The epidemic form of the hemolytic uremic syndrome (HUS) is characterized by renal failure, thrombocytopenia, and hemolytic anemia. Infection with a verocytotoxin-1 (VT-1) producing Escherichia coli, mainly O157:H7, has been strongly implicated in the etiology of the epidemic form of HUS. Endothelial damage of glomeruli and kidney arterioles has a pivotal role in the pathogenesis of HUS. Histopathologic studies of the kidney in HUS patients reveal swollen and detached endothelial cells, deposits of fibrin, and influx of inflammatory cells, all within the glomerulus. A number of observations indicate that inflammatory mediators, in particular tumor necrosis factor-α (TNF-α), contribute to this pathologic process in HUS. Endothelial cells respond strongly to TNF-α by acquiring new properties, albeit that local variation in the response of endothelial cells to TNF-α in urine of HUS patients strongly to TNF-α by acquiring new properties, albeit that local variation in the response of endothelial cells to TNF-α in urine of HUS patients by acquiring new properties, albeit that local variation in the response of endothelial cells to TNF-α in urine of HUS patients strongly to TNF-α by acquiring new properties, albeit that local variation in the response of endothelial cells to TNF-α in urine of HUS patients strongly to TNF-α by acquiring new properties, albeit that local variation in the response of endothelial cells to TNF-α in urine of HUS patients strongly to TNF-α by acquiring new properties, albeit that local variation in the response of endothelial cells to TNF-α in urine of HUS patients strongly to TNF-α by acquiring new properties, albeit that local variation in the response of endothelial cells to TNF-α in urine of HUS patients strongly to TNF-α by acquiring new properties, albeit that local variation in the response of endothelial cells to TNF-α in urine of HUS patients strongly. 

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MATERIALS AND METHODS

Materials. Purified VT-1 was kindly provided by Dr M.A. Karl­ma (Department of Microbiology, The Hospital for Sick Children, Toronto, Ontario, Canada) (1.0 to 1.2 mg protein/mL). The endo­toxin content of the VT-1 preparation was less than 0.05 endotoxin units (EU)/mL by Limulus amebocyte lysate assay at detection level of 0.05 to 0.10 EU/mL. Percoll was purchased from Pharmacia (Uppsala, Sweden). RPMI 1640 Dutch modification supplemented with 20 mmol/L HEPES and 6.2 g/L sodium bicarbonate was ob­tained from Flow Laboratories (Irving, UK), 1 mmol/L pyruvic acid from Sigma (St Louis, MO), 2 mmol/L L-glutamine from GIBCO (Grand Island, NY), 40 mg/mL gentamycin from Boehringer Mann­heim (Mannheim, Germany), and fetal calf serum (FCS), which was heating inactivated at 56°C for 30 minutes, was obtained from GIBCO. Teflon foil bags were provided by Du Pont de Nemours and Co (Geneva, Switzerland). LPS of E coli O111:B4 was pur­chased from GIBCO. 3H-Leu and Na125I-iodine were from Amer­sham (Amersham, UK) and Iodogen indination from Pierce (Rock­ford, IL). The antirat IgM monoclonal against the Gb3 receptor was a gift from Dr J. Wijdenes (Innotherapy, Besancon, France). Cycloheximide and polymyxin B were obtained from Sigma. Chloro­form, methanol, and hexane were purchased from Merck (Darmstadt, Germany). Plastic coated silica gel F1500 Thin Layer Chromatogra­phy (TLC) plates were obtained from Schleicherand Schuell (Dassel, Germany). Polysorbonylmethacrylate was from Polyscience Inc (Washington, MD). Materials were of analytical grade.

Isolation of human monocytes. Mononuclear cells obtained from apheresis of healthy male volunteers were immediately diluted in ice-cold phosphate-buffered saline (PBS) supplemented with the an­ticogulant Na-citrate 2H2O. Thirty-five milliliters of samples were layered on top of 15 mL of 1.075 g/mL Percoll and centrifuged for 30 minutes, 200g at 4°C. Subsequently the interphase was collected and washed further. Purification of the mononuclear cells was performed by centrifugation centrifugation using a CURAME 3000 elutriator (Dijkstra Vereenigda bv, Lelystad, The Netherlands). Monocyte fractions were over 95% pure (as evaluated in cyto centrifuge preparations after May-Grünwald-Giessma staining) and viability was higher than 95% (as assessed by trypan blue dye exclusion). Purified monocytes were cultured immediately.

