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# Ability of Bacteria Associated with Chronic Inflammatory Disease To Stimulate E-Selectin Expression and Promote Neutrophil Adhesion

RICHARD P. DARVEAU,[1,2]* MARK D. CUNNINGHAM,[1] TINA BAILEY,[1] CARRIE SEACHORD,[1] KENT RATCLIFFE,[1] BRIAN BAINBRIDGE,[2] MARY DIETSCH,[1] ROY C. PAGE,[2] AND ALEJANDRO ARUFFO[1]

*Bristol-Myers Squibb Pharmaceutical Research Institute,[1] and Department of Periodontics, University of Washington,[2] Seattle, Washington*

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***Porphyromonas gingivalis*, *Pseudomonas aeruginosa*, and *Helicobacter pylori* have been shown to be associated with adult periodontal disease, chronic lung infections, and peptic ulcers, respectively. The ability of these bacteria to stimulate E-selectin expression and promote neutrophil adhesion, two components necessary for the recruitment of leukocytes in response to infection, was investigated. Little or no stimulation of E-selectin expression was observed with either *P. gingivalis* or *H. pylori* when whole cells, lipopolysaccharide (LPS), or cell wall preparations added to human umbilical cord vein endothelial cells were examined. *P. aeruginosa* was able to induce E-selectin to near-maximal levels; however, it required approximately 100 to 1,000 times more whole cells or LPS than that required by *E. coli*. Neutrophil-binding assays revealed that LPS and cell wall preparations obtained from these bacteria did not promote endothelial cell adhesiveness by E-selectin-independent mechanisms. In addition, *P. gingivalis* LPS blocked E-selectin expression by LPS obtained from other bacteria. We propose that lack of E-selectin stimulation and the inability to promote endothelial cell adhesiveness are two additional indications of low biologically reactive LPS. We suggest that this property of LPS may contribute to host tissue colonization. In addition, the ability of *P. gingivalis* to inhibit E-selectin expression may represent a new virulence factor for this organism.**

*Porphyromonas gingivalis*, *Pseudomonas aeruginosa*, and *Helicobacter pylori* represent three different gram-negative bacteria known to be associated with chronic inflammatory diseases. *P. gingivalis* has been established as a causative agent of adult-type periodontal disease by both retrospective clinical analysis (40) and nonhuman primate animal studies (14). *P. aeruginosa* has long been known to be a primary agent contributing to the diminution of lung function in children and young adults with cystic fibrosis (19). Recently, eradication of *H. pylori* from the lesions associated with peptic ulcer disease has established a role for this organism in the disease process (12). Persistent colonization of host tissue is a common characteristic of these bacteria and is believed to contribute to a destructive inflammatory response resulting in disease. The mechanisms by which these bacteria can elicit a destructive inflammatory response are varied (16, 19, 27). Little is known, however, about how these bacteria initially overcome the innate host defense mechanism and persist in host tissue.

One of the first steps of a normal host response to bacterial invasion is the trafficking of leukocytes from the vascular compartment to extravascular tissues (35). Leukocyte trafficking to surrounding tissue is initiated by an inflammatory stimulus which induces expression of selectin molecules on the surface of the endothelial cell. Selectins are required for leukocyte emigration at the physiologic shear stresses found in the vascular compartment (35). Recently, a novel congenital leukocyte adhesion deficiency has been described in which a deficiency in the expression of sialyl-Lewis X, a selectin ligand found on leukocytes, resulted in severe and recurrent bacterial infections (7). This study clearly demonstrated the requirement for a functional selectin pathway for the normal clearance of bacteria from host tissue. Currently, three different selectins have been described in humans, two of which (P- and E-selectin) are expressed on the vascular endothelium (15).

In vitro studies suggest that E-selectin is involved in the host response to bacterial infection. Lipopolysaccharide (LPS) has been shown to be a potent inducer of E-selectin expression in human endothelial cells (2). Direct E-selectin stimulation with LPS requires soluble CD14 (9, 30) and, similar to VCAM, probably requires a subsequent interaction with an as-yet-unidentified receptor on the endothelial cell. Indirect stimulation of E-selectin occurs via interleukin 1β (IL-1β) or tumor necrosis factor alpha (TNF-α), which are themselves produced in response to bacterial infection by numerous cell types (6, 31).

The rapid and sensitive response to the presence of LPS by endothelial cells is consistent with the pivotal role these cells play in the recognition and response to bacterial infection. In spite of this, *P. gingivalis*, *P. aeruginosa*, and *H. pylori*, all gram-negative bacteria, are able to grow to large numbers in host tissue. In this investigation, it is demonstrated that *P. gingivalis* and *H. pylori* failed to directly stimulate E-selectin expression or neutrophil adhesion to stimulated endothelial cell layers. In addition, *P. gingivalis* LPS inhibited E-selectin expression by LPS obtained from other bacteria. These observations suggest that an additional bacterial component of a successful colonization strategy in chronic inflammatory disease is the inability to stimulate E-selectin expression or promote neutrophil adhesion.

## MATERIALS AND METHODS

**Reagents and buffers.** Human umbilical cord endothelial cell (HUVEC) growth medium consisted of Media 199 (Gibco Laboratories, Gaithersburg, Md.) plus 4 mM L-glutamine, 90 μg of heparin per ml, 1 mM Na pyruvate, 30 μg of endothelial cell growth stimulant (Biomedical Products, Bedford, Mass.) per ml, and 20% fetal bovine serum (Hyclone Laboratories, Inc., Logan, Utah). M-199

* Corresponding author. Mailing address: Bristol-Myers Squibb Pharmaceutical Research Institute, 3005 First Ave., Seattle, WA 98121. Phone: (206) 727-3511. Fax: (206) 727-3602.

stimulation medium consisted of medium 199 plus 4 mM L-glutamine, 90 μg of heparin per ml, 1 mM Na pyruvate, 1 mg of human serum albumin per ml, and 5% pooled normal human serum (Gemini Bioproducts, Calabasas, Calif.). RPMI 1640 medium was obtained from GIBCO (Grand Island, N.Y.). Anti-human E-selectin monoclonal antibody was obtained from R&D Systems (Minneapolis, Minn.). TNF-α and IL-1β levels in supernatants were measured with Biotrak enzyme-linked immunosorbent assay (ELISA) kits (Amersham, Arlington Heights, Ill.).

**Endothelial cells.** HUVECs were obtained from Clonetics (San Diego, Calif.) and maintained in HUVEC growth medium. Cells were used at the fourth passage for all experiments described in this report. Some initial experiments were conducted on cells in the second or third passage, with no apparent difference in the E-selectin response.

