

A Critical Role for Monocytes and CD14 in Endotoxin-induced Endothelial Cell Activation

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Summary

Vascular endothelium activated by endotoxin (lipopolysaccharide [LPS]) and cytokines plays an important role in organ inflammation and blood leukocyte recruitment observed during sepsis. Endothelial cells can be activated by LPS directly, after its interaction with LPS-binding protein and soluble CD14 in plasma. LPS-LPS-binding protein complexes in blood also interact with monocytes and neutrophils bearing glycosyl-phosphatidylinositol (GPI) anchored membrane CD14 (mCD14), promoting the release of cytokines such as tumor necrosis factor and interleukin 1 (IL-1). These molecules, in turn, have the capacity to activate endothelial cells providing an indirect pathway for LPS-dependent endothelial cell activation. In this work, we address the relative importance of the direct and the indirect pathway of *in vitro* LPS-induced human umbilical vein endothelial cell (HUVEC) activation. Substituting whole blood for plasma resulted in a 1,000-fold enhancement of HUVEC sensitivity to LPS. Both blood- and plasma-dependent enhanced activation of HUVEC were blocked with an anti-CD14 monoclonal antibody. Blood from patients with paroxysmal nocturnal hemoglobinuria, whose cells lack mCD14 and other GPI anchored proteins, was unable to enhance LPS activation of HUVEC above the level observed with plasma alone. IL-10, an inhibitor of monocyte release of cytokines, decreased the blood-dependent enhancement of HUVEC activation by LPS. Blood adapted to small doses of LPS was also less efficient than nonadapted blood in producing this enhancement. Addition of purified mononuclear cells to HUVEC or the transfer of plasma from whole blood incubated with LPS to HUVEC, duplicated the enhancement effect observed when whole blood was incubated with HUVEC. Taken together, these data suggest that the indirect pathway of LPS activation of endothelial cell is mediated by monocytes and mCD14 through the secretion of a soluble mediator(s). The indirect pathway is far more efficient than the direct, plasma-dependent pathway.

Vascular endothelium activated by proinflammatory molecules actively participates in organ inflammation by secreting cytokines, chemokines, and leukocyte adhesion molecules (1). Vascular endothelium is therefore thought to play an important role in the pathogenesis of multiple organ dysfunction such as is observed during sepsis.

Endotoxin, or LPS, has been recognized as a major trigger for host inflammatory responses seen during Gram-negative sepsis (2). Either LPS or Gram-negative bacteria can reproduce the clinical syndrome known as sepsis (2). Two glycoproteins are clearly implicated in the molecular and cellular basis of these events. The first, LPS-binding protein (LBP),¹

present in normal serum, recognizes and binds LPS with high affinity through its lipid A moiety (3). LPS-LBP complexes then activate myeloid cells through the second glycoprotein, membrane-bound CD14 (mCD14), to produce cytokines such as TNF and IL-1 β (4, 5).

LPS also induces endothelial cell responses, but through two different pathways. The direct pathway, which is serum dependent, requires the interaction of LPS with LBP and the soluble form of CD14 (sCD14), present in human serum at concentrations of 2–5 $\mu\text{g/ml}$. LPS-sCD14 complexes interact with an as yet unknown receptor on the endothelial cell surface in order to trigger cell responses (6). The same pathway of LPS activation has been described for some epithelial cell lines (6). Endothelial and epithelial cells apparently do not express mCD14 (6, 7). As mentioned above, LPS in the blood stream triggers the release of numerous cytokines and other proinflammatory molecules by white blood cells. Some of them, such as TNF and IL-1, have a proinflammatory effect on endothelial cells, independently of LPS (8). This is re-

¹ Abbreviations used in this paper: GPI, glycosyl-phosphatidylinositol; HUVEC, human umbilical vein endothelial cell; LBP, LPS-binding protein; mCD14, membrane-bound CD14; PNH, paroxysmal nocturnal hemoglobinuria; sCD14, soluble form of CD14; VCAM, vascular cell adhesion molecule 1.

ferred to as the indirect pathway for LPS-dependent endothelial cell activation. Once activated, endothelial cells secrete cytokines (IL-1 β , IL-6), chemokines (typically IL-8), and upregulate various adhesion molecules for leukocytes such as vascular cell adhesions, molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and selectins (8, 9).

In this work, the relative importance of the direct versus the indirect pathway for endothelial cell activation by LPS in vitro was studied. We show that the presence of blood cells will amplify the endothelial cell response to LPS by a factor of 1,000 compared with plasma alone. This enhancement is blocked by mAbs which block mCD14 and sCD14 function. Furthermore, blood from paroxysmal nocturnal hemoglobinuria (PNH) patients, whose white cells lack all glycosyl-phosphatidylinositol (GPI) anchored proteins including CD14, does not amplify the endothelial response to LPS. Because purified mononuclear cells or plasma from blood incubated with LPS replaced whole blood, we suggest that it is the monocyte which serves as the source of a soluble mediator(s) acting as agonist(s) for the indirect activation of endothelial cells by LPS.

