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

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The epidemic form of the hemolytic uremic syndrome (HUS), beginning with an acute gastroenteritis, has been associated with a verocytotoxin-producing *Escherichia coli* infection. The endothelial cell is believed to play an important role in the pathogenesis of HUS. Endothelial cell damage by verocytotoxin-1 (VT-1) in vitro is potentiated by the additional exposure of inflammatory mediators, such as tumor necrosis factor-α (TNF-α). Preincubation of human umbilical vein endothelial cells (HUVEC) with TNF-α resulted in a 10- to 100-fold increase of specific binding sites for 125I-VT-1. Furthermore, interleukin-1 (IL-1), lymphotoxin (TNF-β), and lipo polysaccharide (LPS) also markedly increase VT-1 binding. Several hours' exposure to TNF-α was enough to enhance the number of VT-1 receptors on the endothelial cells for 2 days. The TNF-α-induced increase in VT-1 binding could be inhibited by simultaneous addition of the protein synthesis inhibitor cycloheximide. Glycolipid extracts of TNF-α-treated cells tested on thin-layer chromatography demonstrated an increase of globotriaosylceramide (GbO₃sCer), a functional receptor for VT-1, which suggests that preincubation of human endothelial cells with TNF-α leads to an increase in GbO₃sCer synthesis in these cells. We conclude from this study that TNF-α and IL-1 induce one (or more) enzyme(s) that is (are) rate-limiting in the synthesis of the glycolipid VT-1 receptor, GbO₃sCer. These in vitro studies suggest that, in addition to VT-1, inflammatory mediators play an important role in the pathogenesis of HUS.

**MATERIALS AND METHODS**

Purified VT-1 was prepared in the laboratory of Dr Karmali (1.2 mg protein/mL; CD₂₋ᵥ vero-cells; titer 10⁻⁸ to 10⁻⁹). Endotoxin content of the VT-1 preparation was less than 0.05 EU/mL by Limulus amoebocyte lysate assay (E-Toxic, Sigma Chemicals, St Louis, MO) at detection level of 0.05 to 0.10 EU/mL. Monoclonal antibody PHI against VT-1 was a generous gift of Dr C. Lingwood (Department of Microbiology, Hospital for Sick Children, Toronto, Canada). M199 medium supplemented with 20 mmol/L HEPES was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics were from Costar (Cambridge, MA). A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al. Human serum was obtained from the local blood bank and was prepared from fresh blood of healthy donors, pooled, and stored at 4°C; it was not heat-inactivated before use. Newborn calf serum (NBCS) and fetal calf serum (FCS) were from GIBCO (Grand Island, NY) and Boehringer Mannheim (Mannheim, Germany), respectively; they were heat-inactivated before use (at 56°C for 30 minutes). Heparin was purchased from Leo Pharmaceuticals (Weesp, The Netherlands). Penicillin/streptomycin was from Boehringer Mannheim (Mannheim, Germany). Human fibronectin was a gift of J.A. van Mourik, Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Pyrogen-free human serum albumin (HSA) was purchased from the Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). α-Thrombin was obtained from Sigma Chemicals.
Human recombinant TNF-α and lymphotxin (TNF-β) were gifts from Jan Tavernier (Biogent, Gent, Belgium). The TNF-α preparation contained 2.45 × 10^7 U/mg protein and less than 40 ng lipopolysaccharide (LPS) per mg protein; the specific activity of TNF-β was 1.6 × 10^7 U/mg protein. Human recombinant IL-6 (expressed in yeast cells) was prepared in the Laboratory of Molecular Biology, State University, Gent, Belgium; it was biologically active and had a specific activity of 1.4 × 10^7 U/mg protein as tested on 7TD1 cells. Human recombinant IL-1α and IL-1β were gifts of S. Gillis (Immunex, Seattle, WA); they had a specific activity of 10^8 U/mg. LPS of E. coli serotype O128-B12 was obtained from Sigma Chemicals. ^35S-methionine and Na^125I-iiodine were purchased from Amersham (Amersham, UK). Iodo-gen iodination reagent was obtained from Pierce (Rockford, IL). Anti-Tp se- rum, ^18I serum that contains antibodies against P^k (GbO^3cer), P (globotetraosylceramide [GbO^4cer]), and P1, blood group anti- gens, was obtained from DiaMed (Morat, Murten, Switzerland). Choloroforom, methanol, and hexane was obtained from Merck (Darmstadt, Germany). Plastic-coated silica gel F1500 thin-layer chromatography (TLC) plates were from Schleicher and Schuell (Dassel, Germany). Polysobutylmethacrylate was obtained from Polysciences (Washington, MD). A standard mixture of pure neutral glycolipids containing Ga^l+1-Ceramide(Cer)(CMH), Ga^l+1-4Glc^l+1-Cer( CDH), Ga^l+1-4Gal^l+1-Glc^l+1-Cer(GbO^5cer), Ga^l-4GalNa^l+1-3Gal^l-4Gal^l+1-Glc^l+1-Cer(GbO^6cer), and Ga^lNa^l-3Gal^l-4Glc^l+1-4Glc^l+1-Cer(Forsman pentasaccharide) was from BioCarb (Lund, Sweden). X-O MAT x-ray film was from Eastman Kodak (Rochester, NY).

