uptake, as described elsewhere [22], after 24 h of exposure to varying concentrations of Stx-1. Cells were incubated in 50 μg/mL neutral red in medium 199 with 5% fetal bovine serum for 3 h at 37°C and rinsed in 1% formaldehyde and 1% $CaCl_2$, followed by the addition of 50% ethanol and 1% acetic acid. Absorbance was determined at 450 nm.

Leucine incorporation. ^{3}H incorporation was determined, as described elsewhere [22], after 4 h of exposure to varying concentrations of Stx-1. Cells grown in 24-well plates were incubated with 1 μCi/mL [^{3}H]leucine for 20 min at room temperature, rinsed, and solubilized in 0.1% SDS. Protein was precipitated with 10% tricarboxylic acid and collected on GF/C filters (Whatman), and counts per minute were determined.

Gb3 content. Gb3 content was determined as described elsewhere [22]. Cells were extracted in chloroform-methanol-water and separated on high-performance thin-layer chromatography silica plates (Mallinckrodt Baker). The plates were dried, immersed in 0.5% polyisobutylmethacrylate in acetone, and sequentially incubated with Stx-1, anti–Stx-1 monoclonal antibody (purified from a hybridoma cell line, 13C4), and ^{125}I-labeled goat anti–mouse IgG (DuPont NEN). Gb3 concentrations were calculated by densitometry and standardized to total protein. Before centrifugation, a cell aliquot was solubilized in 0.1 *N* NaOH and mixed with Bradford reagent (Bio-Rad), and protein concentration was determined by measuring absorbance at 590 nm.

Stx-1 binding. Cells grown in 96-well plates were used for ^{125}I-labeled Stx-1–binding assays, as described elsewhere [22]. ^{125}I-labeled Stx-1 (17,000 cpm; Stx-1 iodinated according to the Iodobead manufacturer's protocol [Pierce]) in 100 μL of medium 199 containing 5% fetal bovine serum and 25 m*M* HEPES plus varying concentrations of unlabeled Stx-1 was added, and cells were incubated 24 h at 4°C. Cells were rinsed with ice-cold Hanks' balanced saline solution and solubilized in 0.1 *N* NaOH, and counts per minute were determined.

GalT6 activity. GalT6 activity was determined as described elsewhere [22]. In brief, cells were homogenized in 500 μL of 50 m*M* morpholinoethanesulfonic acid (MES; pH 6.5). Dried LacCer (Matreya; 25 nmol) was added to sodium cholate in water (250 μg) and dried under vacuum, and the dried mixture was incubated for 60 min at 4°C. A total volume of 100 μL of 50 m*M* MES (pH 6.5) containing 10 m*M* $MnCl_2$, 100 μM 5′-adenylimidodiphosphate, 250 μM cold UDP-galactose, 44 μM UDP-[^{14}C]galactose (Amersham Biosciences; 150,000–400,000 cpm), and 125 μg of total cellular protein was added to the dried LacCer–sodium cholate; the samples were vortexed and incubated at 37°C for 1 h, and the reaction was stopped by addition of 1 mL of 2:1 chloroform-methanol. A Folch partition was established by addition of 200 μL of 0.1*M* KCl, and the upper phase was reextracted by addition of 500 μL of 2:1 chloroform-methanol and the lower phase by addition of 500 μL of 1:1 methanol–0.1 *M* KCl. The lower phases (containing the neutral lipids) were combined, dried under vacuum, and subjected to chromatography as described for Gb3.

GalT2 activity. Cells were sonicated in 500 μL of 50 m*M* HEPES (pH 6.8). Protein content was determined using the Bradford assay. Dried GlcCer (Matreya) was vortexed with 2.5 μL of 10% Triton X-100 and incubated at room temperature for 30 min. A total volume of 100 μL of 50 m*M* HEPES (pH 6.8) containing 5