Materials and Methods

Cells. Human umbilical vein endothelial cells (HUVEC) were obtained, identified as described (10), and maintained using 199 medium (BioWhittaker, Inc., Walkersville, MD) supplemented with 20% fetal bovine serum (HyClone Laboratories, Logan, UT), 90 μ g/ml heparin (Sigma Chemical Co., St. Louis, MO) (11) and 30 μ g/ml endothelial cell growth supplement (UBI, Lake Placid, NY) (12). Second to fourth passages were used for the assays.

Reagents. *Escherichia coli* 0111:B4 and *Salmonella minnesota* wild-type LPS were obtained from List Biological Laboratories (Campbell, CA). Re595 LPS was produced as described (13). Heat-killed *Staphylococcus aureus* was a gift from T. Kirkland (University of California at San Diego, San Diego, CA). Recombinant human IL-10 (hIL-10) and a rat anti-hIL-10 mAb were kindly provided by Dr. K. Moore, DNAX (Palo Alto, CA). The anti-CD14 mAbs used were 28C5 and 18E12 (gift of D. Leturcq and A. Moriarty, R. W. Johnson Pharmaceutical Research Institute, San Diego, CA); MY4, MY4-RD1, and isotype control (Coulter Corp., Hialeah, FL); UCHM1 (Sera-Lab, Accurate Chemical & Scientific Corp., Westbury, NY); and 63D3 (American Type Culture Collection, Rockville, MD). Anti-CD18 mAb IB4 was a gift from K. Arfors (Medical Biology Institute, La Jolla, CA); FITC-labeled anti-CD11b mAb and its isotype control were purchased from Amac Inc. (Westbrook, ME); anti-hVCAM-1 mAb from R&D Systems (Minneapolis, MN); and peroxidase-conjugated goat anti-mouse IgG Ab from Cappel Laboratories (Durham, NC). IL-8 levels in supernatants were measured using commercial ELISA kits (R&D Systems). Recombinant human TNF- α was obtained from Cetus Corp. (Emeryville, CA).

Blood. Normal human blood was obtained from several healthy individuals by venipuncture using pyrogen-free syringes and needles. The blood was anticoagulated with 0.2% EDTA (Vacutainer[®], Baxter, Irvine, CA) for FACS[®] studies or with 10 U/ml heparin (Lymphomed, Deerfield, FL) for functional assays, and assayed within 1 h after sampling. Plasma was obtained by centrifugation of heparinized blood. Purified neutrophils and mononuclear cells from normal human blood were obtained using a standard Ficoll-Paque density gradient as described elsewhere (14). Analysis by stan-

dard microscopy revealed that each cell population was >95% pure. Blood from two patients with PNH was obtained and treated the same way as normal human blood. A blood profile, including blood smear and cell counts, was obtained on the day of the assays for the normal donors and PNH patients. At the time of the study, the two patients were clinically free of infection or thromboembolic disease, did not show any signs of active hemolysis, and had not received blood transfusions in the past 6 mo. Soluble CD14 plasma levels were measured using a sandwich ELISA. Briefly, microtiter plates coated with 28C5 mAb were incubated with dilutions of serum or purified recombinant soluble CD14. Biotinylated 18E12 mAb was added and detected using a streptavidin-horseradish peroxidase conjugate (Zymed Laboratories, Inc., South San Francisco, CA) and *o*-phenylenediamine (OPD) substrate (Sigma Chemical Co.). Plates were read at 490 nm. LBP plasma levels were assessed using a similar ELISA, but instead of the mAbs, a polyclonal goat anti-human LBP IgG Ab was used as capture Ab, and the same polyclonal Ab biotinylated as the detecting Ab. Purified native human LBP was used as standard.

Endothelial Cell Activation Assay. HUVECs were plated in 96-well microtiter plates as described (6) 2–3 d before the assay. Confluent HUVECs were washed three times with endotoxin-free RPMI supplemented with 1 mg/ml human serum albumin (dilution buffer), incubated with different dilutions of *E. coli* 0111:B4 or *S. minnesota* wild-type LPS, together with 4% normal or PNH blood, or 2% of the corresponding plasma. Final volume was 0.2 ml/well. Experiments were done in triplicate and repeated at least once. 18E12 mAb was added to either blood or plasma to block CD14, and IB4 mAb was used for CD18 inhibition. In other experiments, hIL-10 and anti-hIL-10 were added to the LPS-blood mixture. All reagents were simply mixed together in the wells, without any preincubation period for the inhibitors. In one experiment, purified neutrophils and mononuclear cells were resuspended in dilution buffer at a concentration corresponding to 4% whole blood. 2% normal human plasma and different dilutions of 0111:B4 LPS were added to the purified cells and the mixtures were transferred to HUVEC monolayers in 96-well plates. In another experiment, 4% normal human whole blood was incubated with different doses of LPS for 6 h. Blood was then centrifuged, supernatants were collected and incubated in parallel with 4% whole blood mixed to the same doses of LPS as those used with whole blood. After 6-h incubation, supernatants were harvested and kept at -20°C for IL-8 determination. At the end of all experiments, the endothelial cells were washed five times with dilution buffer and VCAM-1 expressed by the HUVEC was determined by direct ELISA on the cell surface using a first anti-hVCAM-1 mAb and a second peroxidase-conjugated goat anti-mouse Ab (6).

