Lipopolysaccharide (LPS)-Binding Protein and Soluble CD14 Function as Accessory Molecules for LPS-induced Changes in Endothelial Barrier Function, In Vitro

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Abstract

Bacterial LPS induces endothelial cell (EC) injury both in vivo and in vitro. We studied the effect of *Escherichia coli* 0111:B4 LPS on movement of ^{14}C-BSA across bovine pulmonary artery EC monolayers. In the presence of serum, a 6-h LPS exposure augmented ($P < 0.001$) transendothelial ^{14}C-BSA flux compared with the media control at concentrations ≥ 0.5 ng/ml, and LPS (10 ng/ml) exposures of $\geq$ 2-h increased ($P < 0.005$) the flux. In the absence of serum, LPS concentrations of up to 10 μg/ml failed to increase ^{14}C-BSA flux at 6 h. The addition of 10% serum increased EC sensitivity to the LPS stimulus by > 10,000-fold. LPS (10 ng/ml, 6 h) failed to increase ^{14}C-BSA flux at serum concentrations < 0.5%, and maximum LPS-induced increments could be generated in the presence of $\geq$ 2.5%. LPS-binding protein (LBP) and soluble CD14 (sCD14) could each satisfy this serum requirement; either anti-LBP or anti-CD14 antibody each totally blocked ($P < 0.00005$) the LPS-induced changes in endothelial barrier function. LPS–LBP had a more rapid onset than did LPS–sCD14. The LPS effect in the presence of both LBP and sCD14 exceeded the effect in the presence of either protein alone. These data suggest that LBP and sCD14 each independently functions as an accessory molecule for LPS presentation to the non-CD14–bearing endothelial surface. However, in the presence of serum both molecules are required. (*J. Clin. Invest.* 1994. 93:692–702.) Key words: endotoxin • adult respiratory distress syndrome • vascular permeability

Introduction

Gram-negative bacteremia and its attendant endotoxemia can serve as clinical antecedents for adult respiratory distress syndrome (1). Bacterial LPS induces acute pulmonary vascular endothelial cell (EC)[1] injury in experimental animals (2). Multiple host responses have been postulated as mechanisms for LPS-induced lung injury, including: systemic and pulmonary hemodynamic alterations, complement cleavage products, arachidonate metabolites, platelet-activating factor, granulocytes, toxic oxygen intermediates, and proteinases (2), as well as cytokines (3). LPS has been shown to augment the movement of ^{125}I-albumin across bovine pulmonary arterial EC monolayers in the absence of hydrostatic pressure changes, granulocyte effector cells, alveolar macrophages, or numerous nonendothelial-derived host mediators (4). Some LPS-induced EC responses are serum dependent (4, 5). Recently, an LPS-binding protein (LBP) has been demonstrated in the sera of various species (6–9). LBP binds to the lipid A component of LPS (10), and this LPS–LBP complex recognizes CD14 on host cells of monocyte/macrophage lineage (11). The endothelium is reportedly a non-CD14–bearing surface (12, 13). Monocyte-derived CD14, which is attached to the plasma membrane via a phosphatidylinositol glycan anchor (14, 15), can also be present in soluble form in normal serum (14, 16). This soluble CD14 (sCD14) may participate in LPS presentation to the EC (17, 18). In this report, an endotoxin-sensitive pulmonary vascular EC line (4, 5) was used to focus on the direct effect of LPS on endothelial barrier function and the influence of serum constituent(s) on this LPS–EC interaction. More specifically, we present evidence that the acute phase protein, LBP, and the monocyte differentiation antigen, CD14, can each function as accessory molecules for LPS-induced changes in endothelial barrier function. That monocytic cells shed or release a molecule into the intravascular compartment which in concert with an hepatocyte-synthesized protein can regulate LPS presentation to the non-CD14–bearing EC surface is novel.

Methods

Reagents. LPS phenol-extracted from *Escherichia coli* serotype 0111:B4 (Sigma Chemical Co., St. Louis, MO) was suspended in PBS at 1 mg/ml, and this stock solution was stored at −20°C. Rabbit and human LBP were purified from acute phase sera as described (6, 7). Purified goat preimmune IgG as well as polyclonal anti-human LBP from immune serum were prepared using ammonium sulfate precipitation (50%) and DEAE-cellulose chromatography at pH 7.7, according to standard methods (19). The goat had been immunized with human LBP expressed using the baculovirus, SF-9 insect cell system (20). Human sCD14 and mAb 28C5 specific for human CD14 were obtained from A. Moriarty and D. Leturcq (R. W. Johnson Pharmaceutical Research Institute, La Jolla, CA). Purified murine IgG_{2b} myeloma protein (Sigma Chemical Co.) was used as a control for the IgG_{2b} mAb, 28C5. The acute phase protein, human C-reactive protein (CRP) (Polysciences Inc., Warrington, PA) was used as a control for LBP. Two phosphatidylinositol-anchored proteins, human decay-accelerating factor (DAF) provided by Dr. M. E. Medof (Department of Pathology, Case-Western Reserve University, Cleveland, OH) and bovine folate-binding protein (Bio Pacific Inc., Emeryville, CA) were used as con-

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1. *Abbreviations used in this paper:* CRP, C-reactive protein; DAF, decay-accelerating factor; EC, endothelial cell; HS, human serum; HUVEC, human umbilical vein endothelial cell; LBP, LPS-binding protein; LDH, lactate dehydrogenase; sCD14, soluble CD14.

trols for sCD14. CD14-depleted human serum (HS) was prepared using affinity chromatography. Briefly, anti-human CD14 mAb 28C5 (1 mg) was immobilized using a Reactigel[R] matrix (Pierce Chemical Co., Rockford, IL) and was equilibrated with PBS. Pooled-type AB HS (Sigma Chemical Co.) (10 ml) was passed through the column. sCD14 bound to the immobilized mAb was eluted using glycin-HCl, pH 2.5. After reequilibration with PBS, the first flow through was again passed through the column, and the second flow through was considered as CD14-depleted HS. sCD14 concentrations in the starting material were 2 μg/ml HS and < 1 ng/ml in the CD14-depleted serum.

Endothelial cell culture. Bovine pulmonary artery EC (American Type Culture Collection, Rockville, MD) were grown at 37°C under 5% CO_2 in DME (Sigma Chemical Co.) and were enriched with 20% FBS (Hyclone Laboratories Inc., Logan, UT), 4 mM L-glutamine, nonessential amino acids, and vitamins in the presence of penicillin (50 U/ml) and streptomycin (50 μg/ml) (Sigma Chemical Co.). EC were washed and were gently detached with a brief (1–2 min) trypsin (0.5 mg/ml) (Sigma Chemical Co.) exposure with gentle agitation, followed immediately by neutralization with FBS-containing medium. The cells were counted and were suspended in medium for immediate seeding of assay chambers (4×10^5 cells/ml) or 6-well tissue culture plates (3.0×10^5 cells/ml). Cultures were determined to be endothelial by uniform morphology and quantitative determination of angiotensin-converting enzyme activity with commercially available ^{3}H-benzoyl-Phe-Ala-Pro substrate (Ventrex Laboratories, Portland, ME).

Assay of transendothelial albumin flux. Transendothelial ^{14}C-BSA flux was assayed as we have described (21). Polycarbonate filters (13-mm diameter, 0.4-μm pore size) (Nucleopore Corp., Pleasanton, CA) were treated with 0.5% acetic acid (50°C, 20 min), were washed in distilled H_2O, and were immersed in boiling pig skin gelatin (Fisher Scientific Co., Pittsburgh, PA) (5 mg/liter distilled H_2O) for 60 min. The filters were then dried, were glued to polystyrene chemotactic chambers (ADAPS Inc., Dedham, MA), and were gas sterilized with ethylene oxide. These chambers, which served as the upper compartment for the assay chambers, were inserted into wells of 24-well plates, each well containing 1.5 ml medium and serving as the lower compartment of the assay chamber. Each upper compartment was seeded with 2×10^5 EC in 0.5 ml medium and was cultured for 72 h (37°C, 5% CO_2). We used ^{14}C-BSA (Sigma Chemical Co.) with a specific activity of 30.1 μCi/mg protein as the tracer molecule. The baseline barrier function of each monolayer was determined by applying an equivalent and reproducible amount of ^{14}C-BSA (1.1 pmol/0.5 ml) to each upper compartment for 1 h at 37°C, after which 0.5 ml from the lower compartment was added to 4.5 ml scintillation fluid (Optifluor; Packard Instruments Co., Inc., Downers Grove, IL) and was counted in a liquid scintillation analyzer (Tri-Carb 1500; Packard Instruments Co., Inc.). Only EC monolayers retaining ≥ 95% of the ^{14}C-BSA were studied. The monolayers were then exposed to LPS at varying concentrations for increasing exposure times. Simultaneous controls with medium alone were performed. Transfer of ^{14}C-BSA across EC monolayers was again assayed.

