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Activation of Human Endothelial Cells by Viable or Heat-Killed Gram-Negative Bacteria Requires Soluble CD14

ROBERT F. NOEL, JR., THOMAS T. SATO, CYNTHIA MENDEZ, MARION C. JOHNSON, AND TIMOTHY H. POHLMAN*

Department of Surgery, University of Washington School of Medicine, Seattle, Washington

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In response to bacterial lipopolysaccharides (LPS; endotoxin), endothelial cells are converted to an activation phenotype expressing both proinflammatory and procoagulant properties that include the induction of leukocyte adhesion molecules and tissue factor expression. LPS-induced endothelial cell activation requires a soluble form of the monocyte LPS receptor, sCD14. We evaluated the capacity of multiple strains of gram-negative and gram-positive bacteria to induce endothelial E-selectin and tissue factor expression through sCD14-dependent pathways with cultured human umbilical vein endothelial cells (HUVE). Both viable and heat-killed gram-negative bacteria (*Bacteroides fragilis*, *Enterobacter cloacae*, *Haemophilus influenzae*, and *Klebsiella pneumoniae*) but not viable or heat-killed gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, and *Streptococcus pneumoniae*) induced prominent E-selectin surface expression detected by enzyme-linked immunosorbent assay. Tissue factor activity on HUVE, indicated by factor X activation, was induced in response to gram-negative bacteria but not in response to gram-positive bacteria. Gram-negative bacteria induced transcriptional activation in HUVE, indicated by the appearance of E-selectin-specific mRNA and by the demonstration of activation of NF-κB, a *trans*-activating factor necessary for E-selectin and tissue factor gene transcription. In contrast, neither E-selectin mRNA nor activation of NF-κB was detected in HUVE treated with gram-positive bacteria. Endothelial cell activation by gram-negative bacteria in each of these assays was inhibited with a monoclonal antibody (*60bd*) against CD14. Furthermore, CHO-K1 cells, transfected with human recombinant CD14, responded to all strains of gram-negative bacteria (viable or heat killed), indicated by CHO-K1 NF-κB activation. We conclude that gram-negative bacteria induce endothelial cell activation through a common sCD14-dependent pathway.

CD14, a glycosyl-phosphatidylinositol-anchored glycoprotein, is now recognized as a receptor for bacterial lipopolysaccharides (LPS; endotoxin) (22, 39, 40, 42, 43, 48, 49, 52), mediating the activation of mononuclear phagocytes by LPS. Paroxysmal nocturnal hemoglobinuria is a rare disorder characterized molecularly by a deficiency of glycosyl-phosphatidylinositol anchors (51). Phagocytes from patients with paroxysmal nocturnal hemoglobinuria respond weakly to LPS with minimal release of inflammatory cytokines (10), confirming the requirement of CD14 for normal phagocyte function. Furthermore, a 60-kDa glycoprotein, LPS-binding protein (LBP), functions as a cofactor in CD14-LPS interactions (35), binding to the lipid A portion of LPS with high affinity (23); LBP-LPS complexes then engage CD14 at a second domain in the C-terminal half of LBP (38). LBP, an acute-phase reactant, is required for LPS toxicity in vivo (13).

In vivo and in vitro, circulating inflammatory mediators, derived from activated mononuclear phagocytes, convert endothelial cells to a phenotype characterized by proinflammatory and procoagulant properties (7, 27, 28). Activated endothelial cells release inflammatory cytokines (interleukin-1 [IL-1], IL-6, IL-8, monocyte chemotactic protein-1, and platelet-activating factor) and express surface adhesion molecules (E-selectin, P-selectin, intercellular adhesion molecule [ICAM], and VCAM) that are involved in the recruitment and activation of leukocytes (29). Tissue factor is expressed by activated endothelium that, in concert with the down-regulation of thrombomodulin expression and an increased expression of tissue plasminogen activator inhibitor-1, results in a prothrombotic endothelial cell surface (29). Thus, endothelial cell activation may promote leukocyte infiltration and microvascular thrombosis contributing to the pathogenesis of disseminated intravascular inflammation and coagulation (5).

Very low amounts of LPS also induce endothelial cell activation in vitro. LPS-induced endothelial cell activation requires serum containing a soluble form of CD14 (1, 32, 33). Unlike mononuclear phagocytes, endothelial cells do not express CD14 on the plasma membrane (1, 3, 11). Depleting soluble CD14 (sCD14) from serum, however, diminishes LPS effects on endothelial cells, while reconstitution of CD14-depleted serum with immunopurified sCD14 restores LPS activity on endothelial cells (11, 17). Activation of endothelial cells by LPS, therefore, appears to require the specific formation of LPS-CD14 complexes in serum. LBP is not required for sCD14-mediated activation of endothelial cells in vitro (33).

Although the requirement for sCD14 in LPS-mediated activation of human endothelial cells is established, less is known about the interaction of sCD14 with bacteria or, specifically, whether sCD14 mediates endothelial cell activation with LPS while LPS is an integral part of the bacterial outer membrane. In this study, we demonstrate that viable as well as heat-killed gram-negative bacteria induce an activation phenotype in human umbilical vein endothelial cells (HUVE), indicated by HUVE surface expression of both E-selectin and tissue factor activity. Heat-killed or viable gram-positive bacteria, in contrast, do not induce any phenotypic changes in HUVE. We also show that gram-negative bacteria induce transcriptional activation in HUVE, as suggested by the appearance of E-selectin mRNA and by the finding of nuclear translocation of the

* Corresponding author. Mailing address: Department of Surgery, University of Washington School of Medicine, Harborview Medical Center, 325 Ninth Ave., Seattle, WA 98104. Fax: (206) 223-3656. Electronic mail address: tpohlman@u.washington.edu.

transcription factor NF-κB in HUVE treated with various types of gram-negative bacteria. Furthermore, we demonstrate that the activation of HUVE by different strains of gram-negative bacteria is mediated predominately by a common sCD14-dependent pathway.

