Neutrophil modulation by *Actinobacillus actinomycetemcomitans* II. Phagocytosis and development of respiratory burst

Malka Ashkenazi[1], Robert R. White[2] and David K. Dennison[1]

Departments of [1]Periodontics and [2]Microbiology, Dental Branch, University of Texas Health Science Center, Houston, TX., U.S.A.

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Compromised neutrophil function has been found in a number of patients with localized juvenile periodontitis (LJP), although the pathogenic mechanism is unknown. Since infection with *Actinobacillus actinomycetemcomitans* is frequently found in patients with LJP, we have evaluated *in vitro* the effect of a bacterial extract of *A. actinomycetemcomitans* on the development of the respiratory burst by neutrophils. Pre-incubation of neutrophils with bacterial extract increased H_2O_2 induced by FMLP and zymosan in a dose-dependent fashion. Substitution of FMLP for bacterial extract produced similar results. Moreover, FMLP and bacterial extract had an additive effect on superoxide production following phagocytosis of zymosan. In contrast, bacterial extract significantly *decreased* PMA-stimulated H_2O_2, but pre-incubation with FMLP instead of bacterial extract failed to decrease PMA-stimulated H_2O_2. Bacterial extract did not change the percentage of cells activated by FMLP, opsonized zymosan, or PMA. Heat-treated bacterial extract induced effects similar to non-treated extract. Bacterial extract treated with proteinase K or phenol extraction increased FMLP or zymosan stimulated H_2O_2 equivalent to non-treated bacterial extract. In contrast, proteinase K or phenol extraction abolished the inhibitory effect of bacterial extract on PMA-stimulated H_2O_2 production. The bacterial extract component(s) that inhibits PMA-stimulated H_2O_2 is therefore a protein(s), resistant to 56°C, and is not endotoxin. The partially activated state of PMNs exposed to *A. actinomycetemcomitans* extract, combined with their reduced ability to respond to a protein kinase C-dependent stimulus, may partially explain the abnormalities noted in LJP patients.

Key words: neutrophils – *Actinobacillus actinomycetemcomitans* – juvenile periodontitis – respiratory burst

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Introduction

Localized juvenile periodontitis (LJP) is an early onset periodontitis, usually localized to molars and incisors (1–3). It is characterized by destruction of the alveolar bone of the permanent first molar and incisor teeth. The etiological mechanisms involved in this entity have not been completely elucidated. There is a large body of evidence to suggest that infections with *Actinobacillus actinomycetemcomitans* play a major role in the pathogenesis of the disease (2–5). First, an increased prevalence of the organism in patients with LJP, and their families, has been well documented (2–5). Secondly, these patients exhibit elevated antibody levels to *A. actinomycetemcomitans* in their serum, gingival fluid and saliva (6–8). Thirdly, elimination of *A. actinomycetemcomitans* from periodontal pockets improved the periodontal health (9). Fourthly, histopathologic investigations have shown that *A. actinomycetemcomitans* invades the gingival connective tissue in lesions of localized juvenile periodontitis (4). Moreover, *A. actinomycetemcomitans* possesses numerous virulence factors such as leukotoxin, collagenase, endotoxin, epitheliotoxin and a fibroblast-inhibiting factor all of which may be relevant to periodontal disease (10–13). Previous