In other experiments, serum-dependence for LPS-induced changes in endothelial barrier function was studied. EC were seeded onto gelatin-impregnated filters and were cultured in DME enriched with 20% FBS as above. Over the last 16 h of the 72-h incubation, the monolayers were serum-starved in DME alone. At 72 h, baseline barrier function was established using the permeability tracer ^{14}C-BSA in fresh medium containing no FBS but supplemented with BSA (34 g/liter) yielding a final protein concentration equivalent to DME enriched with 10% FBS. The BSA was included to control for potential nonspecific protein–LPS interactions and to minimize nonspecific binding of reagents to plastic. Then, the monolayers were incubated with media containing LPS 10 ng/ml, increasing FBS concentrations, and reciprocally decreasing BSA concentrations so that the same final protein concentration was maintained. Endothelial barrier function again was determined using ^{14}C-BSA with the same fixed BSA concentration used above. Endothelial barrier function also was assayed for serum-starved monolayers after 6-h exposures to increasing LPS concentrations (10^{-1}–10^5 ng/ml) again, in the presence of the same fixed BSA concentration used above, in the absence of FBS. To address whether LBP or sCD14 could be operative in the serum-dependent LPS effect, rabbit LBP (6), human LBP (7), or human sCD14 were each used in selected experiments in lieu of FBS. LBP (mol wt = 60,000) was used at concentrations (1.2 μg/ml) equimolar to LPS 100 ng/ml (estimated average mol wt = 5,000) (10). Human CRP and DAF as well as bovine folate-binding protein were used as protein controls. To establish a dose–response relationship between LBP and LPS-induced barrier dysfunction, increasing concentrations of rabbit LBP together with a fixed LPS concentration (100 ng/ml) were studied. Similar studies with varying concentrations of human sCD14 and the same LPS exposure were also performed. In experiments where LBP was coadministered with sCD14, lower concentrations were used.

In one set of experiments, serum-starved monolayers were studied for barrier function in the presence of 10% HS. The HS was obtained from the same individual, and was immediately aliquoted and frozen. The monolayers were exposed to LPS 100 ng/ml for 6 h with or without preincubation with either murine monoclonal anti-human CD14 antibody 28C5 (10 μg/ml) or goat anti-human LBP IgG (1 mg/ml) (19). Simultaneous controls for each antibody preparation alone as well as for species-matched irrelevant antibodies were also performed. In another set of experiments, serum-starved monolayers were exposed to LPS 100 ng/ml or media alone for 6 h in the presence of either 10% HS or 10% CD14-depleted HS from the same donor. These two sera had LBP concentrations of 27 and 23 μg/ml, respectively.

Effect of LPS on EC viability. To determine whether LPS-induced changes in endothelial barrier function could be explained by EC injury or loss of viability, LPS-exposed and medium control monolayers were serially studied for lactate dehydrogenase (LDH) release (22) and ^{51}Cr release (21) as we have described previously. EC were seeded into the wells of 24-well plates (2×10^5 cells/well) and were cultured for 72 h to achieve confluence. For the LDH release assay, the medium was decanted and was replaced with medium containing LPS 10 ng/ml or medium alone for increasing exposure times. Supernatants were harvested, and the monolayers were solubilized (1% Triton X-100, 0.5 h). LDH activity was assayed in the medium and in the cell lysate (22). Test samples were mixed with reconstituted LD-L reagent (50 mM lactate, and 7 mM NAD, pH 8.9) (Sigma Chemical Co.), and $\Delta A_{340\,nm}$ was measured at 37°C over 1.5 min in a spectrophotometer (Gilford Response II; Ciba Corning Diagnostics, Oberlin, OH). LDH release was expressed as a percentage of total LDH (LDH in medium plus LDH in cells). Correction was made for LDH in FBS. For the ^{51}Cr release assay, confluent monolayers were labeled with [^{51}Cr]sodium chromate (Amersham Corp., Arlington Heights, IL), 12 μCi/well, for 3 h at 37°C (21). The washed monolayers were incubated with either LPS 10 ng/ml or medium alone for increasing exposure times after which the supernatants were counted. To differentiate between actual EC release of ^{51}Cr and EC detachment into the supernatants, aliquots of washes were centrifuged (300 *g*, 10 min) before counting. All washed monolayers were solubilized with 1% Triton X-100 (Sigma Chemical Co.) to induce maximum release. The lysates were centrifuged, and the supernatants were counted for ^{51}Cr activity. EC injury was expressed as: (^{51}Cr supernatant)/(^{51}Cr supernatant plus ^{51}Cr cell lysate) × 100%.

Statistical methods. The mean response for each experimental group was compared with its respective control by Student's *t* test. Analyses of variance were used to compare the mean responses among experimental and among control groups. A *P* value of < 0.05 was considered significant.

Results

Dose-dependent effect of LPS on transendothelial ^{14}C-BSA flux. A 6-h LPS exposure increased transendothelial ^{14}C-BSA flux in a dose-dependent manner (Fig. 1). The mean (±SE) pretreatment transendothelial ^{14}C-BSA flux was 0.018±0.001

