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Leptospira icterohemorrhagiae and Leptospire Peptidolgycans Induce Endothelial Cell Adhesiveness for Polymorphonuclear Leukocytes

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We have examined the effect of the virulent *Leptospira interrogans* strain Teramo, serotype *icterohemorrhagiae*, on the adherence of human neutrophilic polymorphonuclear leukocytes (PMN) to cultured human umbilical vein endothelial cells (HEC). Selective pretreatment of HEC with intact or sonicated leptospires caused a dose- and time-dependent increase of HEC-PMN adhesion (13.2% ± 2.5% adherence to untreated HEC versus 46.3% ± 5.6% adherence to HEC pretreated for 4 h with 10^8 intact leptospires per ml [mean ± standard error of six experiments; $P < 0.001$]). In contrast, selective leptospire pretreatment of PMN or the addition of leptospires during the adherence assay did not alter HEC-PMN adherence. Leptospire induction of endothelial-cell adhesiveness occurred without detectable HEC damage and was prevented by RNA and protein synthesis inhibitors and by monoclonal antibodies to the CD11/CD18 adhesion complex of neutrophils and to the endothelial-leukocyte adhesion molecule 1 (ELAM-1) of endothelial cells. Similar results were obtained with pretreatment of HEC with interleukin-1 or with the lipopolysaccharide (LPS) of the gram-negative bacterium *Escherichia coli*. The possibility that contamination by the LPS of gram-negative bacteria could be involved in the induction of HEC adhesiveness was ruled out by the observation that the LPS inhibitor polymyxin B, which abolished the proadhesive effect of *E. coli* LPS, was ineffective in inhibiting leptospire- as well as interleukin-1-induced adherence. Similarly, leptospire LPSs seemed to have no role in the increase of endothelial-cell adhesiveness, since pretreatment of HEC with a leptospire LPS extract (phenol-water method) or with a leptospire total lipid extract failed to induce the proadhesive phenotype for neutrophils. Instead, peptidoglycans extracted from our leptospires actively stimulated the endothelial proadhesive activity for neutrophils (16.5% ± 2.1% adherence to untreated HEC versus 51.2% ± 2.9% adherence to HEC pretreated for 4 h with 1 μg of peptidoglycan per ml; [mean ± standard error of four experiments; $P < 0.001$]). This peptidoglycan-induced activity was inhibited by monoclonal antibodies to the CD11/CD18 adhesion complex and to ELAM-1 but not by polymyxin B. We conclude that peptidoglycans from pathogenic leptospires are among the molecules that can directly activate vascular endothelial cells to increase their adhesiveness for neutrophilic granulocytes. These observations may contribute to a better understanding of the mechanisms whereby non-gram-negative bacteria modulate the local and systemic inflammatory reaction.

The adherence of circulating neutrophils to vascular endothelial cells is essential for their migration into tissues. Basically, two types of neutrophil–endothelial-cell adherence mechanisms are known, namely, (i) an endothelial-cell-dependent mechanism in which endothelial cells are induced by various stimuli to display a proadhesive phenotype for neutrophils, consisting of the expression of adherence molecules, and (ii) a neutrophil-dependent mechanism in which other types of stimuli induce expression and/or activation of adherence molecules on the surface of these cells (5, 13, 16, 18, 26). In bacterial inflammations, the adherence of neutrophils to the vascular endothelium and their subsequent migration into inflammatory sites may be influenced in both ways. For example, the chemotactic formyl-peptides released by certain types of bacteria are known to cause the activation of adherence molecules on the surface of neutrophils (13, 18), whereas the lipopolysaccharide (LPS) of gram-negative bacteria induces de novo synthesis and expression of adherence molecules on endothelial cells (29, 31). Besides LPS, however, no other bacterial component, either from gram-negative bacteria or non-gram-negative bacteria, that may be able to induce the endothelial proadhesive phenotype for neutrophils is known. In a recent study, we found that peptidoglycans (PG) from the non-gram-negative bacteria leptospires induce a series of biological effects in vitro that are reminiscent of the effects of gram-negative bacterial LPS, e.g., complement activation and stimulation of leukocyte phagocytosis and lymphocyte mitogenesis (9). Since generalized vascular alterations that closely resemble those observed in gram-negative bacterial endotoxemia are the histologic hallmark of severe leptospirosis (2, 11, 19), we explored the possibility that such changes could be correlated with an LPS-like effect of leptospire PG on endothelial cells.

