Effects of *Porphyromonas gingivalis* and *Escherichia coli* Lipopolysaccharides on Mononuclear Phagocytes

FRANK A. ROBERTS,^{1,2†} GLORIA J. RICHARDSON,² AND SUZANNE M. MICHALEK^{2*}

Department of Periodontics, School of Dentistry,¹ and Department of Microbiology,² The University of Alabama at Birmingham, Birmingham, Alabama 35294

Received 7 October 1996/Returned for modification 26 November 1996/Accepted 28 May 1997

The mononuclear phagocyte plays an important role in the regulation of microbe-induced inflammation, in part through its ability to secrete mediators, particularly cytokines, in response to microorganisms and their products. To evaluate the effects of the microbial flora associated with chronic adult periodontitis on cytokine induction, lipopolysaccharide (LPS) from the periodontopathogen *Porphyromonas gingivalis* was used to stimulate naive and phorbol ester-primed U937 monocytic cells, as well as elutriated human peripheral blood monocytes. We assessed the effect of this LPS, in comparison to that of LPS from *Escherichia coli*, on cell proliferation, cytokine induction, and surface expression of the LPS receptor CD14. *P. gingivalis* LPS stimulated proliferation of U937 cells at concentrations of greater than 1 ng/ml, while *E. coli* LPS inhibited proliferation. Phorbol myristic acid (PMA)-treated U937 cells and elutriated monocytes responded to *E. coli* LPS activation by producing tumor necrosis factor alpha (TNF- α) mRNA and protein; however, *P. gingivalis* LPS induced greater numbers of TNF- α mRNA-positive cells and higher ($P < 0.05$) levels of protein than did *E. coli* LPS. Both cell types expressed interleukin-1 beta (IL-1 β) mRNA and protein in response to either LPS treatment. Compared with *E. coli* LPS, *P. gingivalis* LPS induced significantly ($P < 0.05$) higher numbers of IL-1 mRNA-positive U937 cells and elutriated monocytes, as well as production of significantly more ($P < 0.05$) IL-1 protein by the monocytes. The PMA-treated U937 cells and the monocytes produced high levels of IL-1 receptor antagonist mRNA and protein which were only marginally affected by the LPS preparations. While *E. coli* LPS induced expression of CD14 on the surface of PMA-primed U937 cells and monocytes, *P. gingivalis* LPS exhibited a significantly ($P < 0.05$) greater ability to enhance receptor levels. Our results indicate that *P. gingivalis* LPS can activate the mononuclear phagocyte for proliferation, cytokine production, and CD14 expression, providing evidence for the potential of this bacterial component to act as a critical regulatory factor in the chronic inflammatory response associated with periodontitis.

The mononuclear phagocyte is one of the principal cell types involved in the inflammatory response which occurs under conditions of chronic infection (40). Periodontitis is an infectious process characterized by chronic inflammation affecting the supporting structures of the teeth. If left untreated, it may eventually lead to total destruction of the connective tissue and alveolar bone (39). Specific gram-negative anaerobic bacteria, such as *Porphyromonas gingivalis*, have been suggested to play a primary role in the initiation and exacerbation of this condition (21). Moreover, the host response to the periodontal microbial flora has been implicated in the pathology of periodontitis (27, 29). The immune cells present in the periodontium, which could contribute to local host responses, include T, B, and phagocytic cells.

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria, including that of *P. gingivalis* (34), and has the ability to activate host cells, especially mononuclear phagocytes (24). This activation is mediated by the binding of LPS to cell surface receptors, e.g., the LPS receptor CD14 (18, 36). Host responses can also be regulated by cytokines produced by several cell types in response to stimulation with LPS. In this regard, the macrophage plays an important role in regulating local inflammatory responses to

bacterial insult by the production of cytokines, particularly proinflammatory interleukin-1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α) and the anti-inflammatory IL-1 receptor antagonist (IL-1ra) (2, 8, 9).

LPS from the periodontopathogen *P. gingivalis* differs biochemically from classical LPS derived from *Escherichia coli* (12). The differences involve variations in phosphorylation and acylation patterns in the biologically active component, lipid A, and relative decreased toxicity of *P. gingivalis* LPS compared with *E. coli* LPS in lethal endotoxemia animal models (25, 28). Furthermore, studies have shown that, in contrast to *E. coli* LPS, *P. gingivalis* LPS is capable of inducing some immune responses in the LPS-hyporesponsive C3H/HeJ inbred mouse strain (12, 20). Mechanisms underlying the toxic and immunomodulating effects of *Porphyromonas* LPS in vivo are not well understood.

To determine the contribution of *P. gingivalis* LPS to chronic inflammatory periodontal disease, this study was aimed at testing the hypothesis that *P. gingivalis* LPS is functionally different from *E. coli* LPS in its ability to activate the mononuclear phagocyte. Therefore, we examined the effects of *E. coli* and *P. gingivalis* LPSs on proliferative responses, cytokine mRNA and protein induction, and LPS receptor expression in the U937 monocytic cell line and elutriated human peripheral blood monocytes. Our studies demonstrate that LPS from the periodontopathogen *P. gingivalis*, compared to *E. coli* LPS, displays an enhanced ability to induce monocyte proliferation and cytokine expression which may be related to increased expression of membrane CD14.

(This report was submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the

* Corresponding author. Mailing address: The University of Alabama at Birmingham, 845 South 19th Street-BBRB 258/5, Birmingham, AL 35294-2170. Phone: (205) 934-3470. Fax: (205) 934-1426. E-mail: sue_michalek@micro.microbio.uab.edu.

† Present address: Department of Periodontics, University of Washington, Seattle, WA 98195-7444.

Department of Microbiology in the Graduate School, The University of Alabama at Birmingham, Birmingham.)

