Neutrophil modulation by *Actinobacillus actinomycetemcomitans* I. Chemotaxis, surface receptor expression and F-actin polymerization

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Localized juvenile periodontitis is an early onset periodontitis, usually localized to molars and incisors. Patients usually present with decreased chemotaxis of systemic neutrophils (PMNs) and infection with *Actinobacillus actinomycetemcomitans*. The pathogenic mechanisms involved have not been clarified. The purpose of this study was to determine if an extract of *A. actinomycetemcomitans* could induce changes in PMN chemotaxis similar to those reported in LJP patients. It was demonstrated that the bacterial extract was chemotactic for neutrophils. When neutrophils were pre-incubated with the bacterial extract, chemotaxis toward zymosan-activated serum, FMLP and the bacterial extract was inhibited in two different chemotaxis assays (Boyden chamber and under-agarose). Bacterial extract had no effect on random migration in either assay. Pre-incubation with the extract induced increased expression of CD11b/CD18 (Mac-1), Gp110, and FMLP receptors and increased F-actin polymerization following FMLP or PMA stimulation compared to cells not treated with the extract. Treatment of the bacterial extract with proteinase K or phenol extraction reversed the PMN chemotaxis inhibition activity, but increased significantly the random migration of PMNs. Heating the bacterial extract to 56°C had no effect on its activity. The component(s) in the bacterial extract that inhibits chemotaxis is therefore a protein(s), not sensitive to 56°C, and is not endotoxin. This study suggests that *A. actinomycetemcomitans* may contribute to the pathogenesis of localized juvenile periodontitis by inhibiting chemotaxis. Interference with chemotaxis by *A. actinomycetemcomitans,* however, occurs through a mechanism other than inhibition of actin assembly, reduction of CD11b/CD18 or Gp110 expression, or blockage/downregulation of FMLP receptors.

Key words: neutrophil – *Actinobacillus actinomycetemcomitans* – juvenile periodontitis – chemotaxis

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Introduction

Localized juvenile periodontitis (LJP) is an early onset periodontal disease in adolescents and young adults (1–3). It is characterized by destruction of the alveolar bone of the permanent first molar and incisor teeth. The mechanisms involved in this destructive process have not been completely elucidated. Several lines of evidence suggest that infection with *Actinobacillus actinomycetemcomitans* may contribute to the pathogenesis of LJP (2, 4, 5). As many as 90% of patients with LJP harbor *A. actinomycetemcomitans* in high numbers in subgingival lesions (2, 4). Secondly, *A. actinomycetemcomitans* possesses numerous virulence factors which may be relevant to the disease. They include; leukotoxin, collagenase, endotoxin, epitheliotoxin

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and a fibroblast-inhibiting factor (6–9). Thirdly, LJP patients develop high levels of serum, salivary and crevicular fluid antibodies specifically directed to antigens of *A. actinomycetemcomitans* (10–12). Moreover, clinical improvement has been reported following combined mechanical debridement and antibiotic therapy directed toward elimination of *A. actinomycetemcomitans* from diseased sites (13). Finally, failure to eliminate *A. actinomycetemcomitans* from lesions is associated with progressive loss of alveolar bone (4, 14).

In terms of host defense factors, previous studies have suggested that patients with LJP present with neutrophil defects, which may play a role in the pathogenesis of the disease. In several studies, about 70% of patients with LJP have depressed chemotaxis, with poor neutrophil response to FMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine) and to the complement fragment C5a (15–18). A reduced amount of glycoprotein 110, a neutrophil membrane matrix component, has been also found in these patients (14, 19, 20). Neutrophil defects in patients with prepubertal periodontitis have been attributed to a deficiency of a surface adhesion glycoprotein CD11b/CD18 (Mac-1 deficiency) (21).

The cause of the alterations in neutrophil function in LJP patients has not as yet been clarified. The familial tendency of the disease suggests that the defects may result from genetically-related abnormalities. Also, microorganisms may contribute to the observed neutrophil abnormalities. Bacteria can elaborate substances that are cytotoxic for neutrophils (22, 23) and that can modulate specific neutrophil functions such as chemotaxis (4, 5, 24). Since *A. actinomycetemcomitans* is frequently found in patients with LJP, a thorough analysis of the effects of this bacterium on normal neutrophil function is warranted. We have therefore evaluated *in vitro* the effects of a bacterial extract of *A. actinomycetemcomitans* on various aspects of neutrophil chemotaxis.

Material and Methods

Preparation of bacterial extract

A. actinomycetemcomitans strain 29522, serotype b, was obtained from the American Type Tissue Culture Collection (ATCC) and was cultivated in 12 liters of NIH thioglycollate broth in an humidified atmosphere containing 90% air and 10% CO_2 at 37°C for 48 hours. Bacteria were harvested by centrifugation ($16\,000 \times g$, 15 min), washed three times with 0.2 M sucrose buffer (0.2 M sucrose in 0.0175 M sodium phosphate buffer, pH 7.4), disrupted ultrasonically with a Sonifier Cell Disruptor (Model W185, Heath System) in an ice-bath, using a cycle of 2-min sonication at full power with 1 min of cool down for a total of 10 min of ultrasonic exposure. The disrupted cells were then centrifuged ($12\,000 \times g$, 10 min), and the supernatant was decanted and centrifuged at $100\,000 \times g$ for 60 min. The resulting supernatant, the "bacterial extract", was lyophilized and stored at −20°C. Before usage, bacterial extract was suspended in Hanks' balance salt solution (HBSS, GIBCO, Grand Island, NY) and was used at final concentrations of 0.15, 1.5 and 15 μg protein/ml depending on the experiment. The protein concentrations of the bacterial extracts, determined by Bradford protein assay (Bio-Rad Laboratories, Richmond, CA) ranged from 100 to 200 μg protein/mg dry weight. To evaluate the effect of heat treatment, bacterial extract was heated to 56°C for 30 min.