**Bacterial strains and incubation conditions.** *Escherichia coli* ATCC 29552, which contains the O111:B4 serotype LPS, and *E. coli* ATCC 25922 were obtained from the American Type Culture Collection. Other *E. coli* strains examined included JM 83, MC1061, and MC4100 and were obtained from John Somerville, Bristol-Myers Squibb. *P. gingivalis* strains and *Bacteroides forsythus* 9610 were obtained from Aaron Weinberg, Department of Periodontics, University of Washington; these included *P. gingivalis* ATCC 33277 and strains 381, A7A1-28, A7436, and 5083. *P. aeruginosa* ATCC 27313 and ATCC 27316, *H. pylori* ATCC 43504, *Neisseria flavescens* ATCC 13120, *Fusobacterium nucleatum* ATCC 25586, *Eikenella corrodens* ATCC 23834, *Leptotrichia buccalis* ATCC 14201, and *Haemophilus parainfluenzae* BMS C128 were obtained from our in-house bacterial stock collection. Strains were examined for purity, properly identified, and stored at −70°C. Each week, new cultures were made from frozen bacterial stocks to avoid repetitive subculturing. Except for *H. pylori*, bacteria were grown on brucella agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% defibrinated sheep blood, vitamin K, and hemin as described previously (23). *H. pylori* was grown on agar medium consisting of Trypticase soy broth (BBL Microbiology Systems) with 1.5% agar (Bacto Agar, Difco) supplemented with 10% fetal bovine serum and 0.1% yeast extract (Difco). Cultures were incubated either overnight or for up to 72 h at 37°C under either microaerophilic, anaerobic, or aerobic conditions as appropriate.

**Preparation of LPS and bacterial cell walls.** LPS from *E. coli* O111:B4 and LPS from *P. aeruginosa* HABS10 were obtained from Sigma Chemical Co. (St. Louis, Mo.); LPS was purified from *P. gingivalis* 33277 and A7A1-28 by the phenol-water method (38); LPS was purified from *B. forsythus*, *P. gingivalis* 5083, and *H. pylori* ATCC 43504 by the cold Mg-ethyl alcohol procedure (5). All LPS preparations were suspended in distilled water. LPS preparations were determined to be free from contaminating nucleic acid and protein by determining the optical densities at 280 and 260 nm and subjected to gas chromatographic analysis for sugar and fatty acid compositions (4, 33). The compositions of all preparations were consistent with published data (10, 20). Cell walls were prepared from bacteria grown as described above. Cells were scraped from plates and suspended in M-199 stimulation medium without serum. The cells were broken by passage through a French pressure cell at 15,000 lb/in$^2$. Unbroken cells were removed by centrifugation at 2,200 × *g* for 10 min. Cell walls were collected by centrifugation of the low-speed supernatant at 30,000 × *g* for 20 min at 4°C. Cell walls were suspended in M-199 stimulation medium. A portion of the sample was suspended in M-199 stimulation medium without serum or albumin for total protein determinations. Protein was determined by the Bradford method (Coomassie Protein Assay Reagent; Pierce Chemical Co., Rockford, Ill.) as described in the manufacturer's instructions.

**Human E-selectin expression assay.** HUVECs ($1.4 \times 10^4$ per well) were plated in a fibronectin-precoated 96-well flat-bottom plate (Costar, Pleasanton, Calif.) in HUVEC growth medium the day before stimulation by bacterial cells or bacterial cell products. On the day of the assay, bacterial cultures were suspended (from plate-grown cells) in M-199 stimulation medium and diluted in the same medium to the desired cell number by calculation from a predetermined conversion factor. Conversion factors for each bacterial strain were determined by plate count analysis performed in triplicate by standard procedures for bacterial enumeration. LPS and cell wall preparations were suspended in M-199 stimulation medium at the concentrations indicated in the figures. HUVECs were washed with M-199 stimulation medium without serum, and bacterial preparations were then added to the HUVEC monolayer and incubated for 4 h at 37°C under 5% $CO_2$. After the stimulation interval, the medium was removed, and the cells were washed twice in cold phosphate-buffered saline (PBS), fixed with 0.5% glutaraldehyde (in cold PBS), and placed at 4°C for 10 min. The cells were washed three times with PBS containing 3% pooled goat serum (Sigma) and 0.02 M EDTA (blocking buffer), and after the last wash, 0.2 to 0.3 ml of blocking buffer was added to each well and the plates were stored overnight at 4°C (this blocking step was complete after 1 h, but for convenience, overnight incubations were employed routinely). Blocking buffer was removed, 0.1 ml of anti-E-selectin monoclonal antibody (R&D Systems) at 0.25 μg/ml in blocking buffer was added to each well, and the plate was incubated at 37°C for 1 h. The plates were washed three times in blocking buffer, and 0.1 ml of F(ab′)2 goat anti-mouse immunoglobulin G-specific horseradish peroxidase-conjugated second-step antibody (Jackson Immunoresearch Labs, West Grove, Pa.) diluted in blocking buffer was added to each well. The plates were incubated at 37°C for 1 h and washed four times with blocking buffer, and 0.1 ml of chromagen reagent (3,3′,5,5′-trimethylbenzidine dihydrochloride in substrate buffer; Genetic Systems, Redmond, Wash.) was added. The reaction was stopped with 0.1 ml of 1 N $H_2SO_4$ per well, and the plates were read in an ELISA reader (BioTek Instruments, Winooski, Vt.). When combinations of *P. gingivalis* LPS and *E. coli* LPS were to be examined, they were premixed before addition to the HUVEC monolayer. Endothelial cell viability was determined on duplicate plates after the 4-h incubation by the intracellular esterose hydrolysis of calcein-acetomethyl ester method as described by the manufacturer (Molecular Probes, Inc., Eugene, Oreg.).

**Human neutrophil preparation.** Blood was obtained from normal healthy human volunteers by venipuncture with heparin-containing syringes. Neutrophils were isolated by density gradient centrifugation with Polymorphprep (Nycomed Pharma AS, Oslo, Norway) as described by the manufacturer. Contaminating erythrocytes were lysed as described previously (18). A portion of the neutrophil preparation was stained and checked for purity, and the remaining cells were suspended to $4 \times 10^6$ cells per ml for fluorescent labeling. Neutrophils were labeled with 2′,7′-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein, acetomethyl ester (BCECF-AM) (Molecular Probes) as described in the guidelines given in the manufacturer's instructions. Specifically, neutrophils were incubated with 10 mM BCECF-AM in dimethyl sulfoxide for 15 min in the dark, an equal volume of RPMI 1640 medium containing 5% fetal bovine serum was added, and the cells were centrifuged. Neutrophils were washed in PBS, suspended at $2 \times 10^6$ cells per ml in RPMI 1640 medium containing 1% fetal calf serum (FCS), and kept in the dark.