MATERIALS AND METHODS

Endothelial cell culture. HUVE were obtained by collagenase digestion (Worthington Biochemical, Freehold, N.J.) as described previously (50). Cells were maintained in RPMI 1640 (Whittaker M. A. Bioproducts, Walkersville, Md.) supplemented with 20% adult bovine serum (JRH Biosciences, Lenexa, Kans.), endothelial cell growth supplement (25 mg/ml; Collaborative Research, Lexington, Mass.), heparin (90 mg/ml; Sigma), and antibiotics (penicillin G, streptomycin, and amphotericin B). Third-passage HUVE, maintained at 37°C in a 5% CO_2 atmosphere, were used in all experiments. For experiments with viable bacteria, HUVE monolayers were washed extensively the night before with medium not containing antibiotics and maintained in antibiotic-free medium prior to use.

CHO-K1 cultures. Chinese hamster ovary-K1 (CHO-K1) cells stably transfected with human recombinant CD14 or cells transfected with the expression vector not containing CD14 were obtained as a generous gift from Richard J. Ulevitch, Research Institute of Scripps Clinic, La Jolla, Calif. CHO-K1 cells expressing CD14 were selected by fluorescence-activated cell sorting utilizing fluorescein isothiocyanate-conjugated monoclonal antibody (MAb) *MY4* (19). Activation of CD14-transfected CHO-K1 signaling pathways in response to LPS or gram-negative bacteria was determined by assay for NF-κB translocation to CHO-K1 cell nuclei. MAb *60bd* directed against CD14 (J. Harlan, University of Washington, Seattle) was prepared from ascites fluid and added to HUVE, at a 1:100 dilution, 15 min prior to treatment with bacteria.

Bacterial culture. Isolates of *Bacteroides fragilis*, *Enterobacter cloacae*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Streptococcus pneumoniae* were obtained from the Department of Microbiology at the University of Washington Medical Center. Because the relationship between the number of bacteria in a volume of medium and the optical density at 600 nm of light transmission through the medium will vary substantially among different bacteria, calibration curves were generated by determining the optical density at 600 nm at three different time points in the exponential growth phase of each species examined. The number of bacteria in each sample was determined by plating dilutions onto agar plates. *B. fragilis* cultures were grown to the exponential phase in thioglycolate broth (Difco Laboratories, Detroit, Mich.) while isolated in an anaerobic environment utilizing a BBL GasPak Pouch (Becton Dickinson, Cockeysville, Md.). *Streptococcus* cultures were plated on tryptic soy agar-based, blood-agar plates. Heat-killed bacteria were first incubated to the exponential phase of growth and then heated to 80°C for 30 min. Cultures were washed twice in phosphate-buffered saline (PBS), suspended in RPMI 1640, and stored at −70°C until use. Outer membranes from *B. fragilis* were purified as described previously by Kasper and Seiler (18). Pelleted organisms were washed in PBS and subjected to mild mechanical shearing through a 25-gauge needle and disruption in a Dounce homogenizer. This suspension was centrifuged at 12,000 × *g* at 4°C for 20 min to remove intact organisms. The supernatant was then centrifuged at 80,000 × *g* at 4°C for 180 min. Both procedures were repeated twice. The final pellet was separated from loosely associated lipids by precipitation of outer membrane structures with 0.2 N NaCl in 80% (vol/vol) ethanol. The outer membrane pellet was then washed twice with chloroform-methanol (2:1, vol/vol) and centrifuged at 16,000 × *g* at 4°C. Both pellets were combined and dried under nitrogen. The dried chloroform-methanol-insoluble material was then resuspended in 6 N NaOH containing 0.05 M glycine, 1 mM EDTA, and 0.5% sodium deoxycholate and passed over a Sephadex G-100 column (Pharmacia). The void volume containing outer membrane was collected, lyophilized, resuspended in PBS, and frozen at −70°C. Purified *B. fragilis* capsular polysaccharides A and B were obtained as a generous gift from Andrej Wientraub, Karolinska Institute, Huddinge, Sweden. The lyophilized material was dissolved in PBS and frozen for storage.

Enzyme-linked immunosorbent assay (ELISA). HUVE were plated into 96-well tissue culture plates (Costar) and grown to confluence. Monolayers were treated with viable bacteria or heat-killed bacteria at various log concentrations. As a positive control for HUVE activation, purified *Escherichia coli* LPS (100 ng/ml; Sigma) was used. All HUVE cultures were treated with either bacteria or LPS for 4 h and then washed twice with RPMI 1640 supplemented with 5% fetal calf serum. HUVE were then incubated with MAb against E-selectin (*P6E2*; gift of John Harlan, University of Washington) for 60 min at 37°C. HUVE were incubated for an additional 60 min with a goat anti-mouse, peroxidase-conjugate antibody (Tago Laboratories, Burlingame, Calif.). The plates were washed with PBS and allowed to develop for 20 min with *o*-phenylenediamine–peroxide prior to reading the A_{490} with a kinetic microplate reader (Molecular Devices, Menlo Park, Calif.).

Tissue factor assay. HUVE were plated into 96-well tissue culture plates and grown to confluence. Monolayers were treated for 4 h with LPS or various log concentrations of the different bacteria, washed twice with PBS, and incubated for 3 h with factor IX complex, Proplex T (contains factors VII and X; Baxter Healthcare Corporation, Glendale, Calif.), and S-2222 (chromogenic substrate for factor Xa; Kabi Pharmacia, Franklin, Ohio) in RPMI 1640 (without phenol red) at 37°C. Plates were read for A_{405} with a kinetic microplate reader.