Whole Blood Assay. LPS activation of whole blood was tested using normal and PNH blood as described (15). Briefly, 250 μ l of heparinized blood was incubated 4.5 h in 1 ml pyrogen-free polypropylene tubes (Sarstedt, Inc., Sparks, NV) with 0111:B4 or Re595 LPS. In some experiments hIL-10 was added. At the end, blood was resuspended using 750 μ l of dilution buffer (1:4 dilution) and then centrifuged. TNF activity in supernatants was determined using the sensitive WEHI 164 cell line clone 13-derived bioassay (16). Typically the limit of detection of this assay is 0.25 U/ml of TNF activity. Results obtained with supernatants were multiplied by four and expressed as TNF U/ml of whole blood (sp act of the rhTNF standard, 5×10^7 U/mg).

Adaptation. In some experiments, we tested blood adapted to small doses of 0111:B4 LPS. Whole normal heparinized blood was incubated with doses of LPS ranging from 1 pg/ml to 2.5 ng/ml in sterile, LPS-free siliconized glass tubes for 11 h at 37°C in 100%

humid atmosphere containing 5% CO₂. Blood was then centrifuged to collect the cells and the TNF activity in the supernatant plasma was measured. The collected blood cells were then washed three times with dilution buffer and finally resuspended by adding 1.2 volume of the cell pellet using fresh plasma from the same donor kept at 4°C during the adaptation period. This adapted whole blood was then challenged with LPS using LPS doses ranging from 0.03 to 100 ng/ml. To obtain mock-adapted blood, blood from the same donor was stored at 37°C, centrifuged, and resuspended in the same manner as adapted blood except that addition of LPS was omitted. This mock-adapted blood was challenged with LPS in parallel with the adapted samples. LPS- and mock-adapted blood were further tested for their ability to support indirect HUVEC activation in the presence of a challenge dose of 0111:B4 LPS.

FACS[®] Analysis. For analysis of CD14 and CD11b surface expression by myeloid cells, whole blood from normal donors and PNH patients was incubated 45 min with PE-labeled anti-CD14 (MY4) and FITC-labeled anti-CD11b (IOM1) or their isotype controls. Blood was then washed three times with PBS, pH 7.3 (Irvine Scientific, Santa Ana, CA), incubated for 2 min with red cell lysis buffer (Coulter Corp.), fixed, washed three more times with PBS, and finally resuspended in 1 ml PBS for FACS[®] analysis, according to the protocol of the manufacturer (red cell lysis kit, Coulter Corp.). White blood cells were analyzed using the FACScan[®] (Becton Dickinson Immunocytometry Systems, San Jose, CA), compensated for dual colors, and gated for myeloid cells (excluding lymphocytes from the final analysis). Percent expression of a surface marker was assessed using quadrants in the two colors mode. Thresholds were determined using the PE and FITC isotype controls. Monocytes and neutrophils were also gated and analyzed separately. Anti-CD14 mAb recognizing different epitopes were used for CD14 staining of normal and PNH blood cells. These included 28C5, 18E12, MY4, 63D3, and UCHM1. In these experiments, we used an indirect method with a FITC-labeled goat anti-mouse IgG second Ab. Omission of the first Ab and isotype controls served as negative controls, whereas anti-CD18 IB4 mAb served as positive control.

Results

Blood-dependent Enhancement of LPS-induced Endothelial Cell Activation. We first determined the relative importance of whole blood compared with plasma to support an LPS-induced response of endothelial cells (HUVEC). As shown in Fig. 1 A, in the presence of whole blood, 1,000-fold less LPS was required to achieve the level of HUVEC activation (assessed by VCAM-1 upregulation) observed with plasma alone. Endothelial cells coincubated with whole blood became fully activated with picomoles of LPS, whereas nanomolar quantities of LPS were needed when only plasma was present. Substitution of blood for plasma also induced a 15–50-fold increase in HUVEC sensitivity to LPS when IL-8 was measured (data not shown). In the absence of HUVEC, only small amounts of IL-8 (<10%) were measured in the same volume of blood stimulated with the same range of LPS doses, suggesting an endothelial cell origin for most of the IL-8 in the whole blood experiments (data not shown). To determine the importance of the CD14 molecule in the LPS-dependent activation of endothelial cells, the anti-CD14 mAb 18E12 was added to plasma or blood incubated with LPS and HUVEC. In both experiments with plasma and with blood,