In the present paper, we show that intact leptospires as well as leptospire PG induce a proadhesive activity for neutrophils on cultured human endothelial cells (HEC) which is linked to protein synthesis-dependent expression of specific adhesion molecules on the endothelial cell surface, thus closely resembling the effect on HEC by the LPS of gram-negative bacteria.

MATERIALS AND METHODS

Cell culture. HEC were prepared by collagenase treatment of the vessels as described elsewhere (20) and maintained in endotoxin-free RPMI 1640 medium (Gibco-BRL, Edimburgh, United Kingdom) supplemented with 10% newborn bovine serum (Flow Laboratories Ltd., Irvine, United Kingdom) and 10% fetal calf serum (Biochrom KG, Berlin, Germany; NBS-FCS). Passaged HEC were maintained in RPMI 1640 medium supplemented with 20% NBS-FCS containing heparin (90 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and endothelial cell growth factor (50 μg/ml) as described by Thornton et al. (35). Endothelial cell

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growth factor was prepared from bovine hypothalamus by the method of Maciag et al. (23).

Neutrophil isolation. Peripheral blood was obtained by venipuncture from healthy donors. The blood was collected in syringes containing 15% (vol/vol) sterile acid-citrate-dextrose (ACD) solution (100 mM disodium citrate, 128 mM glucose [pH 5.0]), and the neutrophils were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient centrifugation, 3% dextran sedimentation, and hypotonic saline lysis of contaminating erythrocytes (6). This procedure resulted in a preparation containing greater than 95% neutrophils, which exceeded 95% viability by trypan blue dye exclusion. Isolated peripheral blood neutrophils were washed with phosphate-buffered saline (PBS; Gibco-BRL) and suspended at a final concentration of 7×10^5 cells per ml in PBS containing 5 mM glucose, 1 mM $CaCl_2$, and 1 mM $MgCl_2$.

Leptospire strain and cultivation. The *Leptospira interrogans* strain used in this study was named Teramo, serovar copenhageni, serogroup icterohemorrhagiae, and was from the *Leptospira* Reference Collection of the Institute of Microbiology, University of Trieste. Leptospires were grown in liquid PLM-5 medium (Intergen, Purchase, N.Y.) (21) to a density of about 10^8 cells per ml, harvested by centrifugation at 10,000 × *g* for 30 min, washed twice in PBS, and resuspended to approximately 5×10^9 cells per ml.

PG extraction. PG was extracted by the procedure of Unemoto et al. (37) as described previously (9). Briefly, leptospires were washed twice in PBS and resuspended in 1% (wt/vol) sodium dodecyl sulfate (SDS) in distilled water. The suspension was shaken gently at 37°C for 18 h and then centrifuged at 110,000 × *g* for 60 min. After a second treatment with SDS, the pellet was washed with 6 M urea, resuspended in distilled water, and centrifuged at 110,000 × *g*, and the resulting pellet was suspended in 0.01 M Tris-HCl buffer (pH 7.4) containing 1 mg of trypsin per ml. The mixture was incubated at 37°C for 18 h, washed with distilled water by centrifugation at 110,000 × *g* for 90 min, and then combined with 1 mg of pronase (Sigma) per ml in 0.01 M Tris-HCl (pH 7.4). After digestion at 37°C for 18 h, the pellet was recovered by centrifugation, washed three times with distilled water, and lyophilized. For amino acid determination, the sample was hydrolyzed with 6 N HCl at 108°C for 16 h and then assayed by gas chromatography by the method of MacKenzie and Tenashuk (24). The assay showed a relative molar ratio of the most abundant amino acids, i.e., alanine, glutamic acid, glycine, and *meso*-aminopimelic acid, that meets the biochemical criteria for leptospire PG reported by Yanagihara et al. (40). A 10-μg sample of PG was checked for the presence of LPS by SDS-polyacrylamide gel electrophoresis and silver staining by the method of Tsai and Frasch (36), which detects less than 5 ng of rough LPS; no contamination by LPS was found. Moreover, the sample did not give any clotting activity by the *Limulus* test (Haemachem, St. Louis, Mo.), which is sensitive to endotoxin on the order of picograms per milliliter to a dilution of 10 ng/ml.