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studies have suggested that patients with LJP present polymorphonuclear leukocyte (PMN) function defects, which may play a role in the pathogenesis of the disease. These include depressed *in vitro* chemotaxis of blood neutrophils (14–17), and reduced numbers of binding sites for the chemotactic peptide FMLP (formyl-methionyl-leucyl-phenylalanine), C5a (a complement component; 15, 18-20), and glycoprotein 110 (a neutrophil membrane matrix component; 16, 17, 21). In contrast, others have found either normal or enhanced chemotaxis (22–24), as well as normal surface glycoprotein structure using surface glycoprotein labeling and polyacrylamide gel electrophoresis (25). The reports on the phagocytic and bactericidal capacity of PMNs in patients with LJP are also conflicting. Several investigators have found that phagocytic activity of blood and gingival neutrophils appears to be abnormal in LJP patients (14, 26–29), while others have found normal phagocytic function (30-35). An *in vitro* study by Van Dyke *et al.* (36) showed that culture supernatants and bacterial extracts of *A. actinomycetemcomitans* had no effect on neutrophil phagocytosis. Depressed bactericidal activity of PMNs in patients with LJP have been noted (26, 31), although others have found a normal oxidative capacity in patients with LJP (29, 34, 35, 37). Moreover, Asmen in 1988 (38) and Shapira in 1989 (32) found that PMNs from JP patients have the potential to produce higher quantities of oxyradicals than healthy controls following stimulation with opsonized bacteria.

Since intact phagocytosis and bactericidal capacity is essential for periodontal health, and studies have shown that bacteria can inhibit specific neutrophil function such as phagocytosis (39) and chemotaxis (40), we decided to evaluate *in vitro* the effects of bacterial extract of *A. actinomycetemcomitans* on the phagocytic and bactericidal functions of neutrophils from healthy adults. Specifically, we evaluated the effect of bacterial extract on normal human PMN H_2O_2 production resulting from stimulation with FMLP or PMA, or resulting from zymosan phagocytosis.

Material and Methods

Preparation of bacterial extract

Bacterial extract of *A. actinomycetemcomitans* strain 29522 was prepared by sonication and ultracentrifugation as previously described (40). To evaluate the effect of heat treatment, bacterial extract was heated to 56°C for 30 min. To further characterize the active component(s), the bacterial extract was treated with proteinase K to remove protein components (BE-ProK), or treated with phenol to extract the lipopolysaccharide component (BE-LPS) as described (40). BE-ProK and BE-LPS were used at a final concentration of 150 μg dry weight ml^{-1}.

Isolation of human blood PMNs

Blood PMNs were isolated from healthy donors by dextran sedimentation, as previously described (41), and suspended in ice-cold Hanks' balanced salt solution (HBSS; GIBCO Laboratories, Grand Island, NY) at a final concentration of 3.3×10^6 cell/ml. Cells were stored on ice until use.

Oxidative burst development (H_2O_2 production)

The oxidative burst development was evaluated by stimulating the cells and measuring the amount of H_2O_2 produced. The oxidative respiratory burst is one of the principal mechanisms of PMNs to produce agents capable of killing ingested organisms. We chose H_2O_2 production as a meaure of oxidative burst development because H_2O_2 [formed by the dismutation of two molecules of superoxide (O_2^-)] is the main precursor of the oxidizing radical (OH^-), oxidizing halogen (HOCl), and chloramines (RNHCl). To test the ability of PMNs to respond following exposure to bacterial products, we evaluated the effect of bacterial extract on the production of H_2O_2 by pre-incubating the cells with bacterial extract, and then stimulating them with N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP; Sigma, St Louis, MO) or phorbol 12-myristate 13-acetate (PMA; Sigma). FMLP is a physiologic stimulant of the respiratory burst, while PMA is an irreversible activator of protein kinase C which leads to activation of the respiratory burst. Additionally, the effect of incubation with bacterial extract alone was evaluated. H_2O_2 production was determined using the fluorescent probe dichlorofluorescein, as described by Bass *et al.* (42). Briefly, a PMN suspension (15 ml, 3.3×10^6 cells/ml) was "loaded" with 2′7′ dichlorofluorescein diacetate (DCF-DA, Kodak Chemicals, Rochester, NY) at a concentration of 90 μg DCF-DA/ml (15 min, 37°C). [Loading is accomplished when the non-polar DCF-DA enters the cell, and the diacetate group is cleaved by intracellular esterases, trapping the non-fluorescent polar dichlorofluorescein within the cell.] The cells were then pre-incubated with bacterial extract (final concentration of 0.15 μg/ml, 1.5 μg/ml or 15 μg/ml), BE-ProK, or BE-LPS for 15 min at 37°C in a shaking water bath. Following pre-incubation, the cells were stimulated (15 min, 37°C) with PMA (2 μg/ml or 20 μg/ml) or FMLP (final concentration: 10^{-9} M, 10^{-7} M or 10^{-5} M) in a shaking water bath. Controls loaded with DCF were incubated with HBSS