**Methods**

**Cell culture.** Endothelial cells from human umbilical vein (HUVEC) and from human femoral vein were isolated by collagenase treatment, cultured, and characterized as previously described. The endothelial cells were seeded in fibronectin-coated 10-cm^2 wells and cultured in M199 medium supplemented with 20 mmol/L HEPES (pH 7.4), 10% human serum (HS), 10% NBCS, L-glutamine, 5 U/mL heparin, and 150 µg/mL crude preparation of gamma-interferon (IFN-γ), was obtained from Sigma Chemicals. The endothelial cell cultures in 24-well plates were incubated for 3 hours with 0.3 or 1.0 nmol/L 125I-VT-1 in M199 medium plus 0.1% HSA, and total cell protein was solubilized in 400 µL 1 mol/L sodium hydroxide at room temperature. Radioactivity of the endothelial cells was measured in a gamma-counter. Nonspecific binding was determined by assay of 125I-VT-1 binding in the presence of a 100-fold excess of unlabeled VT-1. Cellular specific binding was determined by subtracting the nonspecific binding from the cellular binding of 125I-VT-1 determined in the absence of unlabeled VT-1. To study the effect of anti-Tp serum (blocking of the binding of VT-1 to GbO^3cer), anti-Tp serum in M199 medium plus 0.1% HSA was added 2 hours before and during the binding assay with 125I-VT-1.

**Extraction of glycolipids.** Confluent HUVEC in 162-cm^2 flasks were incubated for 24 hours with or without TNF-α (500 U/mL). Subsequently, the glycolipids were extracted as described by Lingwood et al; in short, the cells were trypsinized, harvested with iced-cold phosphate-buffered saline (PBS), and spun down by 3-minute centrifugation (3,000 rpm) at 4°C. The pellet was washed three times with PBS. The pellet was finally resuspended in PBS, 20 µl of chloroform/methanol (2:1, vol/vol) was added. Cell debris was removed by filtration through glass-wool. One volume of water was added and partitioned. The lower phase was dried and centrifuged at 37°C for 2 hours in 0.4 mol/L KOH in ethanol; 2 vol (vol/vol) of chloroform was added and the mixture was partitioned against 2 vol of water. The lower phase was separated and frozen at −20°C until TLC studies were performed.

**TLC.** The lower phase from the extraction above was dried and resuspended in chloroform/methanol (2:1). Samples were separated on a silica gel TLC plate using chloroform/methanol/water (65:25:4). After separation, the plate was soaked three times for 1 minute in 0.01% polysbutylmethacrylate in hexane and air-dried, followed by overnight incubation in PBS supplemented with 1% BSA and 0.05% Tween 20. Subsequently, the plate was incubated with 50 µl VT-1 solution (15 nmol/L, unlabeled and 1.5 nmol/L 125I-VT-1 in 1% BSA and 0.05% Tween in PBS) for 4 hours at 4°C. The plate was extensively washed with 0.05% Tween 20 and 1% BSA in PBS, air-dried, and exposed overnight to X-O MAT x-ray film.