Preparation of leptospire LPS. One gram of lyophilized leptospires was extracted twice by the phenol-chloroform-petroleum ether technique (15) and then by the phenol-water method (39). Further purification and analytical assays of the extract were performed as described previously (8). Extraction of total lipids from leptospires was performed by the procedure described by Folch et al. (14), starting with 20 mg of lyophilized bacteria.

Adherence assay. First- to third-passage HEC were harvested with 0.05% trypsin and 0.02% EDTA in balanced salt solution (Gibco-BRL). The cells were then plated in 6.4-mm-diameter wells (Costar Cluster, Cambridge, Mass.) at 1.5×10^4 cells per ml in RPMI 1640 medium supplemented with 20% NBS-FCS. Visually confluent monolayers were formed after overnight incubation. Cells were pretreated for 4 h with the following reagents at the concentrations indicated, unless otherwise stated: 10^8 leptospire sonicates per ml, 1 μg of leptospire extracts per ml, 0.5 μg of *Escherichia coli* LPS per ml, 20 U of interleukin-1 (IL-1) per ml, 5 μg of actinomycin D per ml, 5 μg of cycloheximide per ml, and 10 μg of polymyxin B per ml. After incubation, the monolayers were washed twice with 300 μl of PBS. Before the adherence assay, the neutrophils were incubated for 15 min at room temperature in the presence or absence of the anti-CD18 monoclonal antibody (MAb) 60.3 (20 μg/ml), and HEC monolayers were incubated in the presence or absence of the anti-ELAM-1 (anti-endothelial-leukocyte adhesion molecule 1) MAb BB11 or the anti-VCAM-1 (anti-vascular cell adhesion molecule 1) MAb 4B9 (20 μg/ml). Neutrophils were then added (70 μl per well) to HEC monolayers. In some experiments, the neutrophils were incubated at 37°C for time periods of 15 min to 4 h with or without 10^7 to 10^{10} leptospires or leptospire sonicates before they were added to the HEC monolayers. After a 30-min incubation at 37°C, the monolayers were washed twice with 300 μl of PBS to remove nonadherent neutrophils. A colorimetric assay was then used to detect the neutrophils adhering to the monolayers with tetramethyl benzidine (Sigma) as the peroxidase substrate (1), as described previously in detail (12).

Cytolytic assay. HEC lysis by intact leptospires was evaluated in terms of release of ^{51}Cr by the cells essentially as described by Harlan et al. (17). Monolayers were incubated overnight with $Na^{51}CrO_4$ (1 μCi/ml in saline; 200 to 500 Ci/g; Radiochemical Centre, Amersham, United Kingdom) and then washed five times with RPMI 1640 medium to remove unincorporated radioactivity. Afterwards, 250 μl of RPMI 1640 medium with or without leptospires or a cytolysin isolated from *Gardnerella vaginalis* (30) was added to each well. Incubations were carried out at 37°C for different periods of time. Fifty microliters of supernatant fluid was withdrawn carefully from each well, transferred to plastic tubes, and counted in a gamma spectrometer. In each experiment, the maximum release of ^{51}Cr was determined from wells treated with 0.5% Triton X-100 to lyse the cells. Control (spontaneous) release was determined with wells incubated in RPMI 1640 medium alone. Results were expressed as percent specific ^{51}Cr release as follows: percent release = [(cpm test) − (cpm control)]/[(cpm Triton) − (cpm control)], where cpm is counts per minute, and test, control, and Triton refer to test wells, control wells, and Triton X-100-treated wells, respectively.