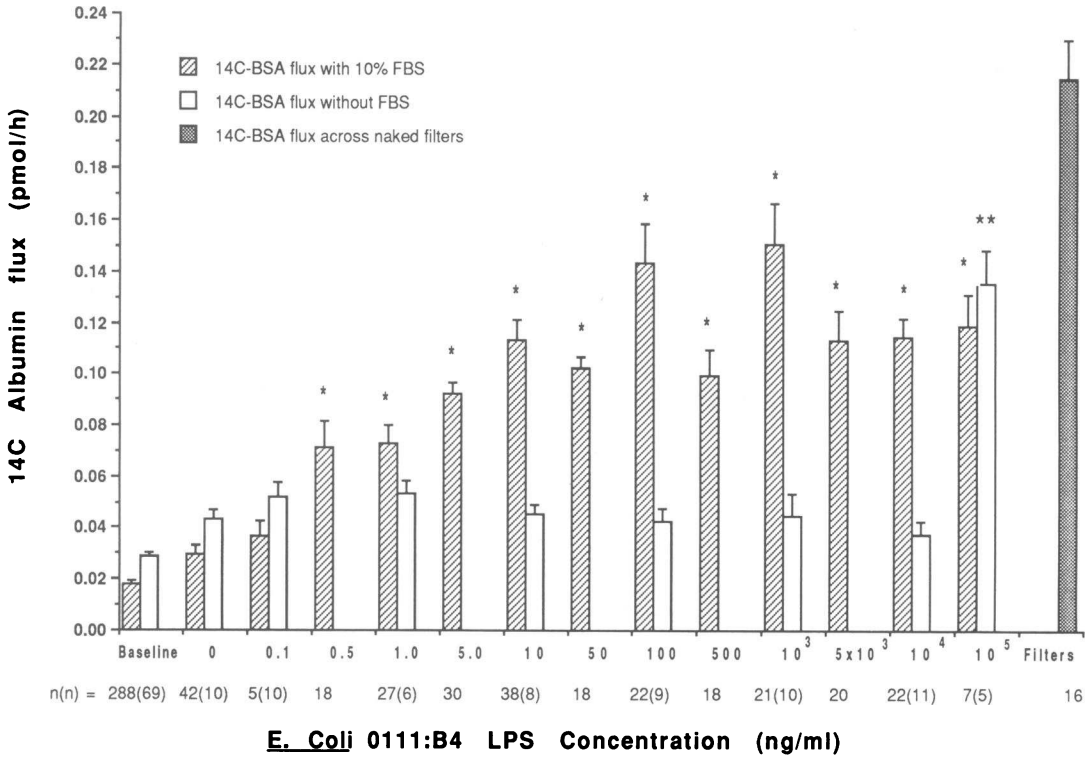

Figure 1. Dose-dependent effect of LPS on transendothelial ^{14}C-BSA flux. Crosshatched bars represent mean (±SE) transendothelial ^{14}C-BSA flux in picomoles per hour immediately after 6-h exposures to increasing concentrations of LPS in the presence of 10% FBS. Open bars represent mean (±SE) ^{14}C-BSA flux across serum-starved monolayers exposed to increasing concentrations of LPS for 6 h in the absence of FBS. Mean (±SE) pretreatment baseline transendothelial ^{14}C-BSA flux across both standard and serum-starved monolayers as well as mean (±SE) ^{14}C-BSA flux across naked filters (*stippled bar*) are also shown. *n*, number of monolayers studied in the presence of FBS. (*n*), number of monolayers studied in serum-free conditions. *Significantly increased compared with the simultaneous media control at $P < 0.001$. **Significantly increased compared with the serum-free media control at $P < 0.0001$.

pmol/h (n = 288), and the mean (±SE) ^{14}C-BSA transfer across naked filters without EC monolayers was 0.215±0.015 pmol/h. The lowest LPS concentration that induced a significant increment in ^{14}C-BSA flux compared with the media control was 0.5 ng/ml. The maximum mean (±SE) ^{14}C-BSA flux of 0.151±0.016 pmol/h was seen with LPS 1,000 ng/ml, although the LPS-induced effect seemed to plateau or saturate at doses > 10 ng/ml. The LPS effect in the absence of FBS is discussed below.

Time-dependent effect of LPS on transendothelial ^{14}C-BSA flux. The effect of LPS on endothelial barrier function was also time dependent (Fig. 2). Transendothelial ^{14}C-BSA flux was assayed immediately after increasing exposure times to LPS 10 ng/ml or media alone. There were no significant differences between ^{14}C-BSA flux across media control monolayers throughout the 6-h study period. LPS 10 ng/ml failed to induce significant increments in ^{14}C-BSA flux compared with simultaneous media controls after 0.5- and 1-h exposures. Only LPS exposures of ≥ 2 h significantly increased ^{14}C-BSA flux with further time-dependent increments throughout the 6-h study period. These studies demonstrated an LPS stimulus-to-EC response lag time of > 1 h but < 2 h.

Effect of LPS on EC injury or death. Two assays were used to determine whether an LPS exposure (10 ng/ml) that compromises endothelial barrier function also might induce EC injury or death over the same time period. LPS-exposed and media control monolayers were studied serially for LDH release and ^{51}Cr release (Table I). The total cell counts in either group did not change over the study period (data not shown). LDH release was not significantly different between experimental and control groups throughout the study period. In fact, even LPS 100 ng/ml failed to significantly increase LDH release compared with media controls at 6 h (4.05±1.37% vs 2.22±0.81%). When the more sensitive ^{51}Cr release assay was used, LPS exposure significantly augmented EC ^{51}Cr release compared with simultaneous media controls at 4 and 6 h. This assay detects defects in the plasma membrane that permit passage of molecules ≤ 1,000 D. If one uses LDH release as a more stringent detection system for membrane integrity and cell viability, these data suggest that a ≤ 6-h LPS exposure at 10 ng/ml induces EC membrane perturbation without frank loss of viability.

Influence of serum on LPS-induced changes in endothelial barrier function. The effect of LPS on endothelial barrier function was serum dependent. LPS concentrations ≤ 10^4 ng/ml failed to increase ^{14}C-BSA flux across serum-starved monolayers in the absence of FBS (Fig. 1, *open bars*). Only LPS 10^5 ng/ml significantly increased ^{14}C-BSA flux compared with the simultaneous media control. This serum-free 6-h LPS exposure at 10^5 ng/ml significantly increased EC cytotoxicity compared with media controls on the basis of LDH release (40.76±6.01 vs 8.40±1.08%, n = 12). The LPS-induced barrier dysfunction induced by a high LPS concentration in the absence of FBS was associated with EC death, unlike barrier changes seen with low LPS concentrations in the presence of FBS (Table I). Transendothelial ^{14}C-BSA flux across serum-

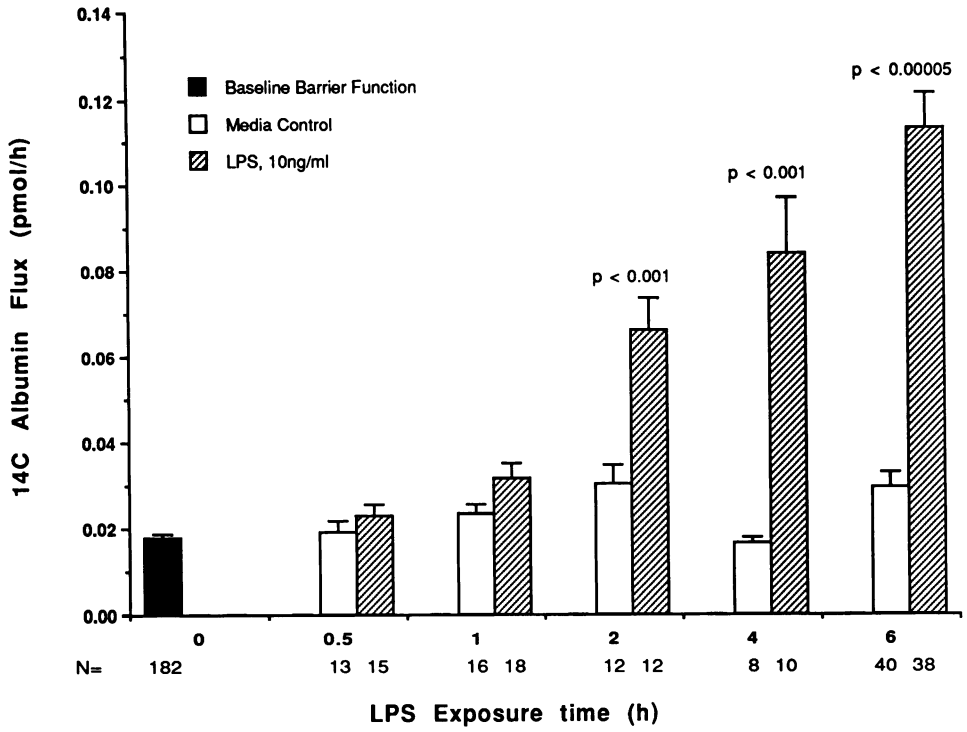

Figure 2. Time-dependent effect of LPS on transendothelial ^{14}C-BSA flux. Vertical bars represent mean (±SE) transendothelial ^{14}C-BSA flux in picomoles per hour immediately after increasing exposure times to LPS 10 ng/ml (*crosshatched bars*) and simultaneous media controls (*open bars*). Mean (±SE) pretreatment baseline is shown by the closed bar.

starved monolayers was measured after a 6-h exposure to a fixed LPS concentration (10 ng/ml) or media, both in the presence of increasing FBS concentrations (Fig. 3). The mean (±SE) pretreatment transendothelial ^{14}C-BSA flux was 0.014±0.001 pmol/h (n = 349). LPS 10 ng/ml failed to significantly increase ^{14}C-BSA flux at serum concentrations < 0.5% compared with the media controls containing equivalent FBS concentrations. The maximum transendothelial ^{14}C-BSA flux after LPS exposure (10 ng/ml, 6 h) was seen in the presence of ≥ 2.5% FBS. At the intervening FBS concentrations, i.e., 0.5–2.5%, the LPS effect on ^{14}C-BSA flux increased with increasing FBS concentrations.