To further characterize the active component(s) in the bacterial extract, the bacterial extract was treated with proteinase K to obtain a protein depleted fraction (BE-ProK), or with phenol-water to obtain endotoxin enriched fraction (BE-LPS).

A. actinomycetemcomitans extract dissolved in water was treated to remove proteins, using Protease XI-A (from Tritirachium album) attached to 4% cross-linked beaded agarose (Proteinase K beads, Sigma, St. Louis, MO). Briefly, proteinase K beads (in 25-fold excess according to specific activity) were added to *A. actinomycetemcomitans* extract and incubated at 37°C for 30 min. Proteinase K beads were removed by centrifugation at $3000 \times g$ for 30 min, and the supernatant was dialyzed against water for 24 h with three changes. The supernatant was negative for proteins by Bradford assay analysis (Bio-Rad).

An endotoxin enriched fraction from the bacterial extract was obtained using the phenol-water procedure according to Nowotny (25). Briefly, lyophilized bacterial extract of *A. actinomycetemcomitans* dissolved in distilled water was mixed with an equal volume of 90% ultrapure phenol (BRL, Gaithersburg, MD) in a 70°C water bath for 10 min with continuous stirring. Subsequently, the solution was cooled, centrifuged at $3000 \times g$ for 30 min, and the water phase containing lipopolysaccharides was collected and dialyzed against water for 3 d (six changes). The remaining dialysate was concentrated using vacuum distillation and was then diluted with cold methanol containing 2% $MgCl_2$ to obtain a final concentration of 75% methanol with constant stirring at 4°C. Following centrifugation ($3000 \times g$, 30 min) the sedimented LPS fraction was dissolved in water, then diluted with cold methanol (final 66% methanol). Centrifugation and dissolution with cold methanol was repeated twice. After the final methanol treatment,

the LPS fraction was dissolved in water and centrifuged at 110 000 × *g* for 2 h, and the nucleic acid-containing supernatant discarded. The viscous lower layer was lyophilized, weighed, and resuspended at a stock concentration of 1 mg/ml.

Isolation of human blood polymorphonuclear cells (PMNs)

Blood was collected from healthy donors by venipuncture in 10 ml vacutainers (Becton Dickinson, Rutherford, NJ) which contained 0.1 ml of 15% EDTA. Ten ml of blood were mixed with 1.5 ml of 6% dextran (M.W. 229,000, Sigma), in HBSS free of Ca^{2+} or Mg^{2+} (HBSS-CMF, GIBCO) at pH 7.2 and allowed to sediment at room temperature for 30 min. The leukocyte-rich plasma (LRP) layer was collected and PMNs processed for further study. For the detection of cell surface receptors, the LRP layer was washed twice with ice-cold HBSS-CMF, and centrifuged (250 × *g*, 5 min) to obtain PMNs. PMNs were resuspended in ice-cold HBSS at a final concentration of 3.3×10^6 cell/ml. For chemotaxis assay, the LRP layer was aspirated, layered on a ficoll-hypaque gradient (Pharmacia, Piscataway, NJ), and centrifuged at 2000 × *g* for 10 min at room temperature. Contaminating erythrocytes were lysed by exposing the pellet to hypotonic solution (10 ml, 0.2% NaCl) for 30 s, and the isotonicity was restored by adding 10 ml 1.6% NaCl. The cells were then washed twice with ice-cold HBSS-CMF.

Pre-treatment of PMNs

For the chemotaxis assays, PMNs were resuspended in 1 ml of ice-cold HBSS, bacterial extract, or heated bacterial extract at a final concentration of 5×10^7 cells/ml, and incubated for 15 min at 37°C in a shaking water bath. Following incubation the cells were washed twice with ice-cold HBSS-CMF and resuspended in HBSS containing 2% bovine serum albumin (Sigma) at a final concentration of 5×10^6 cells/ml, for Boyden chamber assays. For under-agarose assays, cells were resuspended at a final concentration of 2.5×10^7 cells/ml in MEM containing 10% heat inactivated serum. Cells were stored on ice until use. Viability of PMNs was determined following pre-incubation with bacterial extract by trypan blue exclusion (25).

Boyden chamber chemotaxis

Chemotaxis was determined as described by Boyden (26) with the modification of Snyderman and Pike (27), using blind well chemotaxis chambers. A Millipore filter with a pore size of 3 μm, and a thickness of 150 μm (Millipore Corporation, Bedford, MA) separated two compartments. Chemotaxis was evaluated by placing in the lower compartment 15 μg/ml bacterial extract, 15 μg/ml heated bacterial extract, 10^{-7} M FMLP, or 10% zymosan activated serum (ZAS; prepared by incubating 10 mg of freeze-dried zymosan A in 1 ml of serum of 45 min at 37°C). HBSS was placed in the lower compartment in control assays. PMNs (1×10^6 in 200 μl) were placed in the upper compartment. The chambers were then incubated for 45 min at 37°C in 5% CO_2. Following incubation the filters were removed, fixed, stained and cleared according to Boyden (26), and mounted on glass slides. Chemotaxis was measured as the distance from the top of the filter to the leading front of cells, as described by Zigmond and Hirsch (28). Random migration was calculated as the distance traveled by the cells when HBSS alone was placed in the lower chamber. Data presented represent the results of triplicate replications on 3 different days.