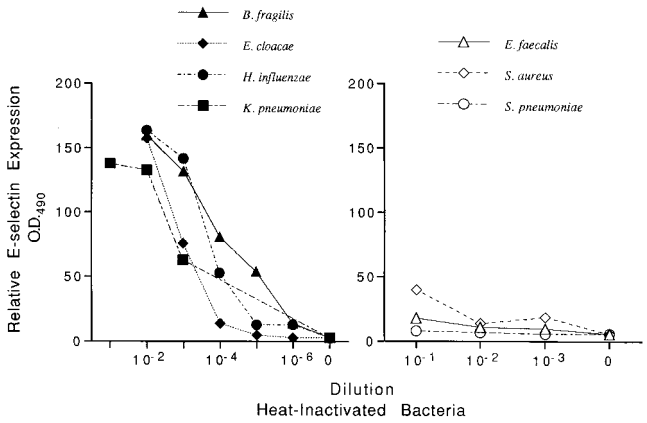

FIG. 1. Gram-negative bacteria induce E-selectin expression on HUVE. Cultures of gram-negative organisms (closed symbols) or gram-positive organisms (open symbols) were heat inactivated at 80°C for 30 min and then diluted in HUVE culture medium to the dilutions indicated on the abscissa for both plots. Washed bacteria were added to HUVE for 4 h. Monolayers were then washed, and the relative amount of E-selectin expression on HUVE was determined by ELISA at 490 nm. Control HUVE monolayers were incubated with culture medium containing no bacteria, and background activity was determined in undisturbed wells. OD_{490}, optical density at 490 nm.

Northern (RNA) blotting. Following 4 h of treatment, total cellular RNA was isolated from confluent HUVE monolayers with acid guanidinium thiocyanate as described previously (6). Cultures were scraped into 1.8 ml of solution D (4 M guanidine isothiocyanate, 0.5% sarcosyl, 25 mM sodium citrate, 0.72% β-mercaptoethanol) and incubated at 4°C with 180 μl of 2 M sodium acetate, 1.8 ml of phenol and 360 μl of chloroform-isoamyl alcohol prior to centrifugation at 12,000 × *g*. Supernatants were precipitated with isopropanol, resuspended in 300 μl of solution D, and reprecipitated in isopropanol. Pellets were washed once in 70% ethanol and suspended in 0.5% sodium dodecyl sulfate. Ten-microgram samples of RNA were electrophoresed in denaturing 1.25% formaldehyde–agarose gels and transferred to nylon membranes (Nytran; Schleicher & Schuell, Inc., Keene, N.H.). The membranes were subjected to Northern blot analysis via hybridization with E-selectin and β-actin cDNA (gifts of L. Osborn, Biogen, Cambridge, Mass., and S. Busby, ZymoGenetics, Seattle, Wash., respectively) labeled with [^{32}P]dCTP by random priming.

Electrophoretic gel mobility shift assay (EGMSA). Nuclear protein extracts were obtained by the method of Dignam et al. with modifications as described previously (9, 24). Confluent HUVE monolayers were treated for 1 h at 37°C. All subsequent procedures were performed at 4°C. HUVE were washed and scraped into buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 1.5 mM $MgCl_2$, 10 mM KCl). Following a brief centrifugation, cell pellets were resuspended in buffer A containing 0.1% Nonidet P-40 (Sigma). Nuclei were pelleted and resuspended in buffer C (20 mM HEPES, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol). Following centrifugation, supernatants containing crude nuclear protein extracts were diluted with buffer D (20 mM HEPES, 0.05 M KCl, 0.2 mM EDTA, 20% glycerol), and protein concentrations were determined (bicinchoninic acid protein assay; Pierce, Rockford, Ill.).

Samples with 20 μg of nuclear protein extracts, 2 μg of poly(dI-dC)·(dI-dC) (Pharmacia), and 5×10^5 to 10×10^5 cpm of ^{32}P-end-labeled synthetic oligonucleotide were incubated for 20 min at 25°C and electrophoresed on 4% native polyacrylamide gels. The NF-κB consensus binding sequence used in the probe is represented as 5′-GCCATTGGGGATTTCCTCTTT-3′.

RESULTS

Induction of E-selectin expression. Bacterial dose-response curves were generated with serial log dilutions of heat-killed bacteria to induce E-selectin expression on HUVE as detected by ELISA utilizing an E-selectin-specific MAb, *P6E1* (Fig. 1). All gram-negative bacteria examined in this study, including *B. fragilis*, *Enterobacter cloacae*, *H. influenzae*, and *K. pneumoniae*, induced E-selectin surface expression in a dose-dependent fashion with dilutions of heat-killed bacterial cultures (5×10^8 CFU/ml, prior to heating cultures to 80°C for 30 min) as high

as $1:10^6$. There was a substantial difference in the relative amounts of E-selectin expression induced by different gram-negative bacteria after significant dilution, particularly at 1,000- to 10,000-fold dilutions. *H. influenzae* and the obligate anaerobe *B. fragilis*, both diluted 1,000-fold, induced near-maximal activity in this assay, whereas *Enterobacter cloacae* and *K. pneumoniae* induced half-maximal E-selectin expression at this dilution. At a dilution of $1:10^5$, only *B. fragilis* induced measurable E-selectin compared with that induced by equivalent dilutions of *Enterobacter cloacae*, *H. influenzae*, and *K. pneumoniae*. In contrast, heat-killed, gram-positive bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, and *Streptococcus pneumoniae*) did not activate E-selectin expression with even minimal dilutions of cultures (5×10^8 CFU/ml, prior to heating cultures to 80°C for 30 min).