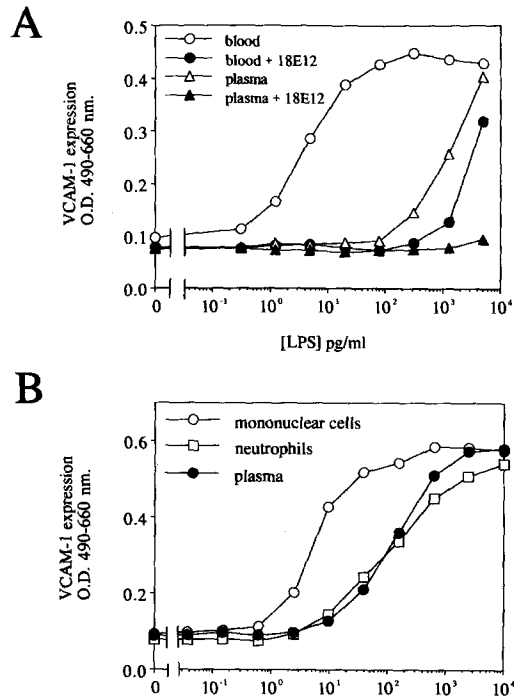


Figure 1. (A) Upregulation of VCAM-1 by HUVEC incubated 6 h with *S. minnesota* wild-type LPS in presence of 4% whole blood (O), 2% plasma (Δ), 4% blood plus 25 μg/ml anti-CD14 mAb 18E12 (●), or 2% plasma plus 25 μg/ml 18E12 (▲). (B) Upregulation of VCAM-1 by HUVEC incubated 6 h with 0111:B4 LPS in presence of purified blood mononuclear cells (equivalent to 4% whole blood [O]), purified neutrophils (□), or 2% plasma (●).

LPS-induced activation of endothelial cells was strongly inhibited by 18E12 (Fig. 1 A). Addition of purified mononuclear cells (containing monocytes) to plasma was sufficient to reconstitute the response noted with whole blood. In contrast, addition of purified neutrophils to plasma had no enhancing effect (Fig. 1 B). However, purified mononuclear cells added to plasma did not completely restore the effect of whole blood. This problem is thought to be due to the hyporesponsiveness of monocytes to LPS, once isolated from whole blood. This has been a constant finding in our laboratory.

Ability of PNH Blood to Enhance LPS Activation of Endothelial Cells. To further address the question of the participation of membrane-bound CD14 in this blood-dependent enhancement of endothelial cell sensitivity to LPS, we tested the ability of blood from normal donors or PNH patients to cause an upregulation of VCAM-1 in HUVEC stimulated with LPS. Typically, blood cells from PNH patients lack GPI-anchored membrane proteins and therefore membrane CD14 is poorly expressed if not absent (17–19). To confirm the diagnosis of PNH in our patients and define their level of mCD14 expression, we stained blood cells with PE-labeled anti-CD14 mAb MY4. As shown in Fig. 2, neutrophil expression of mCD14 was absent in the first PNH patient and minimally expressed in the second (<15% of positive cells) compared with blood

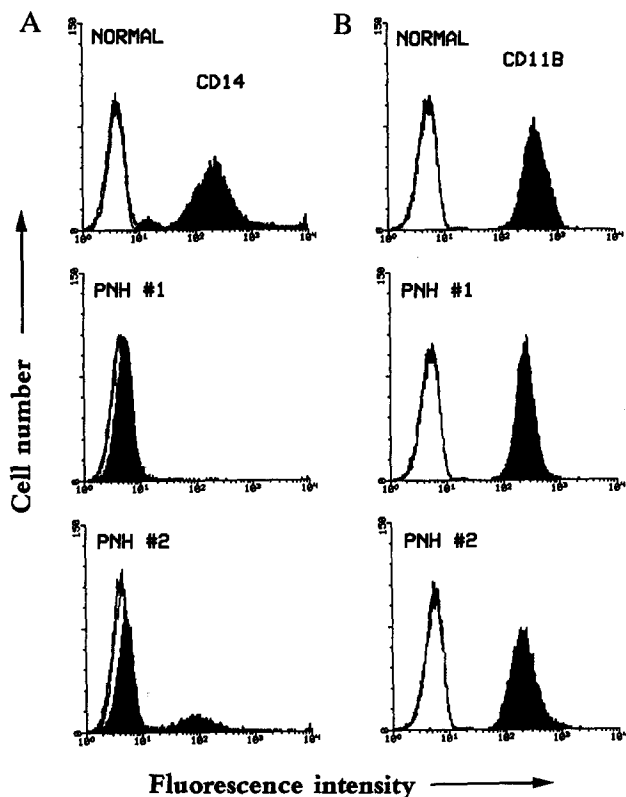


Figure 2. FACS[®] analysis of neutrophils from whole blood obtained from a normal individual and two patients with PNH incubated with (A) PE-labeled anti-CD14 mAb MY4 (filled curves), or PE-labeled isotype control or no Ab (unfilled curves); (B) FITC-labeled anti-CD11b mAb IOM1 (filled curves) or FITC-labeled isotype control or no Ab (unfilled curves).