The effect of bacterial extract of *A. actinomycetemcomitans* on chemotactic activity was examined by pre-incubating the cells with bacterial extract (15 μg/ml, 37°C, 15 min) or with HBSS (control). The cells were then washed twice, and chemotaxis was determined as described above.

Under agarose chemotaxis

We also evaluated the effect of bacterial extract on PMN chemotaxis using the under agarose technique (29, 30), to establish if alterations in chemotaxis of PMNs were due to artifacts specific to the Boyden chamber assay. Briefly, four pairs of parallel wells (2 × 5 mm each, 4 mm apart) were cut into agarose gel (1.2% agarose in MEM, 5% autologous serum) in 60 × 15 mm petri dishes. One well of each pair was filled with either 20 μl of a stimulus (10^{-7} M FMLP, 100% ZAS, 15 μg/ml bacterial extract) or HBSS. The other well of the pair was filled with 20 μl of PMNs at a concentration of 2.5×10^7 cells/ml. Quadruplicate petri dishes were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air for 4 h. Following incubation, each petri dish was flooded with 37% formaldehyde (Sigma) for 5 min, the agarose was removed and the dish was air-dried. The fixed cells were stained with Diff-Quik (American Scientific Product, McGaw Park, IL). After drying, each petri dish was viewed using a histology slide projector, and the distance from the well edge to the leading front of PMNs was measured (30). The effect of bacterial extract on chemotaxis was examined by pre-incubating the cells with 1 ml of 15 μg/ml bacterial extract for 15 min at 37°C, followed by two washes. Control cells were pre-incubated with HBSS.

Expression of CD11b and Gp110

The effect of the bacterial extract on the expression of adhesion receptors following stimulation with FMLP and PMA was evaluated. CD11b/CD18 expression was determined using the monoclonal antibody specific for the α chain (CD11b) as previously described (32). Expression of Gp110, a neutrophil membrane matrix component, was determined using the monoclonal antibody NCD-1. Briefly, 300 μl of the PMN suspension (3.3×10^6 cells/ml) were pre-incubated with HBSS (control), bacterial extract (final concentration of 0.15 μg/ml, 1.5 μg/ml or 15 μg/ml), heated bacterial extract (15 μg/ml), BE-ProK (150 μg/ml), or BE-LPS (150 μg/ml) for 15 min at 37°C, in a shaking water bath. Following pre-incubation, the cells were stimulated with FMLP (final concentration of 10^{-5} M, 10^{-7} M, 10^{-9} M), PMA (20 μg/ml, 2 μg/ml) or HBSS (control) for an additional 15 min at 37°C. A parallel non-stimulated sample was placed on ice. After incubation, the reaction was stopped by placing the tubes on ice for 2 min. The remainder of the experiment was performed at 0–4°C to prevent further mobilization of CD11b. Cell suspensions were washed (centrifugation: $2000 \times g$, 2 min), resuspended in 100 μl HBSS and incubated for 30 min with 1 μg OKM-1 (Ortho Diagnostics, Raritan, NJ; Murine monoclonal antibody to the CD11b. CD11b is the α sub-unit of the CD11b/CD18 complex found on PMNs and is related to the complement receptor CR3, the iC3B receptor) or with NCD-1 monoclonal antibody (kindly provided by Dr. W. Smith). After incubation, cells were washed, resuspended in HBSS containing fluorescein-labeled goat anti-mouse IgG $F(ab')_2$ fragment (Boehringer Mannheim Biochemicals, Indianapolis, IN), and were incubated for 30 min. Subsequently, the cells were washed twice, fixed with 500 μl of 1% formalin in phosphate-buffered saline, and evaluated by flow cytometric analysis, as described below.

Identification of FMLP receptors

FMLP receptors on the surface of PMNs were labeled by incubating cells at 4°C with 10^{-8} M fluorescein derivatized formyl-Nle-Leu-Phe-Nle-Tyr-Lys (F-1314; Molecular Probes, Eugene, OR) for 30 min as described by Sklar *et al.* (33). The effect of the bacterial extract on the expression of FMLP receptors was evaluated by pre-incubating the cells (300 μl, 3.3×10^{-6} cells/ml) with HBSS (control), with bacterial extract (15 μg/ml), BE-ProK or BE-LPS (final concentration of 150 μg/ml or 300 μg/ml) for 15 min at 37°C, in a shaking water bath. Cells were subsequently held on ice until analysis. A parallel non-stimulated sample was placed on ice. In competitive assays, cells were incubated with 10^{-5} M non-fluorescent FMLP and with 10^{-8} M fluorescein derivatized formyl-Nle-Leu-Phe-Nle-Tyr-Lys (30 min, 4°C). Cells were subsequently held on ice until analysis.