Role of LBP in LPS-induced changes in endothelial barrier function. The ability of LBP to promote the LPS-induced EC response was studied (Fig. 4). LPS only in the presence of either FBS or LBP significantly increased transendothelial ^{14}C-BSA flux. The addition of BSA or CRP failed to support the LPS effect. When monolayers were exposed for 6 h to a fixed LPS concentration (100 ng/ml) in the presence of varying rabbit LBP concentrations, the LPS/LBP effect increased as the LBP concentration increased (Fig. 5). LBP concentrations ≥ 0.1 μg/ml in the presence of LPS significantly increased albumin flux compared to LPS alone; LBP alone at 3 μg/ml had no effect. The maximal LPS/LBP effect was seen in the presence of 1.0 μg/ml LBP, a close approximation of the estimated equimolar concentration of LBP for LPS at 100 ng/ml (10). LBP at > 1 μg/ml was not associated with any further increment in the LPS/LBP effect. These data are compatible with an optimal LPS/LBP ratio of 1:1 in our experimental system. When HS was used to take advantage of anti-human LBP antibodies, anti-LBP antisera completely blocked the ability of serum to support the LPS-induced endothelial barrier dysfunction (Fig. 6). In a serum-free reconstituted LPS–human sCD14 system, anti-LBP antibody had no effect, excluding cross-reactivity of the anti-LBP antibody with sCD14. Therefore, the single protein LBP could substitute for serum in the promotion of the LPS-induced changes, and in the presence of serum LBP was required.

Role of sCD14 in LPS-induced changes in endothelial barrier function. Similarly, the ability of sCD14 to alter LPS-induced changes in endothelial barrier function in the absence of serum or LBP was studied (Fig. 7). LPS with sCD14 in the

Table I. Measurements of Endothelial Cell Injury or Death after LPS Exposure

Time	1 h	(n)	2 h	(n)	4 h	(n)	6 h	(n)
LDH release (%)*								
Media control	4.93±1.74	(9)	1.98±0.77	(9)	2.97±0.79	(9)	2.22±0.81	(23)
LPS (*10 ng/ml*)	2.14±0.72	(9)	3.30±1.87	(9)	2.98±1.33	(9)	4.40±0.99	(23)
^{51}Cr release (%)‡								
Media control	5.83±0.51	(10)	8.13±0.28	(10)	8.76±0.23	(10)	13.00±0.50	(10)
LPS (*10 ng/ml*)	6.73±0.29	(10)	7.60±0.44	(10)	13.10±0.42§	(10)	16.26±1.33§	(10)

* Mean (±SE) percentage of LDH release calculated as (LDH in medium)/(LDH in medium plus LDH in cell lysate) × 100%. ‡ Mean (±SE) percentage of ^{51}Cr release calculated as ^{51}Cr activity in supernatant/total ^{51}Cr activities in supernatant and cell lysate × 100%. § Significantly increased compared with simultaneous media controls at $P < 0.04$.

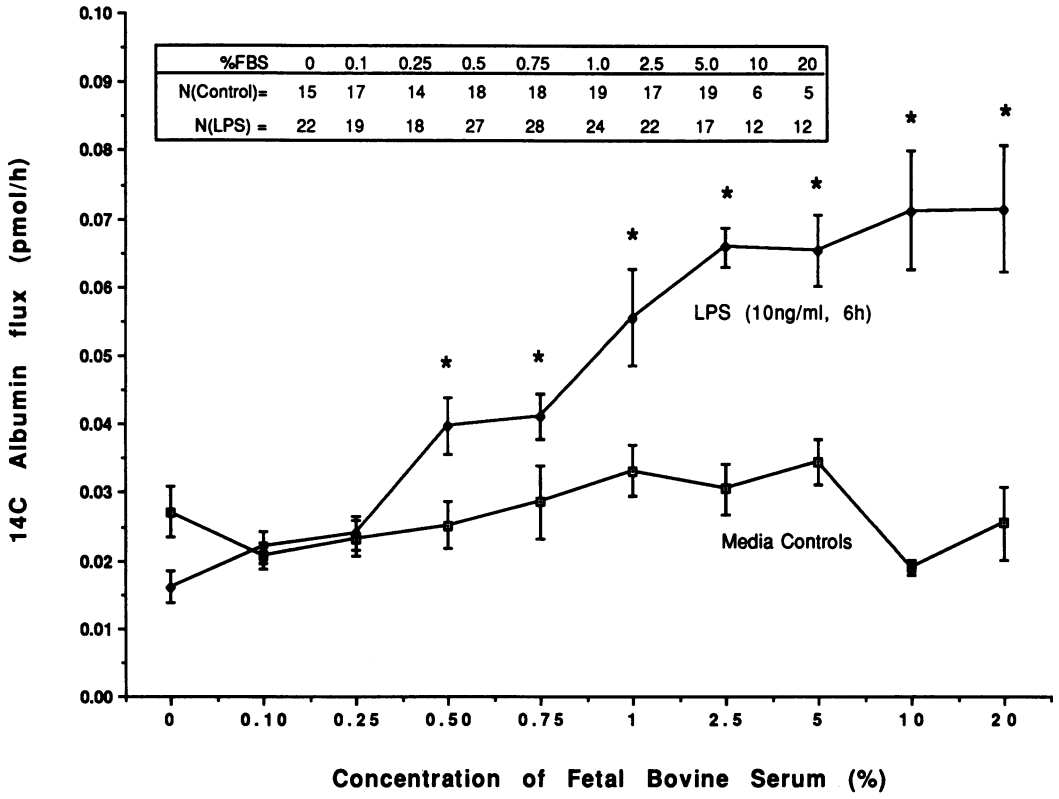

Figure 3. Serum-dependent effect of LPS on transendothelial ^{14}C-BSA flux. Transendothelial ^{14}C-BSA flux across serum-starved monolayers exposed to LPS 10 ng/ml or media for 6 h in the presence of increasing FBS concentrations. Each symbol represents mean (±SE) transendothelial ^{14}C-BSA flux in the presence of a specific FBS concentration. Mean (±SE) pretreatment baseline transendothelial ^{14}C-BSA flux is shown by the open bar. Inset contains *n* values for both LPS-exposed monolayers and the media controls in the presence of increasing FBS concentrations. *Significantly increased compared with the simultaneous media controls containing an equivalent FBS concentration at $P < 0.045$.