**RESULTS**

**Cytotoxicity of VT-1 Toward Human Endothelial Cells**

VT-1 caused a decrease in cell viability of endothelial cells when they had been preincubated with the inflammatory mediator TNF-α (Fig 1A). VT-1 had no significant effect on the cell viability of untreated cells. Only in two of seven independent cell cultures of HUVEC did 24 hours' incubation with 17 nmol/L VT-1 alone cause a small degree of cell detachment (21% and 24%). Even upon prolonged incubation with 17 nmol/L VT-1 alone (72 hours), we found a moderate cell detachment (32% ± 17%, mean ± SD) in
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**Figure 1.** Toxicity of VT-1 for confluent HUVEC preincubated for 24 hours without or with 500 U/mL TNF-α. (A) Number of viable cells after an 8- and 24-hour incubation period with VT-1. After a preincubation period of 24 hours without (open symbols) or with (closed symbols) TNF-α, cells were incubated without VT-1 (circles), with 17 pmol/L VT-1 (triangles), or with 17 nmol/L VT-1 (squares) for 8 and 24 hours, and the number of viable cells was counted in a hemocytometer. (B) Incorporation of 35S-methionine in 35S-proteins was determined by trichloroacetic acid precipitation after an 8- or 24-hour incubation period with the various concentrations of VT-1 given above.

![Graph A](image1.png)

![Graph B](image2.png)

Fig 1. Toxicity of VT-1 for confluent HUVEC preincubated for 24 hours without or with 500 U/mL TNF-α. (A) Number of viable cells after an 8- and 24-hour incubation period with VT-1. After a preincubation period of 24 hours without (open symbols) or with (closed symbols) TNF-α, cells were incubated without VT-1 (circles), with 17 pmol/L VT-1 (triangles), or with 17 nmol/L VT-1 (squares) for 8 and 24 hours, and the number of viable cells was counted in a hemocytometer. (B) Incorporation of 35S-methionine in 35S-proteins was determined by trichloroacetic acid precipitation after an 8- or 24-hour incubation period with the various concentrations of VT-1 given above.

only four of six independent cell cultures. Under all conditions, no cytotoxicity was found when VT-1 was inactivated by heat treatment before addition or when VT-1 was preincubated with monoclonal antibodies to VT-1. Preincubation of the cells with thrombin (0.1 to 1 U/mL) did not change the sensitivity of endothelial cells for VT-1 (three independent experiments; not shown). Similar results were obtained when endothelial cells from adult human vein (vena femoralis) were used (Fig 2A).

Verocytotoxin may inhibit eukaryotic protein synthesis by depurination of a single adenine residue from the 28S RNA component of the ribosome, which may result in the inhibition of the interaction of elongation factor-1 with the ribosome. Cellular protein synthesis was measured by assaying the incorporation of 35S-methionine into total cellular protein. Incubation of the cells with VT-1 alone did not affect the protein synthesis over a 24-hour period (Fig 1B). However, when the cells were preincubated for 24 hours with TNF-α, the protein synthesis rate was decreased dramatically by VT-1, whereas the protein synthesis was not decreased by TNF-α alone. As shown in Fig 2B, the observed decrease depended on the concentrations of both VT-1 and TNF-α.

**Binding of VT-1 to Human Endothelial Cells**

To evaluate whether the enhanced VT-1 sensitivity of endothelial cells caused by TNF-α was due to an increase in VT-1 receptors on the cell surface, binding experiments with 125I-VT-1 were performed. TNF-α induced an increase in VT-1 binding after a 6- to 8-hour lag period; this increase continued for up to 48 hours (Fig 3). The increase was due to an increase in specific binding of 125I-VT-1, as nearly all 125I-VT-1 binding could be displaced by an excess of unlabeled VT-1. No change in VT-1 binding was observed in nontreated cells (Fig 3). In six independent experiments with confluent HUVEC of different donors, 24-hour incubation with 500 U/mL TNF-α resulted in a 38-fold increase in specific VT-1 binding (range, 13- to 90-fold).

Subconfluent HUVEC displayed a higher basal VT-1 binding than highly confluent cells, as is shown in Fig 4 for a representative experiment. When the same cells were incubated for 18 hours with TNF-α, the binding of VT-1 increased for all cells, but the relative increase was larger in highly confluent HUVEC. In further experiments we have used highly confluent cells.

The effect of TNF-α on 125I-VT-1 binding was concentration-dependent (Fig 5A). The binding saturated at approximately 10 nmol/L VT-1 (Fig 5B). From analysis of Scatchard plots of the binding curves (Fig 5C, Table 1), it was concluded that the number of specific VT-1 binding sites increased by one or two orders of magnitude after 24 hours of incubation with TNF-α, whereas the apparent affinity of VT-1 binding to HUVEC did not significantly change (Table 1). Similarly, in human femoral vein endothelial cells, the number of VT-1 binding sites increased 14-fold after a 24-hour incubation period with 100 U/mL TNF-α from 2 × 10^5 VT-1 binding sites/cell in control cells to 29 × 10^5 VT-1 binding sites in TNF-α-treated cells.