F-actin polymerization

Polymerization of the monomeric form of actin to the polymeric form (F-actin) is an obligatory event for leukocyte motility and chemotaxis. The polymerization of F-actin was quantitatively determined following stimulation with FMLP and PMA using NBD-phallacidin which stains the F-actin (34). PMN suspensions (300 μl, 3.3×10^6 cells/ml) were pre-incubated with bacterial extract (15 μg/ml) or with HBSS for 15 min at 37°C. Following incubation, the cells were exposed to FMLP (final concentration: 10^{-9} M, 10^{-7} M or 10^{-5} M), PMA (10 μg/ml or 1 μg/ml) or to bacterial extract (final concentrations of 15 μg/ml or 1.5 μg/ml) for 2 min. Control groups were incubated with HBSS. The reaction was stopped by fixation with 300 μl of HBSS solution containing 3.5% formalin, and 0.188 mg/ml lysophosphatidyl choline. Immediately prior to analysis by flow cytometry, cells were incubated for 45 min with NBD-phallacidin (2.5 units/10^6 cells, Molecular Probes, Eugene, OR) at room temperature in the dark.

PMN analyses using flow cytometry

Samples of PMNs were measured using an Ortho Cytofluorograf System 50-H with a 2150 computer (flow cytometer, Ortho Diagnostics, Westwood, MA). Cells were illuminated by a 5 watt argon laser emitting 400 milliwatts light at 488 nm. PMNs analyzed for fluorescence were selected using forward and right-angle light scatters as previously described (31). A green fluorescence histogram was collected for 10 000 PMNs for each sample analyzed.

Statistical analysis

Paired t-test was used to determine the significant of the differences between cells before and after stimulations. Student's t-test was used to determine the significant of the differences between two different stimulations.

Results

Effect of bacterial extract on chemotaxis using Boyden chamber assay

Bacterial extract and heated bacterial extract at a concentration of 15 μg/ml were found to be

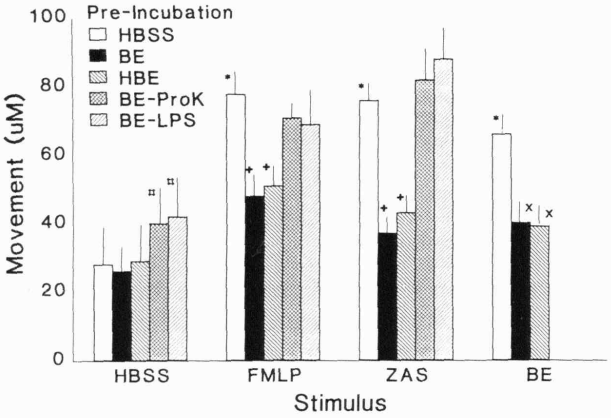


Fig. 1. Chemotaxis of PMNs in Boyden chambers following pre-incubation with bacterial extract (BE). PMNs were pre-incubated with bacterial extract of *A. actinomycetemcomitans*, with Proteinase K-treated BE (BE-ProK; 150 μg/ml), or with phenol extraction of BE (BE-LPS; 150 μg/ml). Control cells were pre-incubated with HBSS. Cells were then placed in the chemotaxis chamber opposite 10^{-7} M FMLP, 10% ZAS, BE (15 μg/ml) or HBSS. Results are presented as the distance moved by the leading front of the cells, and represents the mean $\pm$SEM of 3 experiments each done in triplicate. Significant chemotaxis was noted towards FMLP, ZAS, and BE (* = $p < 0.0001$) compared to random migration (HBSS). Pre-incubation with bacterial extract of *A. actinomycetemcomitans* (15 μg/ml) decreased the chemotaxis toward FMLP, ZAS, and bacterial extract itself (+ = $p < 0.001$; x = $p < 0.02$), but had no effect on random migration. In contrast, pre-incubation with BE-ProK or BE-LPS increased random migration (# = $p < 0.001$).

chemotactic agents (Fig. 1); chemotaxis of PMNs toward bacterial extract was over two times more than toward HBSS ($p < 0.0001$). Pre-incubation of PMNs with *A. actinomycetemcomitans* bacterial extract prior to assay in Boyden chambers caused a significant decrease in the directed locomotion toward FMLP (38% reduction, $p < 0.001$) and ZAS (51% reduction, $p < 0.001$). In addition, PMNs pre-incubated with bacterial extract or heated bacterial extract had significantly decreased chemotaxis toward bacterial extract (39% and 37% reductions respectively, $p < 0.02$). Pre-incubation with bacterial extract heated to 56°C caused the same degree of chemotactic inhibition as did non-heated extract (for heat-treated versus non-heated: FMLP 53% versus 59%; ZAS 48% versus 57%; and bacterial extract 79% versus 75%). In contrast, pre-incubation with BE-ProK or BE-LPS did not result in a significant change in chemotaxis toward FMLP (decreased by 9% and 12% respectively, $p < 0.9$) or toward ZAS (increased by 8% and 16% respectively, $p < 0.1$). Pre-incubation of PMNs with *A. actinomycetemcomitans* bacterial extract or heated bacterial extract had no effect on random migration as compared with PMNs pre-incubated with HBSS alone. In contrast, pre-incubation of the cells with BE-ProK or BE-LPS caused increases in the random migration of the PMNs (43% and 50% respectively, $p < 0.001$)

Effect of bacterial extract on under agarose chemotaxis

Pre-incubation of PMNs with bacterial extract did not significantly alter the non-directed locomotion of the PMNs in the under agarose assay, but significantly decreased the directed locomotion toward FMLP and ZAS (53% and 80%, respectively; $p = 0.001$), as compared with PMNs pre-incubated with HBSS alone. Pre-incubation with bacterial extract was found also to inhibit the directed locomotion toward bacterial extract by 23%. Bacterial extract at a concentration of 15 μg/ml was found to be a chemotactic agent; PMNs moved toward bacterial extract significantly more ($p < 0.02$) than toward HBSS (Fig. 2).