MATERIALS AND METHODS

Reagents. LPS from *P. gingivalis* ATCC 33277 was prepared by hot phenol extraction (23). LPS from *E. coli* K235 was prepared by phenol-water extraction (22). No protein was detected in the LPS preparations by spectrophotometric methods, by silver staining of samples resolved in polyacrylamide gels, or by silver staining of samples electrophoretically resolved in a polyacrylamide gel, electroblotted onto a nitrocellulose membrane, and stained with a solution of colloidal gold (Enhanced Colloidal Gold Total Protein Detection Kit; Bio-Rad Laboratories, Hercules, Calif.). Stock solutions of LPS were prepared (1 mg/ml) in pyrogen-free, distilled H₂O, boiled (15 min), and vortexed prior to use. Dulbecco modified Eagle medium (DMEM) and RPMI 1640 medium (Cellgro; Mediatech, Washington, D.C.), fetal calf serum (FCS; Hyclone Labs, Logan, Utah), phosphate-buffered saline (PBS; 9.25 mM Na₂HPO₄, 3.25 mM NaH₂PO₄ · H₂O, 145 mM NaCl), and elutriation components contained less than 0.3 ng of endotoxin per ml as measured by a chromogenic *Limulus* amebocyte lysate assay (Whittaker Bioproducts, Inc., Walkersville, Md.).

Preparation of U937 cells and elutriated monocytes. The well-characterized U937 cell line (35) was maintained at 37°C in an atmosphere of 5% CO₂ in air in 25-cm² tissue culture flasks (Falcon) by twice weekly splitting of cells 1:5 in RPMI medium containing 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 U of streptomycin per ml (complete RPMI medium). The cells were incubated with medium or 10 ng of phorbol myristate (PMA; Sigma, St. Louis, Mo.) per ml for 48 h to induce adherence and differentiation into macrophage-like cells (15, 31). Following PMA treatment, the medium or the LPS was added and the cells were incubated for an additional 1 to 24 h.

Leukopacks containing peripheral blood mononuclear cells were obtained from healthy volunteers (from 24 to 38 years of age) by leukaphoresis. To isolate monocytes, the mononuclear cells were subjected to counterflow centrifugal elutriation (37, 38). Briefly, the peripheral blood mononuclear cells were washed in PBS, pH 7.0, containing 100 µg of DNase per ml, 250 µg of gentamicin per ml, and 2 U of heparin per ml and then pumped at an initial flow rate of 10 ml/min into a spinning (500 × g, 18°C) elutriation chamber (J-6M Elutriation Centrifuge; Beckman Instruments, Inc., Palo Alto, Calif.). The exiting cells were collected in 50-ml aliquots according to size distribution (Coulter Electronics, Hialeah, Fla.). The aliquots with a greater than 95% monocyte profile were pooled and counted. Monocytes (10⁶/ml) were cultured in 48-well tissue culture plates (500 µl/well) or 25-cm² tissue culture flasks (5 ml/flask; Falcon; Becton Dickinson & Co., Lincoln Park, N.J.) in serum-free DMEM for 2 h at 37°C in 5% CO₂ in air. Following adherence of cells, an equal volume of DMEM supplemented with heat-inactivated FCS, glutamine, penicillin, and streptomycin was added to each culture to yield final supplement concentrations of 10% FCS, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 U of streptomycin per ml (complete DMEM). The cells were kept in culture for 24 h prior to use in the experimental protocol.

Proliferation of U937 cells. U937 cells were cultured in 96-well flat-bottom tissue culture plates (Falcon) in quadruplicate at 10⁵ cells per well in complete RPMI medium. The cells were incubated (37°C, 5% CO₂) alone or with the concentrations of LPS indicated in Results for 24 to 96 h. Cultures were pulsed with [³H]thymidine ([³H]TdR; 0.5 µCi/well; Amersham Life Science, Inc., Arlington Heights, Ill.) during the last 18 h of incubation. Cells were harvested onto glass fiber filters with a cell harvester (PhD; Cambridge Technology, Inc., Cambridge, Mass.), and the amount of [³H]TdR taken up was measured in a liquid scintillation counter (Beckman Instruments, Inc.). The proliferative responses of the monocyte cells are expressed as a proliferation index (PI) calculated as follows: PI = (E - C)/C, i.e., the PI is the mean level of [³H]TdR uptake by cultures containing LPS minus the mean level of [³H]TdR uptake by the respective control cultures, which contained no LPS, divided by the control [³H]TdR uptake.

TNF-α, IL-1β, and IL-1ra ELISA. PMA-treated U937 cells and monocytes were cultured in 48-well flat-bottom tissue culture plates (Falcon) in triplicate at 10⁶ cells per well in complete medium. Cultures were incubated (37°C, 5% CO₂) alone or with the indicated concentrations of the LPS preparations, and supernatants were collected at designated time points. Antigenic TNF-α, IL-1β, and IL-1ra were quantified by using an enzyme-linked immunosorbent assay (ELISA) as previously described (32). Flat-bottom 96-well microtiter plates (Titertek; ICN Biomedicals, Inc., Horsham, Pa.) were coated with 100 µl of goat anti-human TNF-α (1 µg/ml), IL-1β (1 µg/ml), or IL-1ra (3 µg/ml) antibody (R&D Systems, Minneapolis, Minn.) in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 0.02% NaN₃) per well for 16 h at 4°C and then washed with PBS containing 0.05% Tween 20 (wash buffer). To block nonspecific binding sites, 5% heat-inactivated FCS in PBS was added to the wells and the plates were incubated for 2 h at room temperature. Supernatants (undiluted or diluted 1:4, 1:20, or 1:40) or cytokine standards (R&D Systems; see below) were added, and the mixtures were incubated for 16 h at 4°C. The plates were washed five times, 100 µl of mouse monoclonal anti-human TNF-α (R&D Systems), IL-1β (R&D Systems), or IL-1ra (Bachem Bioscience, Inc., King of Prussia, Pa.) antibodies at 1 µg/ml in 1% FCS-PBS was added per well, and the plates were incubated for 1 h at room temperature. The plates were washed five times, and 100 µl of peroxidase-conjugated goat anti-mouse immunoglobulin G (Southern Biotech-