**Neutrophil adhesion assay.** The basic procedure described by Magnuson et al. (18) was followed. HUVEC monolayers were prepared as described above for the E-selectin expression assay with the exception that $4 \times 10^4$ HUVECs per well were added to a 96-well plate. HUVEC monolayers were stimulated for 4 h with LPS or cell wall preparations as described above for the E-selectin expression assay. After the 4 h of stimulation, the HUVEC monolayers were washed with PBS containing 5% FCS, and labeled neutrophils (0.1 ml of the stock solution representing $2 \times 10^5$ cells per well) were added. The neutrophil-HUVEC cell preparation was covered with foil and placed on a shaker with mild agitation for 30 min at ambient temperature. After 30 min, the nonadherent neutrophils were removed by careful aspiration and washed twice with PBS containing 5% FCS. After washing, 0.1 ml of a solution containing 50 mM Tris (pH 8) and 1% sodium dodecyl sulfate was added to the HUVEC monolayers, and the plate was read on a fluorescence reader (Baxter Scientific Products, Philadelphia, Pa.) with excitation at 485 nm and emission at 535 nm. The percentage of total neutrophils which adhered in each assay was determined by constructing a standard curve with various amounts of lysed neutrophils plotted against fluorescent intensity. Routinely, at near-maximum binding (20,000 U), approximately 50% of the neutrophils were bound. When combinations of *P. gingivalis* LPS and *E. coli* LPS were to be examined, they were premixed before addition to the HUVEC monolayer.

**RNA blot by hybridization analysis of E-selectin transcripts from HUVECs.** HUVECs ($\sim 6 \times 10^6$) were stimulated for 4 h at 37°C, under 6% $CO_2$. Total RNA was obtained by standard procedures (32), electrophoresed through a 1% agarose gel, transferred to nylon membranes, hybridized with a radiolabeled probe prepared from a ~1.7-kb cDNA fragment encoding the extracellular domain of human E-selectin (3), and autoradiographed for 24 h.

## RESULTS

**Bacterial stimulation of E-selectin expression.** Initially, the ability of whole bacteria to stimulate E-selectin expression on HUVECs was examined (Fig. 1). Although *E. coli* cells were a potent inducer of E-selectin expression, *H. pylori* and *P. gingivalis* were very poor stimulators of E-selectin expression. At the highest concentrations of these bacteria added to the assay, only low levels of E-selectin were observed. Similar to *E. coli*, *P. aeruginosa* induced nearly maximal levels of E-selectin expression in the assay; however, approximately 3-logs more *P. aeruginosa* bacteria were required. The degree of E-selectin stimulation was consistent among different species in a single genus. For example, four additional strains of *E. coli* and a *Salmonella typhimurium* strain (Table 1) displayed dose-response curves similar to that shown for *E. coli* in Fig. 1. Five different strains of *P. gingivalis* were examined and failed to stimulate E-selectin expression. Strains examined but not shown in Fig. 1 included a monkey strain (strain 5083) previously used to demonstrate that *P. gingivalis* can function as a primary pathogen in periodontal disease (14), a strain (A7436) that is particularly virulent in a rodent model of infection (11), and two other strains, designated A7A1-28 and 381. Two different strains of *P. aeruginosa* yielded similar dose-response curves (Fig. 1 and Table 1). Microscopic examination of the

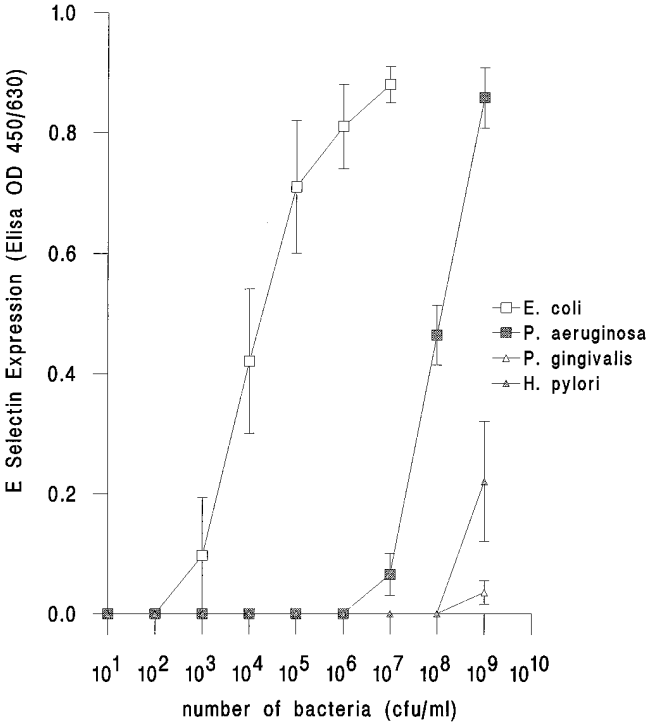


FIG. 1. Stimulation of E-selectin expression with whole bacteria. Bacterial suspensions were added to a monolayer of fourth-passage HUVECs. After 4 h of incubation, the plates were washed and assayed for the presence of E-selectin by an ELISA employing an anti-E-selectin monoclonal antibody. Bacterial cells were diluted in tissue culture medium to the indicated cell number by calculation from a predetermined conversion factor. Endothelial cell viability was determined on duplicate plates after the 4-h incubation by the calcein-AM method (see text). *E. coli* ATCC 29552, *P. gingivalis* ATCC 33277, and *H. pylori* ATCC 43504 were examined in five separate experiments. *P. aeruginosa* ATCC 27313 was examined in two separate experiments. The mean and interassay standard deviation from the mean are presented. OD, optical density.

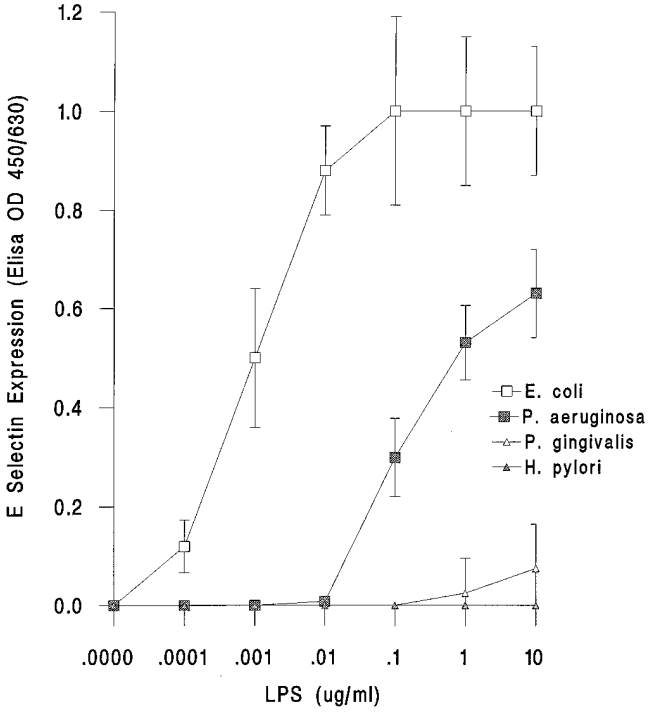


FIG. 2. Stimulation of E-selectin expression with LPS. LPS preparations obtained as described in the text (*E. coli* O111:B4, *P. aeruginosa* HABS10, *P. gingivalis* ATCC 33277, and *H. pylori* ATCC 43504) were added to a monolayer of fourth-passage HUVECs. After 4 h of incubation, the plates were washed and assayed for the presence of E-selectin as described in the text. LPS preparations were suspended in distilled water; duplicate calcein-AM assays revealed no toxicity. Each assay was performed on at least four separate occasions. The mean and interassay standard deviation from the mean are presented. OD, optical density.