from a normal donor. Similar results were obtained by gating on the monocyte population. CD14 staining patterns were identical when five different anti-CD14 mAbs recognizing distinct epitopes were used (data not shown). In contrast, CD11b (Fig. 2) and CD18 (data not shown), transmembrane subunits of the CD11b/CD18 (MAC-1) integrin, were expressed at normal levels in PNH myeloid cells. Monocyte and neutrophil counts in the two PNH patients were not sig-

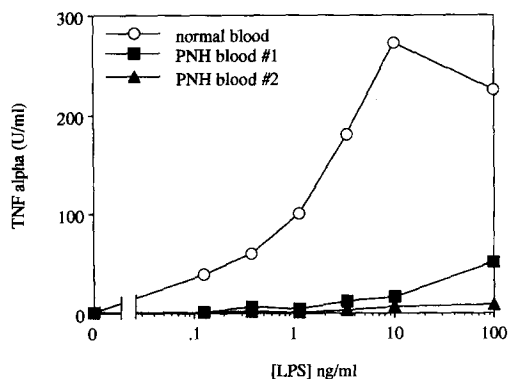


Figure 3. Bioactive TNF- α production in whole blood from a normal individual (O) and two patients (▲ and ■) with PNH incubated 4.5 h with *S. minnesota* Re595 LPS.

nificantly different from normal donors. Functional studies using blood from these patients showed that their level of TNF production was very low in response to Re595 LPS (Fig. 3) or 0111:B4 LPS (data not shown), compared with normal blood. Importantly, whole blood from PNH patients was fully responsive to heat-killed *S. aureus*, a CD14-independent agonist for TNF production by monocytes (3). In blood-HUVEC cocubation experiments, the ability of PNH blood to participate in the upregulation of VCAM-1 by HUVEC stimulated with LPS was much lower than normal blood, only barely distinguishable from the curves obtained with their corresponding plasma (Fig. 4). However, plasmas from PNH patients were as potent as normal plasma to enable HUVEC activation by LPS, suggesting normal activity of soluble CD14 and LBP in these plasmas (Fig. 4). Indeed, levels of 3.8, 5.4, and 2.2 $\mu\text{g/ml}$ of soluble CD14 were measured in the plasma from three PNH patients (two from San Diego and one from Seattle), as compared with 1.6, 2, and 2.8 $\mu\text{g/ml}$ in the normal donors used in these studies. Plasma LBP levels were also comparable, 36, 19, and 12 $\mu\text{g/ml}$ for the PNH patients and 26, 25, and 16 $\mu\text{g/ml}$ in the normal donors.

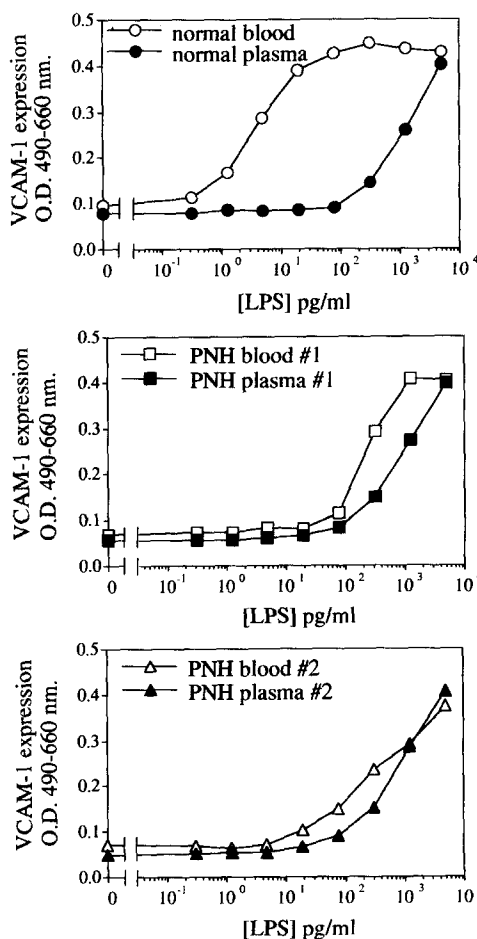


Figure 4. Upregulation of VCAM-1 by HUVEC incubated 6 h with *S. minnesota* wild-type LPS in the presence of 4% whole blood (open symbols) or 2% plasma (closed symbols) from a normal donor (A) and two PNH patients (B and C).