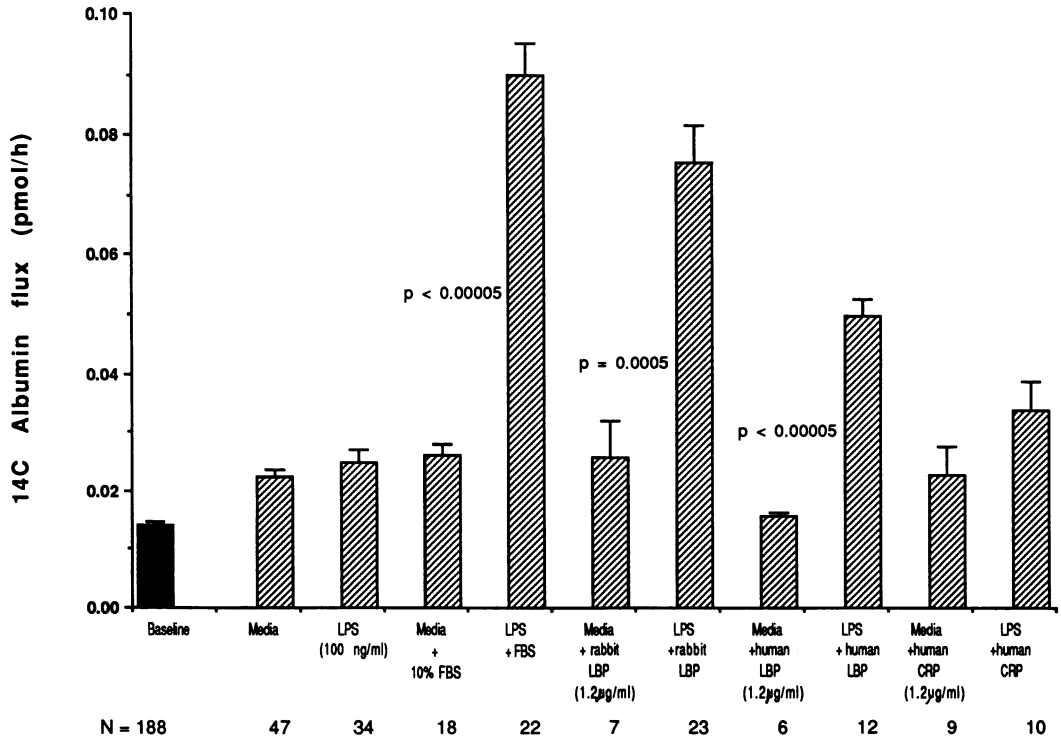

Figure 4. Effect of LBP on LPS-induced changes in endothelial barrier function. Transendothelial ^{14}C-BSA flux was determined across serum-starved monolayers exposed for 6 h to serum-free media, LPS 100 ng/ml in serum-free media, media enriched with 10% FBS, LPS 100 ng/ml in media with 10% FBS, LPS 100 ng/ml with an equimolar concentration of rabbit LBP (1.2 µg/ml) in serum-free media, LPS 100 ng/ml with an equimolar concentration of human LBP in serum-free media, LPS 100 ng/ml with human CRP (1.2 µg/ml), or serum-free media with either rabbit or human LBP or CRP, alone. Vertical cross-hatched bars represent mean (±SE) transendothelial ^{14}C-BSA flux in picomoles per hour immediately after stimulation, and mean (±SE) pretreatment baseline is shown by the closed bar.

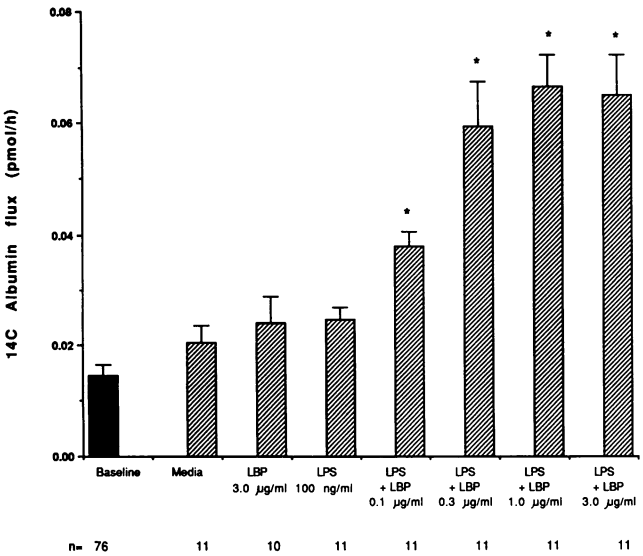

Figure 5. Dose-dependent effect of LBP on LPS-induced changes in endothelial barrier function. Transendothelial ^{14}C-BSA flux was determined across serum-starved monolayers exposed for 6 h to serum-free media, LPS 100 ng/ml in serum-free media, LPS 100 ng/ml in the presence of increasing concentrations of rabbit LBP, or rabbit LBP (3 μg/ml) alone. Vertical crosshatched bars represent mean (±SE) transendothelial ^{14}C-BSA flux in picomoles per hour immediately after stimulation, and the mean (±SE) pretreatment baseline is shown by the closed bar. *Significantly increased compared with LPS in the absence of LBP at $P < 0.002$.

absence of serum or LBP significantly increased ^{14}C-BSA flux compared with either LPS or sCD14 controls. The addition of BSA, DAF, or folate-binding protein each failed to support the LPS effect. When monolayers were preincubated with sCD14 for 0.5 h and then washed before LPS treatment, no increase in ^{14}C-BSA flux could be demonstrated (data not shown). When monolayers were exposed for 6 h to a fixed LPS concentration (100 ng/ml) in the presence of varying sCD14 concentrations, the LPS/sCD14 effect increased as a function of sCD14 concentration (Fig. 8). sCD14 concentrations ≥ 25 ng/ml in the presence of LPS significantly increased albumin flux compared with LPS alone, and the effect plateaued at sCD14 concentrations of ≥ 500 ng/ml. At concentrations of ≥ 250 ng/ml, sCD14 in the absence of added LPS induced a slight but significant increment in albumin flux compared with the media control. However, the sCD14 preparations used in these experiments contained 0.3–16 ng LPS/μg sCD14 (i.e., up to 4 ng LPS/250 ng sCD14). Again, using HS to take advantage of anti-human CD14 antibodies, anti-CD14 antibody completely blocked the ability of serum to support the LPS-induced endothelial barrier dysfunction (Fig. 6). In a serum-free reconstituted LPS–human LBP system, anti-CD14 antibody had no effect, excluding cross-reactivity of the anti-CD14 antibody with LBP. To further substantiate the accessory function of sCD14, monolayers were exposed to LPS in the presence of either HS or HS depleted of sCD14 by immunoaffinity chromatography, both from the same donor. The normal HS supported the LPS effect, whereas the CD14-depleted HS with

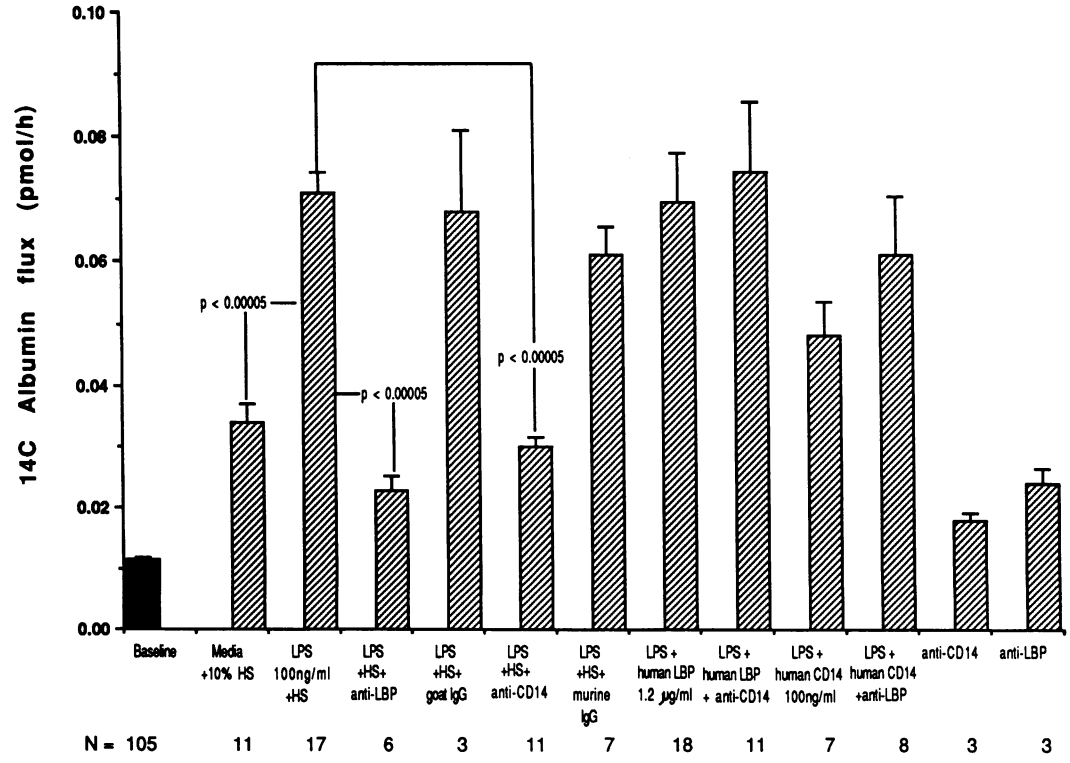

Figure 6. Effect of anti-LBP and anti-CD14 immunoblockade on LPS-induced changes in endothelial barrier function. Transendothelial ^{14}C-BSA flux was determined across serum-starved monolayers exposed for 6 h to media enriched with 10% HS, LPS 100 ng/ml in media with 10% HS, LPS in media with 10% HS in the presence of anti-human LBP antibody (1 mg/ml), LPS 100 ng/ml in media with 10% HS in the presence of anti-human CD14 antibody (10 μg/ml), LPS 100 ng/ml and human LBP (1.2 μg/ml) in serum-free media, LPS 100 ng/ml and human LBP (1.2 μg/ml) in serum-free media in the presence of anti-human CD14 antibody, LPS 100 ng/ml and human sCD14 100 ng/ml in serum-free media, LPS 100 ng/ml and human sCD14 in serum-free media in the presence of anti-LBP antibody, or media with either antibody or their species-matched controls alone. Vertical crosshatched bars represent mean (±SE) transendothelial ^{14}C-BSA flux in picomoles per hour immediately after stimulation, and mean (±SE) pretreatment baseline is shown by the closed bar.