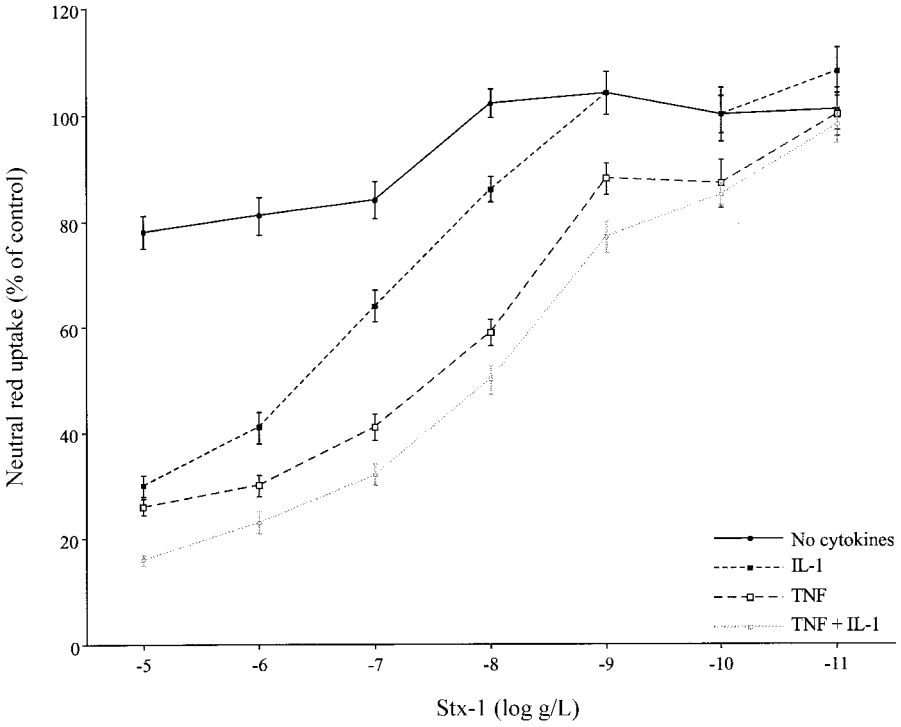

Figure 1. Cytotoxic effect of Shiga toxin 1 (Stx-1; 24-h exposure) on human brain microvascular endothelial cells (HBECs), as determined by neutral red stain uptake. HBECs were preincubated with medium alone or with medium containing 100 U/mL tumor necrosis factor (TNF) and 100 U/mL interleukin (IL)–1 for 24 h before addition of Stx-1 (n = 12 for each data point).

m*M* $MgCl_2$, 5 m*M* $MnCl_2$, 55.7 μM UDP-galactose, 44 μM UDP-[^{14}C]galactose (150,000–400,000 cpm), and 150 μg of cell sonicate was added to the GlcCer (final concentration, 100 n*M*)–detergent mixture, vortexed, and incubated at 37°C for 1.5 h. A Folch partition was established, and the neutral lipids were subjected to chromatography as described for GalT6.

GlcCer synthase activity. Cells were sonicated in 200–500 μL of 50 m*M* HEPES (pH 7.4). Protein content was determined using the Bradford assay. Dried ceramide (Matreya) was vortexed with 2.5 μL of 10% Triton X-100 and incubated at room temperature for 30 min. A total volume of 100 μL of 50 m*M* HEPES (pH 7.4) containing 5 m*M* $MgCl_2$, 5 m*M* $MnCl_2$, 55.7 μM UDP-glucose, 44 μM UDP-[^{14}C]glucose (Amersham; 150,000–400,000 cpm), and 150 μg of cell sonicate was added to the ceramide (final concentration, 100 n*M*)–detergent mixture, vortexed, and incubated at 37°C for 1.5 h. A Folch partition was established, and the neutral lipids were subjected to chromatography as described for GalT6.

α-Galactosidase activity. α-Galactosidase activity was determined as described elsewhere [23]. Cells were suspended in 3 mg/mL sodium taurocholate and 28 m*M* citric acid–44 m*M* Na_2HPO_4 (pH 4.4), sonicated, and centrifuged, and the supernatant was analyzed. For the reaction, supernatant was incubated with 5 m*M* *p*-nitrophenyl-α-galactopyranoside for 10–90 min, followed by addition of 0.2 *M* Na_2CO_3, and absorbance was measured at 400 nm.