To exclude the possibility that heat treatment altered the conformation or activity of membrane constituents of these organisms, HUVE monolayers were stimulated with live bacteria in the exponential phase of growth at concentrations equal to those used with heat-killed bacteria. As shown in Fig. 2A, the same strain of *E. coli*, as a representative example, induced equivalent amounts of E-selectin expression when the organism was added to HUVE as viable bacteria or heat-killed bacteria. Viable gram-positive bacteria were also examined to determine if the heat used to kill the gram-positive organisms in this study altered cell wall structures, rendering the organisms inactive on HUVE. As shown in Fig. 2B, live *Streptococcus pneumoniae* did not induce E-selectin expression at any concentration up to 10^7 CFU/ml compared with equivalent concentrations of live *E. coli*. When treated with live *Streptococcus pneumoniae* or *E. coli* at concentrations greater than 10^7 CFU/ml, HUVE monolayers began to show phase-contrast microscopic evidence of cytotoxicity, with complete disruption of the monolayer when bacteria were added at 10^9 CFU/ml (data not shown).

Pretreatment of HUVE with MAb *60bd*, specific for human CD14, significantly inhibited the induction of expression of E-selectin by all gram-negative bacteria. Similarly, MAb *60bd* significantly inhibited HUVE activation induced by *E. coli* LPS (100 ng/ml; $P < 0.01$) (Fig. 3). The relative amount of E-selectin expression induced by 10^7 CFU of these different species of gram-negative bacteria per ml was equivalent to the level of E-selectin expression induced by 100 ng of *E. coli* LPS per ml.

Since we had shown previously that *B. fragilis* LPS does not induce endothelial cell activation (21), the outer membrane of *B. fragilis* was purified to determine if activation of HUVE by *B. fragilis* organisms was due to a structure localized to the outer membrane component of the cell wall. As shown in Fig. 4, the outer membrane fraction purified from *B. fragilis* organisms was a potent inducer of HUVE activation equivalent to the amount of E-selectin expression induced by *E. coli* LPS (100 ng/ml), IL-1 (10 U/ml), or tumor necrosis factor (100 ng/ml). However, the specific activity of a putative activating factor in the outer membrane cannot be determined until a factor is identified. In addition, MAb *60bd* inhibited the induction of HUVE activation by *B. fragilis* outer membrane (data not shown). It is possible that the HUVE-activating factor(s) associated with the outer membrane was a capsular polysaccharide(s) that copurified during preparation of outer membrane fragments. We therefore examined purified capsular polysaccharides A and B, which constitute most of the mass of *B. fragilis* capsule (46), for the capacity to induce HUVE activation. Neither one of these bacterial polysaccharide preparations had any activity on HUVE (data not shown).

Induction of tissue factor. To examine whether procoagulant activity is induced by gram-negative organisms in parallel with proinflammatory activity, the ability of heat-killed bacteria to induce HUVE tissue factor expression was evaluated by an assay for factor Xa. Factor Xa is the immediate product of tissue factor activity, produced by the serine protease activity of tissue factor complexed with factor VIIa. Heat-killed *B. fragilis*, *Enterobacter cloacae*, *H. influenzae*, and *K. pneumoniae* all strongly induced tissue factor activity on HUVE at 4 h, at levels equal to or higher than those on HUVE stimulated with 100 ng of purified *E. coli* LPS per ml. However, gram-positive bacteria did not induce detectable tissue factor activity over the background level of the assay. Preincubation of HUVE with MAb *60bd* significantly abolished the induction of tissue factor activity by all gram-negative bacteria (Figure 5). ($P < 0.01$).

Transcription activation. E-selectin expression induced by *E. coli* LPS involves the accumulation of E-selectin-specific mRNA by transcriptional activation of the E-selectin gene