endothelial cell layer after bacterial stimulation revealed no change in endothelial cell shape or loss in cell number at the bacterial concentrations employed in the assay. A more quantitative estimate of endothelial cell viability was determined by measuring the hydrolysis of calcein-acetomethyl ester. This reagent detects hydrolysis mediated by an esterase present in eukaryotic but not bacterial cells (31a). Poor E-selectin stimulation by *H. pylori* and *P. gingivalis* could not be attributed to endothelial cell toxicity since even high concentrations of these bacteria were not toxic as assayed by these parameters. IL-1β was added as an additional control of the ability of endothelial cells to express E-selectin in the presence of bacteria. At IL-1β concentrations ranging from 0.03 to 20 ng/ml, $10^9$ whole *P. gingivalis* cells did not reduce the E-selectin ELISA signal. In contrast, calcein-AM hydrolysis assays revealed that concentrations of *E. coli* of $10^8$ CFU/ml and greater were toxic.

TABLE 1. E-selectin stimulation: effect of different strains of bacteria[a]

| Bacteria (CFU) | E-selectin stimulation by[b]: | | | |
|---|---|---|---|---|
| | *E. coli* ATCC 25922 | *E. coli* JM83 | *S. typhimurium* A568 | *P. aeruginosa* ATCC 27316 |
| $10^9$ | ND[c] | ND | ND | 0.84 ± 0.01 |
| $10^8$ | ND | ND | ND | 0.36 ± 0.05 |
| $10^7$ | ND | ND | ND | 0.07 ± 0.05 |
| $10^6$ | 0.71 ± 0.20 | 0.83 ± 0.07 | 0.66 ± 0.14 | 0 |
| $10^5$ | 0.71 ± 0.06 | 0.74 ± 0.05 | 0.44 ± 0.22 | 0 |
| $10^4$ | 0.52 ± 0.08 | 0.38 ± 0.11 | 0.21 ± 0.04 | 0 |
| $10^3$ | 0.20 ± 0.10 | 0.19 ± 0.06 | ND | |
| $10^2$ | 0.03 ± 0.04 | ND | ND | |

[a] The examination of E-selectin expression by ELISA was performed as described in the text and legend to Fig. 1.

[b] Each value represents the mean ± standard deviation (in ELISA optical density units) from at least three separate experiments for each strain examined. *E. coli* ATCC 25922 is a clinical isolate, whereas *E. coli* JM83 is a laboratory strain. Other laboratory strains (MC1061 and MC4100) yielded similar results in two separate experiments.

[c] ND, not determined.

**LPS and cell wall stimulation of E-selectin expression.** The potential of isolated LPS preparations to directly stimulate E-selectin expression was examined (Fig. 2). Similar to what has been reported in other studies (9, 13), serum was required to obtain a significant E-selectin response to *E. coli* LPS. The response to *E. coli* LPS was potent, with as little as 1 ng yielding significant expression. In contrast, but similar to the data obtained with whole cells, LPS obtained from *P. gingivalis* and *H. pylori* did not induce E-selectin expression. Also, similar to what was observed with whole cells, *P. aeruginosa* required significantly more LPS to obtain a level of E-selectin expression equivalent to that of *E. coli*. In addition to the data presented in Fig. 2, LPS obtained from three additional strains of *P. gingivalis* (A7A1-28, A7436, and 5083) and LPS obtained from *B. forsythus* (a gram-negative anaerobe implicated in periodontal disease) also failed to stimulate E-selectin expression (a minimum of three separate experiments was performed

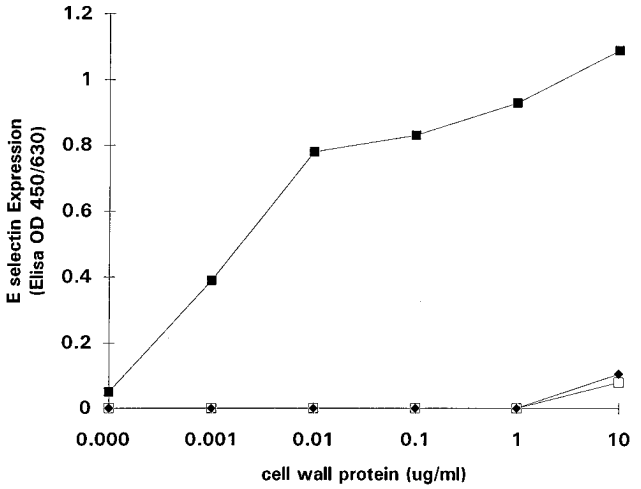


FIG. 3. Stimulation of E-selectin expression with bacterial cell wall preparations. Bacterial cell walls prepared as described in the text (*E. coli* ATCC 29552 [■], *P. gingivalis* ATCC 33277 [□], and *H. pylori* ATCC 43504 [◆]) were added to a monolayer of fourth-passage HUVECs. After 4 h of incubation, the plates were washed and assayed for the presence of E-selectin as described in the text. A duplicate calcein-AM assay revealed no endothelial cell toxicity. Each experiment was performed on at least three separate occasions with similar results. The data are presented as the average of a typical experiment performed in triplicate. OD, optical density.

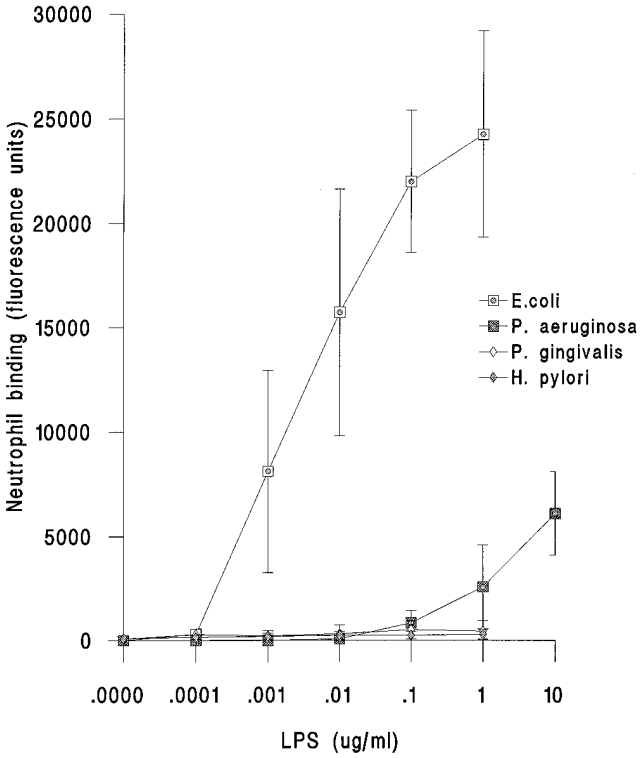


FIG. 4. Neutrophil adherence to HUVECs treated with different LPS preparations. Human neutrophil adherence assays were performed as described in the text. HUVECs were pretreated for 4 h with either *E. coli* ATCC 29552, *P. gingivalis* ATCC 33277, *P. aeruginosa* HABS10, or *H. pylori* ATCC 43504 preparations at the indicated concentrations. At maximum binding (20,000 fluorescence units), approximately 50% of the neutrophils added were bound. Three separate experiments were performed. The mean and interassay standard deviation from the mean are presented.