Northern blot analysis. Total RNA was isolated from confluent cells, electrophoresed on a 0.9% formaldehyde gel, transferred to a nylon membrane, and prehybridized for ⩾3 h at 60°C in 50% formamide, 5× standard saline citrate, 5× Denhardt's solution, 1% SDS, and 100 μg/mL salmon sperm DNA. Fresh solution was added for hybridization along with radioactively labeled probe. For probes, cDNA was made from human proximal tubule cell total RNA by use of oligo dT mRNA primer and SuperScript II reverse transcriptase (Invitrogen). The cDNA was then used as a template for polymerase chain reaction amplification of the coding region of the gene, using specific primers as follows: for GalT6 (GenBank accession no. AB037883), forward primer 5′-GATCTGGGGATACCATGTCCAAG-3′ and reverse primer 5′-CAGTAGCGGGCATGCAGCTGG-3′, which yields a product size of 1040 bp; for α-galactosidase (GenBank accession no. XM037096), forward primer 5′-GGCTAGAGCACTGGACAATGGA-3′ and reverse primer 5′-CTGCGATGGTATAAGAGCGAGG-3′, which yields a product size of 1021 bp; for GalT2 (GenBank accession no. AF097159), forward primer 5′-AACGGTACAGATTATCCCGAAGG-3′ and reverse primer 5′-TGGAGCTAACTCTGGCATGAGG-3′, which yields a product size of 912 bp; and for ceramide glucosyltransferase (GenBank accession no. D50840), forward primer 5′-GCTGTGGCTGATGCATTTCATGG-3′ and reverse primer 5′-CAGTTCTCCAGCTTATAGTTGGG-3′, which yields a product size of 1070 bp. All products were purified, sequenced, and cloned into pGEM-T cloning vector (Promega). The inserts were sequenced again to ensure cloning fidelity and to confirm orientation. Probes were digested with an appropriate restriction enzyme to give the antisense strand, and riboprobes were made by [^{32}P]UTP incorporation with either T7 or SP6 RNA polymerase (Invitrogen). The radioactive probes were purified over a G-50 column, and specific activity was calculated. The probe was added

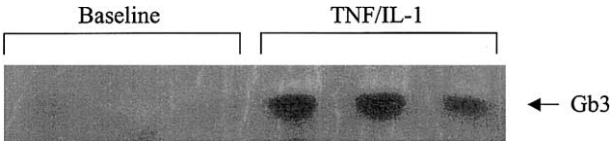

Figure 2. High-performance thin-layer chromatography of galactose-α1,4-galactose-β1,4-glucose-ceramide (globotriaosylceramide; Gb3) content in human brain microvascular endothelial cells (HBECs). HBECs were exposed to medium alone or to medium containing 100 U/mL tumor necrosis factor (TNF) and 100 U/mL interleukin (IL)–1 for 24 h, and then neutral lipids were assayed.

to hybridization solution at 10 ng/mL with a specific activity $\geq 10^9$ dpm/μg and incubated overnight at 60°C. Blots were washed in decreasing concentrations of standard saline citrate and increasing temperature until background was removed. Labeled blots were subjected to autoradiography and densitometry.

mRNA half-life determination. Confluent cells in 6-well plates were treated with 10 μg/mL actinomycin D for 0–24 h, followed by determination of CGT, GalT2, and GalT6 mRNA levels, as described for Northern blot analysis.

Statistical analysis. Data were analyzed by 1-way analysis of variance with the Bonferroni correction. When only 2 data points were compared, the unpaired Student's *t* test was used. $P < .05$ was considered to be significant. Data are expressed as mean ± SE.

Results

Our first set of experiments was designed to confirm that the inflammatory cytokines TNF and IL-1 increased sensitivity of HBECs to Stx-1. As shown in figure 1, HBECs were not substantially killed by Stx-1 (24-h exposure) at concentrations $\leq 10^{-5}$ g/L (140 p*M*). Preincubation with TNF (100 U/mL or 3.5 ng/mL) and IL-1 (100 U/mL or 0.75 ng/mL) for 24 h greatly increased Stx-1 cytotoxicity in HBECs (LD_{50}, $\sim 10^{-8}$ g/L, or 140 f*M*). Dose-response analyses for TNF and IL-1, from 1 to 100 U/mL for each cytokine alone and for both together, also were done (data not shown). TNF and IL-1 separately (100 U/mL) increased Stx-1 cytotoxicity, but to a lesser degree than the cytokines together (figure 1). Lower concentrations of the cytokines increased Stx-1 cytotoxicity; the effect was evident at 10 but not 1 U/mL. Hence, both TNF and IL-1 increase sensitivity of HBECs to Stx-1, although the effect of the combined cytokines is greater than the effect of either cytokine alone. Similar results were obtained for inhibition of protein synthesis. TNF in combination with IL-1 (100 U/mL for 24 h) greatly increased the inhibitory effect of Stx-1 on protein synthesis in HBECs. With regard to cytotoxicity, the cytokines also inhibited protein synthesis when given separately, but the effect was greater when they were given in combination.