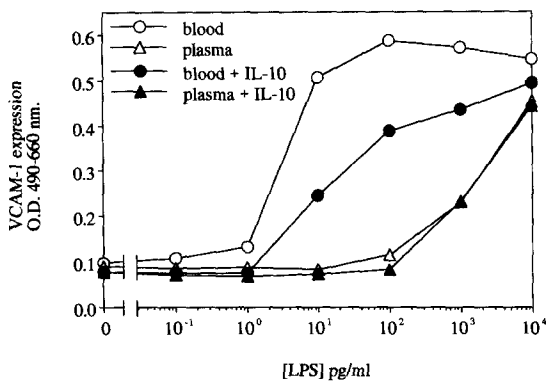


Figure 5. Upregulation of VCAM-1 by HUVEC incubated 6 h with 4% whole blood (O), 2% plasma (Δ), 4% whole blood containing 40 ng/ml human recombinant IL-10 (\bullet) or 2% plasma containing 40 ng/ml human recombinant IL-10 (\blacktriangle).

Effect of hIL-10. IL-10, produced by a variety of cells, including T h cells and macrophages, has been described as a deactivating substance for monocytes/macrophages (20, 21). In experiments not shown here, hIL-10 completely inhibited LPS-induced TNF production in whole human blood or by rabbit peritoneal elicited macrophages with doses of hIL-10 as low as 20 ng/ml. To assess the importance of the cytokine secretion by monocytes in endothelial cell activation, we incubated hIL-10 with LPS, blood, and HUVEC. As shown in Fig. 5, 40 ng/ml hIL-10 had a clear inhibitory effect, although not as dramatic as anti-CD14 mAb. No further inhibitory effect was observed when hIL-10 levels were raised to 1 μ g/ml (data not shown). An anti-hIL-10 mAb added to hIL-10 restored the LPS-induced activation of HUVEC,

indicating the specificity of hIL-10 effects (data not shown). However, hIL-10 had no direct effect on LPS-induced HUVEC activation when only plasma was present (Fig. 5).

Adaptation of Whole Blood to LPS and the Effect of Adaptation on LPS-induced Activation of Endothelial Cells. Monocytes and macrophages adapted to low doses of LPS become hyporesponsive to a new challenge of LPS as assessed by TNF secretion (22). We tested the ability of adapted versus mock-adapted blood to enhance HUVEC activation. TNF activities found after 11-h incubation of whole blood with various primary doses of 0111:B4 LPS are shown in Table 1. After washing and resuspension of the cell pellet in fresh plasma, adapted blood was partially resistant to a new LPS challenge, as compared with mock-adapted blood (see Table 1). This decrease in TNF production in adapted blood was confirmed at different doses of challenge LPS, ranging from 0.03 to 100 ng/ml (data not shown). We then tested if whole adapted blood would still enhance endothelial cell activation in response to a challenge dose of LPS. As shown in Table 1, blood adapted to small doses of LPS was less potent than mock-adapted blood to coactivate HUVEC with LPS. Fig. 6 shows the ability of blood adapted to 2.5 ng/ml LPS, mock adapted blood and plasma to induce activation of HUVEC stimulated with LPS. In experiments not shown here, we have determined whether endothelial cells could be adapted to small doses of LPS in presence of serum. Using IL-8 as a marker for endothelial cell activation, no adaptation phenomenon was observed in these cells, i.e., "adapted" and nonadapted endothelial cells respond the same to a challenge dose of LPS.

Role of a Soluble Factor Versus Direct Cell-Cell Interactions in Blood-dependent HUVEC Activation by LPS. Besides my-

Table 1. Effect of Blood Adapted to Primary Doses of LPS on TNF Production in the Same Blood Challenged with a New Dose of LPS, and VCAM-1 Expression in HUVEC Coincubated with Adapted Blood after a New Challenge Dose of LPS

Primary dose of LPS (pg/ml)	0 (= mock adapted)	1	10	100	500	2500
Primary TNF response (U/ml)	<1	<1	<1	7	34	79
After 1 ng/ml LPS challenge of whole blood						
Secondary TNF response in whole blood (U/ml)	47	38	23	16	8	3
Decrease in TNF activity (compared with mock-adapted blood [%])	-	19	51	66	83	94
After 3 pg/ml LPS challenge of blood incubated with HUVEC						
Decrease in VCAM expression in HUVEC incubated with adapted blood (compared with mock-adapted blood [%])*	-	4	21	38	45	56

* Calculated with OD 490-660 nm.