with each LPS at 1 μg/ml). No stimulation of E-selectin was observed when these LPS preparations were examined with or without the addition of human serum. Cell walls obtained from *P. aeruginosa* ATCC 27316 yielded a similar, significantly reduced response. Cell walls obtained from *H. pylori* ATCC 43504 or *P. gingivalis* ATCC 33277 also were unable to elicit E-selectin expression (Fig. 3). Calcein-AM hydrolysis assays confirmed that these preparations were not toxic to the endothelial cells during the assay.

**LPS and cell wall effect on endothelial cell adhesion to neutrophils.** Neutrophil adhesion assays were performed to determine if these bacterial preparations promoted leukocyte adhesion to endothelium through E-selectin-independent mechanisms. In the assay, human endothelial cells were preexposed to bacterial preparations before the addition of fluorescently labeled neutrophils to evaluate the direct effects of bacterial products on endothelial cells. Although *E. coli* LPS was a potent inducer of neutrophil adhesion (18) (Fig. 4), no adhesion was detected when *P. gingivalis* or *H. pylori* LPS was examined, and significantly lower neutrophil adhesion occurred after endothelial cell exposure to *P. aeruginosa* LPS. In addition, in two separate experiments, the ability of cell wall preparations obtained from *E. coli* ATCC 29552, *P. gingivalis* ATCC 33277 and A7A1-28, *P. aeruginosa* ATCC 27316, and *H. pylori* ATCC 43504 to induce neutrophil adhesion to endothelial cells was examined. *E. coli* cell walls promoted neutrophil adhesion at 10 ng of protein per ml (greater than 20,000 fluorescence units), whereas no significant neutrophil adhesion was observed (i.e., less than 5,000 fluorescence units) when 10 μg of cell wall protein per ml obtained from each of the bacteria listed was examined.

**Indirect activation of E-selectin expression by *P. gingivalis* LPS.** The ability of *P. gingivalis* LPS to induce E-selectin expression by the indirect pathway was examined (Fig. 5). When added to a human adherent monocyte cell population (see Materials and Methods), *P. gingivalis* ATCC 33277 LPS at 1 μg/ml was able to stimulate the secretion of both IL-1β and TNF-α. *E. coli* was able to stimulate similar levels of these cytokines at 1 ng/ml.

***P. gingivalis* LPS inhibition of direct E-selectin expression and neutrophil adhesion.** The ability of *P. gingivalis* LPS to inhibit the expression of E-selectin by other LPS preparations was examined. Preparations of *E. coli* O111:B4 and *P. gingivalis* ATCC 33277 LPS were mixed at various ratios and then added to endothelial cells (Fig. 6a). *P. gingivalis-E. coli* LPS mixtures in which the *P. gingivalis* LPS was 10- to 100-fold greater than the *E. coli* LPS were able to significantly block the stimulation of E-selectin. However, when similar mixing experiments were performed with *P. gingivalis* LPS and TNF-α, no inhibition of E-selectin expression was observed (Fig. 6b). *P. gingivalis* LPS also blocked E-selectin expression by cell wall preparations obtained from a variety of different bacteria normally found in supragingival plaque (Fig. 7). Lack of E-selectin expression and inhibition of *E. coli* LPS stimulation of E-selectin was confirmed by RNA blot hybridization analysis of E-selectin transcripts (Fig. 8).

*P. gingivalis* LPS was also an effective blocker of *E. coli* LPS-stimulated neutrophil adhesion. When preparations of *E. coli* and *P. gingivalis* LPS were mixed at various ratios and preincubated with endothelial cells in the neutrophil adhesion assay, *P. gingivalis* LPS was able to completely block *E. coli*-stimulated adhesion (Fig. 9). Inhibition of neutrophil adhesion was *P. gingivalis* LPS dose dependent at two different *E. coli* LPS stimulation concentrations.

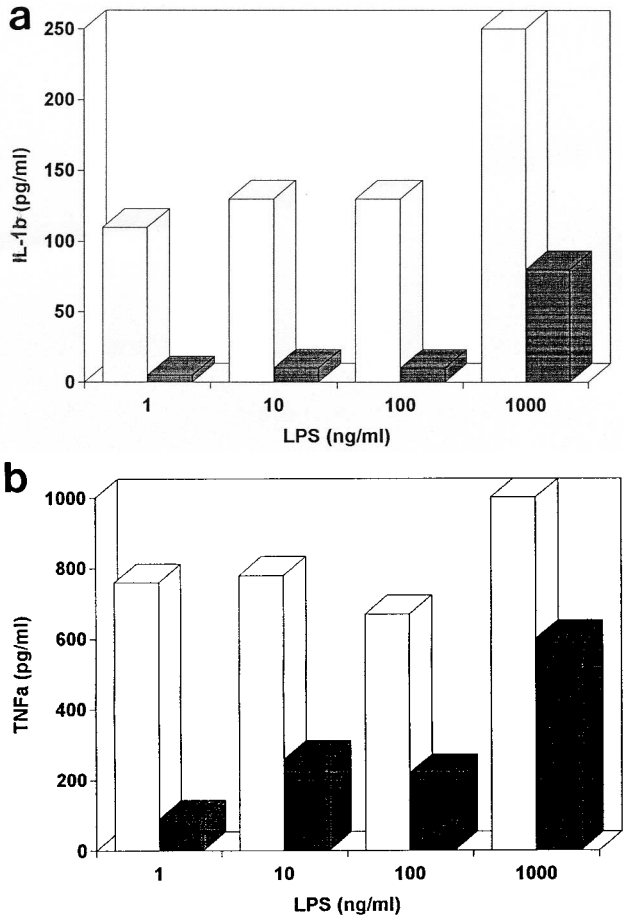


FIG. 5. Stimulation of IL-1β (a) and TNF-α (b) by *E. coli* ATCC 29552 LPS (□) and *P. gingivalis* ATCC 33277 LPS (■). TNF-α and IL-1β were assayed with Biotrak ELISA kits (see text). A human adherent monocyte cell population was obtained by incubation of $8 \times 10^6$ peripheral blood lymphocytes (isolated from normal donors with Ficoll-Hypaque) in a 24-well plate. Nonadherent cells were rinsed free, and the adherent cells were incubated with and without the indicated concentrations of LPS for 24 h as described in the text. Each assay was performed twice with similar results. The results from a typical experiment are shown.