Culture and stimulation of human monocytes. Previous investi­gations have shown that monocytes cultured in teflon foil bags are not functionally impaired and show minimal activation. For this reason all monocytes used for experiments were cultured in teflon foil bags. The culture medium consisted of RPMI 1640 Dutch Modi­fication with 20 mmol/L HEPES, 2 mmol/L L-glutamine, 1 mmol/L pyruvic acid, 40 mg/mL gentamycin, and 5% fetal calf serum. Cells were cultured at a concentration of 3.0 × 106 cells/mL in a humidified incubator with 5% CO2 in air at 37°C.

Monocytes were either incubated under basal conditions or stimu­lated by preincubation with different concentrations of LPS and/or VT-1 for different periods of time. Under basal conditions the nonadherent monocytes cultured in teflon foil bags were nonstimulated as was confirmed by their morphology (small rounded cells with typical horseshoe-shaped nucleus and a small cytoplasm/nucleus ratio) and low/undetectable cytokine production in the condi­tioned medium (IL-1β, IL-6, IL-8, and TNF-α; compare Table 3).

Isolation of VT-1 and the binding of 125I-VT-1 to human monocytes. VT-1 was radiolabeled with Na125I according to the lodogen procedure.27 The radioactive specific activity of 125I-VT-1, as determined by self-displacement analysis,28 ranged from 3.3 to 10 μCi/μg of protein.

After preincubation with or without LPS, the monocytes were cooled to 4°C for 1 hour and gently kneaded, after which the mono­cytes were obtained by needle aspiration with a 19-gauge needle. Subsequently the monocytes were washed twice in PBS/0.1% bovine serum albumin (BSA) and transferred into tubes that were precoated with PBS/1% BSA to avoid aspecific binding of the labeled toxin. The cells were incubated for 3 hours with 0.1 to 15 mmol/L 125I-VT-1 in PBS/1% BSA at 0°C. Cell-associated 125I-VT-1 was determined after washing the cells for three times in excess of PBS/1% BSA. Aspecific binding was assessed in parallel incubation by determining the 125I-VT-1 binding in the presence of a 75-fold excess of unlabeled VT-1. Cellular specific binding was calculated by subtracting the nonspecific binding determined in the presence of a 75-fold excess of unlabeled VT-1 from the cellular binding of 125I-VT-1. All determina­tions were done in duplicate. Data were analyzed using the method described by Scatchard.

To study the ability of the monoclonal antibody (MoAb) against the Gb3 receptor to inhibit binding of 125I-VT-1 to human monocytes, the monocytes were incubated with the MoAb diluted into PBS/1% BSA 2 hours before and during the assay of 125I-VT-1 binding.

Glycolipid extraction and TLC. Human monocytes cultured in teflon foil bags with and without LPS for 36 hours were procured and washed twice with ice-cold PBS. Glycolipids were extracted as described by Lingwood et al.29 In short, the pellet was resuspended in PBS and 20 vol of chloroform/methanol (2:1, vol/vol) were added. Cell debris was removed by performing filtration through glass-wool. One volume of water was added and partitioned. The lower phase was dried and incubated 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.

For TLC the lower phase was dried and resuspended in chloro­form/methanol (2:1, vol/vol). Samples were separated on a silica gel TLC plate using chloroform:methanol:water (65:25:4, vol/vol/vol). After separation the plate was soaked three times for 1 minute in 0.01% polysorbonylmethacrylate in hexane and air dried, followed by night incubation in PBS/1% BSA and 0.05% Tween 20. Subsequently, the plate was incubated with 50 mL, 1.5 mmol/L 125I-VT-1 in 1% BSA and 0.05% Tween-20 in PBS during 4 hours at 4°C. The plate was washed extensively with PBS supplemented with 1% BSA and 0.05% Tween-20 and air dried and analyzed by a Fuji BAS 1000 phosphor-imager (Fuji, Leiden, The Netherlands).

Protein synthesis assay. Protein synthesis was determined by assaying the incorporation of 3H-leucine (2.0 μCi/mL) in 3H-proteins during an 8- to 48-hour incubation period with different concentra­tions of VT-1. The incubation was followed by harvesting and wash­ing with ice-cold PBS. Subsequently, cellular proteins as well as proteins present in the media were precipitated by adding (10% and 20%, respectively) trichloroacetic acid. Precipitated proteins were dissolved in 0.3 mL 0.3 N NaOH; 60 μL 1.5 N HCl was added and radioactivity was counted in a liquid scintillation counter.