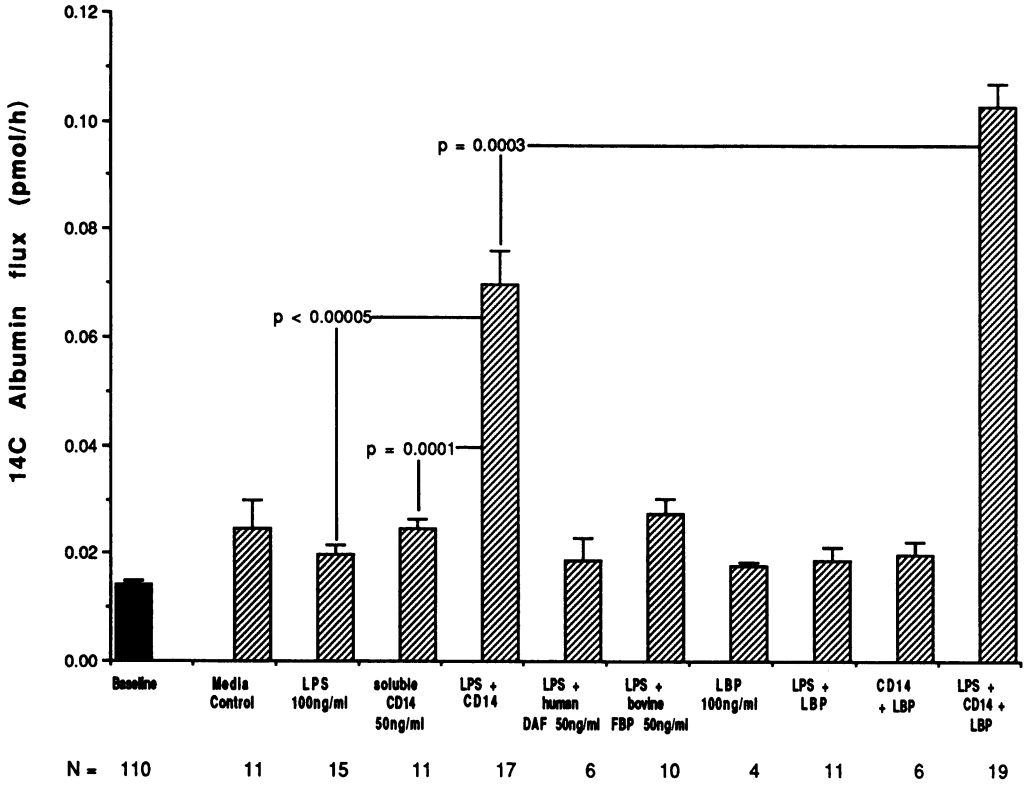

Figure 7. Effect of sCD14 on LPS-induced changes in endothelial barrier function. Transendothelial ^{14}C-BSA flux was determined across serum-starved monolayers exposed for 6 h to serum-free media, LPS 100 ng/ml serum-free media, human sCD14 50 ng/ml, LPS 100 ng/ml, LPS with sCD14 50 ng/ml, LPS 100 ng/ml with human DAF 50 ng/ml, LPS 100 ng/ml with bovine folate-binding protein (*FBP*) 50 ng/ml, human LBF 100 ng/ml, LPS 100 ng/ml with LBP 100 ng/ml, sCD14 and LBP, and LPS with both sCD14 and LBP. Vertical crosshatched bars represent mean (±SE) transendothelial ^{14}C-BSA flux in picomoles per hour immediately after stimulation, and mean (±SE) pretreatment baseline is shown by the closed bar.

suprathreshold concentrations of LPB could not (Fig. 9). LBP significantly enhanced the LPS–sCD14-induced increments compared with those seen after exposure to LPS–sCD14 alone (Fig. 7). Therefore, sCD14 functioned as an accessory molecule for LPS in the absence of LBP, and immunoblockade with anti-CD14 antibody prevented LPS-induced changes only in the presence of serum. The apparent functional differences of LBP and sCD14 in serum and purified reconstituted systems are discussed below.

Kinetic analysis of LPS-induced changes in endothelial barrier function in the presence of LBP, sCD14, or both. LBP, sCD14, or both accessory molecules together were studied for their ability to promote the LPS-induced changes in barrier function over time (Fig. 10). All reagents were introduced at the beginning of the incubation period and remained throughout the indicated time intervals when barrier function was immediately assayed. LPS in the presence of either LBP or both LBP and sCD14 (50 ng/ml) significantly increased ^{14}C-BSA

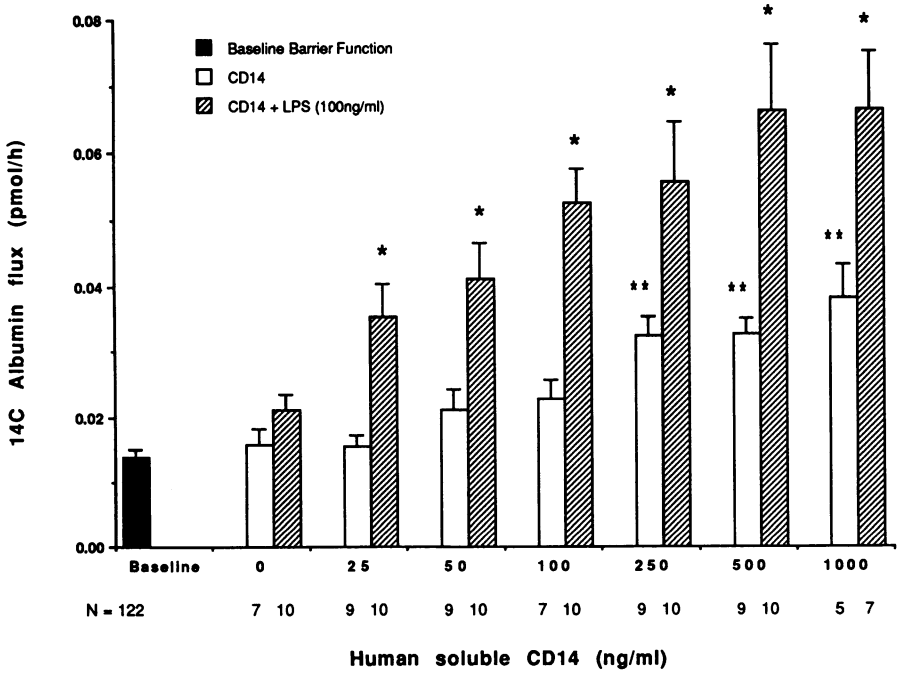

Figure 8. Dose-dependent effect of sCD14 on LPS-induced changes in endothelial barrier function. Transendothelial ^{14}C-BSA flux was determined across serum-starved monolayers exposed for 6 h to serum-free media, LPS 100 ng/ml increasing sCD14 concentrations, or these same sCD14 concentrations in the presence of LPS 100 ng/ml. Vertical bars represent mean (±SE) transendothelial ^{14}C-BSA flux in picomoles per hour immediately after stimulation, and mean (±SE) pretreatment baseline is shown by the closed bar. *Significantly increased compared with sCD14 in the absence of LPS at $P < 0.02$. **Significantly increased compared with the media control at $P < 0.002$.