To demonstrate that cytokine up-regulation of Stx-1 cytotoxicity and inhibition of protein synthesis was associated with increased Stx-1 receptor expression, HBECs Gb3 content and ^{125}I-labeled Stx-1 binding were ascertained. TNF (100 U/mL for 24 h) increased ^{125}I-labeled Stx-1 binding by 661% ± 81% (mean ± SD; $n = 8$; $P < .025$, vs. unstimulated) and Gb3 content by 189% ± 13% ($n = 3$; $P < .05$, vs. unstimulated). IL-1 (100 U/mL for 24 h) increased ^{125}I-labeled Stx-1 binding by 507% ± 35% ($n = 8$; $P < .005$, vs. unstimulated) and Gb3 content by 321% ± 65% ($n = 3$; $P < .025$, vs. unstimulated). TNF and IL-1 together (100 U/mL for 24 h) had a substantially greater effect than when given separately: ^{125}I-labeled Stx-1 binding increased by 1565% ± 89%, and Gb3 content increased by 1409% ± 409% (figures 2 and 3). These results indicate that up-regulation of Stx-1 sensitivity by TNF and IL-1 is clearly associated with a marked increase in Gb3 expression.

The next set of experiments investigated the mechanisms responsible for cytokine-stimulated Gb3 expression in HBECs. If levels of both TNF and IL-1 are increased (at least at the local cellular level) in patients with HUS, then it seems most relevant to examine the combined effect of these cytokines on Gb3 metabolism in HBECs. Each of the 3 enzymes involved in Gb3 synthesis (CGT, GalT2, and GalT6), as well as the enzyme involved in Gb3 degradation (α-galactosidase), was evaluated. The combination of TNF and IL-1 (100 U/mL for 24 h) increased CGT, GalT2, and GalT6 enzyme activity (figures 4 and 5). In contrast, α-galactosidase activity was not changed by cytokine treatment (figure 5). A similar pattern was observed with enzyme steady-state mRNA levels. TNF in combination with IL-1 (100 U/mL for 24 h) augmented CGT, GalT2, and GalT6 mRNA content (figures 6 and 7). Note that the Northern blots (figure 6) show rather faint bands for GalT2

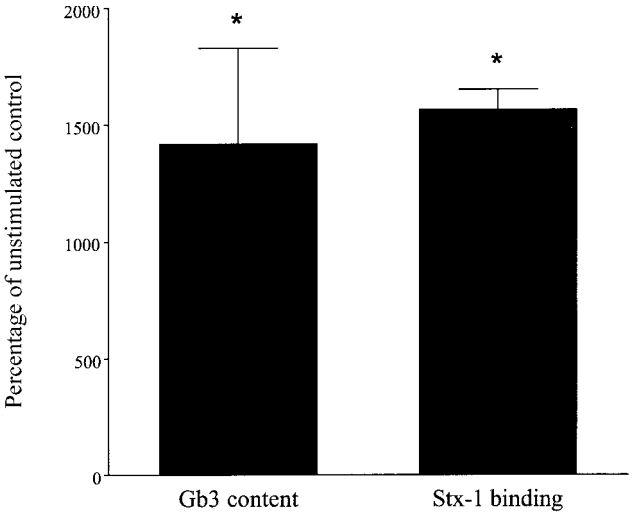

Figure 3. Total cell galactose-α1,4-galactose-β1,4-glucose-ceramide (globotriaosylceramide; Gb3) content in and ^{125}I-labeled Shiga toxin 1 (Stx-1) binding (24-h exposure at 4°C) to human brain microvascular endothelial cells (HBECs). HBECs were pretreated with medium alone or with medium containing 100 U/mL tumor necrosis factor and 100 U/mL interleukin-1 for 24 h, and then Gb3 content ($n = 4$) or ^{125}I-labeled Stx-1 binding ($n = 6$) was determined. *$P < .001$, vs. medium alone.