at 37°C or on ice. Additional controls were pre-incubated with HBSS or with FMLP (10^{-7} M) instead of bacterial extract, to determine whether FMLP could mimic the effect induced by bacterial extract. Both reactions were stopped by placing the cells on ice. Intracellular producton of H_2O_2 which caused hydrolysis of DCF to fluorescent dichlorofluorescein, was measured by flow cytometric analysis within 2 hours. To evaluate the active component(s) of the bacterial extract in altering the H_2O_2 production, heated bacterial extract (15 μg protein/ml), BE-ProK, or BE-LPS were also used.

In order to further characterize the response caused by bacterial extract we performed a competitive inhibition assay using bacterial extract and PMA. PMN suspensions pre-incubated with bacterial extract were stimulated with increasing concentrations of PMA (final concentrations: 0.02 μg/ml, 0.2 μg/ml, 2 μg/ml, 10 μg/ml, 15 μg/ml, 20 μg/ml, 30 μg/ml, 40 μg/ml or 80 μg/ml) without removal of bacterial extract. Control cells were washed twice following the pre-incubation with bacterial extract, and then were stimulated with increasing concentrations of PMA as described above (15 min 37°C).

Phagocytosis

Phagocytosis of zymosan was evaluated indirectly by measuring the respiratory burst development following phagocytosis (43) using the fluorescent probe dichlorofluorescein as was described by Bass (42). The effect of the bacterial extract on phagocytosis was evaluated by pre-incubating the cells with bacterial extract, and then stimulating them with zymosan. Briefly, PMN suspensions loaded with DCF-DA as described above were pre-incubated with bacterial extract (final concentration: 0.15 μg/ml, 1.5 μg/ml or 15 μg/ml), heated bacterial extract (final concentration: 0.15 μg/ml or 15 μg/ml), or with both bacterial extract (15 μg/ml) and FMLP (10^{-7} M) for 15 min at 37°C in a shaking water bath. Parallel controls loaded with DCF were pre-incubated with HBSS at 37°C or on ice. Following pre-incubation, the cells were stimulated with different concentrations of zymosan (final concentration: 20 mg/ml, 2 mg/ml or 1 mg/ml) opsonized by different concentrations of serum (100% serum, 10% serum, or 0% serum, as described below). To evaluate the active component(s) of the bacterial extract in altering the phagocytosis of zymosan, PMNs were pre-incubated with BE-ProK or BE-LPS and stimulated by zymosan (20 mg/ml) opsonized by 100% serum. Additional controls were preincubated with FMLP (final concentration: 10^{-5} M, 10^{-7} M, or 10^{-9}M) instead of bacterial extract to determine whether FMLP could mimic the effect induced by bacterial extract. All reactions were stopped by placing the cells on ice. Intracellular production of H_2O_2, which caused hydrolysis of DCF to the fluorescent analog dichlorofluorescein, was measured by flow cytometric analysis within 2 h. In addition, the percentage of cells producing H_2O_2 following phatocytosis of zymosan was evaluated by flow cytometric analysis. This was done to determine if the bacterial extract had any effect on the percentage of the cells producing H_2O_2.

Opsonization of the zymosan

Ten milligrams of zymosan A (Sigma) were washed with HBSS, and resuspended in 1 ml of 100% fresh serum (from a clot tube collected from the PMN donor), 10% fresh serum (diluted with HBSS) or in 0% serum (HBSS). The suspension was then incubated for 45 min at 37°C, in a shaking water bath. Following incubation, the suspension was centrifuged (2000 × *g*; 2 min), the pellet was washed twice, and resuspended in HBSS and used at a final concentration of 1 mg/ml, 2 mg/ml or 20 mg/ml.