nology Associates, Inc., Birmingham, Ala.) diluted 1:4,000 in 1% FCS-PBS was added per well, and the plates were incubated for 1 h at room temperature. The plates were washed five times, and 100 µl of chromogen ABTS substrate [0.25% 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Sigma Chemical Co., St. Louis, Mo.] in substrate buffer (0.1 M C₆H₅O₇, 0.1 M Na₂HPO₄ [pH 4.2], 0.0075% H₂O₂) was added to the wells. The plates were incubated at room temperature, and color development was read at 414 nm in a Vmax microplate reader (Molecular Devices Corp., Menlo Park, Calif.). Standards used in the assay were twofold dilutions of recombinant human TNF-α (1 ng/ml to 8 pg/ml), IL-1β (4 to 500 pg/ml), or IL-1ra (2 ng/ml to 16 pg/ml). The sensitivities of the ELISAs were 50, 15, and 50 pg/ml, respectively. Dilutions of supernatants giving optical density readings in the linear portion of the appropriate standard curve were used to quantitate the levels of each cytokine in the samples.

In situ hybridization. In situ hybridization was performed by a modification of previously described procedures (4, 17). The probes used were antisense, single-stranded RNA molecules produced by transcription from a T7 or SP6 primer of a cDNA insert cloned into pBluescript (Stratagene, La Jolla, Calif.). The specific nucleotide sequences of the individual probes are as follows: TNF-α, 344 to 917; IL-1β, 445 to 1055; IL-1ra, 289 to 1102 (nucleotides numbered from the start of translation). The riboprobes were labeled with 0.35 mM digoxigenin-labeled UTP (Boehringer GmbH, Mannheim, Germany) and trace labeled with 0.01 mM [³H]UTP (Amersham) with a known specific activity. A small sample of the purified probe was used to determine the amount of ³H incorporated, from which the total amount of probe was calculated. Both sense and antisense probes were used at 400 pg/µl/kb of probe complexity.

The stimulated U937 cells and elutriated monocytes were cultured as described above, in complete medium in 25-cm² flasks at 10⁶ cells/ml. Following treatment, nonadherent cells were harvested by centrifugation at 400 × g for 10 min at 4°C (non-PMA-treated U937 cells) and adherent cells were harvested by gentle scraping. Cytospin preparations of treated cells were fixed in 3% paraformaldehyde for 1 h, acetylated with 0.25% acetic anhydride-triethanolamine for 15 min, and prehybridized with a solution of 50% formamide, 1× SSC (150 mM NaCl, 15 mM Na citrate), 1× Denhardt's solution, 0.05% herring sperm DNA, 0.025% yeast tRNA, and 10% dextran sulfate for 1 h. The cells were incubated overnight at 50°C with 15 µl of hybridization buffer containing a heat-denatured riboprobe for the cytokine of interest. The unhybridized riboprobe was removed by a 45-min incubation with 20 µg of RNase A per ml in STE buffer (500 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA) and high-stringency washing with 2× SSC-50% formamide at 50°C (three times) and with 0.5× SSC at 25°C (twice).

Cells positive for cytokine mRNA were detected with an alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer GmbH) diluted 1:500 in 1% normal horse serum. The presence of antibody was detected by color development after the addition of a nitroblue tetrazolium substrate (Boehringer GmbH). Cells detected with digoxigenin-labeled RNA probes were quantitated with bright-field microscopy by direct counting. Five fields of positive and negative cells were counted at a magnification of ×10, and the values were expressed as the number of positive cells per 100 cells counted.

Flow cytometry analysis for CD14. Stimulated U937 cells and elutriated monocytes were cultured as described above and then characterized for the human LPS receptor CD14 by use of mouse monoclonal antibody MY4 (Coulter Clone, Hialeah, Fla.). The cells were harvested by gentle scraping and phenotypically characterized following incubation of aliquots of cell preparations with either fluorescein isothiocyanate-labeled MY4 or the immunoglobulin G2b isotype-matched control (Coulter Clone). Analysis was performed with a FACStar (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) fluorescence-activated cell sorter.

Statistical analysis. A comparison of the significance of the effects of LPS on cytokine and CD14 induction in U937 cells and monocytes was made with Student's *t* test and Fisher's PLSD statistical methods by using Statview 4.5 software (Abacus Concepts, Berkeley, Calif.) on a PowerMac 8500 computer (Apple Computer, Inc., Cupertino, Calif.).

RESULTS

Viability and proliferation. The U937 cell and elutriated monocyte preparations used in the studies were >90% viable in the presence or absence of LPS treatment as assessed by trypan blue exclusion. LPS-induced proliferation of U937 cells was dose dependent (Fig. 1). At low doses, both *E. coli* and *P. gingivalis* LPSs induced proliferation of the U937 cells at 24, 48 (data not shown), 72, and 96 h. Cultures incubated with higher doses of *E. coli* LPS ([³H]TdR uptake, 13,600 cpm) showed less proliferation than did control cell cultures ([³H]TdR uptake, 17,100 cpm). Cultures incubated with higher doses of *P. gingivalis* LPS, however, had significantly (*P* < 0.01) stronger proliferative responses (maximum [³H]TdR uptake, 22,900 cpm). The U937 cells were initially nonadherent with either LPS treatment alone and then loosely attached to the plastic by

FIG. 1. Effects of *P. gingivalis* (Pg) and *E. coli* (Ec) LPSs on proliferative responses of U937 cells. U937 cells were incubated with medium or with the indicated concentrations of *P. gingivalis* or *E. coli* LPS for 72 or 96 h. Cultures were pulsed with [3 H]TdR during the last 18 h of incubation. The graph indicates the PI; $PI = (E - C)/C$ is the indexed level of [3 H]TdR uptake by LPS-treated or control medium-treated U937 cells at the indicated time points. The amount of [3 H]TdR taken up in control cultures ranged from 16,000 to 18,000 cpm. The data represent the mean $PI \pm$ the standard error of the mean of quadruplicate samples per experiment and three separate experiments.