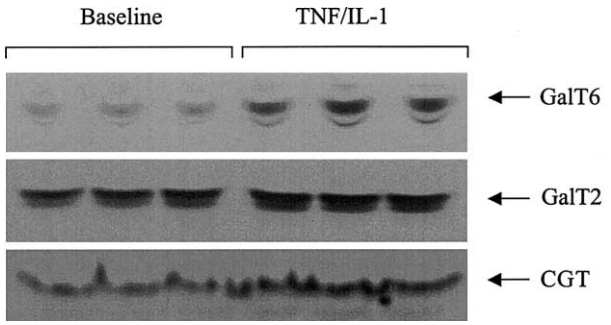

Figure 4. High-performance thin-layer chromatography of galactose-α1,4-galactose-β1,4-glucose-ceramide (globotriaosylceramide; Gb3) synthase (GalT6), lactosylceramide synthase (GalT2), and ceramide glucosyltransferase (CGT) activities in human brain microvascular endothelial cells (HBECs). HBECs were preincubated with medium alone or with medium containing 100 U/mL tumor necrosis factor (TNF) and 100 U/mL interleukin (IL)–1 for 24 h, and then enzyme activity was determined. GalT6 bands are Gb3, GalT2 bands are lactosylceramide, and CGT bands are glucosylceramide. Standards confirming glycosphingolipid location were run on the same gels (not shown).

and CGT; mRNA for these enzymes was quite difficult to detect, even by means of polymerase chain reaction using a variety of primer combinations. The Northern blot analysis with use of riboprobes yielded the best results, although far from the intensity of the GalT6 message. Finally, α-galactosidase mRNA levels were not affected by the inflammatory cytokines (figure 7).

To assess whether TNF and IL-1 (100 U/mL for 24 h) induced alterations in mRNA stability, mRNA half-lives were determined for CGT, GalT2, and GalT6 in HBECs. The half-life for CGT mRNA was ~6 h; this did not change with cytokine treatment. The half-life for GalT2 mRNA was ~7 h; this also was not affected by cytokines. GalT6 mRNA half-life was ~8 h; again, cytokines had no effect. Consequently, these data suggest that TNF and IL-1 up-regulation of CGT, GalT2, and GalT6 mRNA levels is most likely related to increased gene transcription.

Discussion

CNS involvement in patients with HUS can have serious and even fatal consequences. Such CNS injury typically involves, at least on a histologic level, microvascular brain endothelial cells. As mentioned earlier, previous studies have reported that, in contrast to the histologic findings of CNS endothelial cell injury in HUS, HBECs were highly resistant to the cytotoxic effects of Stx [11, 12]. The present study found that HBECs were virtually insensitive to Stx-1 cytotoxicity and inhibition of protein synthesis and that these cells had relatively little Gb3 expression and Stx-1 binding. Importantly, we noted that TNF and IL-1 greatly enhanced Stx-1 cytotoxicity and inhibition of protein synthesis in association with parallel increases in Gb3 expression and Stx-1 binding. Thus, taken together with previous findings of augmentation by inflammatory cytokines of endothelial cell responsiveness to Stx [11, 12], the results of the present study suggest that HBEC injury may critically depend on TNF and/or IL-1 modulation of the activity of Stx-1.

We noted that IL-1 and TNF each increased HBEC Gb3 expression and Stx-1 binding and that these increases were comparable. The cytokines together, however, markedly augmented HBEC Gb3 expression and Stx-1 binding (by ~15-fold). Whether such a combined cytokine effect occurs in patients with HUS is unknown; however, data suggest that Stx can stimulate TNF and IL-1 production by a variety of cell types. Stx increases release of both TNF and IL-1 by murine macrophages [16], nonadherent human monocytes [17], human proximal tubule cells [14], and human glomerular epithelial cells [15]. Furthermore, lipopolysaccharide, which is likely to be present in the serum of patients with HUS, also stimulates both TNF and IL-1 production by a variety of cell types [24]. Consequently, it seemed most relevant to the pathogenesis of brain endothelial cell injury in patients with HUS to examine the combined effect of TNF and IL-1 on HBEC Gb3 expression.