Cytokine production. Human monocytes, cultured in teflon foil bags, were incubated with either LPS (range, 10 μg/mL to 1 ng/ mL) or VT-1 (range, 1 mmol/L to 20 mmol/L) for 4, 12, 24, and 36 hours. Unstimulated controls were exposed to medium alone. After the indicated periods of time the cells were procured and centrifuged. The supernatants were collected and stored at −20°C until assayed for the presence of cytokines. In additional experi­ments, cycloheximide and polymyxin B were simultaneously added to confirm respectively necessity of protein synthesis and absence of contamination with LPS, TNF-α as well as IL-1β was assayed by radioimmunossay.36,37 The sensitivity of both assays was 40 pg/mL. IL-6 was measured by an enzyme-linked immunossorbent assay (ELISA) as described by Barrera et al.38 Materials were a gift from Dr J. Wijdenes (Innotherapy, Besancon, France).
The sensitivity of this ELISA was 20 pg/mL. IL-8 was also determined by an ELISA (R&D Systems Inc, Abingdon, UK), with a sensitivity of 18.1 pg/mL.

Detection of mRNA. Human monocytes (1 x 10⁶ cells/mL) were cultured in teflon foil bags for 12 hours in the presence of either control media or media supplemented with LPS (1 ng/mL) or VT-1 (10 nmol/L). After the indicated period of time the cells were obtained and pelleted by centrifugation at 200 g at 4°C. Cells were subsequently washed twice with ice-cold PBS. Total cellular RNA was extracted according to the RNA-zol B method. RNA amount and purity was assessed by measuring the optical densities at 260 to 280 nm.

cDNA was synthesized in 20 µL of reaction volumes containing 0.5 µg of monocyte total RNA, 5 µg/mL of oligo (dT)₁₂₋₁₅, 10 mmol/L dithiothreitol, 0.5 mmol/L dNTPs, 50 mmol/L Tris-HCl, pH 8.3, 20 U RNAsin (Promega, Madison, WI), 75 mmol/L KCl, 3 mmol/L MgCl₂, and 200 U of Moloney murine leukemia virus reverse transcriptase (RT) (GIBCO-BRL, Life Technologies BV, Breda, The Netherlands). After a 10-minute incubation at 20°C followed by a 45-minute incubation at 42°C, samples were heated at 95°C for 10 minutes and then quickly chilled on ice.

DNA amplification was performed with 2 µL of the RT reaction mixture in 1 x polymerase chain reaction (PCR) buffer (20 mmol/L Tris-HCl pH 8.3, 50 mmol/L KC1, 1.5 mmol/L MgCl₂, 0.01% gelatin) supplemented with 125 µmol/L dNTPs, 30 pmol each of 5'- and 3'- specific primers for IL-6, and β-actin (5' GCT ACG GCC TGC CTT ACG 3' and 5' GAG GCC AGG ATG GAG CC 3') and 2.5 U of Taq polymerase (GIBCO-BRL) in a final volume of 80 µL. All primers used for PCR were 50% GC rich and did not exhibit 3'-complementarity between primers pairs. Primers were designed to produce amplicons spanning RNA-splicing sites. The mixture was overlaid with 60 µL of mineral oil. Initial denaturation step consisted of 5 minutes at 94°C followed by amplification in 25 (β-actin) and 24, 26, 28, 30, and 32 (IL-6) segmental cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 90 seconds (Perkin-Elmer Cetus instrument, Norwalk, CT). All components used in cDNA synthesis reaction and PCR were checked for possible contaminations (in corresponding reactions lacking RNA or reverse transcriptase). Reaction products (20 µL) were analyzed by 1.5% agarose (1.5% agarose, 0.01% ethidium bromide in Tris acetate/EDTA buffer) gel electrophoresis.

Fig 1. Bacterial LPS increases the specific binding of 125I-VT-1 to nonadherent human monocytes. (A) Saturability of specific 125I-VT-1 binding to human monocytes. Increasing concentrations (0.1 to 15 nmol/L) of 125I-VT-1 were incubated for 3 hours at 4°C with 2 x 10⁵ human monocytes cultured in teflon foil bags either under basal conditions (□) or after stimulation with LPS (0.1 µg/mL) for 36 hours (■). Values are the mean ± SD of monocytes from three different donors. (B) The effect of LPS preincubation on the binding of 125I-VT-1 to human monocytes. Human monocytes were cultured in teflon foil bags with LPS (0.1 µg/mL) and without LPS for various periods of time (0 to 42 hours), after which the cells were obtained and counted. Subsequently the binding of 0.5 nmol/L 125I-VT-1 to 2 x 10⁵ human monocytes was assessed. Similar data were obtained with monocytes of a second donor. (C) Scatchard plot analysis of 125I-VT-1 binding to human monocytes of one representative donor out of three. Cells were cultured in teflon foil bags either under basal conditions (inset: □) or stimulated with LPS (0.1 µg/mL: ■) for 36 hours. Inset: x-axis, B (mol/cell) x 10⁻¹⁹; y-axis, B/F (L/cell) x 10⁻¹⁰.