96 h. Addition of PMA to U937 cultures for the initial 48 h of culture resulted in adherence of the cells ($64\% \pm 10\%$) and a marked reduction in proliferation ([3 H]TdR uptake, 2,100 cpm) with a $PI = -0.88$.

TNF- α production. In the absence of PMA differentiation, control U937 cells did not produce any detectable TNF- α (data not shown). Although detectable levels of TNF- α were present in supernatants of PMA-induced U937 cells, significantly ($P < 0.05$) larger amounts of TNF- α were released within 4 h after PMA-treated U937 cells were stimulated with either LPS preparation (Fig. 2). *P. gingivalis* LPS stimulated the greatest release of TNF. Low doses of *P. gingivalis* LPS had very little effect on the PMA-differentiated U937 cells, while larger amounts of *P. gingivalis* LPS induced a dose-response induction of TNF- α (Fig. 3). The ability of *E. coli* LPS to induce TNF- α production reached a plateau at a dose of 10 ng/ml.

The elutriated human monocytes stimulated with either LPS preparation produced significantly ($P < 0.05$) more TNF- α than did control cultures by 24 h (Fig. 4A). Monocytes treated with 0.1 and 5 μ g of *P. gingivalis* LPS per ml produced significantly ($P < 0.05$) higher levels of TNF than did cells treated with the same concentrations of *E. coli* LPS.

IL-1 β production. Undifferentiated U937 cells produced no detectable IL-1 β following either LPS treatment, or the kinetics of IL-1 β production by PMA-activated cells stimulated with LPS differed from that of TNF- α production. U937 cells treated with only PMA produced significantly more ($P < 0.05$) IL-1 β than did control cultures (medium only) at 4 to 24 h (22 to 44 pg/ml versus less than 15 pg/ml). The addition of *E. coli* or *P. gingivalis* LPS to PMA-treated U937 cells increased the IL-1 β levels in the supernatant in a dose- and time-dependent manner (27 to 142 pg/ml). *E. coli* LPS (100 ng/ml) stimulated

FIG. 2. Kinetics of TNF- α production by PMA-treated U937 cells in response to *E. coli* (Ec) or *P. gingivalis* (Pg) LPS. U937 cells were cultured with control medium or 10 ng of PMA per ml for 48 h. Following incubation, control medium or LPS was added to a final concentration of 1.0 or 0.1 μ g/ml and incubation was continued for various times. Culture supernatants were collected and cytokine release was analyzed by ELISA. The values are mean cytokine levels $\pm$ the standard error of the mean of triplicate samples and are representative of three separate experiments.

a peak response at 12 h, whereas the same dose of *P. gingivalis* LPS stimulated a weaker response which peaked by 8 h. *E. coli* LPS (100 ng/ml) induced significantly ($P < 0.05$) more IL-1 β (120 pg/ml) than the same dose of *P. gingivalis* LPS by 24 h (92 pg/ml). Higher concentrations of IL-1 were present in supernatants of cultures stimulated with 1 μ g *P. gingivalis* LPS per ml (150 pg/ml) than in cultures stimulated with a similar dose of *E. coli* LPS (110 pg/ml).

FIG. 3. Dose response of TNF- α production by PMA-treated U937 cells stimulated with *E. coli* (Ec) or *P. gingivalis* (Pg) LPS. U937 cells were cultured with 10 ng of PMA per ml for 48 h. Following incubation, medium, *P. gingivalis* LPS, or *E. coli* LPS was added at the indicated final concentrations and incubation was continued for 24 h. Culture supernatants were collected, and cytokine release was analyzed by ELISA. The values shown are mean cytokine levels $\pm$ the standard error of the mean of triplicate samples and are representative of three separate experiments.

The elutriated monocytes also responded to both LPS preparations (Fig. 4B). By 24 h, supernatants from cultures of elutriated monocytes incubated with *P. gingivalis* LPS contained significantly ($P < 0.05$) more IL-1 than did those from cultures incubated with *E. coli* LPS.

IL-1ra production. The kinetics and dose response of U937 cells to LPS were characterized by the absence of IL-1ra without PMA priming and high levels of IL-1ra in the supernatant of cells treated with PMA alone (19,380 to 29,000 pg/ml). *E. coli* LPS at 1 μg/ml was able to marginally increase IL-1ra levels in culture supernatants at 12 h. *P. gingivalis* LPS (0.1 μg/ml) induced supernatant levels of IL-1ra consistently above levels in cells treated with PMA alone by 4 to 12 h (30,600 to 33,800 pg/ml). These levels returned to the baseline by 24 h. The induction of IL-1ra release by PMA-treated U937 cells stimulated with LPS was not dose dependent. The various doses of *P. gingivalis* LPS tested did not increase IL-1ra levels in the supernatant above those produced by treatment with PMA only (data not shown).

Unstimulated, elutriated monocytes produced relatively high levels of IL-1ra (Fig. 4C). These cells were stimulated to release more IL-1ra after incubation with either LPS for 24 h. *E. coli* and *P. gingivalis* LPSs (100 ng/ml) induced significantly ($P < 0.05$) higher levels of IL-1ra than those seen in control cultures. At 5 μg/ml, *P. gingivalis* LPS induced the production of IL-1ra levels which were significantly ($P < 0.05$) above the background and above the level produced following treatment with a similar dose of *E. coli* LPS.