The combination of TNF and IL-1 increased CGT activity and steady-state mRNA levels in HBECs. Taken together with the finding that cytokine treatment did not alter CGT mRNA half-life, these data suggest that TNF and IL-1 in combination increase CGT gene transcription. Although no data exist on

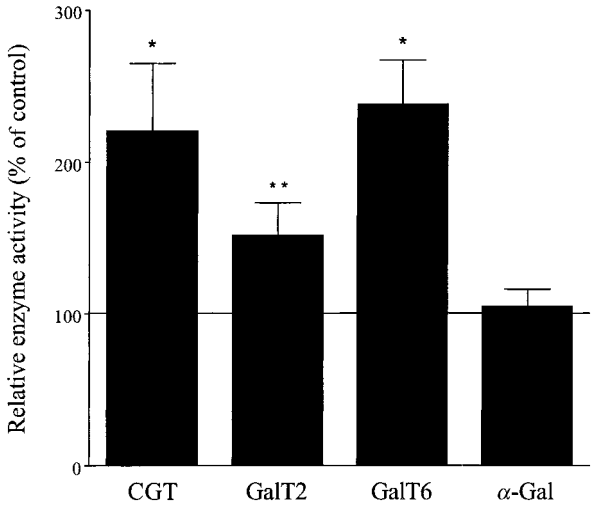

Figure 5. Relative galactose-α1,4-galactose-β1,4-glucose-ceramide (globotriaosylceramide) synthase (GalT6), lactosylceramide synthase (GalT2), ceramide glucosyltransferase (CGT), and α-galactosidase (α-Gal) enzyme activities in human brain microvascular endothelial cells (HBECs). HBECs were preincubated with medium alone or with medium containing 100 U/mL tumor necrosis factor and 100 U/mL interleukin-1 for 24 h, and then enzyme activity was determined. Ordinate values are expressed as percentage of unstimulated enzyme activity ($n = 3$ for each data point). *$P < .005$, vs. unstimulated control; **$P < .025$, vs. unstimulated control.

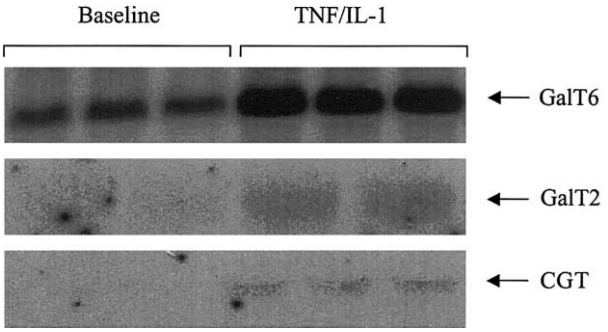

Figure 6. Northern blots of galactose-α1,4-galactose-β1,4-glucose-ceramide (globotriaosylceramide; Gb3) synthase (GalT6), lactosylceramide synthase (GalT2), and ceramide glucosyltransferase (CGT) mRNA levels in human brain microvascular endothelial cells (HBECs). HBECs were preincubated with medium alone or with medium containing 100 U/mL tumor necrosis factor (TNF) and 100 U/mL interleukin (IL)–1 for 24 h, and then mRNA content was determined. All products are predicted size.

the regulation of CGT in the brain, there is precedent for cytokine and lipopolysaccharide modulation of this enzyme. Both TNF and IL-1 have been reported to increase CGT mRNA levels in hamster liver in vivo and in HepG2 cells (a human hepatoma line), whereas lipopolysaccharide increased CGT activity and mRNA content in HepG2 cells and liver [25]. Another report noted a biphasic response to TNF in KYM-1 cells (a human rhabdomyosarcoma cell line), in which CGT activity first decreased and then increased [26]. In this latter study, ceramide levels were elevated during both decreased and increased CGT activity; however, interpretation of these findings is complicated, because the cells were undergoing apoptosis. Others have noted that CGT activity is elevated by treatments (including inflammatory cytokines) that increase intracellular ceramide levels [27]. Notably, on computer analysis, the human CGT promoter contains an NF-κB–recognition site (765 bp proximal to the transcriptional start site). Because elevation of intracellular ceramide is associated with activation of NF-κB [27], this suggests a possible mechanism of cytokine regulation of CGT gene transcription. In addition, the human CGT promoter contains AP-1 sites, although far upstream (closest site, approximately -1100). Ultimately, which sites in the CGT promoter are responsive to cytokine stimulation remains to be determined.