Effect of bacterial extract on cell surface expression of CD11b, Gp110 and FMLP receptors

CD11b expression following various stimulations (bacterial extract, FMLP, or PMA) is shown in Table 1 and is expressed as mean fluorescent intensity. Bacterial extract, FMLP and PMA increased the cell surface expression of CD11b. Bacterial extract at a concentration of 15 μg/ml significantly increased (136% after 30 min, $p < 0.05$) the expression of CD11b as compared with control. In

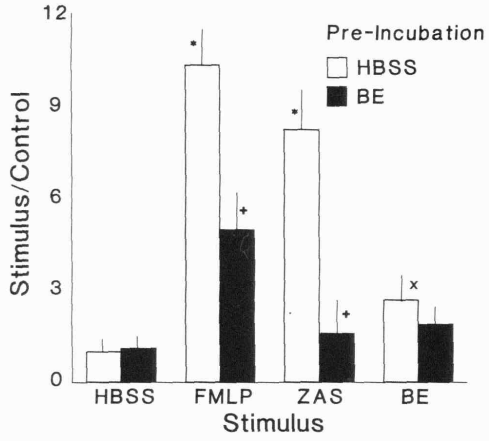


Fig. 2. Chemotaxis of PMNs under agarose following pre-incubation with bacterial extract (BE). PMNs were pre-incubated with HBSS or with bacterial extract of *A. actinomycetemcomitans* and chemotaxis was evaluated toward HBSS, 10^{-7} M FMLP, 100% ZAS, and bacterial extract (15 μg/ml). Results are presented as the distance moved by the leading front cells toward the stimulus divided by the distance moved toward HBSS, and represents the mean $\pm$SEM of 3 experiments, each done in quadruplicate, FMLP, ZAS and BE were chemotactic stimulants (* = $p < 0.001$, x = $p < 0.02$). Pre-incubation with bacterial extract of *A. actinomycetemcomitans* (15 μg/ml) decreased the chemotaxis toward FMLP and ZAS (+ = $p < 0.001$), but did not decrease random migration.

contrast, sub-optimal concentrations of bacterial extract (0.15 or 1.5 μg/ml) did not significantly affect the CD11b expression by PMNs. FMLP, at final concentrations of 10^{-9} M, 10^{-7} M, or 10^{-5} M increased CD11b expression by 2%, 72% and 54%, respectively. The optimal concentration of FMLP for inducing CD11b expression was 10^{-7} M (72% increase); lower and higher concentrations induced significantly lower expression. PMA (20 μg/ml) induced comparable levels of CD11b expression as 10^{-7} M FMLP. Lower concentrations of PMA (0.2 μg/ml) did not change the CD11b as compared with control. Extension of the incubation time with the stimulants (HBSS, FMLP, PMA or bacterial extract) from 15 to 30 min did not significantly increase the expression of CD11b.

Pre-incubation of the cells with bacterial extract (15 μg/ml) did not significantly change the CD11b expression stimulated by optimal stimulation with FMLP (15% and 14% for 10^{-7} M and 10^{-5} M, respectively) as compared to stimulation by FMLP alone. No change in CD11b expression was found when the cells were pre-incubated with bacterial extract and subsequently were stimulated with 20 μg/ml PMA. In contrast, pre-incubation of the cells with bacterial extract (15 μg/ml) followed by sub-optimal concentrations of stimulants (10^{-9} M FMLP or 0.2 μg/ml PMA) increased (75% and 73%, respectively; $p<0.01$) the CD11b expression as compared to stimulation by FMLP or PMA alone. Lower concentrations of bacterial extract did not significantly alter the expression of CD11b following stimulation with FMLP or PMA. Heated bacterial extract (15 μm/ml), BE-ProK (150 μg/ml), and BE-LPS (150 μg/ml) induced comparable expression of CD11b as compared with bacterial extract.

Pre-incubation of PMNs with bacterial extract (15 μg/ml), increased the expression of Gp110 by 64% ($p<0.01$) (Fig. 3). BE-ProK and BE-LPS (150 μg/ml) increased the expression of Gp110 by 62% ($p<0.01$) and 43% ($p<0.05$), respectively. No significant change in Gp110 expression was found when the cells were pre-incubated with bacterial extract (15 μg/ml), BE-ProK (150 μg/ml), or BE-LPS (150 μg/ml) and subsequently were stimulated with 10^{-7} M FMLP as compared to stimulation with FMLP alone. Pre-incubation with bacterial factors did not significantly change the Gp110 expression produced by PMA stimulation (20 μg/ml). Pre-incubation of PMNs with 15 μg/ml bacterial extract increased FMLP receptor expression by 30%. Pre-incubation of PMNs with BE-Prok (150 μg/ml) did not have a significant effect on FMLP receptor expression as compared with control. In contrast, BE-LPS (150 μg/ml) increased