In addition to TNF-α, IL-1α, IL-1β, TNF-β, and bacterial LPS induced a more than 10-fold increase in specific VT-1 binding to HUVEC (Fig 6). Thrombin induced only a threefold increase, whereas IL-6 (5 or 50 U/mL) did not...
Fig 2. Toxicity of VT-1 for confluent human femoral vein endothelial cells preincubated with 500 U/mL TNF-α for 24 hours. (A) After a preincubation period of 24 hours without (open symbols) or with (closed symbols) TNF-α, cells were incubated without VT-1 (circles), with 17 pmol/L VT-1 (triangles), or with 17 nmol/L VT-1 (squares) for 8 and 24 hours, and the number of viable cells was counted. (B) Incorporation of \(^{35}\text{S}\)-methionine in \(^{35}\text{S}\)-proteins. Cells were preincubated for 24 hours without TNF-α (double-hatched bars), with 50 U/mL TNF-α (hatched bars), or with 500 U/mL TNF-α (black bars). After preincubation, cells were incubated for 24 hours with 17 pmol/L, 170 pmol/L, or 17 nmol/L VT-1 or without VT-1 (control).

significantly alter VT-1 binding. The increase induced by 10 μg/mL LPS was twofold less than that induced by 500 U/mL TNF-α. LPS increased VT-1 binding in a concentration-dependent manner (Fig 7). As can be seen in Fig 7, 24 hours’ incubation with small amounts of TNF-α and LPS resulted in an additional increase in the binding of VT-1 to HUVEC.

As the half-life of TNF-α in plasma is relatively short (several minutes),\(^{25}\) we have evaluated how long after exposure of HUVEC to TNF-α an effect on the VT-1 binding can be established. To that end, HUVEC were incubated for various time periods with 20 U/mL TNF-α, vigorously washed, and subsequently incubated for another

Fig 3. Effect of TNF-α pretreatment on the binding of VT-1 to confluent HUVEC. After preincubation of the cells with 500 U/mL TNF-α (triangles) at 37°C for the indicated time, the cells were washed and binding of 0.3 nmol/L \(^{125}\text{I}\)-VT-1 was assayed at 0°C in the presence (open symbols) or absence (closed symbols) of 30 nmol/L unlabeled VT-1. Binding of VT-1 to control cells that were preincubated without TNF-α is indicated by squares.

Fig 4. Effect of cell density on the specific binding of VT-1 to HUVEC. During the assay of VT-1 binding, subconfluent cells had a 70% to 80% cell density of that of confluent cells. Highly confluent cells were maintained for 3 days at confluent density before the start of the experiment. Before assay of the binding of 0.3 nmol/L \(^{125}\text{I}\)-VT-1, the cells were preincubated with 500 U/mL TNF-α (□) or without it (■). Nonspecific binding (determined in the presence of 30 nmol/L unlabeled VT-1) was 5% or less in all conditions.
period without TNF-α, after which the VT-1 binding was assayed. The results of two independent experiments are given in Table 2. Six hours of incubation with TNF-α was enough to detect an increase in VT-1 binding after a 24- or 48-hour incubation. This suggests that the increased sensitivity of endothelial cells for VT-1 can last for at least 1 day after the disappearance of inflammatory mediators.