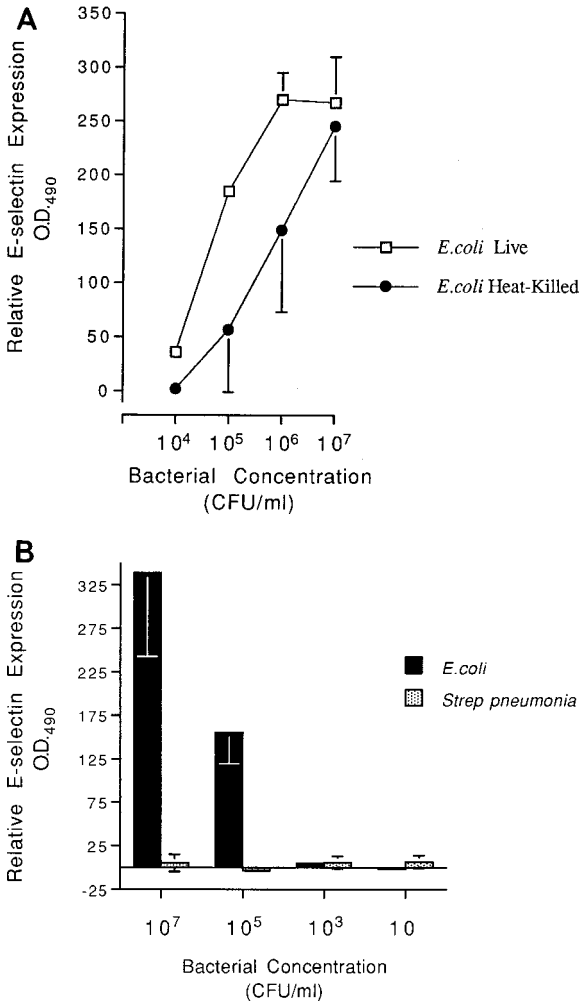

FIG. 2. E-selectin expression induced by viable bacteria. (A) *E. coli* was grown to the exponential phase and either heat killed and diluted (closed circles), or simply diluted (open boxes), to the concentrations indicated on the abscissa axis. Dilutions of heat-killed or viable bacteria were added to HUVE monolayers for 4 h, the HUVE monolayers were washed, and E-selectin expression was determined by ELISA at 490 nm. (B) *E. coli* (closed bars) and *Streptococcus pneumoniae* (stippled bars) were each grown to the exponential phase and then diluted to the concentrations shown on the abscissa. Dilutions of live bacteria were then added to HUVE for 4 h. Following washing, the monolayers were examined for E-selectin expression by ELISA. OD_{490}, optical density at 490 nm.

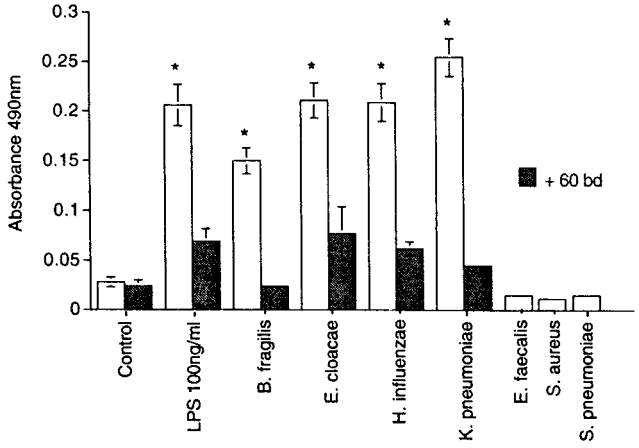

FIG. 3. Inhibition of E-selectin expression induced by gram-negative bacteria. HUVE monolayers were treated with medium alone (open bars) or medium containing MAb *60bd* (stippled bars; final dilution, 1:100, from ascites fluid) for 15 min. Either gram-negative or gram-positive bacteria as indicated or *E. coli* LPS (100 ng/ml) was then added for 4 h. In control wells, only culture medium was added. E-selectin expression was determined at the end of the 4-h incubation period by ELISA at 490 nm as described in Materials and Methods. *, $P < 0.01$.

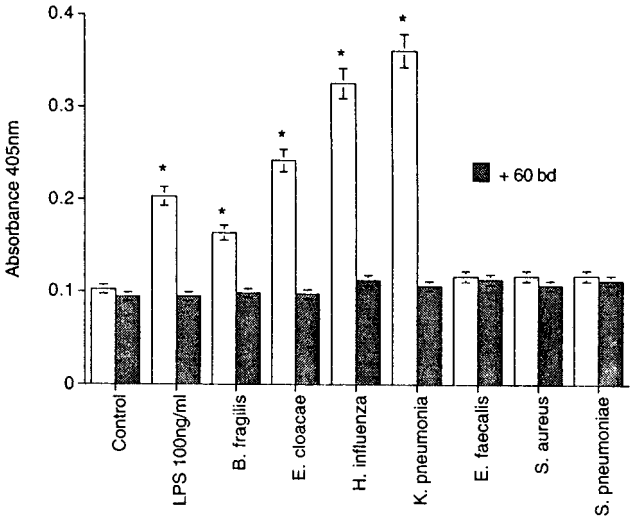

FIG. 5. Tissue factor expression induced by gram-negative bacteria is inhibited by anti-CD14 MAb *60bd*. HUVE monolayers were treated with medium alone or medium containing MAb *60bd* (final dilution, 1:100) for 15 min. At the end of the 15-min incubation period, medium alone, medium containing LPS (100 ng/ml), or medium containing either heat-killed gram-positive bacteria or heat-killed gram-negative bacteria was added to triplicate wells for 4 h. At the completion of the 4-h incubation period, cells were washed once and assayed for the conversion of factor X to factor Xa. *, $P < 0.01$.

(24). To determine if E-selectin mRNA formation occurs in response to gram-negative or gram-positive bacteria, total cellular RNA was isolated from HUVE after a 4-h exposure to *B. fragilis*, *Enterobacter cloacae*, *H. influenzae*, *K. pneumoniae*, *Enterococcus faecalis*, *Staphylococcus aureus*, or *Streptococcus pneumoniae*. Northern blot analysis of E-selectin mRNA demonstrated accumulation of E-selectin-specific transcripts in response to treatment with gram-negative bacteria. However, gram-positive bacteria did not induce E-selectin transcript accumulation. Pretreatment of HUVE with MAb *60bd* completely inhibited induction of E-selectin gene transcription by gram-negative bacteria (Fig. 6).