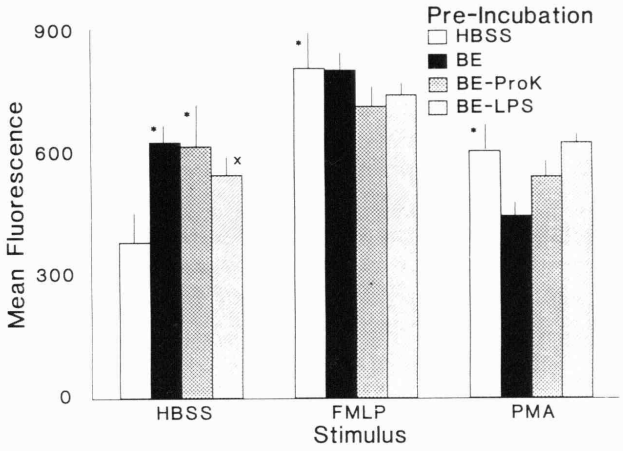


Fig. 3. Gp110 receptor expression of PMNs following incubation with bacterial extract. PMNs were pre-incubated with bacterial extract of *A. actinomycetemcomitans* (15 μg/ml), bacterial extract treated with Proteinase K (BE-ProK; 150 μg/ml), or extracted by phenol (BE-LPS; 150 μg/ml). Following pre-incubation, the cells were stimulated with FMLP or PMA. Results represent mean fluorescence intensity ± SEM of 2 determinations. Cells pre-incubated in HBSS and stimulated with FMLP or PMA had increased expression of Gp110 (* = $p<0.01$). BE, BE-ProK and BE-LPS increased the expression of Gp110 (* = $p<0.01$, x = $p<0.05$).

Table 1. CD11b expression following pre-incubation with bacterial extract or heat-treated bacterial extract and stimulation with FMLP or PMA

Stimulus follow 1st incubation	Primary – Incubation					
	HBSS	BE[a] 0.15 μg/ml	BE 1.5 μg/ml	BE 15 μg/ml	HBE 0.15 μg/ml	HBE 15 μg/ml
none (37°C 15 min)	43 ± 32[b]	147 ± 25	112 ± 5	184 ± 27+	185 ± 7+[c]	237 ± 11+
none (37°C 30 min)	160 ± 40	152 ± 32	150 ± 8	245 ± 22+	223 ± 11+	281 ± 11+
FMLP 10^{-9}	163 ± 22	168 ± 35	140 ± 15	286 ± 43++	240 ± 10+	305 ± 4+
FMLP 10^{-7}	275 ± 39*[d]	262 ± 48	232 ± 47	323 ± 47	337 ± 39+	358 ± 43+
FMLP 10^{-5}	247 ± 34*	219 ± 45	189 ± 34	288 ± 53	ND	331 ± 4+
PMA 0.2 μg/ml	164 ± 29	ND	ND	284 ± 54++	ND	331 ± 46
PMA 20 μg/ml	285 ± 39*	ND	ND	266 ± 38	296 ± 49	295 ± 49

[a] BE = bacterial extract; HBE = BE heated to 56°C for 30 min; ND = not determined.
[b] The results represent the mean fluorescence of cells following stimulation ± SEM of four experiments (except for heat-treated bacterial extract which represents the mean ± SEM of two experiments).
[c] Significantly different (+ = $p<0.05$, ++ = $p<0.01$) from samples stimulated with same agent, but pre-incubated with HBSS.
[d] Significantly different from cells pre-incubated with HBSS and without added stimulant ($p<0.05$).

Table 2. FMLP receptor expression by PMNs following pre-incubation with bacterial extract

Pre-incubation	Fluorescence[a]
HBSS	365 ± 45
BE[b]	474 ± 35*[c]
BE-ProK	325 ± 38
BE-LPS	446 ± 52*

[a] Results represent mean channel fluorescence ± SEM of 3 determinations.

[b] Pre-incubation was done with bacterial extract (BE; 15 μg/ml) of *A. actinomycetemcomitans*, BE treated by proteinase K (BE-ProK; 150 μg/ml), or extracted by phenol (BE-LPS; 150 μg/ml) for 15 min at 37°C. FMLP receptors were detected using 10^{-8} M fluorescein derivatized formyl-Nle-Leu-Phe-Nle-Tyr-Lys. Specificity of binding was shown in a 66% reduction of mean fluorescence with the addition of 10^{-5} M FMLP during staining to samples pre-incubated with HBSS and stained with 10^{-8} M fluorescein derivatized formyl-Nle-Leu-Phe-Nle-Tyr-Lys.

[c] Significantly different from HBSS ($p < 0.05$).

the FMLP receptor expression by 22% (Table 2). Competitive binding by the FMLP receptor was demonstrated by adding 10^{-5} M FMLP with 10^{-8} M FITC-F-Nle-Leu-Phe-Nle-Tyr-Lys. This resulted in a 66% reduction in receptor binding.