The combination of TNF and IL-1 also appeared to increase GalT2 gene transcription: combined cytokine treatment increased HBEC GalT2 activity and steady-state mRNA levels but did not change GalT2 mRNA half-life. Very few data are available on potential cytokine regulation of GalT2. One study noted that treatment with TNF increased GalT2 activity in human umbilical vein endothelial cells [28]. This study did not, however, assess TNF effects on GalT2 mRNA. Computer analysis shows that the human GalT2 promoter contains several transcription-factor DNA-binding domains known to be involved in inflammatory cytokine signaling, although these are relatively far upstream of the transcriptional start site. There is an NF-κB–binding site at position -1050, an AP-1 site at position -880, and an Sp1 site at -780 (although the latter site has been implicated in TNF-mediated down-regulation of gene transcription [29]). Notably, TNF treatment of human umbilical vein endothelial cells increased NF-κB–binding activity in association with increased intracellular LacCer concentrations [28]. With regard to CGT, the GalT2 promoter domains that confer functionality are not yet clarified.

GalT6 activity and steady-state mRNA levels were also elevated by the combination of TNF and IL-1. As for CGT and GalT2, there was no effect of cytokines on GalT6 mRNA half-life, which implies that gene transcription may have a role. Notably, the increased GalT6 activity occurred in the setting of no change in α-galactosidase activity or mRNA levels, which indicates that a net increase in the driving force for Gb3 accumulation occurred. The GalT6 gene was recently cloned [18]; its regulation is largely unknown. TNF can induce GalT6 enzyme activity in human umbilical vein endothelial cells [30], although the cytokine's effect on GalT6 mRNA levels has not been assessed. The GalT6 promoter contains an AP-1 domain 160 bp proximal to the transcription start site; no NF-κB or other transcription factor–binding sites known to be involved in TNF or IL-1 signaling were identified on computer analysis.

In summary, this study demonstrates that inflammatory cytokines can markedly increase Gb3 expression by HBECs via

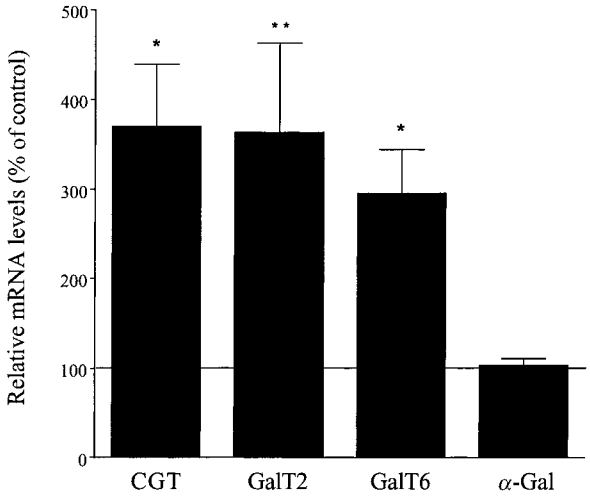

Figure 7. Relative galactose-α1,4-galactose-β1,4-glucose-ceramide (globotriaosylceramide) synthase (GalT6), lactosylceramide synthase (GalT2), ceramide glucosyltransferase (CGT), and α-galactosidase (α-Gal) mRNA levels in human brain microvascular endothelial cells (HBECs). HBECs were preincubated with medium alone or with medium containing 100 U/mL tumor necrosis factor and 100 U/mL interleukin-1 for 24 h, and then mRNA content was determined. Ordinate values are expressed as percentage of unstimulated mRNA levels ($n = 4$ for each data point). *$P < .005$, vs. unstimulated control; **$P < .025$, vs. unstimulated control.

increased activity of all of the enzymes involved in Gb3 glycosphingolipid precursor synthesis. To our knowledge, this is the first study demonstrating the coordinated and simultaneous upregulation of CGT, GalT2, and GalT6. Such a finding suggests that Gb3, and possibly its metabolites, could play an important and as-yet-unidentified role in mediating the biologic effects of inflammatory cytokines. In addition, these studies suggest that agents designed to block Gb3 accumulation, possibly inhibiting activity of any of the 3 enzymes, may be of benefit in ameliorating or preventing brain endothelial cell injury in patients with HUS.

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