DNA amplification was performed with 2 µL of the RT reaction mixture in 1 x polymerase chain reaction (PCR) buffer (20 mmol/L Tris-HCl pH 8.3, 50 mmol/L KC1, 1.5 mmol/L MgCl₂, 0.01% gelatin) supplemented with 125 µmol/L dNTPs, 30 pmol each of 5'- and 3'- specific primers for IL-6, and β-actin (5' GCT ACG GCC TGC CTT ACG 3' and 5' GAG GCC AGG ATG GAG CC 3') and 2.5 U of Taq polymerase (GIBCO-BRL) in a final volume of 80 µL. All primers used for PCR were 50% GC rich and did not exhibit 3'-complementarity between primers pairs. Primers were designed to produce amplicons spanning RNA-splicing sites. The mixture was overlaid with 60 µL of mineral oil. Initial denaturation step consisted of 5 minutes at 94°C followed by amplification in 25 (β-actin) and 24, 26, 28, 30, and 32 (IL-6) segmental cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 90 seconds (Perkin-Elmer Cetus instrument, Norwalk, CT). All components used in cDNA synthesis reaction and PCR were checked for possible contaminations (in corresponding reactions lacking RNA or reverse transcriptase). Reaction products (20 µL) were analyzed by 1.5% agarose (1.5% agarose, 0.01% ethidium bromide in Tris acetate/EDTA buffer) gel electrophoresis.
EFFECTS OF VEROCYTOTOXIN-1 ON HUMAN MONOCYTES

Table 1. Binding of Verocytotoxin-1 (VT-1) to Human Monocytes

<table>
<thead>
<tr>
<th>Cells</th>
<th>Addition</th>
<th>Binding Sites/Cell</th>
<th>Apparent kd (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>None</td>
<td>0.07 × 10⁵</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>LPS (0.1 µg/mL)</td>
<td>2.0 × 10⁵</td>
<td>2.3</td>
</tr>
<tr>
<td>Donor 2</td>
<td>None</td>
<td>0.08 × 10⁵</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>LPS (0.1 µg/mL)</td>
<td>1.8 × 10⁵</td>
<td>1.6</td>
</tr>
<tr>
<td>Donor 3</td>
<td>None</td>
<td>0.06 × 10⁶</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>LPS (0.1 µg/mL)</td>
<td>1.8 × 10⁶</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Specific binding of ¹²⁵I-VT-1 (0.1 to 15 nmol/L) to human monocytes of three different donors using human monocytes cultured in teflon foil bags and exposed to either culture medium alone (controls) or supplemented with LPS (0.1 µg/mL). The total number of specific binding sites and the apparent kd were calculated according to the method described by Scatchard.

RESULTS

LPS increases the number of VT-1 receptors on human monocytes. To determine the binding of ¹²⁵I-VT-1 to non-adherent monocytes, monocytes that had been cultured and preincubated in teflon foil bags were used. The amount of specific ¹²⁵I-VT-1 binding to these cells reached a maximum in 2 hours; it increased linearly with the cell concentration (not shown). The binding of ¹²⁵I-VT-1 was saturable (Fig 1A) and was entirely displaced by a 75-fold excess of unlabeled VT-1. All further experiments were performed using 2 × 10⁶ cells in a 100 µL volume and a 3-hour incubation period with ¹²⁵I-VT-1.

Preincubation of the cells with LPS caused a time-dependent increase in VT-1 binding (Fig 1A and B). When the cells were cultured for 42 hours under basal conditions, no change in specific ¹²⁵I-VT-1 binding was observed (Fig 1B). However, when the cells were incubated during the same time period with bacterial LPS an increase in specific ¹²⁵I-VT-1 binding was observed, which became detectable after 18 hours and progressed steadily for another 18 hours (Fig 1B). The response was maximal with 1 ng/mL LPS and half-maximal with 0.1 ng/mL LPS (not shown). Scatchard plot analysis indicated that one type of binding site was involved (Fig 1C, Table 1). Furthermore, it showed that preincubation of monocytes with 0.1 µg/mL LPS for 36 hours caused a 27-fold (range 23- to 30-fold) increase in the number of specific VT-1 binding sites, while the apparent affinity of ¹²⁵I-VT-1 did not change significantly (Table 1).

In addition to LPS, 36-hour preincubation of the cells with 100 U/mL IL-2 and to a lesser extent with 33 ng/mL TNF-α caused an increase in specific VT-1 binding to monocytes (sixfold and twofold, respectively); γ-interferon (150 U/mL) and IL-4 (300 U/mL) did not change this binding (not shown).