Effect of bacterial extract on F-actin polymerization

F-actin polymerization following various stimulations is shown in Fig. 4. FMLP, PMA, and bacterial extract increased the F-actin polymerization in a dose-dependent fashion. The optimal stimulation for inducing F-actin polymerization was 10^{-7} M of FMLP; lower and higher concentrations induced significantly less F-actin polymerization ($p < 0.0001$). PMA (10 μg/ml or 1 μg/ml) and bacterial extract (15 μg/ml) induced lower responses as compared with FMLP (10^{-7} M or 10^{-5} M). Pre-incubation with bacterial extract followed by incubation with various stimuli increased the F-actin polymerization in a dose-dependent fashion as compared with pre-incubation with HBSS, followed by the incubation with the same stimuli. For example, bacterial extract (15 μg/ml) increased the F-actin polymerization following stimulation with FMLP at concentrations of 10^{-5} M, 10^{-7} M, 10^{-9} M by 34%, 15% and 31%, respectively. Similarly, bacterial extract increased the F-actin polymerization following stimulation with PMA at concentrations of 10 μg/ml, and 1 μg/ml by 37% and 42%, respectively. The increase in F-actin polymerization induced by pre-incubation with bacterial extract was more profound when the stimulation was sub-optimal. Pre-incubation of PMNs with BE-ProK or BE-LPS (150 μg/ml or 300 μg/ml) caused similar increases in F-actin polymerization as PMNs pre-incubated with bacterial extract (15 μg/ml). No significant difference was found between the effect of different concentrations (150 μg/ml or 300 μg/ml) of BE-ProK or BE-LPS for inducing F-actin polymerization.

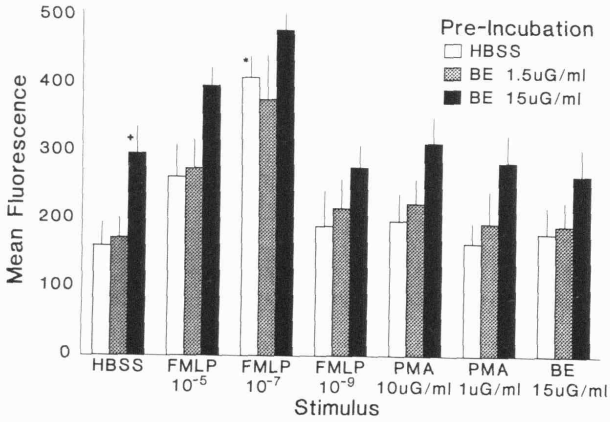


Fig. 4. F-actin polymerization of PMNs following pre-incubation with bacterial extract (BE), quantified by NBD phallacidin fluorescence. PMNs were pre-incubated with bacterial extract of *A. actinomycetemcomitans* (1.5 μg/ml or 15 μg/ml) for 15 min, and then stimulated with FMLP, PMA, or BE. Results represent the mean fluorescence intensity ± SEM of 6 determinations. Pre-incubation with BE (15 μg/ml) increased actin polymerization (+ = $p < 0.05$). Peak actin polymerization was noted with 10^{-7} M of FMLP (* = $p < 0.0001$). In all cases, BE increased F-actin polymerization in a dose-dependent manner.

Discussion

Chemotaxis of PMNs depends on a series of events which include binding of chemotactic factors to leukocyte surface receptors, expression of adhesion glycoproteins such as CD11b/CD18 (Mac-1), adherence to substrate, alteration of intracellular calcium concentration, and cytoskeletal changes including F-actin polymerization. Interference in one or more of these events can lead to decreases in PMN chemotaxis. In this study, we have evaluated the effects of an *A. actinomycetemcomitans* bacterial extract on PMN chemotaxis, and have explored the influence of the bacterial extract on four major components involved in chemotaxis: binding of chemotactic factor by FMLP receptors; expression of two major adhesion glycoproteins; CD11b and Gp110; and F-actin polymerization in response to chemotactic stimuli.

It was demonstrated that the *A. actinomycetemcomitans* bacterial extract was itself chemotactic for neutrophils. However, when the cells were pre-incubated with the extract, chemotaxis toward ZAS, FMLP and the bacterial extract was inhibited in two different chemotaxis assays (Boyden chamber and under agarose chemotaxis). This inhibition by the bacterial factor could result from an inability to sense a chemotactic gradient, or from a more general interference with the events leading

to chemotaxis. The latter include aggregation, hyper-adhesion, and down regulation or inhibition of intracellular events such as F-actin polymerization/depolymerization.

One of the first obligatory events for directed locomotion is detection of a chemotatic agent such as formyl peptides released from bacteria. FMLP is a peptide that has been used as a chemotactic agent in numerous investigations of *in vitro* neutrophil function. Van Dyke originally showed that FMLP receptors were decreased in a number of patients with localized juvenile periodontitis (17). These observations can be extended to suggest that the reduced receptor expression is partly responsible for the decreased chemotaxis observed in these patients. Accordingly, we questioned if the reduced chemotaxis that occurs in PMNs exposed to bacterial extract was the result of decreased FMLP receptor expression. Another method for inhibition of chemotaxis by the bacterial extract could have been blockade or alteration of the receptors for the chemotactic agent. Our observation of *increased* FMLP binding following exposure to bacterial extract argues against the hypotheses that disruption of this critical first step is the cause of inhibited chemotaxis. In addition, the fact that FMLP stimulated CD11b expression and F-actin polymerization were not affected by pre-incubation with bacterial extract further argues that these receptors were functionally intact.

Shortly after binding of chemotactic factors by the chemotactic receptor, an increased expression of surface adhesion glycoproteins (CD11b/CD18) occurs, leading to increased adhesion of the neutrophil to the substratum and enabling the PMN to move toward the chemotactic source. The biological importance of this phenomenon has been demonstrated in patients with impaired PMN adhesion, e.g., CD11b/CD18 (Mac-1) deficiency. As a result of this deficiency, they suffer from generalized prepubertal periodontitis, a severe periodontal disease with oral ulcerations (21). Since the bacterial extract of *A. actinomycetemcomitans* was able to induce increased CD11b/CD18 expression it is apparent that the chemotactic alteration did not arise from a reduction of this adhesion glycoprotein.