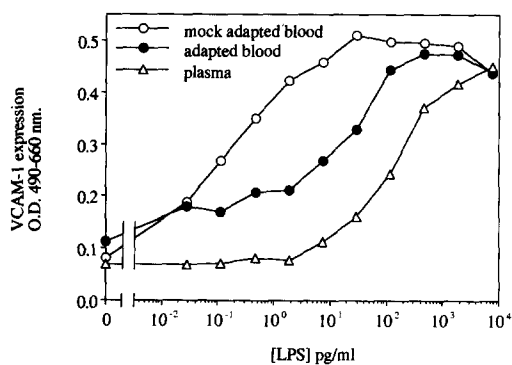


Figure 6. Upregulation of VCAM-1 by HUVEC incubated 6 h with various doses of *E. coli* 0111:B4 LPS and 4% whole mock-adapted blood (○), 4% whole blood preadapted for 11 h with 2.5 ng/ml 0111:B4 LPS (●) or 2% plasma (△).

eloid cell-derived soluble mediators, myeloid cell-endothelial cell contacts through adhesion molecules might be important for an indirect endothelial cell activation by LPS. The CD18 subunit, part of the MAC-1, and LFA-1 integrins, plays a critical role in the firm adhesion of myeloid cells to endothelial cells (23). Anti-CD18 mAb IB4, which totally blocks the CD18-dependent adhesion of myeloid cells to endothelial cells (24), was used to address this question. IB4 had no inhibitory effect on VCAM-1 upregulation (Fig. 7) or IL-8 production (data not shown) by HUVEC, suggesting a minor role for CD18-dependent adhesion of myeloid cells to endothelial cells in LPS-induced activation of HUVEC. We then addressed the involvement of a soluble factor(s) produced in blood upon LPS stimulation versus a direct contact between blood cells and endothelial cells as the trigger of endothelial cell activation. We found that cell-free, conditioned plasma from blood incubated with doses of LPS in the picomolar range was sufficient to reconstitute the level of HUVEC activation observed with whole blood (Fig. 8). Thus, direct contact of blood cells to endothelium in the presence of LPS does not seem to be required to produce the enhancing effect. In contrast, a stable and soluble factor(s) produced in whole blood mediates the amplification noted with whole blood.

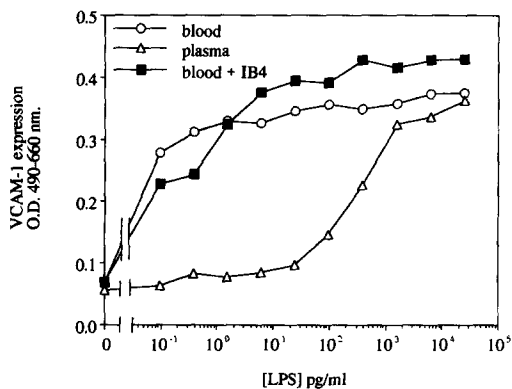


Figure 7. Upregulation of VCAM-1 by HUVEC incubated 6 h with various doses of *E. coli* 0111:B4 LPS and 4% whole blood (○), 4% whole blood containing 50 μ g/ml anti-CD18 mAb IB4 (■), or 2% plasma (△).

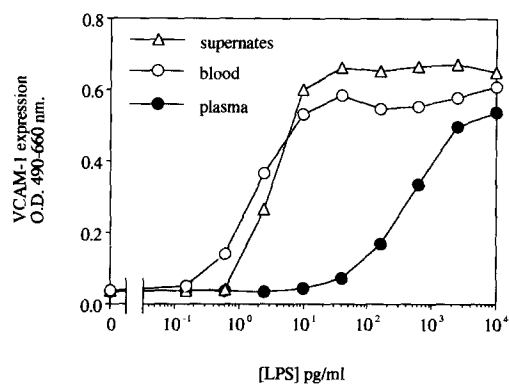


Figure 8. Upregulation of VCAM-1 by HUVEC incubated 6 h with 0111:B4 LPS and 4% blood (○), 2% plasma (●) or with plasma harvested from 4% blood preincubated 6 h with 0111:B4 LPS (supernates, △).

Discussion

This work addresses the question of the relative importance of the direct and the indirect pathway for endothelial cell activation by LPS via soluble CD14. We found that when endothelial cells were incubated with whole blood, they were fully activated with picomolar doses of LPS, whereas nanomolar quantities of LPS were required for the same level of activation when only plasma was used. These picomolar levels of LPS are consistent with the levels measured in plasma from endotoxemic or septic patients (25, 26). The first indication of the crucial role of CD14-bearing cells in blood to produce this enhancement came from blockade experiments involving anti-CD14 mAb. Indeed, endothelial responses to LPS incubated with blood could be almost completely blocked by the anti-CD14 mAb.

Another important demonstration that membrane-bound CD14 and myeloid cells had a central role in this enhanced sensitivity of endothelial cells to LPS came from experiments where blood from patients with PNH was substituted for normal blood. PNH blood cells lack GPI-anchored proteins because of a mutation in an enzyme critical in the early synthesis of GPI anchors (27, 28). As demonstrated in this work and by others (18, 19), CD14 is missing or poorly expressed in myeloid cells from PNH patients. However, normal to elevated levels of soluble CD14 were found in the plasma of these patients, as is the case for other GPI-linked proteins found in soluble forms in PNH plasma (29, 30). These particular features of PNH blood gave us a unique opportunity to test the involvement of cell-associated CD14 in the indirect pathway of LPS-induced endothelial cell activation. We found that PNH blood was hyporesponsive to LPS to produce TNF. In blood from another PNH patient, both TNF- α and IL-6, as well as IL-8 mRNA were produced at lower than normal levels in response to LPS (Martin, T. R., University of Washington, Seattle, WA, personal communication). These observations reflect the poor mCD14 expression by PNH myeloid cells. However, the involvement of GPI-anchored molecules other than mCD14 cannot be totally ruled out. Using heat-killed *S. aureus*, a CD14-independent agonist for monocyte/macrophage activation, we demonstrated that PNH blood cells were not deficient in TNF production per se. PNH blood