Characterization of the ligand to which VT-1 binds on monocytes. To investigate if globotriaosylceramide (Gb3), which has been shown to be the receptor for VT-1 on endothelial cells,39 lymphocytes,36 and in human kidney,37 was also the receptor on monocytes, the cells were preincubated with LPS and an MoAb specifically recognizing Gb3. The anti-Gb3 MoAb inhibited the binding of ¹²⁵I-VT-1 to stimulated monocytes in a concentration-dependent way, although no complete inhibition was reached at the concentrations...
completely inhibited by the protein synthesis inhibitor cycloheximide. This indicates that the synthesis of VT-1 receptors is increased rather than the fact that it only reflects a redistribution of membrane. In favor of an increased synthesis of proteins VT-1 binding to monocyte glycolipids involves a neutral glycolipid with an Rf value slightly higher than Gb,| (Fig 2, lanes A and B). Because Gb, does not bind VT-1, this may present other Gb, forms, possibly Gb, with short-chain fatty acids or α-OH-Gb, which both display a lower Rf value than the classical form of Gb, | . This pattern was consistently found with different preparations of monocytes obtained from three different donors.

The increase in the binding of VT-1 to the glycolipid extracts of LPS-stimulated monocytes (Fig 2, lanes A and B) indicates that the synthesis of VT-1 receptors is increased rather than the fact that it only reflects a redistribution of VT-1 receptors from intracellular stores toward the plasma membrane. In favor of an increased synthesis of proteins involved in Gb, synthesis is the observation that the LPS-induced increase in VT-1 binding to monocytes was completely inhibited by the protein synthesis inhibitor cycloheximide (Fig 3). When LPS-stimulated monocytes were preincubated with cycloheximide only for 1 hour before harvesting, no change in VT-1 binding was found, excluding a direct effect of cycloheximide on the receptor availability or the binding assay itself.

Effect of VT-1 on protein synthesis and viability of monocytes. To evaluate whether human monocytes, which possess a Gb, | -receptor with a reduced Rf value, are sensitive to the toxic effect of VT-1, like other eukaryotic cells containing the classical Gb, 21 protein synthesis was estimated from the incorporation of 3H-leucine in newly synthesized proteins. No effect of VT-1 at concentrations ranging from 1 to 10 nmol/L on protein synthesis by monocytes was observed over a 48-hour time period. Table 2 summarizes the data on the effect of 24-hour incubation with 10 nmol/L VT-1 or 1 ng/mL LPS on nonstimulated and LPS-stimulated monocytes obtained from three different donors. VT-1 exerted no effect on the overall protein synthesis by nonstimulated monocytes and LPS-stimulated monocytes of two donors, and had only a minor effect on protein synthesis in the LPS-stimulated cells of one donor (84% of control). No toxicity of 10 nmol/L VT-1 on nonstimulated and LPS-stimulated monocytes was observed, as determined by trypan blue exclusion test.

Effect of VT-1 on the production of cytokines by human monocytes. When human monocytes cultured in teflon bags were exposed to various concentrations of VT-1 for different periods of time, they started to produce considerable amounts of IL-1β, IL-6, IL-8, and TNF-α (Fig 4; Table 3). The time course of the production of these cytokines during incubation with 10 nmol/L VT-1 is given in Fig 4 for monocytes of three different donors. The release of all four cytokines was enhanced after 4 hours and reached a maximum at 12 hours for TNF-α, whereas (dependently of the donor) the release of IL-1β, IL-6, and IL-8 continued for 12 to 36 hours. The dose-response of cytokine release after 24 hours of incubation with VT-1 is shown in Fig 5. VT-1 concentrations of 1 to 20 nmol/L induced the release

used (47% and 59% inhibition at 5% and 10% [vol/vol] antibody solution, respectively).

To further characterize the nature of the receptor for VT-1 present on human monocytes, neutral glycolipid extracts were prepared from both unstimulated and LPS-stimulated monocytes and TLC of these extracts was performed. After incubation of these thin-layer chromatograms with 125I-VT-1 and extensive washing, the bound 125I-VT-1 was detected by exposure to a phosphor-imager. The radiolabeled VT-1 bound strongly to Gb, and not to Gb, when a standard preparation of neutral glycolipids was tested (Fig 2, lane E). In LPS- and TNF-α-stimulated human endothelial cells, VT-1 binding predominantly occurs to the classical Gb, (two bands probably related to differences in chain length of fatty acids) (Fig 2, lanes C and D). However, the basal and stimulated VT-1 binding to monocyte glycolipids involves a neutral glycolipid with an Rf value slightly higher than Gb, (Fig 2, lanes A and B). Because Gb, does not bind VT-1, this may present other Gb, forms, possibly Gb, with short-chain fatty acids or α-OH-Gb, which both display a lower Rf value than the classical form of Gb, | . This pattern was consistently found with different preparations of monocytes obtained from three different donors.