NF-κB activation, which includes nuclear translocation of this transcription factor from the cytoplasm to the nucleus, and binding of NF-κB to consensus sequences in the 5′-flanking regions of the E-selectin and tissue factor genes are necessary for increasing transcription of E-selectin and tissue factor in response to LPS. Whether gram-negative bacteria or gram-positive bacteria induce NF-κB activation was evaluated by EGMSAs. EGMSA, with a radiolabeled oligonucleotide encompassing the E-selectin consensus sequence for NF-κB binding, demonstrated activation of NF-κB by each species of gram-negative bacteria examined in this study (Fig. 7). As

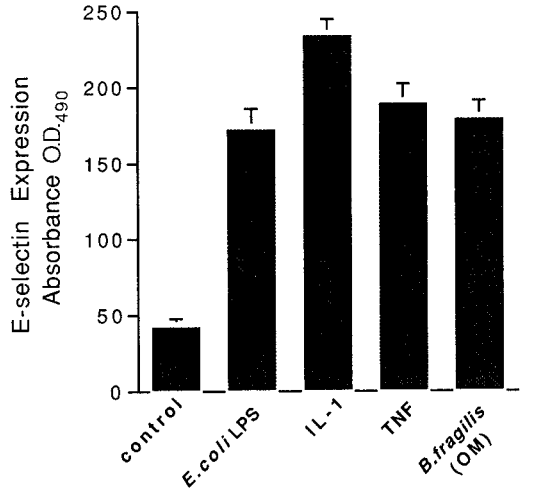

FIG. 4. Induction of E-selectin expression by *B. fragilis* outer membrane. HUVE confluent monolayers were treated for 4 h with medium alone (control), or medium containing *E. coli* LPS (100 ng/ml), recombinant human IL-1 (10 U/ml), recombinant human tumor necrosis factor (TNF; 100 ng/ml), or purified *B. fragilis* outer membrane (diluted 1:1,000 [vol/vol] from pooled void volume samples). After the 4-h incubation period, HUVE were washed twice with RPMI 1640 and then treated with anti-E-selectin MAb *P6E2* to determine relative E-selectin expression on the cell surface in response to the various agonists. Results are from four separate experiments with three replicate wells for each experiment. OD_{490}, optical density at 490 nm.

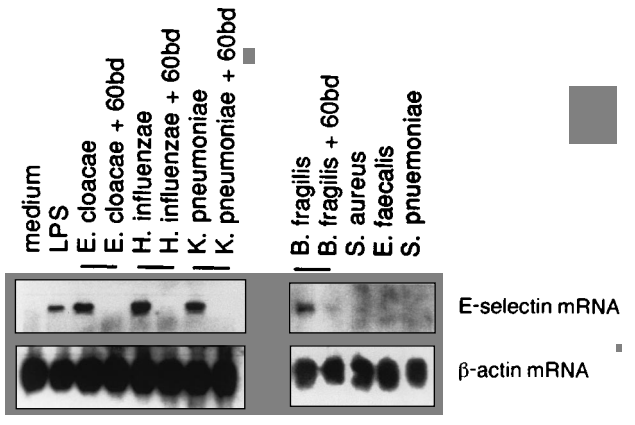

FIG. 6. Northern blot of HUVE treated with gram-negative and gram-positive bacteria. Total cellular RNA was isolated from HUVE, as described in Materials and Methods, after monolayers were treated for 3 h with LPS (100 ng/ml), gram-negative bacteria (*Enterobacter cloacae*, *H. influenzae*, *K. pneumoniae*, or *B. fragilis*), or gram-positive organisms (*Staphylococcus aureus*, *Enterococcus faecalis*, or *Streptococcus pneumoniae*). All organisms were heat killed. Gram-negative organisms were added at 10^5 CFU/ml for each condition, and gram-positive organisms were added at 10^7 CFU/ml for each condition.

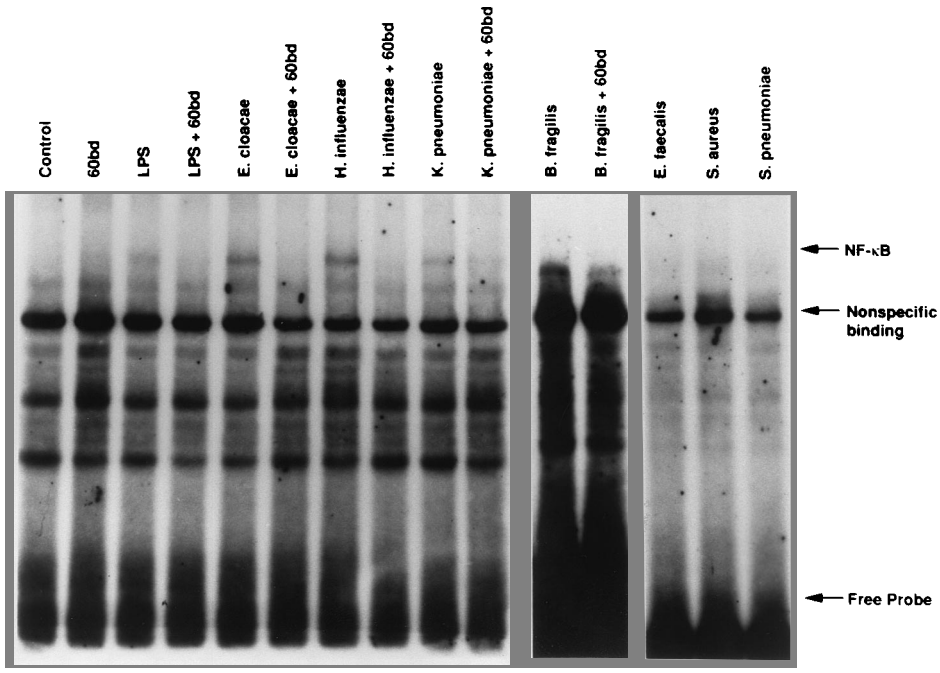

FIG. 7. EGMSA for NF-κB in bacterium-treated endothelium. Nuclear proteins were purified from HUVE after 4 h of treatment with *E. coli* LPS (100 ng/ml), gram-negative bacteria (*Enterobacter cloacae*, *H. influenzae*, *K. pneumoniae*, or *B. fragilis*), or gram-positive organisms (*Staphylococcus aureus*, *Enterococcus faecalis*, or *Streptococcus pneumoniae*), in the presence or absence of MAb *60bd*. Nuclear protein extracts were reacted with an oligonucleotide probe matching the sense strand of the E-selectin 5′-flanking region NF-κB binding site. In this assay with HUVE, a shifted band, labeled nonspecific binding, is always observed in both treated and untreated HUVE and is not considered to have *trans*-activating potential. A shifted band at a higher molecular weight is observed only when HUVE are activated. This band in HUVE most likely represents a p65/p50 heterodimer (23a).

expected, because of the absence of surface expression of E-selectin and tissue factor or mRNA accumulation for E-selectin and tissue factor, NF-κB was not detected in nuclei from HUVE treated with gram-positive bacteria, all at 10^7 CFU/ml. NF-κB activation induced by each of the gram-negative organisms was blocked by MAb *60bd*, also confirming that sCD14 initiates, at least in part, a signaling pathway leading to NF-κB activation.