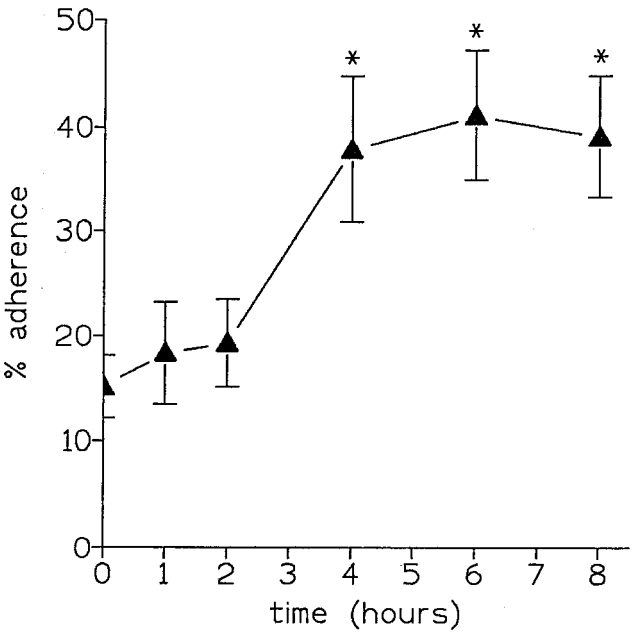

FIG. 1. Time course of leptospire-induced PMN adherence to HEC. HEC monolayers were treated for various time periods with control medium or medium containing intact leptospires (10^8/ml). Values represent means ± standard errors of five experiments. *, $P < 0.005$ compared with leukocyte adherence to HEC treated with control medium. The other results were not significantly different from control values.

MAbs. MAb 60.3 is of the immunoglobulin G2a subclass and recognizes the CD18 subunit (common beta-chain) of the CD11/CD18 antigen complex (3). MAb BB11 is a murine immunoglobulin G2b that recognizes a functional epitope on ELAM-1 (4). MAb 4B9 is a murine immunoglobulin G1 and recognizes a functional epitope on VCAM-1 (12). MAbs were generous gifts of John Harlan, University of Washington, Seattle.

Reagents. *E. coli* O55:B5 LPS, extracted by phenol-water, was obtained from Sigma. Part of the *E. coli* LPS (50 mg) obtained from Sigma was further extracted by the same procedure used for the preparation of leptospire LPS (15, 39). Both *E. coli* LPS preparations were then suspended in PBS at a concentration of 1.5 mg/ml, dispersed by sonication for 5 min at 4°C, and stored in aliquots at −35°C until used. Purified recombinant human IL-1 alpha (1,000 U/ml) was purchased from Boehringer GmbH, Mannheim, Germany.

Statistics. Significance was determined by a paired, one-tailed *t* test.

RESULTS

Leptospires induce endothelial-cell adhesiveness for neutrophils. Treatment of HEC monolayers with intact leptospires followed by washing the monolayers and by the addition of neutrophils increased the level of neutrophil adherence to the monolayers above control values, i.e., adherence to untreated HEC. Neutrophil adherence was dependent on the time of exposure of the monolayers to leptospires (Fig. 1) and on the number of leptospires added to the monolayers. Similar results were obtained with leptospire sonicates and with intact leptospires (Fig. 2). In contrast, no increase of adherence was observed when neutrophils were treated with intact leptospires or leptospire sonicates (up to 10^{10}/ml and up to 4 h) and then added to untreated HEC monolayers for a 30-min adherence assay (results not shown).

The increase of neutrophil adherence to HEC monolayers

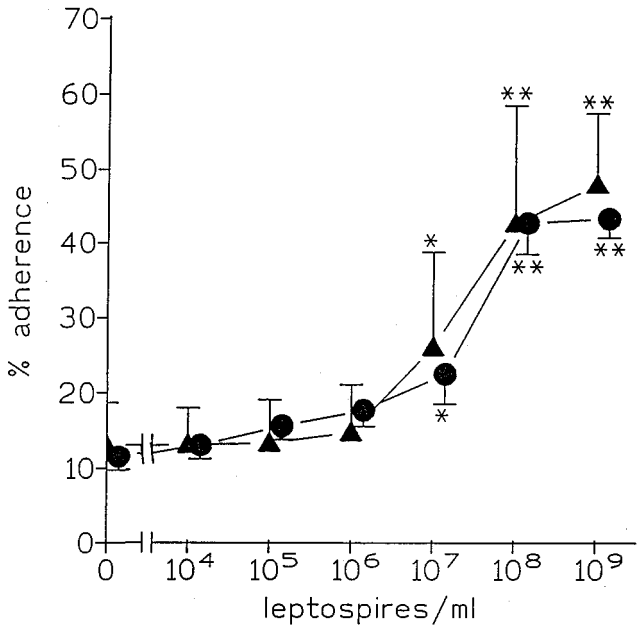

FIG. 2. Dose response of leptospire-induced PMN adherence to HEC. HEC monolayers were pretreated for 4 h with control medium or medium containing intact leptospires (▲) or leptospire sonicates (●). Values represent means ± standard errors of six experiments. *, and **, $P < 0.005$ and $P < 0.001$, respectively, compared with leukocyte adherence to HEC treated with control medium. The other results were not significantly different from control values.