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Table 2. Effects of LPS and VT-1 on the Incorporation of 3H-Leucine in 3H-Proteins by Human Monocytes

<table>
<thead>
<tr>
<th>Cells</th>
<th>Addition</th>
<th>Control</th>
<th>Preincubation With LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor 1</td>
<td>None</td>
<td>0.97</td>
<td>1.36</td>
</tr>
<tr>
<td>VT-1 (10 nmol/L)</td>
<td>1.05</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td>LPS (1 ng/mL)</td>
<td>1.12</td>
<td>1.47</td>
<td></td>
</tr>
<tr>
<td>Donor 2</td>
<td>None</td>
<td>1.39</td>
<td>1.65</td>
</tr>
<tr>
<td>VT-1 (10 nmol/L)</td>
<td>1.59</td>
<td>1.38</td>
<td></td>
</tr>
<tr>
<td>LPS (1 ng/mL)</td>
<td>1.51</td>
<td>2.03</td>
<td></td>
</tr>
<tr>
<td>Donor 3</td>
<td>None</td>
<td>0.62</td>
<td>0.82</td>
</tr>
<tr>
<td>VT-1 (10 nmol/L)</td>
<td>0.81</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>LPS (1 ng/mL)</td>
<td>0.71</td>
<td>0.88</td>
<td></td>
</tr>
</tbody>
</table>

Control cells (control incubation) and LPS-stimulated cells (preincubation with LPS (1 ng/mL for 36 hours) were cultured in teflon foil bags and exposed to control medium alone (None), control medium supplemented with VT-1 (10 nmol/L), or control medium supplemented with LPS (1 ng/mL) during a 24-hour incubation period. Subsequently, the incorporation of 3H-leucine into 3H-proteins was determined as described in Materials and Methods. Data are expressed as incorporation of 3H-leucine into 3H-proteins (μg/2 × 10⁶ monocytes).
EFFECTS OF VERO CYTOTOXIN-1 ON HUMAN MONOCYTES

Fig 4. Time kinetics of cytokine release by human monocytes induced by VT-1. Human monocytes of three different donors were cultured in teflon foil bags (1.5 x 10^6/3 mL) and incubated with VT-1 (10 nmol/L) for various periods of time (0 to 36 hours). At the indicated time points, conditioned media were collected and assayed for the cytokines IL-1β (A), IL-6 (B), IL-8 (C), and TNF-α (D) as described in Materials and Methods. Values from three different donors are given.

of the indicated cytokines, a maximum being reached at 5 to 10 nmol/L VT-1.

The effect of VT-1 was specific and was not caused by LPS contamination in the VT-1 preparation. Heat-inactivation (15 minutes at 90°C; two monocyte preparations of different donors) completely destroyed the ability of the VT-1 preparation (10 nmol/L) to induce the release of three cytokines (IL-1β, 1.9% ± 1.2%; IL-6, 6.1% ± 0.6%; TNF-α, 4.5% ± 2.3% of control). Furthermore, addition of polymyxin B (1 μg/mL) did not reduce the effect of 10 nmol/L VT-1 (99% ± 34%), whereas it prevented the effect of 1 μg/mL LPS on cytokine release (4.0% ± 2.0%). Finally, preexposure of monocytes to LPS (1 ng/mL; 36 hours) followed by a second incubation with 1 ng/mL LPS caused only a significant release of IL-1β after the first exposure to LPS. Nevertheless, the LPS-desensitized monocytes still respond to VT-1 (Fig 6). In five experiments with two different preparations of LPS-desensitized monocytes, IL-1β accumulation in the supernatant amounted to 111% ± 11% of control values after reincubation with 1 ng/mL LPS and 247% ± 21% and 325% ± 101% after incubation with 1 nmol/L VT-1 and 10 nmol/L VT-1, respectively (mean ± SD of monocytes of three different donors).

Table 3. The Cytokine-Inducing Effect of VT-1 and LPS

<table>
<thead>
<tr>
<th>Cytokine Determined</th>
<th>Control (LPS 1 ng/mL)</th>
<th>VT-1 (10 nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>0.03 ± 0.03</td>
<td>0.07 ± 0.04</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.13 ± 0.09</td>
<td>0.10 ± 0.09</td>
</tr>
<tr>
<td>IL-8</td>
<td>12.0 ± 8.5</td>
<td>10.3 ± 6.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.07 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
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Human monocytes of three different donors were cultured in teflon foil bags and exposed to either control medium alone or control medium supplemented with either LPS (1 ng/mL) or VT-1 (10 nmol/L). After 24 hours cells were obtained and conditioned media were collected and assayed for the cytokines: IL-1β, IL-6, IL-8, and TNF-α as described in Materials and Methods. Values are the mean (ng/mL) ± SD of monocytes of three different donors.