Cytokine mRNA expression. Cytopreparations of U937 cells and monocytes were evaluated by in situ hybridization for cytokine mRNA. Neither *P. gingivalis* nor *E. coli* LPS stimulated detectable levels of mRNA for the cytokines in non-PMA-treated U937 cells (Table 1). The PMA-treated U937 cell preparation contained cells with mRNAs for TNF-α, IL-1β, and IL-1ra. The proportion of PMA-treated cells expressing TNF-α and IL-1β mRNAs increased significantly ($P < 0.05$) after 4 h of incubation with LPS. PMA-treated cells stimulated with *P. gingivalis* LPS had more intense staining in a greater proportion of cells for both TNF-α and IL-1β mRNAs than did those stimulated with *E. coli* LPS (Fig. 5A). Neither LPS preparation influenced the level of IL-1ra mRNA expression by U937 cells over treatment with PMA alone.

LPS treatment did not visibly alter the morphology of the adherent elutriated monocytes as assessed by use of Wright's stain (data not shown). Expression of mRNAs for TNF-α, IL-1β, and IL-1ra by elutriated monocytes was significantly ($P < 0.05$) enhanced by the LPS preparations over treatment with medium alone (Table 1 and Fig. 5B). Following treatment with *P. gingivalis* LPS, more monocytic cells stained positively for TNF-α mRNA and IL-1β mRNA than after *E. coli* LPS treatment. The numbers of monocytes expressing IL-1ra mRNA were similar in cultures treated with *E. coli* LPS and those treated with *P. gingivalis* LPS.

Surface CD14 expression. To determine if the differential effects of the two LPS preparations might be associated with variations in LPS receptor expression in U937 cells and monocytes, flow cytometric analysis of surface CD14 levels was per-

FIG. 4. Release of TNF-α, IL-1β, and IL-1ra by elutriated monocytes stimulated with *E. coli* (Ec) or *P. gingivalis* (Pg) LPS. Monocytes were cultured with medium or LPS for 24 h, and then culture supernatants were collected and cytokine levels were analyzed by ELISA. The values shown are mean cytokine levels ± the standard error of the mean of duplicate samples and are representative of four separate experiments. Means were significantly different from those of medium-treated (*) or *E. coli* LPS-treated (†) samples.

TABLE 1. Cytokine mRNA expression by U937 cells or elutriated monocytes after treatment with LPS and/or PMA^a

Cell type and treatment	Cytokine mRNA expression (% positive cells) ^b		
	TNF- α	IL-1 β	IL-1ra
U937			
Medium only	0	0	0
<i>E. coli</i> LPS (100 ng/ml)	0	0	0
<i>P. gingivalis</i> LPS (100 ng/ml)	0	0	0
PMA (10 ng/ml)	38 $\pm$ 6	17 $\pm$ 5	81 $\pm$ 7.5
PMA + <i>E. coli</i> LPS (100 ng/ml)	62 $\pm$ 5 ^c	48 $\pm$ 8 ^c	80 $\pm$ 10
PMA + <i>P. gingivalis</i> LPS (100 ng/ml)	74 $\pm$ 5.5 ^{c,d}	71 $\pm$ 9 ^{c,d}	84 $\pm$ 9
Control probe	0	0	0
Elutriated monocytes			
Medium only	0	0	0
<i>E. coli</i> LPS (100 ng/ml)	38 $\pm$ 5 ^c	52 $\pm$ 6 ^c	72 $\pm$ 9 ^c
<i>P. gingivalis</i> LPS (100 ng/ml)	57 $\pm$ 7 ^{c,d}	69 $\pm$ 7.5 ^{c,d}	67 $\pm$ 8 ^c
Control probe	0	0	0

^a Cells were incubated with medium or PMA for 48 h and then treated with medium or LPS and incubated for an additional 4 h (see Materials and Methods).

^b Values are average frequencies $\pm$ the standard error of the mean (four to six slides per group) of positive cells in five microscope fields (magnification, $\times 10$) per 100 cells counted.

^c Significantly different ($P < 0.05$) from PMA-treated cells or medium-only controls.

^d Significantly different ($P < 0.05$) from samples treated with PMA plus *E. coli* LPS or with *E. coli* LPS.

formed on these cell types. Eleven percent of the untreated U937 cells expressed surface CD14, with a mean fluorescence index (MFI) of 9.6. Addition of LPS alone did not significantly increase the number of positive cells or the fluorescence intensity. PMA-treated U937 cell preparations had higher percentages of CD14-positive cells (31% $\pm$ 4%) and greater mean fluorescence (MFI, 24 $\pm$ 4) than did untreated cells. In contrast to treatment with PMA and *E. coli* LPS, treatment of cultures with PMA and *P. gingivalis* LPS significantly ($P < 0.05$) increased both the percentage of positive cells (45% $\pm$ 7%) and the mean fluorescence (MFI, 35.5 $\pm$ 6).

The majority of the untreated, elutriated monocytes expressed CD14 on their surface, and the levels of expression were not affected by addition of either LPS (Fig. 6). The initial surface expression of CD14 as assessed by MFI was significantly ($P < 0.05$) increased after stimulation of cells with *E. coli* or *P. gingivalis* LPS (100 ng/ml). Higher doses of *E. coli* and *P. gingivalis* LPS further enhanced the mean fluorescence on monocytes as assessed by use of a fluorescence-activated cell sorter.

DISCUSSION

The mononuclear phagocyte plays a critical role in the regulation of the host response during chronic inflammation (40). The ability of microbial LPS to activate the monocytic cell to produce inflammatory cytokines may directly affect the outcome of bacterium-induced inflammation by promoting either an acute tissue reaction, resolution, or chronic destruction (3). The present study investigated whether *P. gingivalis* LPS differs from *E. coli* LPS in the ability to induce TNF- α , IL-1 β , and IL-1ra production by monocytes, which would support its role in promoting chronic inflammatory activation and tissue destruction as characterized in periodontitis. The expression of the LPS receptor CD14 on the monocytes was also evaluated to determine its possible role in the activation process.