did not enhance endothelial cells activation above the level of activation dependent on the direct pathway, i.e., dependent on soluble CD14, suggesting again a crucial role for membrane-bound CD14 and myeloid cells in this system. It is also interesting to notice that soluble CD14 (present in PNH plasma) does not seem to substitute for membrane-bound CD14, despite an interaction of sCD14 with its ligand, LPS, in serum (6). In another system involving a GPI-anchored receptor, the ciliary neurotropic factor (CNTF) receptor α , soluble complexes of CNTF receptor α and its ligand (CNTF) are as potent as the GPI-attached complex to interact with the two other receptor complex components (LIF β and GP130) to transduce a signal to the interior of the cell (31, 32). This does not seem to be the case for the LPS-CD14 pathway. Experiments using purified blood leukocytes allowed us to demonstrate that the enhancing effect of whole blood was a property of the mononuclear cell fraction, but not the neutrophils. Because the whole blood amplification effect is blocked with anti-CD14 mAbs and is absent in PNH blood, we conclude that it is the monocyte that is responsible for this effect and not the lymphocyte present in the mononuclear fraction.

Adhesion of myeloid cells to endothelial cells is a complex, multistep event involving different molecules at the surface of both cell types. Among them, the CD18 molecule, subunit of the MAC-1 and LFA-1 leukocyte integrins, is critical for a firm adhesion of neutrophils and monocytes to endothelial cells (23). Cellular activation dependent on the adhesion process is poorly understood. For example, it is not known if adhesion events are quantitatively important for triggering cells to produce proinflammatory molecules. Using anti-CD18 mAb IB4, our data suggest that CD18-mediated adhesion of white blood cells is not important for the initial activation of endothelial cells to upregulate VCAM-1 or produce IL-8.

Soluble mediators including cytokines released upon LPS stimulation are very likely to participate in the activation observed in endothelial cells incubated with LPS and normal blood (8). Supporting this point, IL-10, which inactivates monocytes for cytokine production (20, 21), partially inhibited the blood-dependent activation of endothelial cells. Interestingly, IL-10 had no effect on endothelial cells directly activated with LPS and plasma, suggesting the absence of an IL-10 receptor in these cells. Moreover, the evidence that the transfer of plasma from blood incubated with LPS is sufficient

to reproduce the enhancing effect observed with whole blood indicates that this amplification effect is likely to be mediated by a soluble and stable mediator(s) produced in blood. Leukocyte-endothelial cell interactions are not required for the enhancement because of the addition of whole blood.

These findings raise the question of the *raison d'être* of the direct pathway of endothelial cell activation, involving LBP and soluble CD14, since the indirect pathway seems to require three orders of magnitude less LPS to achieve the same level of endothelial cell activation. The phenomenon of adaptation of blood to LPS may indicate an answer. Since myeloid cells in prolonged contact with LPS become hyporesponsive (effect demonstrated both *in vitro* [22] and *in vivo* [33]) then, with time, the direct pathway might become important for maintaining endothelial cell activation. Interestingly, the magnitude of the decrease of endothelial cell activation by LPS when incubated with adapted blood was less than the decrease of TNF activity in the same adapted blood challenged with LPS. This suggests that the presence of at least one other mediator besides TNF is responsible for the LPS-dependent indirect endothelial cell activation observed with whole blood. The direct pathway might also play an important role in the extravascular space. During sepsis, increased permeability of the endothelium would allow transudation of plasma. Epithelial cells would be activated in the presence of LPS, LBP, and soluble CD14 to produce IL-8 (6) and upregulate ICAM-1 (Pugin J., unpublished observation), a receptor for neutrophil integrins. Thus, epithelia activated by LPS might play an important role in the initial phase of tissue inflammation by attracting and activating neutrophils *in situ*. Finally, in a disease such as PNH where the indirect pathway is nonfunctional, the direct pathway of endothelial cell activation might be crucial for an inflammatory response to LPS.

In conclusion, we have shown that in the activation of human endothelial cells by LPS in the presence of blood, the indirect pathway, involving mCD14 on monocytes, is quantitatively more important than the direct pathway dependent on LBP and soluble CD14. However, whether the endothelial cells are activated directly or indirectly, the CD14 molecule plays a critical role. Monocyte-derived stable, soluble mediators are responsible for the indirect pathway and further work is being pursued to identify them.

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