Fig 5. Dose-response of cytokine release by human monocytes induced by VT-1. Human monocytes (1.5 x 10^6/3 mL) were cultured in teflon foil bags and exposed to various concentrations of VT-1 (0 nmol/L, □; 1 nmol/L, □; 5 nmol/L, □; 10 nmol/L, □; and 20 nmol/L, □) during a 24-hour incubation period. Cells were procured and conditioned media were collected and assayed for the cytokines IL-1β (A), IL-6 (A), TNF-α (A), and IL-8 (B). Shown are the data of human monocytes of one representative donor out of three.
Fig 6. LPS-desensitized human monocytes do respond to VT-1. Human monocytes were cultured in teflon foil bags and stimulated with LPS (1 ng/mL) for 36 hours. Cells were obtained, washed, and counted. IL-1β concentration in conditioned medium of the LPS-stimulated monocytes was > 10 ng/mL. Subsequently, 2 x 10^6 LPS-stimulated cells were cultured in new teflon foil bags and either cultured in control medium (1) or control medium supplemented with VT-1 1 nmol/L (2), VT-1 5 nmol/L (3), VT-1 10 nmol/L (4), or LPS (1 ng/mL) (5). After 6 ( ), 12 ( ), and 24 hours ( ) cells were obtained and conditioned media were collected and assayed for IL-1β.

SEM). The induction of the release of IL-1β by 10 nmol/L VT-1 was prevented by simultaneous addition of cycloheximide (2 μg/mL) (108% of control). This indicates that protein synthesis is required for the induction of this cytokine. To confirm the induction of the cytokine IL-6 at the mRNA level, human monocytes were incubated with either culture medium or culture medium supplemented with 1 ng/mL LPS or 10 nmol/L VT-1. Total cellular RNA was extracted after a 12-hour incubation period and mRNA levels for IL-6 (Fig 7) were determined by RT-PCR. To determine the linear range of amplification, the number of cycles ran for amplification of IL-6 mRNA was varied (24, 26, 28, 30, and 32 cycles). Figure 7 shows that only a weak band was observed in non-stimulated monocytes (lane 1) after 32 cycles, whereas marked increases in the IL-6 PCR signal become visible after 24 cycles in VT-1 (lane 2) and LPS-stimulated (lane 3) cells. The responses of VT-1 and LPS are rather similar. The housekeeping gene β-actin was simultaneously amplified (25 cycles) in parallel tubes (Fig 7; left panel), indicating equivalent loading of the samples. Corresponding reactions lacking RNA or RT did not show any RT-PCR product. Specificity of the RT-PCR product (260 bp) was confirmed by restriction enzyme digestion.

**DISCUSSION**

Verocytotoxin producing E. coli play an important role in the etiology of the epidemic form of HUS. In this study we have shown that freshly isolated human monocytes bind VT-1 to a Gb3 species that is different from that found on endothelial cells and that the number of VT-1 binding sites is enhanced after incubation of the cells with LPS. Furthermore, we have demonstrated that instead of inhibiting protein synthesis, VT-1 specifically induces human monocytes to synthesize the cytokines IL-1β, TNF-α, IL-6, and IL-8.

Several in vivo studies in humans and mice indicate...
cate that in addition to verocytotoxin, the sensitivity for LPS and the availability of the inflammatory mediator TNF-α also play a role in the development and severity of the disease. Previous studies in vitro have shown that the sensitivity of endothelial cells for VT-1 markedly increases after exposure of cultured endothelial cells to TNF-α, IL-1, or LPS. Because monocytes and macrophages are able to produce and release these inflammatory mediators, we investigated the interaction of human peripheral blood monocytes with VT-1. Human monocytes express small amounts of VT-1 receptors, the number of which increased 23- to 30-fold after exposure of the cells to LPS due to an increase in receptor synthesis. This observation is of interest, because HUS is associated with a bloody diarrhea and plasma titers of antibodies against O157 LPS were elevated in children with HUS. Therefore, it is likely that part of the circulating monocytes become exposed to LPS.