The Nature of TNF-α–Induced VT-1 Binding to Endothelial Cells

GbOse₃cer has been identified as a receptor for VT-1 in various cells. To evaluate whether the increase in specific VT-1 binding was due to an increase in the exposure of GbOse₃cer to the cell surface, we incubated HUVEC with anti-Tja serum (which contains antibodies against the Pk, P [GbOse₃cer], and P₁ blood group antigens). The addition of anti-Tja serum inhibited the binding of VT-1 in a concentration-dependent manner (Fig 8). This finding was confirmed in three other experiments of HUVEC, as well as in femoral vein endothelial cells. Seven different human serum preparations from healthy donors did not decrease the binding of VT-1 to the endothelial cells. To ascertain whether the increase in VT-1 binding resulted from an increase in cellular GbOse₃cer synthesis or from a change in the availability of GbOse₃cer at the cell surface, HUVEC were incubated with and without TNF-α, and glycolipid extracts of cells were subsequently prepared. After TLC of these glycolipids, an increase in VT-1 binding at the spot of GbOse₃cer was clearly demonstrated in the TNF-α-treated cells (Fig 9). Occasionally, a second VT-1 binding band was observed, as demonstrated in Fig 9. Because the GbOse₃cer in our standard sample sometimes appeared as two bands after mixture with the cellular extract, it is likely that the second band here is also (derived from) GbOse₃cer. But the involvement of αOH-GbOse₃cer, which has a similar Rf value as the second band and can also bind VT-1, cannot be excluded. When standard glycolipids were run together with glycolipid extracts of the TNF-α-treated cells, VT-1 binding to the GbOse₃cer of the standard mixture coincided precisely with the induced VT-1 binding glycolipids in the TNF-α-treated cells (not shown). These experiments indicate that the number of

Table 1. Binding of VT-1 to HUVEC

<table>
<thead>
<tr>
<th>Cells</th>
<th>Addition</th>
<th>Binding Sites/ Cell (mol/L)</th>
<th>Apparent kd (mol/L)</th>
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<tr>
<td>HUVEC 1</td>
<td>None</td>
<td>0.03 x 10⁶</td>
<td>0.6 x 10⁻⁹</td>
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<td>TNF-α (500 U/mL)</td>
<td>9.4 x 10⁶</td>
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<tr>
<td>HUVEC 2</td>
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<td>0.8 x 10⁶</td>
<td>1.2 x 10⁻⁹</td>
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<td>TNF-α (500 U/mL)</td>
<td>11.3 x 10⁶</td>
<td>1.9 x 10⁻⁹</td>
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Binding of [¹²⁵I]-VT-1 was performed at various concentrations (0.15 to 50.0 nmol/L) and with excess of unlabeled VT-1, in two independent HUVEC cultures. The number of binding sites for the holotoxin VT-1 and the apparent kd were calculated by analysis of Scatchard plots.
GbOse$_3$cer molecules in/on the cells has been markedly increased after preincubation with TNF-α.

Requirement of Protein Synthesis for TNF-α-Induced VT-1 Binding

The protein synthesis inhibitor cycloheximide was used to evaluate whether protein synthesis was required for the appearance of VT-1 receptors on the cell surface induced by TNF-α. The simultaneous addition of high concentrations of TNF-α and cycloheximide was toxic for the cells. However, it has been reported that partial protection for TNF-α cytotoxicity can be mediated by plasminogen activator inhibitor-2, the mRNA of which is rapidly induced in endothelial cells by TNF-α itself (own unpublished data). Therefore, we have added various concentrations of cycloheximide 3 hours after the addition of 20 U/mL TNF-α, and incubated the cells for another 21 hours. Subsequently, the cells were counted and specific VT-1 binding was determined in parallel wells and calculated on a per cell basis. Whereas cycloheximide did not change the number of VT-1 binding sites per cell in control cells, it prevented the TNF-α-induced increase in VT-1 binding in a concentration-dependent manner (Fig 10). Similarly, in HUVEC, 0.5 and 2.0 μg/mL cycloheximide reduced the TNF-α-induced increase in VT-1 binding sites per cell by 58% and 73%, respectively (not shown). These data suggest that the TNF-α-induced increase in VT-1 receptors involves the induction of de novo synthesis of protein(s), that is (are)

<table>
<thead>
<tr>
<th>TNF-α Preincubation (h)</th>
<th>Subsequent Incubation Without TNF-α (h)</th>
<th>Binding of $^{125}$I-VT-1 (fmol/10$^6$ cells)</th>
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<tbody>
<tr>
<td>Exp 1</td>
<td></td>
<td></td>
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<tr>
<td>0 (control)</td>
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</tr>
<tr>
<td>1</td>
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To evaluate how long confluent HUVEC have to be exposed to TNF-α to express an increase in VT-1 binding after 1 or 2 days, the cells were incubated for various times with 20 U/mL TNF-α, washed vigorously three times, and subsequently incubated without TNF-α for a residual time period until 24 hours (experiment 1) or 48 hours (experiment 2) after addition of TNF-α. Binding of 1 nmol/L $^{125}$I-VT-1 was determined with two different endothelial cell cultures, which had a threefold difference in basal VT-1 binding. Binding assay was performed as described in Materials and Methods.
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Fig 8. Inhibition of 1 nmol/L 125I-VT-1 binding by anti-Tj* serum (concentration range, 0.2% to 4.0%). Cells were incubated for 24 hours without or with TNF-α 500 U/mL. Anti-Tj* serum was added 2 hours before and during incubation with 125I-VT-1. Similar inhibition by 4% anti-Tj* serum was obtained in three other endothelial cell cultures (45% ± 9%, mean ± SD).