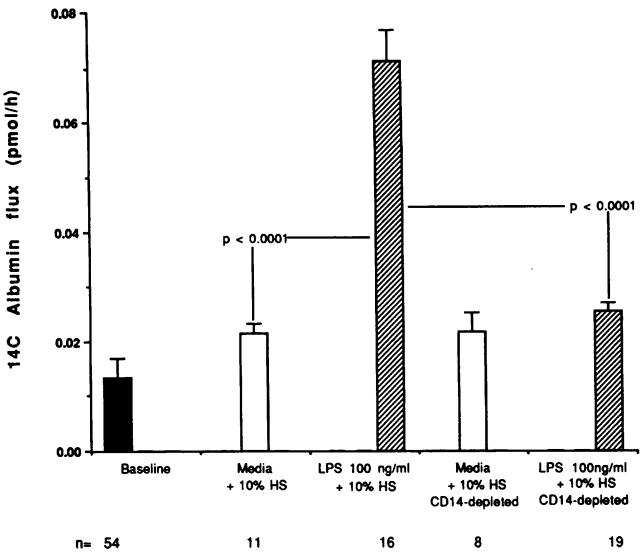

Figure 9. Effect of CD14-depletion on serum-dependent LPS-induced changes in endothelial barrier function. Transendothelial ^{14}C-BSA flux was determined across serum-starved monolayers exposed for 6 h to media enriched with 10% HS, LPS 100 ng/ml in media with 10% HS, media with 10% CD14-depleted HS from the same donor, or LPS 100 ng/ml with 10% CD14-depleted HS. The LBP concentrations in the HS and CD14-depleted HS were 27 and 23 μg/ml, respectively. Vertical bars represent mean (±SE) transendothelial ^{14}C-BSA flux in picomoles per hour immediately after stimulation, and the mean (±SE) pretreatment baseline is shown by the closed bar.

flux compared with LPS alone at all time points. LPS in the presence of sCD14 alone (50 or 500 ng/ml) did not significantly increase ^{14}C-BSA flux until 6 h. At 6 h, LPS in the presence of both LBP and sCD14 (50 ng/ml) induced significantly greater ^{14}C-BSA flux than did LPS with either LBP or sCD14 alone.

Discussion

In this report, we have demonstrated that LPS extracted from *E. coli* 0111:B4 induces dose-, time-, and serum-dependent increments in transendothelial ^{14}C-BSA flux across bovine pulmonary artery EC monolayers. In the presence of 10% FBS, a 6-h LPS exposure at concentrations as low as 0.5 ng/ml increased ^{14}C-BSA flux. Meyrick et al. (4) have previously shown that *E. coli* 055:B5 LPS at one fixed concentration (10 μg/ml) augments movement of ^{125}I-BSA across similarly passaged bovine pulmonary artery EC cells cultured on filters, also in the presence of 10% FBS. Their LPS concentration was ≥ 1,000-fold higher than those used in our study.

Based on the dose–response relationship between LPS concentration and change in endothelial barrier function, LPS 10 ng/ml in the presence of FBS was used to study the time profile for the LPS-induced effect. A minimum LPS exposure of 2 h was necessary for immediate changes. Using LPS 10 μg/ml in a similar experimental system, Meyrick et al. (4) found increased transendothelial albumin flux only at 3 h. This 2–3-h stimulus-to-response lag time is similar to that described for

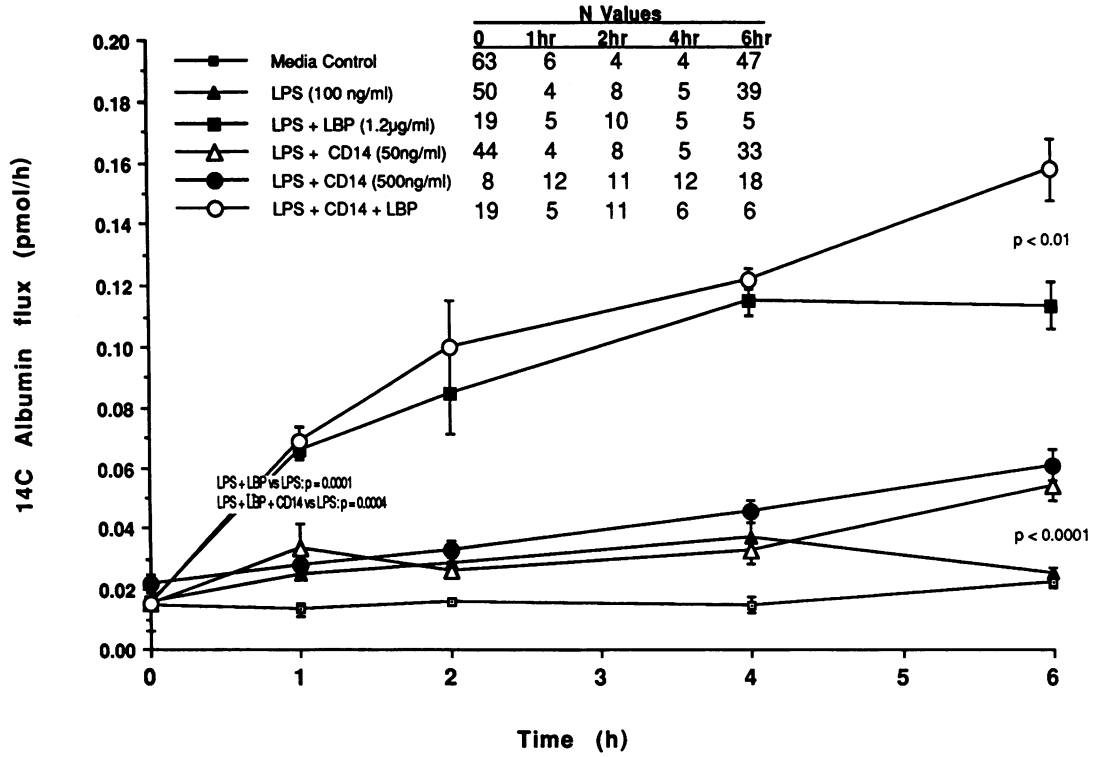

Figure 10. Kinetic analysis of accessory molecule(s) regulation of LPS-induced changes in endothelial barrier function. Transendothelial ^{14}C-BSA flux was determined across serum-starved monolayers immediately after increasing exposure times to serum-free media, LPS 100 ng/ml serum-free media, LPS with LBP (1.2 μg/ml), LPS with sCD14 (50 or 500 ng/ml), and LPS with both LBP and sCD14 (50 ng/ml). Each symbol represents the mean (±SE) transendothelial ^{14}C-BSA flux in picomoles per hour immediately after increasing exposure times.

certain other LPS-induced EC responses, including hyperadhesiveness for neutrophils (23), and increased expression of plasminogen activator inhibitor 1 (24), IL-1 (25), IL-6 (18, 26), IL-8 (18), and PDGF (27). The time requirements for the LPS-induced changes in endothelial barrier function are not unlike those described after either rTNFα (21) or rIL-1 (28) exposures. Compared with LPS, most other established mediators of increased endothelial permeability, including histamine, bradykinin, and leukotriene B4, all display a much more rapid onset of action (29).

To determine whether endothelial barrier dysfunction produced by a $\leq$ 6-h LPS exposure at 10 ng/ml was mediated through EC injury, two cytotoxicity assays were used. LPS (10 ng/ml) failed to increase endothelial cell LDH release over 6 h. LPS failed to increase ^{51}Cr release after 1 or 2 h, but after 4 and 6 h, ^{51}Cr release was minimally but significantly increased. LPS-induced EC cytotoxicity has been well described and is highly dependent on LPS concentration and exposure time, the presence of serum, as well as the species and possibly anatomical origin of the target EC (5, 30–32). Most of these earlier studies used higher LPS concentrations and longer LPS exposure times (4, 5, 30, 31, 33, 34). In our system, LPS induced changes in endothelial barrier function at doses and exposure times (10 ng/ml $\times$ 2 h), which could not be ascribed to cytotoxicity or loss of viability as measured by these assay systems. On the basis of trypan blue exclusion (data not shown) and increased ^{51}Cr release, LPS induced sublethal cell injury by 4 and 6 h.

The LPS-induced changes in endothelial barrier function were profoundly serum dependent. LPS (10 ng/ml, 6 h) failed to increase ^{14}C-BSA flux at serum concentrations < 0.5%, and maximum LPS-induced increments could be generated in the presence of $\geq$ 2.5% FBS. The addition of 10% FBS increased EC sensitivity to the LPS stimulus by > 10,000-fold. LPS-induced EC cytotoxicity (4, 35) and detachment (5) as well as prostacyclin synthesis (4) have all been shown to be serum dependent. LPS functionally interacts with numerous serum constituents; it activates the alternative complement pathway, and the intrinsic clotting and kallikrein–kinin systems (36), and directly binds to Hageman factor (36), high and low density lipoproteins (37), the acute phase protein, LPB (6, 10), and sCD14 (18). We have now demonstrated serum factors necessary for LPS-induced endothelial barrier dysfunction.