The VT-1 receptor on monocytes appeared to be different from the classical Gb₃ described for endothelial cells and lymphocytes. It interacts with MoAbs against Gb₃, but has a relatively low RF value during TLC. The chromatographic behavior is comparable with that of short-chain fatty acyl Gb₃ as described by Kiarash et al and also with that of α-OH-Gb₃, which has been recognized by Jacewicz et al as a VT-1 binding receptor in erythrocyte membrane extracts. The exact nature of the receptor involved in VT-1 binding to human monocytes remains to be clarified. Interestingly, interaction of VT-1 with the receptor on monocytes did not affect the protein synthesis or viability of these cells, despite the fact that they express 2 × 10⁵ receptors per cell after stimulation with LPS. Human umbilical endothelial cells expressing 10 × 10⁵ receptors per cell were highly sensitive to VT-1. However, monocytes do respond to VT-1 by releasing cytokines. Therefore, it is likely that monocytes are less susceptible to the toxic effect of VT-1, although it cannot be excluded that the lower number of VT-1 receptors on monocytes partly contributes to the lack of sensitivity for VT-1 cytotoxicity. Alternatively, we cannot exclude that the increase in VT-1 receptors is limited to a relatively small subpopulation of the monocytes. This may mask a toxic effect, because the majority of the cells are nonresponsive. It remains to be investigated whether the lack of toxicity by Gb₃ with different fatty acyl moieties (Gb₃ short-chain fatty acyl Gb₃ and α-OH-Gb₃) is due to different localization and processing of these VT-1 receptors on the cell membrane. In this context it is of interest to note that different routes of internalization of Gb₃ molecules have been reported, i.e., via coated pits or via caveoli and transport to the Golgi region. A last possible explanation for human monocytes not being susceptible to the toxic effect of VT-1 might be that regulation of ribosomal protein synthesis in human monocytes reacts in a different way to VT-1 compared with that in endothelial cells. Such a different response has been suggested to exist in Chinese hamster ovary (CHO) cells.

VT-1 binding to human monocytes did not cause protein synthesis inhibition, but it caused a considerable release of the cytokines IL-1β, TNF-α, IL-6, and IL-8 in a dose- and time-dependent manner. This observation closely agrees with a recent observation of Tesh et al, who reported that mouse peritoneal macrophages express low to undetectable amounts of Gb₃ and that interaction of VT-1 with these macrophages induces the production of IL-1, TNF-α, and IL-6. Our study and that by Tesh et al show that the induction of cytokine release is accompanied by an increase in cellular mRNA concentrations. In particular, our observation that desensitisation of the monocytes for LPS prevented cytokine release after a second LPS stimulation, while it did not reduce cytokine release by VT-1, strongly points to a stimulation of monocytes by VT-1 independently of LPS stimulation. A similar conclusion was drawn by Tesh et al using mouse peritoneal macrophages from LPS-hyporesponsive C3H/HeJ mice. Peritoneal macrophages from these LPS-hyporesponsive mice did respond in terms of cytokine release when incubated with SLT-1. The fact that in vivo these mice, which have a defective macrophage response after LPS challenge, showed a consistently longer mean time to death after inoculation with Shiga-like toxin II (VT-2), further points to a cooperative effect between LPS and verocytotoxins. Our data on the increase of VT-1 receptors on human monocytes by LPS may be relevant in this context. Firstly, because circulating VT-1 remain below detection level in blood plasma and the toxicity occurs predominantly in the kidney, monocytes may be involved in transporting the toxin from the intestine to the kidney. Secondly, once activated by LPS, monocytes increase their number of VT-1 receptors and probably respond more easily to the tiny concentrations of verocytotoxins that will be reached in HUS.

Our observation that VT-1 induces the release of inflammatory mediators also agrees with in vivo data of HUS patients showing elevated plasma concentrations of IL-8, IL-6, and TNF-α among HUS patients and elevated concentrations of TNF-α and IL-6 in urine of HUS patients. Urinary concentrations being higher than serum concentrations in individual patients points to a predominantly local role for inflammatory mediators. A local role was also suggested by Harel et al, who injected SLT-1 into transgenic mice bearing a chloramphenicol acetyltransferase (CAT) reporter gene coupled to a TNF-α promoter. After injection of SLT-1 in these mice, CAT activity, reflecting stimulation of the TNF-α promoter, was induced in the kidney but not in other tissues.

The locally released cytokines, in particular TNF-α and IL-1β, may increase leukocyte-endothelial cell interaction and enhance the VT-1 receptors on endothelial cells and as such the sensitivity of these cells for the toxin. The accumulation of granulocytes in kidney biopsies of HUS patients has been well described as well as elevated concentrations of IL-8 known to be chemotactic for granulocytes and elevated elastase levels indicating activation of granulocytes. However, the presence of monocytes in such biopsy samples and the activation of monocytes remained unnoted. Recently we have been able to analyze a biopsy sample taken in the acute stage of a HUS patient. This biopsy specimen displayed a significant number of monocytes in the glomeruli. This further underlies a possible involvement of blood monocytes in the pathogenesis of HUS. Our observations
that LPS increase the number of a specific type of VT-1 receptors and that interaction of VT-1 with monocytes induces the synthesis and release of various proinflammatory cytokines may contribute to understanding the complex cellular interactions in HUS.

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