Involved in the synthesis pathway of the glycolipid VT-1 receptor.

Fig 9. VT-1 binding to HUVEC glycolipids. Glycolipids from endothelial cells were extracted, separated by TLC, and assayed for 125I-VT-1 binding. Lane a: standard neutral glycosphingolipids, 2 μg of each glycolipid, stained by orcinol spray. Lanes b-g: Autoradiographs of 125I-VT-1 binding to separated standard or endothelial glycolipids. Lane b: standard neutral glycosphingolipids, 0.2 μg of each glycolipid. Lane c: standard neutral glycosphingolipids, 2 μg of each glycolipid. Lane d and e: glycolipid extracts of 1.6 × 10⁷ HUVEC. Lane d: 500 U/mL TNF-α-treated cells. Lane e: control cells. Lane f and g: glycolipid extracts of 1.44 × 10⁷ cells. Lane f: TNF-α-treated cells. Lane g: control cells. 1, galactosylceramide; 2, lactosylceramide; 3, globotriaosylceramide; 4, globotetraosylceramide; 5, Forssman pentasaccharide; 6, origin of the lane.

DISCUSSION

Infections with verocytotoxin-producing E. coli are associated with HUS, and verocytotoxins are believed to play a role in the etiology of HUS. A functional receptor (GbOse₃cer) for VT-1 has been demonstrated on HUVEC, but it is present in a very low amount. We have demonstrated here that incubation of human endothelial cells with TNF-α, IL-1, or LPS markedly enhances the binding of VT-1 to these cells. Furthermore, we have demonstrated that the TNF-α-induced increase in VT-1 binding is accompanied by an increase in the cellular content of GbOse₃cer, a molecule that has been recognized as a functional receptor for VT-1.

Two recent reports have demonstrated that human endothelial cells also become more sensitive to other related toxins, Shiga-like toxin II and Shiga toxin, when they are simultaneously exposed to TNF-α. Tesh et al. have suggested that this may be due to a higher TNF-α susceptibility of endothelial cells whose protein synthesis was inhibited by VT-1. However, in our study, VT-1 was ineffective in untreated confluent endothelial cells, and
only reduced protein synthesis in these cells when they were preincubated or coincubated with TNF-α. As the incubation with TNF-α resulted in a 10- to 100-fold increase in VT-1 receptors, it is likely that the reduction of protein synthesis and the increased cytotoxicity caused by VT-1 is primarily the consequence of enhanced VT-1 binding to endothelial cells. Once the VT-1 binding sites are increased on the cell surface, the toxicity of TNF-α itself may act synergistically to the effect of VT-1.

Obrig et al. have observed that Shiga toxin has a higher cytotoxicity for subconfluent cells than for confluent cells. Our observation that subconfluent cells bind more VT-1 may correspond with this finding. However, it should be noted that the subconfluent cells were studied 24 hours after exposure to a strong proteolytic agent (trypsin, during passage of the cells), by which the cells certainly have been activated. Furthermore, as the half-life of endothelial cells in the normal adult human body is in the order of magnitude of 100 to 1,000 days, pathophysiologic relevance of VT-1 cytotoxicity toward subconfluent cells will be limited to areas of neovascularization. Although our confluent cells displayed a low basal VT-1 binding, the amount of VT-1 binding was apparently not sufficient to interfere with the viability and protein synthesis of the endothelial cells.