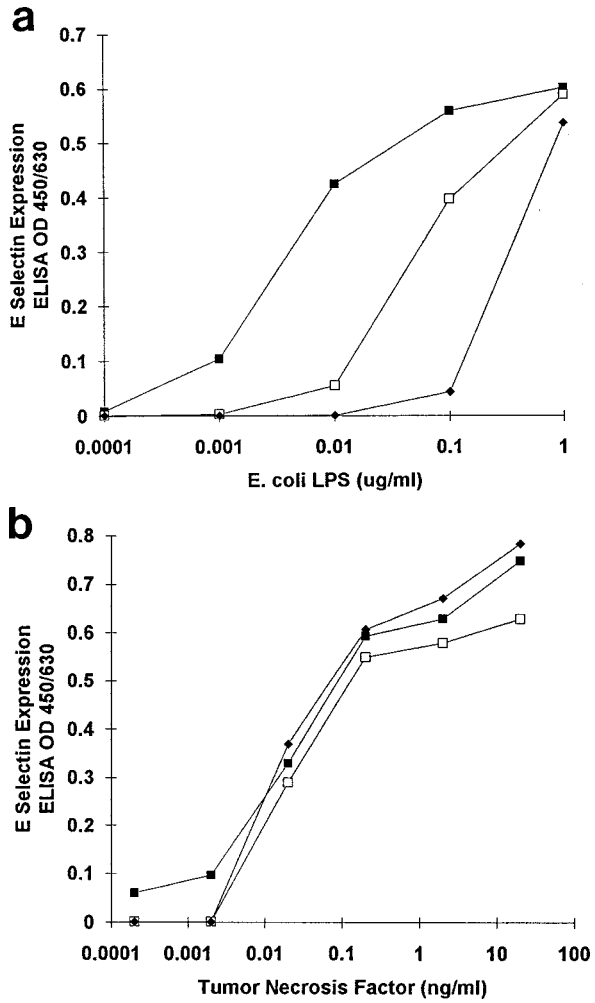


FIG. 6. *P. gingivalis* ATCC 33277 LPS inhibition of *E. coli* LPS but not TNF-α stimulation of E-selectin. *P. gingivalis* LPS (at 1 [□] or 10 [◆] μg/ml) was mixed with preparations of *E. coli* O111:B4 LPS (a) or TNF-α (b) as indicated on the horizontal axis prior to the addition to HUVECs. The assay was also done with no *P. gingivalis* LPS (■). The assay of E-selectin was performed after 4 h as described in the text. Three separate experiments were performed with similar results. The results of a typical experiment are shown. OD, optical density.

## DISCUSSION

This study has demonstrated that *P. gingivalis* and *H. pylori*, two bacteria associated with chronic inflammatory disease, were unable to induce E-selectin expression or promote neutrophil adhesion. *P. aeruginosa*, a chronic colonizer of children and young adults with cystic fibrosis, was not as potent as *E. coli* in stimulating these initial inflammatory events. No significant difference in the ability of whole cells, isolated LPS, or cell wall preparations obtained from these bacteria to stimulate E-selectin expression or neutrophil adhesion was observed. These experiments suggest that the lack of E-selectin expression was not a result of the form in which the LPS was presented to the endothelial cells. It may relate to in vivo conditions where endothelial cells may be exposed to both whole bacteria and soluble components.

*P. gingivalis* and *H. pylori* both have been reported to contain LPS of low biological reactivity (10, 20, 22, 26). LPS of low biological reactivity is determined by comparisons with *E. coli* in the ability to elicit inflammatory cytokine production, mitogenicity, pyrogenicity, Schwartzman reaction, and other indicators of LPS activity. Most of the immunobiological activity of LPS resides in the lipid A portion of the molecule, and studies with defined partial structures of *E. coli* LPS (17) as well as chemically synthesized analogs (36) have shown that reduction in either the degree of fatty acid acylation or number of phosphates significantly decreased cytokine production from human monocytes and yielded a negative Shwartzman reaction. The Shwartzman reaction is an in vivo inflammatory phenomenon which was shown recently to require the participation of endothelial cell adhesions (1). The work presented here suggests that lack of E-selectin expression and the failure to promote neutrophil adhesion are also indicators of LPS of low biological reactivity. The chemical composition of both *P. gingivalis* (10, 25) and *H. pylori* (20) LPS is consistent with these LPSs being less-potent inflammatory mediators. *P. aeruginosa* also has a lipid A composition which differs significantly from that of *E. coli* (39).

It seems paradoxical that bacteria associated with inflammatory disease would contain LPS of low biological reactivity. Initially, it would seem more likely that these bacteria would have an LPS that would be a strong stimulator of inflammatory mediators. Indeed, when we first initiated these investigations,

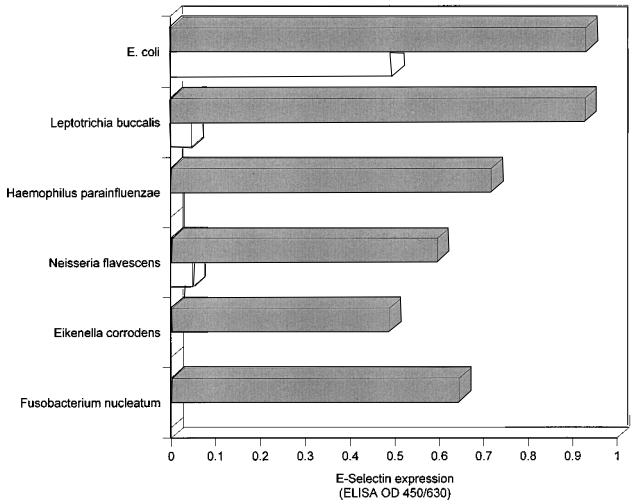


FIG. 7. *P. gingivalis* ATCC 33277 LPS inhibition of oral bacterial cell wall stimulation of E-selectin expression. An E-selectin ELISA was performed as described in the text. Cell wall preparations were obtained from *E. coli* ATCC 29552; *L. buccalis* ATCC 14201; *H. parainfluenzae* BMS C128; *N. flavescens* ATCC 13120; *E. corrodens* ATCC 23834; and *F. nucleatum* ATCC 25586 as described in the text and were added (1 μg of protein per ml) to human endothelial cells (■). In addition, the same amount of cell wall protein was mixed with 10 μg of *P. gingivalis* ATCC 33277 LPS per ml and added to human endothelial cells (□). The results presented are from a typical experiment which was performed on three separate occasions.