The LPS of the periodontopathogen *P. gingivalis* demon-

strates chemical and biological differences from classical *E. coli* LPS (12, 25, 28). *P. gingivalis*, in comparison to *E. coli* LPS, exhibits less endotoxic activity in vivo (25, 28). It has also been shown that *P. gingivalis* LPS may be similar to *E. coli* LPS in terms of mitogenicity, polyclonal B-cell activation, stimulation of IL-1 production (20), and the ability to induce TNF- α (33). However, other investigators have shown that *P. gingivalis* LPS, in contrast to *E. coli* LPS, does not induce epithelial cells to express E-selectin or promote neutrophil adhesion, which may facilitate bacterial colonization of the gingival tissue and persistence (7). Our results show that *P. gingivalis* LPS, compared to *E. coli* LPS, induced similar or higher levels of cytokine production by monocytes and that the higher cytokine expression related to upregulation of CD14 expression.

Both U937 cells and human peripheral blood monocytes were used to evaluate cellular responses to the two LPS preparations. When the abilities of the LPS preparations to stimulate U937 cells were assessed, it was noted that at the higher concentrations of LPS tested, *P. gingivalis* LPS promoted cellular proliferation while a similar amount of *E. coli* LPS was inhibitory. This finding may be explained by a lesser toxicity of *P. gingivalis* LPS than *E. coli* LPS or by different mechanisms of cell activation. In gingival tissue, the initial proliferation of

FIG. 5. Expression of cytokine mRNA by LPS-treated U937 cells and elutriated monocytes. PMA-treated U937 cells (A) or untreated monocytes (B) were incubated with 100 ng of *E. coli* (Ec) or *P. gingivalis* (Pg) LPS per ml for 24 h, harvested by gentle scraping, and spun onto glass slides. Cells were fixed, and cytokine mRNA was detected by in situ hybridization. The fields shown are from representative slides of six (U937 cells) or four (monocytes) separate experiments performed in duplicate.

FIG. 6. Induction of LPS receptor (CD14) expression by LPS-treated, elutriated monocytes. Monocytes were incubated with the indicated amounts of *E. coli* (Ec) or *P. gingivalis* (Pg) LPS for 24 h, harvested, and examined for surface expression of CD14 by flow cytometry. The histograms are representative of four separate experiments, and percentages of CD14-positive cells and MFIs reflecting the relative number of CD14 molecules per cell are shown.

monocytic cells resulting from exposure to the LPS of the colonizing *P. gingivalis* could allow enhanced expression of inflammatory mediators in the region as the disease process progresses.

The U937 cells used in this study required PMA stimulation to become sensitive to LPS stimulation for a cytokine response, whereas the elutriated monocytes used were sensitized without pretreatment. This is likely due to the more advanced state of differentiation of the monocytes compared to the untreated cell line, particularly with regard to expression of LPS receptors (30). Phorbol ester treatment of U937 cells induces them to differentiate into more macrophage-like cells (e.g., more extensive cytoplasm, increased protein synthesis, decreased mitosis, and increased HLA-DR and Fc- γ receptor expression) (19, 26, 31, 35). *P. gingivalis* LPS-stimulated release of TNF- α from both cell types was dose dependent at concentrations of 10 ng/ml to 1 μ g/ml. *E. coli* LPS stimulated maximum TNF- α release at a concentration of 10 ng/ml and did not promote greater TNF- α release at higher concentrations. These results extend the findings of others (33) showing the induction of similar levels of TNF- α by human peripheral blood monocytes stimulated with either *E. coli* or *P. gingivalis* LPS at ≤ 10 ng/ml. The ability of *P. gingivalis* LPS to stimulate TNF- α release at higher doses may allow the establishment of a chronic inflam-

matory process as the anaerobic *P. gingivalis* bacteria multiply in the diseased periodontal pocket. TNF- α released by the mononuclear phagocyte can target other inflammatory and accessory cells, as well as function as an autocrine activator. This response may lead to periodontal destruction via processes including neutrophil chemotaxis and degranulation, fibroblast production of collagenase, and osteoclast activation for bone resorption.

Unlike the TNF- α response, the IL-1 β response to each LPS preparation was the same at all of the concentrations tested. This may reflect differences in the regulation of TNF and IL-1 responses to LPS, a finding that has been previously documented (5, 6). The induction of these two cytokines appears to involve separate second-messenger systems which relay the LPS signal to the monocyte nucleus. The differences in TNF and IL-1 release which we observed in the LPS dose-response experiments might be explained by the separate routes of intracellular signaling which are used to activate mRNA transcription of the two cytokines. IL-1 β may play a dual role in periodontal disease processes, as both a potent inducer of osteoclasts and an activator of B cells. It can therefore elicit both destruction of alveolar bone and protection from pathogenic flora colonization (10, 11, 16).

In our studies, we observed high levels of IL-1ra which were 100 to 200 times those of IL-1 β . This amount of antagonist is required to effectively block the binding of IL-1 to its cellular receptor (14). The inhibition of IL-1 activity can decrease inflammatory destructive processes and can dampen IL-1-associated immune activation (16). IL-1ra production by the mononuclear phagocyte can function to protect tissues from an acute inflammatory response and may be involved in the healing phase of inflammation subsequent to periods of active destruction (1, 13). However, it is not possible to discern from this study how LPS could influence a balance between IL-1 and IL-1ra production.

In situ hybridization experiments were performed to correlate cytokine mRNA induction with the observed protein expression following LPS stimulation of monocytic cells. Studies examining LPS cytokine induction in mononuclear phagocytes usually measure average quantities of cytokine mRNA induced in the entire population of cells. In this study, we utilized sensitive in situ hybridization methods to analyze the production of cytokine mRNAs by individual cells in the U937 and monocyte populations. Addition of PMA to the U937 cells caused expression of detectable levels of TNF- α mRNA, which were increased by *E. coli* LPS and further augmented by *P. gingivalis* LPS. Regulation of TNF- α protein release was associated with mRNA induction as observed by in situ hybridization. *P. gingivalis* LPS induced both TNF transcription and protein release. These findings are compatible with the potential role of *P. gingivalis* LPS in maintaining TNF- α expression in periodontal tissues and thereby promoting inflammation, release of prostaglandins and matrix metalloproteinases, osteoclast activation, and eventual bone loss.

Flow cytometric analyses demonstrated induction of CD14 expression in U937 cells (increased percentage of positive cells and increased MFI) and monocytes (increased MFI) caused by the addition of LPS, with *P. gingivalis* LPS eliciting a greater number of CD14-positive cells and higher MFIs than *E. coli* LPS. Increased expression of LPS receptors on the mononuclear phagocytes may account for their enhanced ability to produce TNF- α when treated with increasing doses of *P. gingivalis* LPS. This idea is supported by the finding that pretreatment of human monocytes with antibody to CD14 (MY4) resulted in a reduction of TNF- α mRNA and protein levels after LPS stimulation (33). As more LPS receptors are avail-

able on the cell surface, the cytokine response to the LPS may be further enhanced. In a periodontal lesion, the cells could respond to increasing levels of LPS and promote chronic activation.

Our studies have shown that there are differences in the manner of activation of mononuclear phagocytes by LPS from the periodontal pathogen *P. gingivalis* compared to *E. coli* LPS. This included differences in promotion of proliferation, cytokine mRNA and protein production, and expression of the LPS receptor CD14. The ability of *P. gingivalis* LPS to promote the expression of cytokines such as TNF- α , IL-1 β , and IL-1ra and upregulation of CD14 expression may be important in the establishment of the chronically activated lesion with subsequent osseous tissue destruction observed in the inflammatory disease periodontitis. An understanding of the role of bacterial LPS in promoting cytokine production by monocytic cells at sites of inflammation will offer the opportunity to develop intervention strategies for the treatment of inflammatory diseases such as periodontitis.

ACKNOWLEDGMENTS

We thank Dee Reynolds, Jimin Li, and R. P. Bucy for expert technical advice on this study and Dawn Ward and Jannet Katz for critical comments and advice on the manuscript.

This work was supported by Public Health Service grants DE 00279 and DE 08228 from the National Institutes of Health. Frank Roberts is the recipient of a Dentist Scientist Award (DE 00279) from the National Institute of Dental Research.