that had been incubated with leptospires was not linked to lytic damage of HEC by leptospires, since treatment of HEC monolayers with leptospire suspensions of up to 10^{10}/ml and for time periods of up to 8 h caused no increase in ^{51}Cr release from the cells, as compared with the results with untreated HEC. Moreover, the same results were obtained regardless of whether neutrophils were present during the last 30 min of incubation, as in the adherence experiments. In contrast, a clear increase of ^{51}Cr release from HEC was observed when the cells were treated with a cytolytic toxin produced by *G. vaginalis* that was used as a positive control for cytolysis in our assays (results not shown).

Inhibition of leptospire-induced polymorphonuclear leukocyte (PMN) adherence to HEC. The cytokine IL-1 and the LPS of gram-negative bacteria have been shown to render HEC proadhesive for neutrophils (5, 29, 31). The effect is dependent on protein synthesis and on the expression of specific adherence molecules for neutrophils on the endothelial-cell surface, namely, ELAM-1, which binds to the neutrophil carbohydrate SLe^x (Sialyl Lewis x) (28, 38), and ICAM-1 (intercellular adhesion molecule 1), which binds to the neutrophil adhesion complex CD11/CD18 (25, 33). As shown in Table 1, blocking MAbs directed to ELAM-1 or to the leukocyte adherence molecule CD18 were effective in inhibiting neutrophil adherence to HEC treated with leptospire sonicates or with the LPS of the gram-negative *E. coli*. In contrast, neutrophil adherence to leptospire-treated HEC was not inhibited by blocking MAbs directed to VCAM-1, the endothelial ligand for the integrin receptor VLA-4 present on the surface of monocytes, lymphocytes, and eosinophils but not neutrophils (12) (three experiments; results not shown). Table 2 shows that the RNA synthesis inhibitor actinomycin D and the protein synthesis inhibitor cycloheximide abrogated the proadhesive effect on HEC of leptospire sonicates, IL-1, or *E. coli* LPS, thus indicating that even in the case of leptospires, the proadhesive activity of HEC was dependent on RNA and protein synthesis. Of note, cycloheximide (as well as actinomycin D)-treated HEC monolayers did not show evidence of cell retraction or detachment by phase-contrast or light microscopy. Further controls carried out with cycloheximide indicated that the inhibitory effect by this agent was reversible, since neutrophils actively adhered to HEC that had been treated for 4 h with the inhibitor and then washed and challenged for another 4-h period with leptospire sonicates ($P < 0.005$; four experiments). Finally, polymyxin B, which in our experiments abolished the proadhesive effect of *E. coli* LPS, was ineffective on the leptospire- as well as the IL-1-induced HEC adhesiveness.

TABLE 1. Effect of anti-ELAM-1 and anti-CD18 MAb on HEC adhesiveness for PMN after treatment with leptospire sonicates or *E. coli* LPS

Treatment of HEC	% Inhibition[a]		
	Anti-ELAM-1	Anti-CD18	Anti-ELAM-1 + anti-CD18
Leptospire-treated HEC	38.1 ± 9.5[b]	58.8 ± 6.5[b]	85.9 ± 4.4[b]
LPS-treated HEC	28.8 ± 13.5	51.0 ± 11.1[b]	88.2 ± 4.3[b]

[a] Means ± standard errors of three experiments.
[b] $P < 0.05$, compared with adherence in the absence of MAbs (controls).

TABLE 2. Effect of actinomycin D, cycloheximide, and polymyxin B on HEC adhesiveness for PMN after treatment with leptospire sonicates, *E. coli* LPS, or IL-1

Agent	% Inhibition[a]		
	Leptospires	*E. coli* LPS	IL-1
Actinomycin D	94.1 ± 5.3[b]	98.5 ± 1.5[b]	89.5 ± 6.7[b]
Cycloheximide	91.9 ± 9.1[b]	86.3 ± 10.1[b]	90.0 ± 5.6[b]
Polymyxin B	2.9 ± 1.9	96.8 ± 5.1[b]	6.6 ± 5.1

[a] Means ± standard errors of three experiments.
[b] $P < 0.05$, compared with leukocyte adherence to HEC treated in the absence of inhibitors.