CD14-transfected CHO cells. To define more precisely the requirement for CD14 in bacterium-associated LPS activation of NF-κB, CHO-K1 fibroblasts, stably transfected with human recombinant CD14 and expressing surface CD14 (obtained from Richard Ulevitch, Scripps Research Institute) were examined for responsiveness to either viable gram-negative or viable gram-positive bacteria (Fig. 8). Previous studies have demonstrated that wild-type CHO-K1 cells are unresponsive to LPS at concentrations up to 100 μg/ml but become markedly responsive to LPS, as determined by EGMSA for NF-κB, after stable transfection with human CD14 (8, 15, 19). Consistent with these studies, NF-κB translocation to the nucleus of CD14-transfected CHO cells was observed when these cells were treated with live *E. coli* organisms in the presence of serum. In contrast, viable *Staphylococcus aureus* induced no to minimal activity for the dilutions of bacteria examined (10^4 to 10^8 CFU/ml). Treating CD14-transfected CHO cells with medium alone did not result in activation of NF-κB. NF-κB activation in wild-type CHO cells was not observed after the cells were treated with either gram-positive or gram-negative organisms, whether heat-killed or viable. These findings suggest that LPS, as an integral constituent of gram-negative bacterial outer membranes, binds specifically to human sCD14.

DISCUSSION

Previous studies have demonstrated that the cell walls of gram-positive (20, 31, 37) and gram-negative bacteria contain unique structures that interact with human endothelial cells

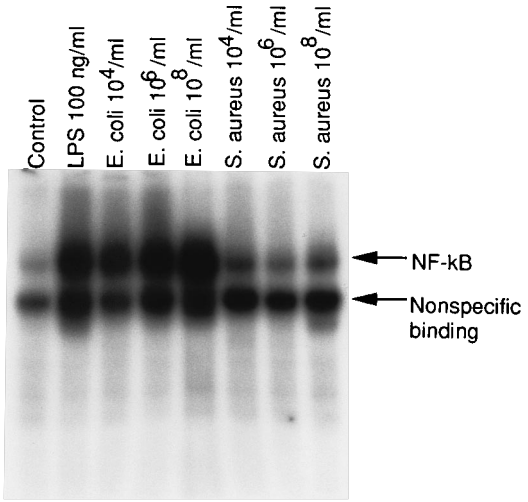

FIG. 8. NF-κB activation in CD14-transfected CHO cells treated with bacteria. Nuclear proteins were purified from human CD14-transfected CHO cells following 4 h of treatment with *E. coli* LPS (100 ng/ml), live *E. coli* organisms (10^4 to 10^8 CFU/ml), or live *Staphylococcus aureus* (10^4 to 10^8 CFU/ml). Nuclear protein extracts were reacted with an oligonucleotide probe, labeled with ^{32}P, matching the sense strand of the E-selectin 5′-flanking region NF-κB binding site.

and thus activate endothelium to a proinflammatory and procoagulant phenotype. Activation of HUVE with highly purified LPS from gram-negative organisms or with cell wall fragments from gram-positive organisms is mediated in both cases by a soluble form of CD14 circulating in serum (i.e., sCD14) (31). Although the isolation of active structures from the complex cell walls of bacteria simplifies analyses, the activity of these structures may be modified significantly by the architecture and composition of the complete cell wall. In addition, capsule may surround the cell wall and may potentially exclude outer membrane structures from interaction with the host. (The polysaccharide capsules of certain gram-negative organisms, however, may also mediate virulence; for example, intra-abdominal abscess formation induced by gram-negative, anaerobic bacteria requires the polysaccharide capsule of the organisms [45]). In this study, we have examined whether viable or heat-killed, gram-positive or gram-negative bacteria in serum activate cultured human endothelial cells by a CD14-dependent mechanism. We show that gram-negative bacteria, with LPS located at the outer aspect of the cell wall, activated HUVE. Gram-positive bacteria, in contrast, with putative activated components (for example, lipoteichoic acid) located within the cell wall, do not activate HUVE.