PMN analyses of phagocytosis and oxidative burst development by flow cytometry

Samples of PMNs were measured using an Ortho Cytofluorograf System 50-H with a 2150 computer (Ortho Diagnostics, flow cytometer. Westwood, MA) (40). Cells were illuminated by a 5 watt argon laser emitting 400 milliwatts of light at 488 nm. PMNs analyzed for fluorescence were selected using forward and right-angle light scatter signals as previously described (41). Green fluorescence histograms were collected for 10 000 PMNs for each sample analyzed.

Statistical analysis

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

Results

H_2O_2 production

I. The effect of bacterial extract on H_2O_2 production induced by FMLP. The effect of bacterial extract on FMLP-stimulated H_2O_2 production is shown in Fig. 1. Bacterial extract alone was found to increase H_2O_2 production in a dose-related fashion, with 15 μg/ml increasing production by 43% compared to pre-incubation with HBSS. A similar dose-depend-

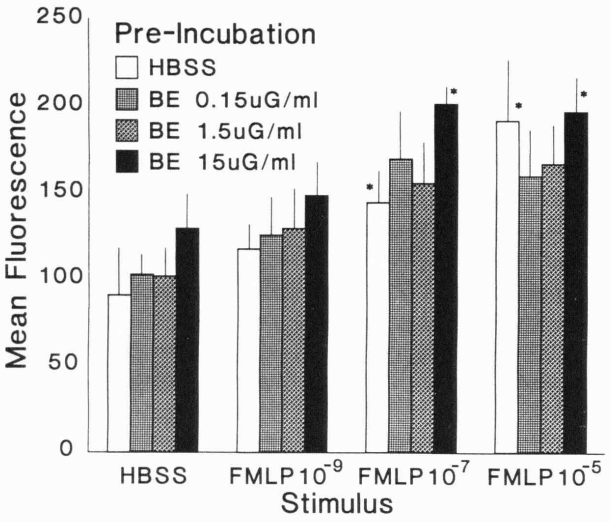


Fig. 1. Effect of pre-incubation with bacterial extract (BE) on H_2O_2 production by PMNs. PMNs were pre-incubated with BE or HBSS (4 experiments), and then stimulated with FMLP. Results represent the mean fluorescence ± SEM of 4 experiments, except for BE (1.5 μg/ml) which represents 2 experiments. Bacterial extract had little effect on the H_2O_2 production by PMNs stimulated with FMLP. Significantly more H_2O_2 was produced by cells treated with BE (15 μg/ml) and stimulated with 10^{-7} M FMLP, or by cells stimulated with 10^{-5} M FMLP with or without pre-incubation with BE (15 μg/ml) than cells pre-incubated with HBSS and not stimulated (* = $p < 0.01$).

ent effect was noted when the cells pre-incubated with bacterial extract were stimulated using FMLP, with a significant increase of 26% ($p = 0.0002$) and 39% ($p = 0.004$) for 10^{-5} M and 10^{-7} M FMLP, but no change with 10^{-9} M FMLP ($p > 0.7$). The maximal effect of bacterial extract was obtained when the cells were pre-incubated with bacterial extract (15 μg/ml) and activated with 10^{-7} M FMLP. Heated bacterial extract induced similar results to those of unheated bacterial extract (Fig. 2), suggesting that the stimulant component(s) in the bacterial extract is heat-stable. Pre-incubation of the cells with FMLP followed by second stimulation with various concentrations of FMLP induced elevated levels of H_2O_2 production as compared with single stimulation by FMLP. The elevated levels were comparable with the levels of H_2O_2 induced by stimulating the cells with bacterial extract and FMLP. