Actinobacillus actinomycetemcomitans Serotype b-Specific Polysaccharide Antigen Stimulates Production of Chemotactic Factors and Inflammatory Cytokines by Human Monocytes

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Serotype b-specific polysaccharide antigen (SPA) was extracted from whole cells of *Actinobacillus actinomycetemcomitans* Y4 by autoclaving and purified by chromatography on DEAE-Sephadex A-25 and Sephacryl S-300. SPA induced the release of monocyte and leukocyte chemotactic factors by human monocytes. Polymyxin B had almost no effect on the release of monocyte chemotactic factor, but a monoclonal antibody against SPA markedly inhibited it. Human monocytes stimulated with SPA exhibited the increased mRNA expression of monocyte chemoattractant protein 1 (MCP-1) and a neutrophil chemotactic factor, interleukin-8 (IL-8). On the other hand, SPA induced the release of IL-1, IL-6, and tumor necrosis factor (TNF) and enhanced the expression of IL-1 α , IL-1 β , IL-6, and TNF alpha (TNF- α) mRNAs. Human monocytes expressed MCP-1 and IL-8 mRNAs when stimulated by human recombinant IL-1 α , IL-1 β , IL-6, and TNF- α , suggesting that these inflammatory cytokines induced by SPA might participate in the production of chemotactic factors in human monocytes.

Actinobacillus actinomycetemcomitans is a small fastidious gram-negative coccobacillus. The organism has been implicated as one of the causative organisms of localized juvenile periodontitis (7, 50) and adult periodontitis (4). *A. actinomycetemcomitans* is recovered in higher prevalence and proportion from subgingival lesions of localized juvenile periodontitis patients than from quiescent periodontal sites (4, 35). Patients with localized juvenile periodontitis exhibit elevated antibody levels to whole cells and cellular components of *A. actinomycetemcomitans* (5, 30, 35, 45).

A. actinomycetemcomitans possesses various cell surface components including a capsular-like serotype-specific polysaccharide antigen (3, 5), lipopolysaccharide (LPS) (22), proteinaceous surface-associated material (43, 44), fimbriae (15), and immunoglobulin Fc receptor (25). It is possible that these cell surface components are important in the adherence to host tissues, destruction of gingival tissue and periodontal ligament, and resorption of alveolar bone (17). We have recently reported that serotype b-specific polysaccharide antigen (SPA) of *A. actinomycetemcomitans* plays a key role in the resistance to phagocytosis and killing by human polymorphonuclear leukocytes (PMNs) (46). Moreover, SPA is known to exhibit the ability to induce the release of interleukin-1 (IL-1) by murine macrophages (38) and to promote osteoclast-like cell formation in mouse marrow cultures (28). However, little is known about the effect of SPA on the production of inflammatory cytokines by human host cells.

Monocytes/macrophages and leukocytes do not only exhibit the abilities to phagocytose and kill pathogens but also release various biologically active substances such as inflammatory cytokines, proteolytic tissue-degrading enzymes, and oxidizing agents. Therefore, the analysis of the infiltration of monocytes

and leukocytes in periodontal lesions is thought to be important in the elucidation of pathogenesis of periodontal diseases. In this study, we compared SPA and LPS from *A. actinomycetemcomitans* serotype b for their ability to induce the release of monocyte and leukocyte chemotactic factors, IL-1, IL-6, and tumor necrosis factor (TNF) by human monocytes. In addition, we have discussed the possibility that inflammatory cytokines such as IL-1, IL-6, and TNF alpha (TNF- α) participate in the production of chemotactic factors in SPA-stimulated monocytes.

MATERIALS AND METHODS

Bacteria. *A. actinomycetemcomitans* Y4 (serotype b) was obtained from Y. Yamamoto (Sunstar Corp., Osaka, Japan). The strain was grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% (wt/vol) yeast extract (THY broth) at 37°C in a 5% CO₂ atmosphere. The cells were harvested by centrifugation, washed three times with pyrogen-free water, and lyophilized.

Preparation of SPA and LPS from strain Y4. Y4 SPA was extracted from lyophilized cells of *A. actinomycetemcomitans* Y4 by autoclaving. The extract was purified by chromatography on DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden) and Sephacryl S-300 (Pharmacia) by the method of Amano et al. (3). LPS was extracted from lyophilized cells of *A. actinomycetemcomitans* Y4 by the hot phenol-water procedure, treated with nuclease, and washed extensively with pyrogen-free water by ultracentrifugation (38). The crude Y4 LPS was purified by chromatography on Sephadex G-200 (Pharmacia) equilibrated with a solution containing 10 mM Tris hydrochloride (pH 8.0), 0.2 M NaCl, 0.25% (wt/vol) deoxycholate, 1 mM EDTA, and 0.02% (wt/vol) sodium azide (16).

MAbs. A monoclonal antibody (MAb) directed against strain Y4 SPA (MAb S5) and one directed against Y4 LPS (MAb L2) were prepared and purified as described by Koga et al. (18). Normal ascitic fluid samples from BALB/c mice, were used as a control.

Reagents. Histopaque-1077, polymyxin B, *N*-formylmethionylleucylphenylalanine (FMLP) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Human recombinant IL-1 β (human rIL-1 β) and human recombinant TNF- α (human rTNF- α) were kindly provided by Otsuka Pharmaceutical Co., Tokushima, Japan, and M. Kohase, National Institute of Health, Tokyo, Japan, respectively. Human rIL-6 was purchased from Collaborative Research, Inc., Waltham, Mass., and mouse rIL-6 and human rIL-1 α were from Genzyme, Cambridge, Mass.

Monocyte preparation. Heparinized peripheral blood drawn from a healthy donor was diluted 1:3 in phosphate-buffered saline (PBS) (pH 7.3). The suspension (36 ml) was placed on 12 ml of Histopaque-1077 in a 50-ml conical tube (Sumitomo Bakelite Co., Tokyo, Japan) and then centrifuged at 400 $\times$ g for 30 min at room temperature. Human peripheral blood mononuclear cells (PBMCs) were collected at the interface and washed with PBS. The washed cells (4×10^6

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TABLE 1. Specific primers synthesized for PCR

mRNA	Size (bp) of PCR-amplified fragment	Synthesized primers (sense and antisense)	Reference
GAPDH	600	5'-CCACCCATGGCAAATTCCATGGCA-3' 5'-TCTAGACGGCAGGTCAGGTCCACC-3'	39
IL-1 α	816	5'-ATGGCCAAAGTTCCAGACATGTTTG-3' 5'-GGTTTTCCAGTATCTGAAAGTCAGT-3'	27
IL-1 β	802	5'-ATGGCAGAAGTACCTGAGCTCGC-3' 5'-ACACAAATTGCATGGTGAAGTCAGTT-3'	27
IL-6	628	5'-ATGAACTCCTTCTCCACAAGCGC-3' 5'-GAAGAGCCCTCAGGCTGGACTG-3'	12
IL-8	289	5'-ATGACTTCCAAGCTGGCCGTGGCT-3' 5'-TCTCAGCCCTCTCAAAACTTCTC-3'	23
MCP-1	290	5'-ATGAAAGTCTCTGCCGCC-3' 5'-TTGCTTGTCAGGTGGTC-3'	49
TNF- α	702	5'-ATGAGCACTGAAAGCATGATC-3' 5'-TCACAGGGCAATGATCCCAAGTAGACCTGCCC-3'	34
TNF- β	610	5'-ATGACACCACCTGAACGTCTCTTC-3' 5'-CGAAGGCTCCAAGAAGACAGTACT-3'	1

cells) were cultured in triplicate in 1 ml of Dulbecco's modified Eagle medium (DMEM; GIBCO Laboratories, Grand Island, N.Y.) containing 100 IU of penicillin per ml, 100 μ g of streptomycin per ml, and 2 mM L-glutamine in a 24-well plate (Falcon 3047; Becton Dickinson and Co., Paramus, N.J.) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. After 2 h of incubation, the nonadherent cells were removed by washing with DMEM, leaving a population of >95% phagocytic cells (40, 48). These adherent monolayers were used as human monocytes.

Cell culture. The adherent cells were incubated with various concentrations of *A. actinomycetemcomitans* Y4 SPA, Y4 LPS, LPS from *Escherichia coli* O55:B5 (*E. coli* LPS) (Difco Laboratories), or SPA component sugars, D-fucose and L-rhamnose, in 1 ml of DMEM containing antibiotics for 16 to 96 h in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The supernatants of these cultures were obtained by centrifugation and sterilized by passage through a filter (0.45- μ m pore size; Millipore Corp., Bedford, Mass.). These samples were stored at -30°C till use. The effect of polymyxin B on the release of monocyte chemotactic factor by monocytes stimulated with Y4 SPA or LPS was examined as follows. Human monocytes were cultured at 37°C for 48 h in 1 ml of DMEM containing various amounts of polymyxin B, antibiotics, and Y4 SPA (25 μ g) or LPS (3.1 μ g), and then the monocyte chemotactic activity of cell-free supernatant was determined as described below. To examine the effects of MABs on the release of monocyte chemotactic factor, human monocytes were cultured at 37°C for 48 h in 1 ml of DMEM containing various amounts of MAb S5, MAb L2, or control ascitic fluid samples, antibiotics, and Y4 SPA (25 μ g) or Y4 LPS (3.1 μ g), and then monocyte chemotactic activity of the cell-free supernatant was determined.

Chemotaxis assay. PBMCs obtained as described above were washed with PBS and suspended to a cell density of 5×10^6 cells per ml in Gey's balanced salt solution containing 2% bovine serum albumin and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Sigma) (Gey's BSA) (pH 7.0). To isolate PMNs, heparinized peripheral blood samples drawn from healthy donors were placed on Histopaque-1077 and then centrifuged at $400 \times g$ for 30 min. After removal of the upper layer containing plasma and PBMCs, erythrocytes in the lowest layer were lysed with a lysing buffer (pH 7.4) to obtain PMNs (32). PMNs were washed with PBS and suspended to a cell density of 3×10^6 cells per ml in Gey's BSA. Chemotaxis assay was performed by using a 96-well chemotaxis assembly (Neuro Probe, Rockville, Md.) (48). FMLP was used as a positive control for chemotaxis assay. Test and control samples were diluted with a mixture of seven parts of Gey's BSA and five parts of Veronal buffer with 0.5 mM MgCl₂ and 0.15 mM CaCl₂ (Gey's BSA-VB²⁺). Each of the wells on a bottom plate was filled with 25 μ l of the test or control sample in Gey's BSA-VB²⁺. A polycarbonate filter sheet (Neuro Probe) with a pore size of 5 μ m for monocytes or 3 μ m for PMNs was then placed on the bottom plate. A gasket and a top plate were fixed in place. Fifty microliters of monocyte suspension (5×10^6 cells per ml) or leukocyte suspension (3×10^6 cells per ml) was added to each well on the top plate. The whole assembly was incubated at 37°C for 90 min for monocytes or for 60 min for PMNs in a humidified incubator. After incubation, the filter was removed, fixed, and stained with Diff-Quik (Kokusai Shiyaku, Kobe, Japan). Ten fields of monocytes and leukocytes which had completely passed through the filter were counted by bright-field microscopy and oil immersion at $\times 1,000$ magnification, and then the total count was divided by 10. Each assay was carried out three times with triplicate filters. Data are expressed as the mean total numbers of migrating cells per oil immersion field $\pm$ standard deviations (SDs) for triplicate assays. In some experiments, data are expressed as the mean

percentage relative to the number of migrating cells of a positive control (10^{-8} M FMLP) $\pm$ SD for triplicate assays.

Bioassays for IL-1, IL-6, and TNF. IL-1 activity was determined by a human melanoma A375.S2 (ATCC CRL 1872) growth inhibition assay, with a known concentration of human rIL-1 β as a standard (26). IL-1 activity was defined by comparing the reciprocals of the dilutions of IL-1-containing test samples and the IL-1 standard (human rIL-1 β), causing 50% cell growth inhibition after 96 h of culture.

IL-6 activity was determined by a proliferation assay with an IL-6-dependent 7TD1 mouse hybridoma cell line which was provided by Jacques Van Snick, Ludwig Institute for Cancer Research, Brussels, Belgium (42). The IL-6 activities in the culture supernatants of human monocytes were estimated by comparison with a standard curve generated with human rIL-6 (42).

TNF activity of a culture supernatant of human monocytes was determined by cytotoxicity in murine fibroblast line L-929 which was obtained from Riken Gene Bank, Tsukuba, Japan (33). A known concentration of human rTNF- α was used as a standard. TNF activity was determined by comparing the reciprocals of the dilutions of TNF-containing test samples and the TNF standard which resulted in a cytotoxicity value of 50% in the L-929 cell bioassay system (24).

Limulus amoebocyte lysate clotting activity. The colorimetric blood endotoxin determination reagent (Toxicolor Test LS-6; Seikagaku Kogyo Co., Tokyo, Japan) was used for the measurement of *Limulus* amoebocyte lysate clotting activity of *A. actinomycetemcomitans* Y4 SPA, Y4 LPS, and *E. coli* LPS. The reagent (100 μ l) was incubated for 30 min at 37°C with 10 μ l of various concentrations of sample. After the reaction was terminated by adding 200 μ l of 0.6 M acetic acid, the A_{405} of each well was determined by a microplate reader.

Primers. Sense and antisense primers for human IL-1 α , IL-1 β , TNF- α , TNF- β , IL-6, and IL-8 were synthesized on the basis of published DNA sequences (1, 12, 23, 27, 34, 49). A pair of primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Stratagene, La Jolla, Calif. (39). The sequences of primers used in this study and the sizes of PCR fragments amplified by using these primers are shown in Table 1.

cDNA probes. A human MCP-1 cDNA plasmid and a human GAPDH cDNA plasmid were provided by T. Yoshimura (Laboratory of Immunology, National Cancer Institute, Frederick, Md.) and S. Sakiyama (Chiba Cancer Center Research Institute and Hospital, Chiba, Japan), respectively. A human IL-8 cDNA clone containing a 0.5-kb insert (5' end) was supplied by H. A. Young (Laboratory of Experimental Immunology, National Cancer Institute). Probes for Northern (RNA) blot assay were labeled with digoxigenin (DIG) by PCR (20). A PCR mixture contained 10 μ l of 10 $\times$ PCR buffer (50 mM KCl, 10 mM Tris hydrochloride buffer [pH 8.3], 1.5 mM MgCl₂, 0.01% [wt/vol] gelatin), 10 μ l of 10 $\times$ PCR DIG mixture (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 100 pmol of each primer, 5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Emeryville, Calif.), and 50 ng of template DNA in a total volume of 100 μ l. PCR consisted of dissociation of DNA (95°C for 1 min), annealing of primers (53°C for 1 min), and primer extension (72°C for 1 min). PCR was performed for 30 cycles with a thermal cycler (Perkin-Elmer Cetus). Aliquots (2 μ l) of each reaction mixture were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Northern blot assay. Human PBMCs were washed with PBS and suspended to a cell density of 4×10^6 cells per ml in RPMI 1640 medium containing antibiotics in 15-ml conical tubes (Sumitomo Bakelite Co.). Strain Y4 SPA or LPS was added to 2.5 ml of the cell suspension to a final concentration of 25 or 3.1 μ g/ml and then cultured for 1 to 18 h in a humidified atmosphere of 95% air and 5%

CO₂ at 37°C. To deplete monocytes, PBMCs were resuspended to a cell density of 4×10^6 cells per ml in RPMI 1640 medium containing antibiotics and allowed to adhere to six-well plates at 37°C for 2 h. Nonadherent cells were collected, washed with PBS, and incubated with Y4 SPA (25 µg/ml) or LPS (3.1 µg/ml) for 4 h. Moreover, human PBMCs were stimulated with human rIL-1α (230 U/ml), human rIL-1β (230 U/ml), human rIL-6 (60 U/ml), or human rTNF-α (190 U/ml). Total cellular RNA was extracted from stimulated or unstimulated cells by the acidic guanidinium-phenol-chloroform method (6). Extracted RNA samples (10 µg each) were electrophoresed on a 1.2% agarose gel containing 0.66 M formaldehyde and capillary blotted onto nylon membranes (Schleicher and Schuell Inc., Keene, N.H.). The RNA bound to the membrane was cross-linked by exposure to UV light and prehybridized for 3 h at 50°C with a high sodium dodecyl sulfate (SDS) hybridization buffer (7% SDS, 50 mM sodium phosphate buffer [pH 7.0], 50% formamide, 2% blocking reagent [Boehringer Mannheim], $5 \times$ SSC [$1 \times$ SSC is 0.15 M sodium chloride and 15 mM sodium citrate], 0.1% [wt/vol] *N*-lauroylsarcosine). Hybridization was performed with 100 ng of DIG-labeled PCR probes per ml in 7% high SDS hybridization buffer at 50°C for 16 h. Blots were washed with $2 \times$ SSC–0.1% SDS for 10 min at room temperature and then washed twice with $0.2 \times$ SSC–0.1% SDS for 20 min at 55°C. The hybridized probes were detected with a DIG luminescence detection kit (Boehringer Mannheim Biochemicals) (14).

Reverse transcriptase PCR (RT-PCR) assay. Human monocytes were enriched by fractionation on a one-step discontinuous Percoll (Pharmacia) gradient (8). The cells were suspended to a cell density of 5×10^6 cells per ml in RPMI 1640 medium. The cell suspension (2.0 ml) was incubated with strain Y4 SPA (25 µg/ml), Y4 LPS (3.1 µg/ml), D-fucose (25 µg/ml), or L-rhamnose (25 µg/ml) for 4 h in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The cells were collected by centrifugation at $850 \times g$ for 10 min at room temperature and subjected to total RNA extraction. Reverse transcription of the RNA samples to cDNA was done with the Ready-To-Go T-Primed First-Strand kit (Pharmacia). Briefly, the RNA samples (5 µg each) were dissolved in 33 µl of diethylpyrocarbonate-treated water and heated to 65°C for 5 min. The samples were then moved to the First-Strand reaction mixture (Pharmacia) and incubated at 37°C for 60 min. A PCR mixture contained 2 µl of cDNA mixture, 5 µl of $10 \times$ PCR buffer, 16 µmol of deoxynucleoside triphosphate, 50 pmol of each primer, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) in a total volume of 50 µl. PCR consisted of dissociation of DNA (94°C for 45 s), annealing of primers (60°C for 45 s), and primer extension (72°C for 1.5 min). PCR was performed for 35 cycles in a thermal cycler. Aliquots (5 µl) of each reaction mixture were electrophoresed on a 2% agarose gel and stained with ethidium bromide. *Bsp*1286I digests of pUC119 were used as standard DNA fragments. Photographic negatives of the gels were prepared with Polaroid type 665 film.

Statistical analysis. Statistical significance of differences in chemotactic activity between control and test samples was determined by Student's *t* test.

RESULTS

Release of monocyte and leukocyte chemotactic factors by human monocytes. SPA and LPS were extracted from whole cells of *A. actinomycetemcomitans* Y4 (serotype b) by autoclaving and the hot phenol-water procedure, respectively, and purified by chromatography. The *Limulus* ameocyte lysate clotting activity of these purified materials was compared with that of LPS from *E. coli* O55:B5 by a colorimetric endotoxin determination. The values of the clotting activity (A_{405}) in Y4 LPS and *E. coli* LPS at 100 µg/ml were 0.346 ± 0.009 and 0.409 ± 0.003 , respectively. The clotting activity was undetectable in Y4 SPA even at a concentration of 1 mg/ml.

SPA and LPS from strain Y4 induced the release of monocyte chemotactic factor by human monocytes (Fig. 1). The optimal concentrations of Y4 SPA and LPS for the release of monocyte chemotactic factor were 25 and 3.1 µg/ml, respectively. SPA component sugars, D-fucose and L-rhamnose, did not induce the release of monocyte chemotactic factor (Fig. 1A). The maximal activity of monocyte chemotactic factor in culture supernatants of human monocytes stimulated with Y4 SPA and LPS was detected after 48 to 72 h of culture (Fig. 2). To determine whether the monocyte migration is due to the presence of a true chemotactic factor or a chemokinetic factor, serial twofold dilutions of the monocyte supernatants were added to either the upper or bottom well or both wells, and then monocyte migration was determined under these conditions. The chemotactic activity is truly chemotactic, since the monocytes migrated only in the presence of a positive stimulatory gradient (Table 2). Figure 3 shows the time course of

FIG. 1. Monocyte chemotactic activities of the culture supernatants of human monocytes stimulated with SPA from *A. actinomycetemcomitans* Y4 (○) (A) or LPSs from *A. actinomycetemcomitans* Y4 (●) and *E. coli* O55:B5 (△) (B). SPA component sugars, D-fucose (▲) and L-rhamnose (□), were included as controls (A). Human monocytes were cultured with various concentrations of Y4 SPA, Y4 LPS, *E. coli* LPS, D-fucose, or L-rhamnose for 48 h. Culture supernatants at a 1:4 dilution were assayed for monocyte chemotaxis. Data are shown as the mean percentages relative to the number of migrating cells of a positive control (10^{-8} M FMLP) $\pm$ SDs for triplicate cultures. The numbers of migrating cells per oil immersion field of a negative control and a positive control were 4.0 ± 1.7 and 45.6 ± 5.7 , respectively. The experiments were performed three times, and similar results were obtained in each experiment.

PMN chemotactic factor activity in culture supernatants of human monocytes stimulated with Y4 SPA (25 µg/ml) or LPS (3.1 µg/ml). The peak activity was reached after 48 h of culture.

The effect of polymyxin B on the release of monocyte chemotactic factor by human monocytes stimulated with Y4 SPA or LPS was examined. The addition of polymyxin B to cultures markedly inhibited the monocyte chemotactic factor release by Y4 LPS-stimulated human monocytes, but it had virtually no effect on the release of monocyte chemotactic factor by Y4 SPA-stimulated cells (Fig. 4). The release of monocyte chemotactic factor by Y4 SPA-stimulated monocytes was significantly inhibited by MAb S5 and was insensitive to MAb L2 and control ascitic fluid samples (Fig. 5). On the other hand, significant inhibition of the monocyte chemotactic factor release by Y4 LPS-stimulated monocytes occurred with MAb L2, but not with MAb S5 and control ascitic fluid samples. As noted above, the Y4 SPA preparation used in this study exhibited no *Limulus* ameocyte lysate clotting activity. These findings indicate that contamination with LPS does not explain the ability of Y4 SPA to stimulate the release of monocyte chemotactic factor.

FIG. 2. Time course of monocyte chemotactic factor release by human monocytes stimulated with *A. actinomycetemcomitans* Y4 SPA or LPS. Human monocytes were cultured without (□) or with Y4 SPA (25 μ g/ml) (○) or LPS (3.1 μ g/ml) (●) for 16 to 96 h. Culture supernatants at a 1:4 dilution were assayed for monocyte chemotaxis. Data are shown as the mean percentages relative to the number of migrating cells of a positive control (10^{-8} M FMLP) $\pm$ SDs for triplicate cultures. The numbers of migrating cells per oil immersion field of a negative control and a positive control were 2.1 ± 0.8 and 43.3 ± 6.6 , respectively. The experiments were performed three times, and similar results were obtained in each experiment.

FIG. 3. Time course of PMN chemotactic factor release by human monocytes stimulated with *A. actinomycetemcomitans* Y4 SPA or LPS. Human monocytes were cultured without (□) or with Y4 SPA (25 μ g/ml) (○) or LPS (3.1 μ g/ml) (●) for 16 to 96 h. Culture supernatants at a 1:4 dilution were assayed for PMN chemotaxis. Data are shown as the mean percentages relative to the number of migrating cells of a positive control (10^{-8} M FMLP) $\pm$ SDs for triplicate cultures. The numbers of migrating cells per oil immersion field of a negative control and a positive control were 7.1 ± 0.9 and 51.3 ± 11.3 , respectively. The experiments were performed three times, and similar results were obtained in each experiment.

Expression of MCP-1 mRNA in PBMCs stimulated with Y4 SPA and LPS. Expression of MCP-1 mRNA was investigated in unseparated human PBMCs by Northern hybridization. Human PBMCs expressed little or no MCP-1 mRNA in the absence of stimulant. Substantial levels of MCP-1 transcripts were detected in PBMCs stimulated with Y4 SPA (25 μ g/ml) or LPS (3.1 μ g/ml) after 4 h of incubation (Fig. 6). On the other hand, monocyte-depleted lymphoid cells did not express MCP-1 mRNA in response to Y4 SPA or LPS (Fig. 7).

Release of IL-1, IL-6, and TNF. Activities of inflammatory cytokines such as IL-1, IL-6, and TNF in culture supernatants of human monocytes stimulated with Y4 SPA or LPS were determined by bioassays. Y4 SPA and LPS induced the release

of IL-1, IL-6, and TNF (Table 3). The IL-6 activity in culture supernatants of monocytes stimulated with Y4 SPA was lower than that in supernatants of cells stimulated with Y4 LPS. The maximal IL-1 activity in culture supernatants of monocytes treated with both stimulants was detected after 72 h of culture (data not shown). The release of IL-6 by monocytes stimulated with Y4 SPA and LPS peaked after 72 and 48 h of incubation, respectively. Moreover, the release of TNF by monocytes treated with both stimulants peaked after 48 h of incubation.

Cytokine gene expression in human monocytes. The expression of mRNAs specific for IL-1 α , IL-1 β , IL-6, IL-8, MCP-1, TNF- α , and TNF- β in human monocytes stimulated with Y4 SPA (25 μ g/ml), Y4 LPS (3.1 μ g/ml), D-fucose (25 μ g/ml), or L-rhamnose (25 μ g/ml) for 4 h was analyzed by RT-PCR. Y4 SPA and LPS markedly induced or enhanced the expression of IL-1 α , IL-1 β , IL-8, and MCP-1 mRNAs (Fig. 8). The levels of IL-6 and TNF- α transcripts were significantly enhanced by Y4 LPS, but not by Y4 SPA. TNF- β transcripts were not detected in any monocyte samples. D-Fucose and L-rhamnose had no effect on the expression of mRNAs of inflammatory cytokines and chemotactic factors by human monocytes (data not shown). Figure 9 shows the results of the time course of TNF- α mRNA expression in human monocytes stimulated with Y4 SPA or LPS. The peak level of TNF- α transcripts was detected after 6 h of incubation with Y4 SPA or LPS.

Induction of MCP-1 and IL-8 mRNA expression in human PBMCs by inflammatory cytokines. As mentioned above, Y4 SPA and LPS enhanced the release of IL-1, IL-6, and TNF, as well as monocyte and leukocyte chemotactic factors, by human monocytes. It is possible that Y4 SPA and LPS induce indirectly the production of these chemotactic factors through the production of such inflammatory cytokines. Therefore, we examined the effects of human rIL-1 α , rIL-1 β , rIL-6, and rTNF- α on the expression of mRNAs for MCP-1 and IL-8.

TABLE 2. Checkerboard analysis of human monocyte migration in culture supernatants of human monocytes stimulated with *A. actinomycetemcomitans* Y4 SPA

Dilution of monocyte supernatant in lower wells ^a	Monocyte migration at the following dilution of monocyte supernatant in upper wells ^b					
	0	1:32	1:16	1:8	1:4	1:2
0	3 $\pm$ 1	3 $\pm$ 1	3 $\pm$ 0	4 $\pm$ 0	3 $\pm$ 1	3 $\pm$ 0
1:32	5 $\pm$ 1	3 $\pm$ 1	5 $\pm$ 2	5 $\pm$ 0	4 $\pm$ 1	3 $\pm$ 0
1:16	6 $\pm$ 2	4 $\pm$ 0	4 $\pm$ 0	5 $\pm$ 0	6 $\pm$ 1	3 $\pm$ 0
1:8	11 $\pm$ 2	7 $\pm$ 1	6 $\pm$ 1	5 $\pm$ 1	6 $\pm$ 1	2 $\pm$ 0
1:4	18 $\pm$ 4	12 $\pm$ 3	10 $\pm$ 1	8 $\pm$ 1	6 $\pm$ 1	4 $\pm$ 0
1:2	32 $\pm$ 1	19 $\pm$ 3	14 $\pm$ 2	13 $\pm$ 2	8 $\pm$ 0	4 $\pm$ 0

^a Various dilutions of supernatants of human monocytes stimulated with Y4 SPA were added to the lower or upper well (with monocytes) of the chemotaxis chambers.

^b Monocyte migration was quantitated by counting the total number of cells migrating completely through the filter in 10 high-power fields in triplicate samples. Data are shown as the mean numbers of countable cells per oil immersion field $\pm$ SDs for triplicate cultures.

FIG. 4. Effects of polymyxin B on the release of monocyte chemotactic factor by human monocytes stimulated with *A. actinomycetemcomitans* Y4 SPA or LPS. Human monocytes were cultured with Y4 SPA (25 µg/ml) (○) or LPS (3.1 µg/ml) (●) in the presence of polymyxin B (0 to 30 µg/ml) for 48 h. Culture supernatants at a 1:4 dilution were assayed for monocyte chemotaxis. The percentage of control of monocyte chemotactic factor release was calculated by the following formula: percent control = $100 \times (\text{chemotactic activity of the culture supernatant of monocytes incubated with stimulant plus polymyxin B}) / (\text{chemotactic activity of the culture supernatant of monocytes incubated with stimulant})$. Data are shown as the mean percentages of control $\pm$ SDs for triplicate cultures. The numbers of migrating cells per oil immersion field for Y4 SPA and LPS were 27.8 ± 3.1 and 31.9 ± 1.3 , respectively. The experiments were performed three times, and similar results were obtained in each experiment. Values significantly different from the values for the control ($P < 0.05$ [**]; $P < 0.01$ [***]) are indicated.

Human rIL-1 α , rIL-1 β , rIL-6, and rTNF- α enhanced the expression of transcripts of these chemotactic factors (Fig. 10). However, the level of IL-8 transcripts induced with human rTNF- α was weak.

DISCUSSION

In the present study, we compared the ability of SPA from *A. actinomycetemcomitans* Y4 to induce the release of chemotactic factors by human monocytes with that of LPS from the same strain. Our bioassays demonstrated that human monocytes stimulated with Y4 SPA or LPS secreted monocyte chemotactic factor and leukocyte chemotactic factor. Moreover, the Northern hybridization and RT-PCR experiments revealed that Y4 SPA and LPS enhanced the expression of mRNAs of MCP-1 and IL-8 that are known as a human monocyte chemoattractant (49) and a human leukocyte chemoattractant (23), respectively. The ability of Y4 SPA to induce the production of chemotactic factors was clearly weaker than that of Y4 LPS, suggesting that SPA of *A. actinomycetemcomitans* may be less important in the infiltration of phagocytes in periodontal disease than LPS of the organism. However, this result cannot rule out the possibility that SPA acts more effectively on host cells than LPS does, because SPA is considered to be localized on the bacterial cell surface as a capsule (3). On the other hand, the lipid A portion of LPS which possesses most of the endotoxic and biological characteristics of intact LPS is embedded in the outer membrane of gram-negative bacteria (13).

IL-1, which is secreted by a variety of cells including monocytes and macrophages, plays a central role in the regulation of immunological and inflammatory reactions (29). Takahashi et

FIG. 5. Effects of MABs on the release of monocyte chemotactic factor by human monocytes stimulated with *A. actinomycetemcomitans* Y4 SPA (A) or LPS (B). Human monocytes were cultured with Y4 SPA (25 µg/ml) or LPS (3.1 µg/ml) in the presence of anti-SPA MAB (MAB S5) (○) (0 to 100 µg/ml), anti-LPS MAB (MAB L2) (●) (0 to 100 µg/ml), or control ascitic fluid samples (□) (0 to 100 µg/ml) for 48 h. Culture supernatants at a 1:4 dilution were assayed for monocyte chemotaxis. The percentage of control of monocyte chemotactic factor release was calculated by the following formula: percent control = $100 \times (\text{chemotactic activity of the culture supernatant of monocytes incubated with stimulant plus MAB}) / (\text{chemotactic activity of the culture supernatant of monocytes incubated with stimulant})$. The numbers of migrating cells per oil immersion field for Y4 SPA and LPS were 28.4 ± 3.0 and 32.1 ± 6.0 , respectively. Data are shown as the means $\pm$ SDs for triplicate cultures. Values significantly different from the values for the control ($P < 0.1$ [*]; $P < 0.05$ [**]) are indicated.

al. (38) reported that Y4 SPA induces the release of IL-1 by P388D₁ murine macrophages. Moreover, they showed that the ability of Y4 SPA to induce the release of IL-1 is higher than that of SPAs from serotypes a and c. The present study indicates that Y4 SPA also exhibits strong ability to induce the release of IL-1 by human monocytes. Human peripheral blood monocytes and monocytic cell lines are known to produce two forms of IL-1, referred to as IL-1 α and IL-1 β exhibiting isoelectric points of 5.0 and 7.0, respectively (29). IL-1 β is more abundant in human monocytes than IL-1 α and is more potent than IL-1 α in stimulating in vitro bone resorption (37). The RT-PCR experiments revealed that Y4 SPA markedly induced the expression of both IL-1 α mRNA and IL-1 β mRNA in

FIG. 6. Expression of MCP-1 mRNA and GAPDH mRNA in PBMCs treated with *A. actinomycetemcomitans* Y4 SPA or LPS. PBMCs (10^7 cells) were stimulated with Y4 SPA (25 $\mu\text{g/ml}$) or LPS (3.1 $\mu\text{g/ml}$) for 0 to 18 h, and then their total RNA was isolated. Northern blot analysis was performed with DIG-labeled MCP-1 and GAPDH PCR probes.

human monocytes, suggesting the possibility that the polysaccharide antigen might participate in bone resorption in periodontal lesions. In this regard, Nishihara et al. (28) have recently reported that Y4 SPA promotes osteoclast-like cell formation by IL-1 α in a mouse bone marrow culture system.

Although LPS is well-known to induce TNF- α (19), few studies have dealt with the effects of microbial polysaccharides on the production of TNF- α by host cells. Mancuso et al. (21) have reported that the type III-specific polysaccharide of group B streptococci induces dose-dependent, transient elevations in plasma TNF- α levels in neonatal rats. Soell et al. (36) have shown that streptococcal rhamnose glucose polymers stimulate human monocytes to produce TNF- α in a dose-dependent manner and that TNF- α release is correlated with binding to

FIG. 7. Expression of MCP-1 mRNA in PBMCs (A) and monocyte-depleted PBMCs (B) stimulated with medium alone (lane 1), Y4 SPA (25 $\mu\text{g/ml}$) (lane 2), or Y4 LPS (3.1 $\mu\text{g/ml}$) (lane 3) for 4 h. To deplete monocytes, PBMCs were resuspended to a cell density of 4×10^6 cells per ml in RPMI 1640 medium containing antibiotics and allowed to adhere to six-well plates at 37°C for 2 h. Nonadherent cells were collected and washed with PBS. Northern blot analysis was performed with DIG-labeled MCP-1 and GAPDH PCR probes.

TABLE 3. Release of IL-1, IL-6, and TNF by human monocytes stimulated with various amounts of *A. actinomycetemcomitans* Y4 SPA or LPS^a

Stimulants	Dose ($\mu\text{g/ml}$)	Activity (U/ml)		
		IL-1	IL-6	TNF
None		3 $\pm$ 0	15 $\pm$ 4	16 $\pm$ 2
Y4 SPA	12.5	66 $\pm$ 11	43 $\pm$ 15	172 $\pm$ 46
	25	230 $\pm$ 34	61 $\pm$ 20	190 $\pm$ 30
	50	206 $\pm$ 20	59 $\pm$ 16	188 $\pm$ 37
	100	235 $\pm$ 31	93 $\pm$ 35	108 $\pm$ 18
Y4 LPS	3.1	118 $\pm$ 35	207 $\pm$ 11	242 $\pm$ 8
	6.3	143 $\pm$ 48	203 $\pm$ 6	198 $\pm$ 12
	12.5	97 $\pm$ 7	274 $\pm$ 40	166 $\pm$ 11
	25	96 $\pm$ 5	240 $\pm$ 16	119 $\pm$ 48

^a Human monocytes ($10^6/\text{ml}$) were stimulated with various amounts of *A. actinomycetemcomitans* Y4 SPA or LPS for 48 h. Activities of IL-1, IL-6, and TNF were measured as described in Materials and Methods. The activities of IL-1, IL-6, and TNF were calculated on the basis of the standard curve by using known concentrations of human rIL-1 β , human rIL-6, or human rTNF- α in the same assay, respectively. Data are shown as the means $\pm$ standard errors for experiments performed in triplicate.

the CD14 antigen. The present study showed that the abilities of Y4 SPA to induce TNF secretion and TNF- α mRNA expression were comparable to those of Y4 LPS. Further work is needed to determine the receptor for Y4 SPA on human monocytes.

It was reported that high levels of IL-6 are produced by gingival mononuclear cells isolated from inflamed periodontal

FIG. 8. Expression of mRNAs specific for various inflammatory cytokines in human monocytes stimulated with medium alone (A), Y4 SPA (25 $\mu\text{g/ml}$) (B), or Y4 LPS (3.1 $\mu\text{g/ml}$) (C) for 4 h. The expression of mRNAs for these cytokines was analyzed by RT-PCR. Lane M, standard DNA fragments (*Bsp*1286I digests of pUC119); lane 1, GAPDH mRNA; lane 2, IL-1 α mRNA; lane 3, IL-1 β mRNA; lane 4, IL-6 mRNA; lane 5, IL-8 mRNA; lane 6, MCP-1 mRNA; lane 7, TNF- α mRNA; lane 8, TNF- β mRNA. The numbers on the left indicate sizes of standard DNA fragments in base pairs.

FIG. 9. Time course of expression of TNF- α mRNA and GAPDH mRNA in human monocytes stimulated with *A. actinomycetemcomitans* Y4 SPA (25 μ g/ml) (A) or Y4 LPS (3.1 μ g/ml) (B). The expression of TNF- α mRNA and GAPDH mRNA was analyzed by RT-PCR. M, standard DNA fragments (*Bsp*1286I digests of pUC119). The numbers on the left indicate sizes of standard DNA fragments in base pairs.

tissues (9). Gingival biopsies from diseased sites of periodontitis patients contain more IL-6 than those from periodontally healthy sites (10). These findings suggest that IL-6 may be considered as a marker for active periodontitis (41). However, the level of IL-6 in human monocytes stimulated with Y4 SPA was low by bioassay and RT-PCR, indicating that this polysaccharide antigen might not play an active role in IL-6 induction in periodontal tissues.

LPS from *A. actinomycetemcomitans* is known to induce or enhance the synthesis of various cytokines by human monocytes (2). It is possible that the ability of Y4 SPA to induce the secretion of chemotactic factors and inflammatory cytokines is the result of contamination with LPS. However, polymyxin B, which is an inhibitor of lipid A, did not suppress the release of monocyte chemotactic factor (Fig. 4) and inflammatory cytokines (47). This polysaccharide preparation did not exhibit any *Limulus* amebocyte clotting activity. Moreover, MAb against

Y4 SPA, but not MAb against Y4 LPS, suppressed significantly the release of monocyte chemotactic factor by human monocytes stimulated with Y4 SPA (Fig. 5). Taken together, the ability of Y4 SPA to stimulate the release of chemotactic factors and inflammatory cytokines is not due to the contamination with LPS.

Yoshimura et al. (49) reported that MCP-1 mRNA is induced in human peripheral blood mononuclear leukocytes by stimulation with LPS or IL-1, but not by treatment with IL-2, TNF, or gamma interferon. Rollins et al. (31) demonstrated that human endothelial cells express MCP-1 mRNA after treatment with IL-1 β , TNF, and gamma interferon. Moreover, Hanazawa et al. (11) showed that MCP-1 mRNA is induced in human gingival fibroblasts treated with IL-1 β and TNF, but not with IL-6. We found that human monocytes expressed MCP-1 mRNA after treatment with human rIL-1 α , rIL-1 β , rIL-6, and rTNF- α . The discrepancy in these results might be due to the difference in host cells used among the experiments.

In this study, we have shown that SPA from *A. actinomycetemcomitans* serotype b induced the release of monocyte and leukocyte chemotactic factors and inflammatory cytokines by human monocytes. Human monocytes expressed mRNA of MCP-1 and IL-8 after incubation with human rIL-1 α , rIL-1 β , rIL-6, and rTNF- α , suggesting that these inflammatory cytokines secreted by SPA-stimulated monocytes might participate in the production of chemotactic factors in the host cells. On the other hand, we reported previously that the SPA of *A. actinomycetemcomitans* sustains the resistance to phagocytosis and killing of the organism by human PMNs (46). These findings suggest that SPA possesses various biological functions in human phagocytes. However, the exact roles of human PMNs and monocytes stimulated with SPA in protection and/or destruction of periodontal tissues in periodontitis remain to be resolved.

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FIG. 10. Effects of IL-1 α , IL-1 β , IL-6, and TNF- α on MCP-1 mRNA expression and IL-8 mRNA expression in human PBMCs. The cells were incubated with medium alone (lane 1), human rIL-1 β (230 U/ml) (lane 2), human rIL-6 (60 U/ml) (lane 3), human rTNF- α (190 U/ml) (lane 4), or human rIL-1 α (230 U/ml) (lane 5) for 4 h, and total RNA was analyzed by Northern blotting with DIG-labeled MCP-1, IL-8, and GAPDH PCR probes.

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