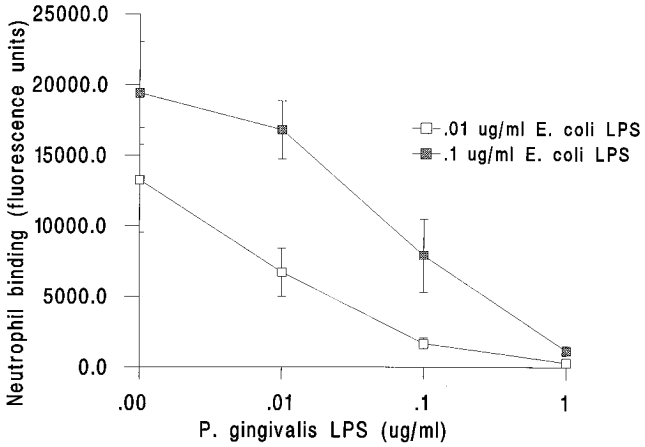


FIG. 9. *P. gingivalis* ATCC 33277 LPS inhibition of *E. coli* LPS-stimulated neutrophil adherence to HUVECs. HUVECs were pretreated for 4 h with two concentrations of *E. coli* O111:B4 LPS (as indicated on the figure) mixed with various concentrations of *P. gingivalis* ATCC 33277 LPS (as indicated on the horizontal axis). Human neutrophil adherence assays were performed as described in the text. At maximum binding (20,000 fluorescence units), approximately 50% of the neutrophils added were bound. Two separate experiments were performed. The mean and intraassay standard deviation from the mean are presented.

we hypothesized that these bacteria would be potent stimulators of E-selectin expression and neutrophil adhesion. However, in addition to causing an inflammatory infiltrate during episodes of disease in the periodontium (28), lung (19), and stomach (12), these bacteria must also be able to evade the initial innate host defense mechanism, colonize, and persist in tissue. The lack of E-selectin expression and neutrophil adhesion exhibited by *P. gingivalis* and *H. pylori* and the poor induction of these parameters by *P. aeruginosa* may contribute directly to the successful persistent colonization aspect of the disease process. It is possible that the lack of direct activation of endothelial cells combined with poor immunobiological activity of LPS allows these bacteria to be poorly detected or perhaps go undetected by the host for long periods of time. This speculation will require additional work defining the relative roles of direct and indirect (cytokine-mediated) activation of inflammation.

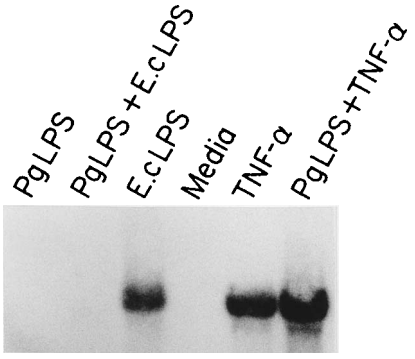


FIG. 8. RNA blot hybridization analysis of E-selectin transcripts from HUVECs. Total RNA (10 mg) was obtained from resting HUVECs (media) or HUVECs stimulated with *P. gingivalis* ATCC 33277 LPS (Pg-LPS; 1 μg/ml), *E. coli* O111:B4 LPS (E.cLPS; 10 ng/ml), TNF-α (1 ng/ml), *P. gingivalis* ATCC 33277 LPS plus *E. coli* O111:B4 LPS (Pg-LPS+E.cLPS; 1 μg/ml and 10 ng/ml, respectively), or *P. gingivalis* ATCC 33277 LPS plus TNF-α (Pg-LPS+TNF-α; 1 μg/ml and 1 ng/ml, respectively). The fourth-subculture HUVECs (~$6 \times 10^6$) were stimulated for 4 h at 37°C, under 6% $CO_2$. RNA was electrophoresed through a 1% agarose gel, transferred to nylon membranes, hybridized with a radiolabeled probe prepared from a ~1.7-kb cDNA fragment encoding the extracellular domain of human E-selectin (3), and autoradiographed for 24 h.

*P. gingivalis* LPS was able to completely inhibit the ability of cell walls obtained from bacteria found in clinically healthy supragingival plaque to directly stimulate E-selectin expression. This may be particularly relevant to host defense in the periodontium. Recently, it has been reported that clinically normal periodontal tissue has elevated levels of E-selectin (21, 24) and the inflammatory cytokine MCP-1 (37). The increased expression of these inflammatory mediators is consistent with observations that describe a state of low-level inflammation in clinically normal tissue (28, 37). It is possible that components of normal supragingival plaque (such as cell walls) diffuse into gingival tissue and elicit the production of these markers of inflammation. This would be consistent with the expression of MCP-1 and IL-8 in clinically healthy tissue in close proximity to bacterial plaque (37). The ability of *P. gingivalis* LPS to block direct E-selectin expression in this environment may contribute to the colonization of the tooth root surface and the proposed bacterial blooms that occur in periodontal disease (34). In addition, inhibition of inflammation normally induced by other bacteria may contribute to the characteristically large numbers of different bacteria found in these lesions (34). In this respect, inhibition of inflammation in the periodontium by *P. gingivalis* LPS would fit the attribute of circumventing or exploiting the host's innate defense mechanism ascribed to successful pathogens (8).

Although this is the first report of LPS inhibition of E-selectin expression, other investigators have shown that deacylated *S. typhimurium* LPS (29) as well as *Bacteroides fragilis* LPS (18) inhibited neutrophil adhesion. Inhibition of inflammation by *B. fragilis* was proposed (18) to be beneficial to the host in areas like the intestine, where large numbers of bacteria reside and a sensitive signal for leukocyte diapedesis may cause more harm than good. Further characterization of host muco-

sal immunity in the periodontium and intestine will be required to determine the role that LPS may have in disease and host defense.