REFERENCES

- Arend, W. P. 1991. Interleukin 1 receptor antagonist. A new member of the interleukin 1 family. *J. Clin. Invest.* **88**:1445-1451.
- Beutler, B. 1995. TNF, immunity and inflammatory disease: lessons of the past decade. *J. Invest. Med.* **43**:227-235.
- Bone, R. C. 1991. The pathogenesis of sepsis. *Ann. Intern. Med.* **115**:457-469.
- Bucy, R. P., L. Karr, G. Q. Huang, J. Li, D. Carter, K. Honjo, J. A. Lemons, K. M. Murphy, and C. T. Weaver. 1995. Single cell analysis of cytokine gene coexpression during CD4⁺ T-cell phenotype development. *Proc. Natl. Acad. Sci. USA* **92**:7565-7569.
- Burchett, S. K., W. M. Weaver, J. A. Westall, A. Larsen, S. Kronheim, and C. B. Wilson. 1988. Regulation of tumor necrosis factor/cachectin and IL-1 secretion in human mononuclear phagocytes. *J. Immunol.* **140**:3473-3481.
- Daniel-Issakani, S., A. M. Spiegel, and B. Strulovici. 1989. Lipopolysaccharide response is linked to the GTP binding protein, Gi2, in the promonocytic cell line U937. *J. Biol. Chem.* **264**:20240-20247.
- Darveau, R. P., M. D. Cunningham, T. Bailey, C. Seachord, K. Ratcliffe, B. Bainbridge, M. Dietsch, R. C. Page, and A. Aruffo. 1995. Ability of bacteria associated with chronic inflammatory disease to stimulate E-selectin expression and promote neutrophil adhesion. *Infect. Immun.* **63**:1311-1317.
- Dinarello, C. A. 1994. The interleukin-1 family: 10 years of discovery. *FASEB J.* **8**:1314-1325.
- Ertel, W., M. H. Morrison, P. Wang, Z. F. Ba, A. Ayala, and I. H. Chaudry. 1991. The complex pattern of cytokines in sepsis. *Ann. Surg.* **214**:141-148.
- Gemmell, E., and G. J. Seymour. 1992. Different responses in B cells induced by *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. *Arch. Oral Biol.* **37**:565-573.
- Genco, R. J. 1992. Host responses in periodontal diseases: current concepts. *J. Periodontol.* **63**:338-355.
- Hamada, S., H. Takada, T. Ogawa, T. Fujiwara, and J. Mihara. 1990. Lipopolysaccharides of oral anaerobes associated with chronic inflammation: chemical and immunomodulating properties. *Int. Rev. Immunol.* **6**:247-261.
- Hammerberg, C., W. P. Arend, G. J. Fisher, L. S. Chan, A. E. Berger, J. S. Haskill, J. J. Voorhees, and K. D. Cooper. 1992. Interleukin-1 receptor antagonist in normal and psoriatic epidermis. *J. Clin. Invest.* **90**:571-583.
- Hannum, C. H., C. J. Wilcox, W. P. Arend, F. G. Joslin, D. J. Dripps, P. L. Heimdahl, L. G. Armes, A. Sommer, S. P. Eisenberg, and R. C. Thompson. 1990. Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. *Nature* **343**:336-340.
- Hass, R., H. Bartels, N. Topley, M. Hadam, L. Kohler, M. Goppelt-Strube, and K. Resch. 1989. TPA-induced differentiation and adhesion of U937 cells: changes in ultrastructure, cytoskeletal organization and expression of cell surface antigens. *Eur. J. Cell Biol.* **48**:282-293.
- Howard, M., and W. E. Paul. 1983. Regulation of B-cell growth and differentiation by soluble factors. *Annu. Rev. Immunol.* **1**:307-333.
- Karr, L. J., A. Panoskaltis-Mortari, J. Li, D. Devore-Carter, C. T. Weaver, and R. P. Bucy. 1995. *In situ* hybridization for cytokine mRNA with digoxigenin-labeled riboprobes. Sensitivity of detection and double label applications. *J. Immunol. Methods* **182**:93-106.
- Kielian, T. L., and F. Blecha. 1995. CD14 and other recognition molecules for lipopolysaccharide: a review. *Immunopharmacology* **29**:187-205.
- Klein, J. B., T. M. Schepers, W. L. Dean, G. Sonnenfeld, and K. R. McLeish. 1990. Role of intracellular calcium concentration and protein kinase C activation in IFN-gamma stimulation of U937 cells. *J. Immunol.* **144**:4305-4311.
- Koga, T., T. Nishihara, T. Fujiwara, T. Nisizawa, N. Okahashi, T. Noguchi, and S. Hamada. 1985. Biochemical and immunological properties of lipopolysaccharide (LPS) from *Bacteroides gingivalis* and comparison with LPS from *Escherichia coli*. *Infect. Immun.* **47**:638-647.
- Mangan, D. F., S. E. Mergenhagen, and S. M. Wahl. 1993. Apoptosis in human monocytes: possible role in chronic inflammatory diseases. *J. Periodontol.* **64**:461-466.
- McIntire, F. C., H. W. Sievert, G. H. Barlow, R. A. Finley, and A. Y. Lee. 1967. Chemical, physical, and biological properties of a lipopolysaccharide from *Escherichia coli* K-235. *Biochemistry* **6**:2363-2372.
- Millar, S. J., E. G. Goldstein, M. J. Levine, and E. Hausmann. 1986. Modulation of bone metabolism by two chemically distinct lipopolysaccharide fractions from *Bacteroides gingivalis*. *Infect. Immun.* **51**:302-306.
- Morrison, D. C., and J. L. Ryan. 1987. Endotoxins and disease mechanisms. *Annu. Rev. Med.* **38**:417-432.
- Nair, B. C., W. R. Mayberry, R. Dziak, P. B. Chen, M. J. Levine, and E. Hausmann. 1983. Biological effects of a purified lipopolysaccharide from *Bacteroides gingivalis*. *J. Periodontal Res.* **18**:40-49.
- Nambu, M., M. Morita, H. Watanabe, Y. Uenoyama, K. M. Kim, M. Tanaka, Y. Iwai, H. Kimata, M. Mayumi, and H. Mikawa. 1989. Regulation of Fc gamma receptor expression and phagocytosis of a human monoblast cell line U937. Participation of cAMP and protein kinase C in the effects of IFN-gamma and phorbol ester. *J. Immunol.* **143**:4158-4165.
- Offenbacher, S., P. A. Heasman, and J. G. Collins. 1993. Modulation of host PGE₂ secretion as a determinant of periodontal disease expression. *J. Periodontol.* **64**:432-444.
- Ogawa, T. 1994. Immunobiological properties of chemically defined lipid A from lipopolysaccharide of *Porphyromonas* (*Bacteroides*) *gingivalis*. *Eur. J. Biochem.* **219**:737-742.
- Page, R. C. 1991. The role of inflammatory mediators in the pathogenesis of periodontal disease. *J. Periodontal Res.* **26**:230-242.
- Pedrinaci, S., C. Huelin, M. Patarroyo, F. Ruiz-Cabello, and F. Garrido. 1989. Studies on CD11a and CD18 molecules with two new monoclonal antibodies: differential myelomonocytic antigen expression of PMA treated HL60 and U937 cell lines. *Hybridoma* **8**:13-23.
- Radzun, H. J., M. R. Parwaresch, C. Sundstrom, K. Nilsson, and M. Eissner. 1983. Monocytic origin of the human hematopoietic cell line U-937 and its convertibility to macrophages evidenced by isoenzyme mapping. *Int. J. Cancer* **31**:181-186.
- Redman, T. K., C. C. Harmon, and S. M. Michalek. 1996. Oral immunization with recombinant *Salmonella typhimurium* expressing surface protein antigen A (SpaA) of *Streptococcus sobrinus*: effects of the *Salmonella* virulence plasmid on the induction of protective and sustained humoral responses in rats. *Vaccine* **14**:868-878.
- Shapira, L., S. Takashiba, S. Amar, and T. E. Van Dyke. 1994. *Porphyromonas gingivalis* lipopolysaccharide stimulation of human monocytes: dependence on serum and CD14 receptor. *Oral Microbiol. Immunol.* **9**:112-117.
- Socransky, S. S., and A. D. Haffajee. 1992. The bacterial etiology of destructive periodontal disease: current concepts. *J. Periodontol.* **63**:322-331.
- Sundstrom, C., and K. Nilsson. 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer* **17**:565-577.
- Ulevitch, R. J., and P. S. Tobias. 1995. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu. Rev. Immunol.* **13**:437-457.
- Wahl, L. M., I. M. Katona, R. L. Wilder, C. C. Winter, B. Haraoui, I. Scher, and S. M. Wahl. 1984. Isolation of human mononuclear cell subsets by counterflow centrifugal elutriation (CCE). I. Characterization of B-lymphocyte-, T-lymphocyte-, and monocyte-enriched fractions by flow cytometric analysis. *Cell. Immunol.* **85**:373-383.
- Wahl, S. M., I. M. Katona, B. M. Stadler, R. L. Wilder, W. E. Helsel, and L. M. Wahl. 1984. Isolation of human mononuclear cell subsets by counterflow centrifugal elutriation (CCE). II. Functional properties of B-lymphocyte-, T-lymphocyte-, and monocyte-enriched fractions. *Cell. Immunol.* **85**:384-395.
- Williams, R. C. 1990. Periodontal disease. *N. Engl. J. Med.* **322**:373-381.
- Zembala, M., and G. L. Asherson (ed.). 1989. Human monocytes. Academic Press, Inc., New York, N.Y.