In our bovine EC system, either rabbit or human LBP alone, in the absence of other serum constituents, promoted the serum-dependent LPS-induced changes in endothelial barrier function. A dose–response relationship between the LBP concentration and this LPS effect was demonstrated. The optimal LPS/LBP ratio was $\approx$ 1:1. In addition, anti–human LBP antibody completely blocked the LPS-induced effect in the presence of HS. These data strongly suggest that LBP can function as an accessory molecule for LPS presentation to the EC surface. LBP is present in normal rabbit serum in concentrations of < 0.5 μg/ml, increasing $\sim$ 100-fold 24 h after induction of an acute phase response (6, 10). In humans, LBP rises from 7.2±4.0 μg/ml in the sera of normal subjects to 220±100 μg/ml in acute phase sera (38). The concentration of LBP employed in our studies (1.2 μg/ml) was well within the expected concentration range found in serum of either species (6, 7). That rabbit and human LBP as well as HS could each present LPS to bovine EC should not be surprising, as the molecule is highly conserved across species (7). Rabbit and human LBP share 69% amino acid identity and 78% nucleotide identity. An EC surface receptor that LPS–LBP complexes recognize is not known. To our knowledge, CD14, a differentiation antigen on monocytic cells (14, 15, 39), has not been demonstrated on EC. Wright et al. (12) found that LBP greatly enhances binding of LPS-coated erythrocytes to monocytes and macrophages, and that this binding could be blocked by anti-CD14 antibodies. No such binding could be demonstrated for human umbilical vein EC (HUVEC). Beekhuizen et al. (13) found no CD14 expression on EC by flow cytometry. More recently, although CD14 transcripts have been demonstrated in EC by polymerase chain reaction, CD14 message and protein could not be found using Northern analysis, ELISA, immunoprecipitation, or flow cytometry (Harlan, J. M., personal communication).

There are two studies that are not consistent with our observation that LBP can support LPS presentation to the EC. Pugin et al. (18) found that LPS–LBP was unable to induce HUVEC expression of adhesion molecules and cytokine production. It is conceivable that EC from different anatomic sites and species differ sufficiently in their cell-surface receptors to explain these differences. Further, the protein synthesis–dependent EC responses of adhesion molecule and cytokine expression used by Pugin et al. (18) differ considerably with the cytoskeletally driven changes in barrier function (22) used in our studies. Recently, Arditi et al. (35) reported that LBP does not support LPS-induced bovine brain microvascular EC cytotoxicity as measured by LDH release after a 24-h LPS exposure. Relevant to the EC type used in this study, Meyrick et al. (30) have previously demonstrated that although LPS induces bovine pulmonary artery EC barrier dysfunction, pulmonary microvascular EC from the same species are relatively LPS resistant. Arditi et al. (35) never showed their LBP to be active, i.e., there was no positive control, making their negative results ambiguous. Similarly, they did not confirm their depletion of LBP from serum. Thus, it might be possible to reconcile the observed differences in behavior of LBP in these three systems, but the explanations are not very satisfying, and the reconciliation of these differences are under investigation.

sCD14 also functioned dose-dependently as an accessory molecule for the LPS-induced EC response in the absence of serum or LBP. Anti–human CD14 antibody totally blocked the LPS-induced effect in the presence of HS, but had no effect in a reconstituted serum-free LPS–LBP system. In other experiments, HS depleted of sCD14 by immunoaffinity chromatography also failed to support the LPS effect. Thus, the CD14 that contributes to the LPS–EC interaction in our system seems to be present in a soluble or circulating form and not on the EC surface. On the basis of a semiquantitative ELISA, normal human plasma contains sCD14 at $\sim$ 6 μg/ml (16). It has now been shown that *Haemophilus influenzae* LPS induces bovine pulmonary artery and brain EC cytotoxicity in the presence of HS that can be blocked by a murine monoclonal anti-CD14 antibody (3C1O); no experiments were performed in a serum-free system (32). More recently, sCD14 has been shown to participate in LPS-induced expression of endothelial leukocyte adhesion molecule-1 by HUVEC, as well as cytotoxicity of bovine pulmonary artery (17) and brain microvascular (32, 35) EC. Another study has demonstrated that sCD14 is involved in the induction by LPS of HUVEC IL-6 and IL-8 biosynthesis, as well as intercellular adhesion molecule-1 and vascular-cell adhesion molecule-1 expression (18). At present, no specific bind-

ing site on EC for LPS–sCD14 complexes is known. Human monocytes express CD14 on their surface, and their adherence to cytokine-stimulated EC can be blocked by anti-CD14 antibody (13). Whether this finding represents an EC receptor for CD14 expressed on the monocyte surface is unknown.

We found that LBP and sCD14, two structurally unrelated molecules, could each singularly promote LPS-induced changes in endothelial barrier function in the absence of serum. Each molecule influenced the LPS effect in a dose-dependent manner. Their contributions to the LPS effect were kinetically distinct; LBP had a much more rapid onset of action than did sCD14 (1 vs 6 h). However, in the presence of serum, antibodies to either LBP or sCD14 completely blocked the LPS effect. These results cannot be explained by cross-reactivity of the antibodies, because antibody to either LBP or sCD14 did not cross-react with the other antigen in a serum-free reconstituted system. Further, immunodepletion of sCD14 did not significantly diminish serum LBP concentrations, and this sCD14-depleted serum that contained suprathreshold concentrations of LBP failed to support the LPS effect. Thus, our data suggest that in a serum-free, reconstituted system, LBP and sCD14 can each independently function as accessory molecules for LPS presentation, whereas in the presence of serum, both molecules are required. Perhaps an additional serum component(s) exists that participates in LPS, LBP, and sCD14 interactions. In serum, other sinks for LPS exist. For example, serum lipoproteins avidly bind up and detoxify LPS (37, 40). For the EC to compete effectively with these other fates for LPS in serum, a more effective presentation mechanism than either LBP or sCD14 alone may be required. In the reconstituted system, LBP and sCD14 together enhance LPS-induced increments in transendothelial ^{14}C-BSA flux more than either protein does alone. Whether LBP and sCD14 function additively or synergistically is unclear. The optimal molar ratios of LBP, sCD14, and LPS required to exert a specific biological response in a specific target tissue have not yet been defined. Alternatively, LPS complexed with both LBP and sCD14 might operate through a third pathway. Our data are compatible with one or more finite receptor populations that recognize LPS in the presence of an accessory molecule(s). Whether these accessory molecules complex to and alter the tertiary structure of the LPS ligand to promote its accommodation by such an EC receptor(s) and/or directly serve as docking proteins within the EC receptor–ligand interaction itself is unknown. Whatever the mechanism(s), this ability of LBP and sCD14 to support the LPS effect cannot be ascribed to a nonspecific protein–LPS interaction; BSA, DAF, CRP, and folate-binding protein each failed to function as an accessory molecule for LPS presentation to the EC.

Humans have evolved over many thousands of years with bacteria and their endotoxins. That cells of monocyte/macrophage lineage can provide a molecule that together with an hepatocyte-derived protein can facilitate LPS–EC interaction demonstrates remarkable host adaptability to a potentially lethal substance found in both gut flora and in the external environment. Through their facilitation of the LPS–EC interaction, these accessory molecules may be central to tissue-specific responses to LPS. sCD14 may be synthesized and shed in situ within monocyte/macrophage-containing sites where hepatocyte-synthesized LBP is excluded. The ability of these accessory molecules to pass through physiological barriers into a given body compartment, the rapidity of their appearance, and their local concentrations all may modulate LPS-induced biological responses at the ubiquitous endothelial surface. LBP facilitates LPS priming of neutrophils (40), and sCD14 participates in LPS-stimulated astrocytoma cell secretion of IL-6 (17) and colonic adenocarcinoma cell secretion of IL-8 (18). Whether LBP and/or sCD14 can serve as accessory molecules for LPS presentation to other host cells is not yet known.

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