Activity of leptospire extracts. The results of the experiments with polymyxin B described in the preceding paragraph suggested that LPS was not responsible for the increased neutrophil adherence to HEC treated with leptospire sonicates. This was confirmed by experiments in which HEC were pretreated with a phenol-water extract of leptospires containing a previously described LPS compound endowed with biological effects, such as activation of complement and of lymphocyte mitogenesis (7, 32). As shown in Table 3, the extract was ineffective in inducing the endothelial-cell proadhesive phenotype for neutrophils. In contrast, pretreatment of HEC monolayers with a preparation of *E. coli* LPS that had been extracted by the same procedure used for the extraction of leptospire LPS actively induced neutrophil adherence to the monolayers ($P < 0.003$; four experiments), suggesting that the negative results obtained with leptospire LPS were not due to inactivation of this LPS during the extraction procedure. Treatment of HEC with a total lipid extract of leptospires, obtained by the chloroform-methanol method, was ineffective in increasing neutrophil adherence to the endothelial monolayers, suggesting that leptospire lipids were not responsible for the proadhesive effect. In contrast, the incubation of HEC monolayers with a preparation of leptospire PG proved to induce endothelial-cell adhesiveness for neutrophils. Moreover, control experiments with boiled PG excluded a possible activation of

TABLE 3. Effect of leptospire extract-treated HEC on neutrophil adherence

Treatment	Neutrophil adherence (%)[a]
Medium (control)	16.5 ± 2.1
Leptospire sonicate	50.5 ± 8.5*
Leptospire LPS (phenol-water extract)	17.2 ± 3.0
Total lipids (chloroform extract)	15.1 ± 2.0
PG (SDS-pronase extract)	51.2 ± 2.9**
PG boiled 15 min	51.4 ± 1.2**
PG + cycloheximide	18.3 ± 1.4
PG + actinomycin D	16.3 ± 1.5
PG + polymyxin B	47.4 ± 6.7*

[a] Means ± standard errors of four experiments on different occasions. * and **, $P < 0.005$ and $P < 0.001$ (paired t test), respectively, compared with leukocyte adherence to HEC treated with control medium. The other results were not significantly different from control values.

HEC by the enzymes used for the extraction of PG, i.e., by potentially contaminating pronase and trypsin. Identical endothelial-cell–leukocyte adherence mechanisms seemed to be involved in the PG-induced adherence as well as in the total leptospire-induced adherence, since equal degrees of inhibition were obtained in the two instances by the specific adherence molecules blocking MAbs. In two experiments, the increase of adherence produced by PG (1 μg/ml) was inhibited by 38.1 and 46.8% by the anti-ELAM-1 MAb BB11 and by 47.3 and 35.6% by the anti-CD18 MAb 60.3, respectively, and by 87.6 and 79.6% when the two MAbs were used in combination. Similarly, the PG-induced adherence was blocked by actinomycin D or cycloheximide as was the total leptospire-induced adherence. Finally, the activity of our PG preparation was not inhibited by polymyxin B, thus excluding contamination by LPS.

DISCUSSION

These results demonstrate that leptospires stimulate neutrophil adherence to vascular endothelial cells. Previous studies have shown that neutrophils adhere to endothelial cells by neutrophil-dependent or endothelial-dependent mechanisms. Neutrophil-dependent adherence is induced by stimuli that directly activate the neutrophil, such as bacterial chemotactic peptides or tumor necrosis factor (13, 18). Endothelial cell-dependent adherence is triggered by endogenous compounds such as thrombin, leukotrienes C4 and D4, IL-1, and tumor necrosis factor as well as by the LPS of gram-negative bacteria. Thrombin and leukotrienes induce an almost immediate increase in endothelial-cell adhesiveness (26), whereas adherence induced by IL-1 and LPS requires de novo protein synthesis (5). Finally, tumor necrosis factor was shown to induce endothelial-cell-dependent adherence by either protein synthesis-dependent or -independent mechanisms (16). In our case, the leptospire-induced adhesiveness was of the endothelial-cell-dependent type, since it was obtained by exposing HEC, but not neutrophils, to the leptospires. Also, it resembled more the adhesiveness induced by IL-1 and LPS than that elicited by thrombin or leukotrienes, since it required a time of contact of leptospires with HEC of at least 2 h. The large number of leptospires (10^7/ml) needed to produce adherence in our assays may apply also to in vivo conditions, i.e., to leptospire infections. This would be suggested by the histologic findings of the greatest inflammation of the vascular vessels in the so-called filter organs, i.e., the liver, the kidney, and the lungs, where leptospires are known to accumulate during illness (2).