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## REFERENCES


1. **Argenbright, L. W., and R. W. Barton.** 1992. Interactions of leukocyte integrins with intercellular adhesion molecule 1 in the production of inflammatory vascular injury in vivo. J. Clin. Invest. **89:**259–272.
2. **Bevilacqua, M. P., J. S. Pober, D. L. Mendrick, R. S. Cotran, and M. A. Gimbrone, Jr.** 1987. Identification of an inducible endothelial-leukocyte adhesion molecule. Proc. Natl. Acad. Sci. USA **84:**9238–9242.
3. **Bevilacqua, M. P., S. Stengelin, M. A. Gimbrone, Jr., and B. Seed.** 1989. Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. Science **243:** 1160–1165.
4. **Bryn, K., and E. Jantzen.** 1982. Analysis of lipopolysaccharides by methanolysis, trifluoroacetylation, and gas chromatography on a fused-silica capillary column. J. Chromatogr. **240:**405–413.
5. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155:**831–838.
6. **Dinarello, C. A.** 1991. Interleukin-1 and interleukin-1 antagonism. Blood **77:**1627–1652.
7. **Etzioni, A., M. Frydman, S. Pollack, I. Avidor, M. L. Phillips, J. C. Paulson, and R. Gershoni-Baruch.** 1992. Recurrent severe infections caused by a novel leukocyte adhesion deficiency. N. Engl. J. Med. **327:**1789–1792.
8. **Falkow, S.** 1991. Bacterial entry into eukaryotic cells. Cell **65:**1099–1102.
9. **Frey, E. A., D. S. Miller, T. G. Jahr, A. Sundan, V. Bazil, T. Espevik, B. B. Finlay, and S. D. Wright.** 1992. Soluble CD14 participates in the response of cells to lipopolysaccharide. J. Exp. Med. **176:**1665–1671.
10. **Fujiwara, T., T. Ogawa, S. Sobue, and S. Hamada.** 1990. Chemical, immunobiological and antigenic characterizations of lipopolysaccharides from *Bacteroides gingivalis* strains. J. Gen. Microbiol. **136:**319–326.
11. **Genco, C. A., C. W. Cutler, D. Kapczynski, K. Maloney, and R. R. Arnold.** 1991. A novel mouse model to study the virulence of and host response to *Porphyromonas* (*Bacteroides*) *gingivalis*. Infect. Immun. **59:**1255–1263.
12. **Genta, R. M., G. M. Lew, and D. Y. Graham.** 1993. Changes in the gastric mucosa following eradication of *Helicobacter pylori*. Mod. Pathol. **6:**281–289.
13. **Hailman, E., H. S. Lichenstein, M. M. Wurfel, D. S. Miller, D. A. Johnson, M. Kelley, L. A. Busse, M. M. Zukowski, and S. D. Wright.** 1994. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. J. Exp. Med. **179:**269–277.
14. **Holt, S. C., J. Ebersole, J. Felton, M. Brunsvold, and K. S. Kornman.** 1988. Implantation of *Bacteroides gingivalis* in nonhuman primates initiates progression of periodontitis. Science **239:**55–57.
15. **Lasky, L. A.** 1993. Combinatorial mediators of inflammation? Curr. Biol. **33:**366–368.
16. **Lee, A., J. Fox, and S. Hazell.** 1993. Pathogenicity of *Helicobacter pylori*: a perspective. Infect. Immun. **61:**1601–1610.
17. **Loppnow, H., H. Brade, I. Dürrbaum, C. A. Dinarello, S. Kusumoto, E. T. Rietschel, and H.-D. Flad.** 1989. IL-1 induction—capacity of defined lipopolysaccharide partial structures. J. Immunol. **142:**3229–3238.
18. **Magnuson, D. K., A. Weintraub, T. H. Pohlman, and R. V. Maier.** 1989. Human endothelial cell adhesiveness for neutrophils, induced by *Escherichia coli* lipopolysaccharide in vitro, is inhibited by *Bacteroides fragilis* lipopolysaccharide. J. Immunol. **143:**3025–3030.
19. **Marshall, B. C., and K. C. Carroll.** 1991. Interaction between *Pseudomonas aeruginosa* and host defenses in cystic fibrosis. Semin. Respir. Infect. **6:**11–18.
20. **Mattsby-Baltzer, I., Z. Mielniczuk, L. Larsson, K. Lindgren, and S. Goodwin.** 1992. Lipid A in *Helicobacter pylori*. Infect. Immun. **60:**4383–4387.
21. **Moughal, N. A., E. Adonogianaki, M. K. Thornhill, and D. F. Kinane.** 1992. Endothelial cell leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression in gingival tissue during health and experimentally-induced gingivitis. J. Periodontal Res. **27:**623–630.
22. **Muotiala, A., I. M. Helander, L. Pyhala, T. U. Kosunen, and A. P. Moran.** 1992. Low biological activity of *Helicobacter pylori* lipopolysaccharide. Infect. Immun. **60:**1714–1716.
23. **Nash, P., and M. M. Krenz.** 1991. Culture media, p. 1226–1288. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy. (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
24. **Nylander, K., B. Danielsen, O. Fejerskov, and E. Dabelsteen.** 1993. Expression of the endothelial leukocyte adhesion molecule-1 (ELAM-1) on endothelial cells in experimental gingivitis in humans. J. Periodontol. **64:**355–357.
25. **Ogawa, T.** 1993. Chemical structure of lipid A from *Porphyromonas* (*Bacteroides*) *gingivalis* lipopolysaccharide. FEBS Lett. **332:**197–201.
26. **Ogawa, T.** 1994. Immunobiological properties of chemically defined lipid A from lipopolysaccharide of *Porphyromonas* (*Bacteroides*) *gingivalis*. Eur. J. Biochem. **219:**737–742.
27. **Page, R. C.** 1991. The role of inflammatory mediators in the pathogenesis of periodontal disease. J. Periodontal. Res. **26:**230–242.
28. **Page, R. C., and H. E. Schroeder.** 1976. Pathogenesis of inflammatory periodontal disease. A summary of current work. Lab. Invest. **34:**235–249.
29. **Pohlman, T. H., R. S. Munford, and J. M. Harlan.** 1987. Deacylated lipopolysaccharide inhibits neutrophil adherence to endothelium induced by lipopolysaccharide in vitro. J. Exp. Med. **165:**1393–1402.
30. **Pugin, J., C.-C. Schurer-Maly, D. Leturcq, A. Moriarty, R. J. Ulevitch, and P. S. Tobias.** 1993. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proc. Natl. Acad. Sci. USA **90:**2744–2748.
31. **Pugin, J., R. J. Ulevitch, and P. S. Tobias.** 1993. A critical role for monocytes and CD14 in endotoxin-induced endothelial cell activation. J. Exp. Med. **178:**2193–2200.

31a. **Ratcliffe, K.** Unpublished observations.

32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, p. 7.37–7.52. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Sims, T. J., B. J. Moncla, R. P. Darveau, and R. C. Page.** 1991. Antigens of *Actinobacillus actinomycetemcomitans* recognized by patients with juvenile periodontitis and periodontally normal subjects. Infect. Immun. **59:**913–924.
34. **Socransky, S. S., and A. D. Haffajee.** 1992. The bacterial etiology of destructive periodontal disease: current concepts. J. Periodont. **63:**322–331.
35. **Springer, T. A.** 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell **76:**301–314.
36. **Takada, H., and S. Kotani.** 1992. Structure-function relationships of lipid A, p. 107–130. *In* D. C. Morrison and J. L. Ryan (ed.), Bacterial endotoxic lipopolysaccharides, vol. I. Molecular biochemistry and cellular biology. CRC Press, Inc., Boca Raton, Fla.
37. **Tonetti, M. S., M. A. Imboden, L. Gerber, N. P. Lang, J. Laisue, and C. Mueller.** 1994. Localized expression of mRNA for phagocyte-specific chemotactic cytokines in human periodontal infections. Infect. Immun. **62:**4005–4014.
38. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure, p. 83–91. *In* R. L. Whistler (ed.), Methods in carbohydrate chemistry. Academic Press, Inc., New York.
39. **Wilkinson, S. G.** 1983. Composition and structure of lipopolysaccharides from *Pseudomonas aeruginosa*. Rev. Infect. Dis. **5:**S941–S949.
40. **Zambon, J. J.** 1990. Microbiology of periodontal disease, p. 147–160. *In* R. J. Genco, H. M. Goldman, and D. W. Cohen (ed.), Contemporary periodontics. The C.V. Mosby Co., St. Louis.