Several hours’ exposure of endothelial cells to TNF-α was enough to induce a detectable increase in VT-1 binding to these cells for 2 days. Therefore, an increase in endothelial VT-1 receptors may persist, even though inflammatory mediators are no longer detectable. No increase in concentrations of TNF-α and IL-1 could be established in the plasma of HUS patients on arrival at the hospital (own unpublished results). However, Siegler et al. recently reported that TNF-α was elevated in the urine of HUS patients. This suggests that TNF-α may indeed have an effect in the kidneys of HUS patients. Possible local sources of TNF-α in the kidney are glomerular macrophages and mesangial cells. It is of interest to note that a study in mice on the toxicity of a related toxin, verocytotoxin-2 (Shiga-like toxin II), has suggested a role of TNF-α in the toxicity of this toxin. Mice defective in macrophage response to LPS, including a defective production of TNF-α, showed a consistently longer mean time to death than mice who were normally responsive. In addition, Barrett et al. found that verocytotoxin-2 induced normal peritoneal macrophages to produce TNF-α. This may indicate that verocytotoxins act at various levels, ie, in activating macrophages to produce TNF-α and in damaging endothelial cells after the induction of their toxin receptors by TNF-α. It would be of great interest to know whether mesangial cells can also be induced to release TNF-α on exposure to verocytotoxins.

In addition to TNF-α, other inflammatory mediators, in particular IL-1, TNF-β, and LPS, also enhance VT-1 binding sites on endothelial cells. The parallel effects of these mediators on the synthesis of many endothelial proteins is well known. Coincubation of LPS with Shiga toxin resulted in one recent study in more sensitive endothelial cells than with Shiga toxin alone, whereas in another study no additional effect was observed. Louise and Obrig reported that coincubation of IL-1 in combination with Shiga toxin did not result in an enhanced cell death. In contrast, unpublished experiments in our laboratory demonstrate that after 24 hours’ preincubation of HUVEC with IL-1α or IL-1β (5 or 50 U/mL), the endothelial viability becomes more sensitive toward VT-1, albeit less dramatically than toward 500 U/mL TNF-α. The variation in these data suggests that IL-1 and LPS, similar to TNF-α, can induce VT-1 receptors on endothelial cells, necessary to make these cells vulnerable to verocytotoxin, and that they act in concert with VT-1 on endothelial cell viability, but less profoundly than high concentrations of TNF-α. The combination of low concentrations of both LPS and TNF-α, which, in our study, caused a larger increase in the binding of VT-1 on the endothelial cells than did incubation with these mediators separately, may be of interest in HUS, because the patient may become exposed to very low concentrations of LPS during the prodromal phase of acute gastroenteritis.

Fibrin has been seen in the histopathology of the kidney in HUS, and the presence of thrombin-antithrombin complexes (TAT), elevated levels of prothrombin fragment F1+2 (a marker of thrombin formation), and increased concentrations of fibrinopeptide A are detectable in the plasma of HUS patients on admission. Therefore, we wondered whether thrombin may make HUVEC more sensitive to VT-1. This was not the case. The small increase in VT-1 binding caused by thrombin may be explained by the fact that thrombin can induce HUVEC to produce IL-1, which in turn will induce more VT-1 binding sites on the cell surface.

The endothelial binding of VT-1, induced by TNF-α, was inhibited by the presence of anti-TJa serum, which indicates that at least a major part of the specific VT-1 binding is a glycosphingolipid (GbOSe2cer). From VT-1 binding to cellular glycolipid extracts, which were separated by TLC, a large increase in the GbOSe2cer content of TNF-α-treated endothelial cells was established. The increase must be due to an increase in GbOSe2cer synthesis, rather than to a change in the availability of existing GbOSe2cer on the cell surface, because the amount of GbOSe2cer increased in the cellular extracts and the increase in binding could be prevented by simultaneous condition of cycloheximide. Therefore, we conclude that TNF-α induces one (or more) enzyme(s) that is (are) rate-limiting in the synthesis of GbOSe2cer.

It is believed that the presence of verocytotoxins is necessary but not sufficient for the HUS development. Our study suggests that one of the additional stimuli needed to develop the syndrome is the exposure of kidney endothelial cells to one or more of the inflammatory mediators TNF-α, IL-1, or LPS. We cannot yet discriminate whether the local availability of inflammatory mediators causes the preferential kidney damage of HUS in children, or whether the sensitivity of the endothelium in human glomeruli is larger than that of other endothelia. Endothelial cells of glomeruli...
have only recently been isolated from animal kidneys, and isolation of these cells from human glomeruli is still in its infancy. However, in severe cases of HUS, not only kidney endothelial cells are affected, but also the endothelium in many other organs, including brain and pancreas. We therefore believe that our observations on the induction of verocytotoxin receptors on HUVEC are important for understanding the etiology of HUS, as well as for a better understanding of the biological actions of inflammatory mediators.

ACKNOWLEDGMENT
We thank Mario Vermeer for his excellent technical assistance.

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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

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