Injury of the vascular endothelium is a rather constant histological finding in fatal leptospire infections of humans (2) as well as in experimental leptospirosis (11, 19). This raises the question whether the increased adhesiveness of neutrophils to the HEC exposed to leptospires could be somehow aspecific, i.e., linked to cell changes preceding or accompanying lytic damage. However, the ^{51}Cr release assay, a commonly used test for endothelial-cell lysis (17), did not indicate any damage of our endothelial cells upon exposure to leptospires, not even when the cells were incubated with leptospires for periods longer than those required to obtain neutrophil adherence.

The following two molecules which function as adhesion receptors for neutrophils have been identified on the surface of HEC treated for at least 2 h with the cytokine IL-1 or TNF or with gram-negative bacterial LPS: (i) ICAM-1, which functions as a ligand for the CD11/CD18 neutrophil integrin (33), and (ii) the selectin ELAM-1 (5), which binds the SLe^x carbohydrate complex on the surface of neutrophils (28, 38). Upregulation of ICAM-1 and ELAM-1 by cytokine- or LPS-stimulated HEC was shown to be dependent on RNA and protein synthesis (5, 29, 31). In our assays, the anti-ELAM-1 MAb BB11 or the anti-CD11/CD18 MAb 60.3 caused a significant inhibition of neutrophil adherence to leptospire-treated HEC, and the two MAbs in combination totally abolished neutrophil adherence, indicating that the same adherence molecules for neutrophils are upregulated in HEC treated for 4 h either with leptospires, the cytokines TNF and IL-1, or LPS. A further similarity is offered by the effect of the protein synthesis inhibitors cycloheximide and actinomycin D, which prevent the proadhesive effect on HEC caused by leptospires as well as by LPS or IL-1, confirming a requirement for protein synthesis in all three instances. Of note, cycloheximide and actinomycin D did not cause morphological changes on the endothelial-cell monolayers. Moreover, the inhibitory effect of cycloheximide was reversible, thus leading us to exclude the possibility that the reagent had caused relevant damaging effects to endothelial cells in addition to inhibiting protein synthesis.

Leptospires have been shown to contain an LPS that differs substantially from the LPS of gram-negative bacteria. The leptospire LPS has a very low fatty acid content (less than 2%), does not show immunological cross-reactivity with the lipid A component of LPS, is not pyrogenic, and is ineffective in inducing the local Schwartzman reaction (8). Nevertheless, the LPS-containing extracts of leptospires were reported to possess some properties that are shared with bacterial LPS, such as complement activation and stimulation of lymphocyte mitogenesis (7, 32). Therefore, an essential point of the present investigation was to ascertain a possible role of leptospire LPS in the induction of leukocyte adherence to HEC. This was excluded by the following observations. (i) The classic LPS inhibitor polymyxin B (10, 27) prevented the adherence induced by the LPS of gram-negative bacteria but not the adherence induced by leptospires. (ii) Leptospire extracts obtained by the classic phenol-water method for the isolation of gram-negative bacterial LPS (39) failed to induce neutrophil–endothelial-cell adherence. (iii) Similarly, a total lipid extract, obtained by the chloroform-methanol method (14), gave negative results, thus excluding any possible involvement of leptospire lipids. In contrast, a PG preparation obtained from the leptospire sonicate (9) actively induced endothelial-cell adhesiveness for neutrophils, and this PG activity was not inhibited by polymyxin B. This result was not totally unexpected since previous studies have already reported a variety of biological activities by bacterial PGs, particularly by muramyl peptide

derivatives, including an adjuvant effect and activation of monocytes and lymphocytes (9, 22, 34). The observation that bacterial PGs are among the rare molecules known to directly activate endothelial cells in terms of increased adhesiveness for PMN may contribute to a better understanding of the mechanisms whereby bacteria modulate the local and systemic inflammatory reactions. Further studies are needed to characterize the basic structure or component of the PG molecule that is responsible for the induction of endothelial-cell adhesiveness for PMN.

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