Van Dyke *et al.* (19) reported that impairment of neutrophil chemotaxis was associated with a decrease in cell surface protein (Gp110) in patients with LJP (19). Since, in our study, the bacterial extract increased the expression of Gp110 in non-stimulated cells, but had no effect on expression of Gp110 in cells stimulated with FMLP, the observed decreased chemotaxis would not seem to be attributable to alterations in Gp110.

Yet another critical component of cell locomotion that could be blocked by the bacterial factor is the conversion of monomeric actin to cytoskeletal associated F-actin. This possibility can be ruled out due to the fact that the chemoattractant FMLP stimulated F-actin polymerization by PMNs pre-incubated with bacterial factor. This suggests that the cytoskeletal components necessary for F-actin assembly are not affected by the bacterial factor.

We have not identified the specific inhibitory factor(s) involved. The bacterial extract is a crude, complex mixture of bacterial components. However, the fact that bacterial extract heated to 56°C retained inhibitory activity, whereas bacterial extract treated with proteinase K or extracted with phenol was depleted of inhibitory activity strongly suggests that the active component in the bacterial extract is a protein(s) not sensitive to 56°C, and is not a phenol extractable endotoxin. Interestingly, the components which increased expression of FMLP, CD11b and Gp110 receptors, are endotoxin-like materials. Further biochemical characterization of the extract is needed to identify the active components.

In a companion paper, we have further investigated the ability of the bacterial extract to alter PMN function by evaluating H_2O_2 production (34). One of the most profound stimuli for H_2O_2 production is phorbol myristate acetate (PMA), an irreversible activator of protein kinase C. We found that the bacterial extract inhibited by 50% the ability of PMA to stimulate H_2O_2 production, thereby suggesting that the mechanism of action of the bacterial extract is through blockade of protein kinase C function. Of significance is the fact that characterized protein kinase C inhibitors block *chemotaxis* as well as development of H_2O_2 production (35).

Van Dyke *et al.* (24) found that *A. actinomycetemcomitans* can elaborate a chemotaxis-inhibiting factor into the culture supernatant, but they failed to demonstrate inhibitory activity in the bacterial extract produced by sonication. This discrepancy with our results may be related to differences in strains of *A. actinomycetemcomitans* used in their study, or possibly to differences in bacterial extract preparation; Van Dyke *et al.* lyophilized the bacterial extract after sonication, whereas we ultracentrifuged the sonicated bacteria to remove particulates and collected the supernatant as the bacterial extract. In addition, Van Dyke *et al.* determined the concentration of their extract according to dry weight, while we determined the bacterial extract concentration according to protein content (which was about 10% of dry weight). Our data showing a dose dependency for the bacterial extract activity demonstrates that no effect is seen

at reduced concentrations. This may explain the discrepancy between our results and those of Van Dyke *et al.*

The fact that the bacterial extract of *A. actinomycetemcomitans* significantly inhibits PMN chemotaxis *in vitro* suggests that the systemic PMN defect reported to occur in LJP patients may be induced by the infecting bacteria; i.e., the bacteria may produce factor(s) that modulate PMN function. This modulation may be expressed as a structural and/or functional variation of surface receptors, and may be manifest as a "defect" in PMN chemotaxis correlated with the amount of "factor" produced by the organisms. Intact functional activity of surface adherence glycoproteins is suggested by Genco *et al.* (20) who found that adherence of neutrophils from LJP patients to nylon wool was significantly elevated. Our finding that the bacterial extract increased CD11b expression would seem to be compatible with Genco's study. Adherence characteristics of PMNs to various substrates following exposure to the bacterial extract is unknown, but presumably correlate with CD11b expression (21). Controversy exists regarding PMN surface receptors. Van Dyke *et al.* (19) found that Gp110 was reduced in LJP patient's PMNs who had defective chemotaxis, while Larjava *et al.* (36) found that PMNs from patients with LJP expressed normal surface glycoproteins using surface labeling and gel electrophoresis. It is unknown if this difference is explained by a correlation with impaired chemotaxis; normal levels of Gp110 were found in LJP patients with normal chemotaxis towards FMLP, while all of Larjava's patients had normal chemotaxis towards casein.

The locomotory defect observed in peripheral blood neutrophils from LJP patients cannot be explained totally on the basis of bacteria associated with the periodontal lesion. In longitudinal studies, depressed chemotaxis was not reversed by therapy which successfully alleviated clinical signs of periodontitis and significantly reduced the levels of *A. actinomycetemcomitans* (16, 37, 38). This may indicate that a "minor" PMN defect exists that is manifest as disease when a "threshold level" of bacteria is reached. This may be applicable to understanding the function of PMNs in the local environment of the periodontal lesion. In the absence of *A. actinomycetemcomitans* or in the presence of very low levels, there may be no apparent damage to the periodontium. On the other hand, at a higher concentration of *A. actinomycetemcomitans*, the bacteria and/or its factor(s) may overcome the protective ability of the LJP PMN. Thus, a high concentration of *A. actinomycetemcomitans* in a patient with "intrinsic" PMN functional defects, as found in gingival lesions of patients with LJP, may lead to local pathology. Evaluation of the dose-responses of bacterial extract comparing PMNs from controls with PMNs from patients with LJP may elucidate the role of bacterial modulators in the disease process.

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