LPS of the outer membrane of gram-negative bacteria are considered to be major virulence factors for these pathogens. The active component of LPS, lipid A, is embedded in the outer leaflet of the outer membrane of the cell wall. In serum, LPS from many species of gram-negative organisms is released from the bacterial outer membrane in the form of mixed micelles composed of LPS, bacterial phospholipids, and membrane proteins (26). The formation of outer membrane blebs may serve a secretory function for the bacterium. This process also increases the accessibility of LPS to mammalian LBPs. Thus, in the presence of LBP, phospholipid micelles containing lipid A are converted from weak toxins to very active toxic moieties comparable to purified LPS (12, 41). With LBP, lipid A can be transferred from the hydrophobic environment of outer membrane phospholipid, through an aqueous environment as an LBP-LPS complex, to CD14. Binding of the LBP-LPS complex to CD14, anchored to the membrane by glycosylphosphatidylinositol, induces rapid phosphorylation of tyrosine residues in mitogen-activated protein kinases. Activation of a distinct 38-kDa protein kinase of the mitogen-activated protein kinase family (16) leads to further phosphorylation events in a protein kinase cascade that ultimately results in new gene transcription and cell activation. Capsular polysaccharides are also at the outer aspect of the cell wall in some species of gram-negative bacteria during different phases of the growth cycle. The solubility of capsular components in an aqueous environment should be expected to obviate the requirement for serum proteins analogous to LBP for mononuclear phagocyte activation or sCD14 for endothelial cell activation. The presence of encapsulating polysaccharides on one species of gram-negative bacteria examined in our study did not appear to activate HUVE even in the presence of serum. We found that characterized capsular polysaccharides A and B from *B. fragilis* (2, 25, 46, 47) did not induce E-selectin surface expression on HUVE even at very high concentrations or if added together.

Gram-positive bacteria are pathogenic as a result of production and release of specific exotoxins and also because of cell wall constituents that likely interact with the host in a fashion similar to that of gram-negative cell wall toxins (4). Exotoxins derived from *Staphylococcus* spp. have been shown to induce the release of platelet-activating factor (37) or endothelial-derived relaxation factor (20) from endothelial cells. Also, heat-killed *Streptococcus* spp. induce cultured endothelial cell procoagulant activity, demonstrating a virulence mechanism that cannot be attributed to heat-labile bacterial exotoxins (14). Recently, Pugin et al. have reported that constituents of gram-positive cell walls activate endothelial cells by a sCD14-dependent pathway (31). Gram-positive cell walls contain several constitutively or transiently expressed LPS-like amphiphilic lipopolymers, including lipoteichoic acid, lipomannan, lipopeptidoglycan, lipopoly-(*N*-acetyl-glucosamine), and lipoteichuronic acid, as examples (34). These molecules share similar properties with LPS in that they are located in association with the single phospholipid cell membrane of gram-positive bacteria, with the lipophilic domain embedded in the membrane. Unlike LPS, however, the hydrophilic domain of gram-positive cell wall amphiphiles often exist covalently linked to peptidoglycans that surround the cell membrane (36). Therefore, the amphiphilic putative cell wall toxins of viable gram-positive organisms are not necessarily exposed to the external environment. Because of the number of potential gram-positive cell wall toxins and the unique position of these structures in the cell wall, the molecular pathways by which gram-positive bacteria induce cellular activation have not been as well defined as those of gram-negative bacteria.

In our study, HUVE activation was not induced by either heat-killed or viable, growth-phase gram-positive bacteria. These results differ from a previous study in which heat-killed *Streptococcus pneumoniae*, with or without a capsule, induced procoagulant expression on HUVE (14). This difference in results may be caused by dissimilar methodologies used in the two studies to determine highly variable procoagulant activity. Live *Streptococcus pneumoniae* as well as the other two viable gram-positive organisms we examined is a strong activator of rabbit alveolar macrophages obtained by bronchoalveolar lavage, even though the same viable organisms had no activity on HUVE (24a). On the basis of our results with viable gram-positive organisms and previously reported data with gram-positive cell wall fragments, we postulate that putative cell wall compounds (possibly amphiphilic structures) interact with sCD14 only after disruption of the intact organism. Thus, activation of cultured endothelium by viable gram-positive organisms may occur after agents that disrupt the cell wall, such as β-lactam antibiotics, are included in the culture medium.

We have shown previously that LPS purified from *B. fragilis* (21) and deacylated LPS derived from *Salmonella typhi* (30) do not activate HUVE to a proinflammatory phenotype. These LPS molecules lack nonhydroxylated long-chain fatty acids esterified to the β-hydroxylated fatty acids substituted on the diglucosamine backbone of lipid A. It was therefore unexpected to observe endothelial cell activation induced by *B. fragilis* organisms. Since *B. fragilis* LPS competitively inhibits *E. coli* LPS induction of HUVE activation, it is unlikely that the HUVE-activating potential observed with *B. fragilis* organisms is due to contaminating, toxic LPS from other organisms. A partially purified outer membrane fraction of *B. fragilis* LPS, containing LPS, phospholipids, and bacterial proteins, retained the activity observed with heat-killed or viable *B. fragilis* organisms. In other experiments, we have shown that activation of HUVE to a proinflammatory phenotype by *B. fragilis* outer membrane can be completely inhibited by an anti-CD14 antibody, *60bd*, suggesting that pattern recognition of bacterial cell wall toxins by CD14 may extend to an unidentified toxin in the cell wall of *B. fragilis*. Of note, isolated porins from *B. fragilis* cell wall outer membrane induce platelet-activating factor release from cultured human endothelial cells (44). Whether *B. fragilis* porin-endothelial interactions are mediated by sCD14 is not known.

We conclude from this study that gram-negative organisms activate endothelium by a common sCD14-dependent pathway. We postulate that gram-negative LPS-sCD14 complexes (or an outer membrane structure other than LPS that is also taken up by sCD14) are derived from the gram-negative bacterial outer membrane and bind with an as-yet-unidentified endothelial cell membrane receptor. This interaction results in NF-κB activation, new gene transcription, and the induction of an endothelial cell activation phenotype. Further studies may determine whether therapies directed against sCD14 or the putative endothelial cell receptor for the bacterium-sCD14 complex could prove beneficial for patients at risk for gram-negative infections. Gram-positive organisms, in contrast, appear to induce endothelial cell activation indirectly through activation of mononuclear phagocytes and the release of proinflammatory cytokines that activate the endothelium to a proinflammatory and procoagulant phenotype.

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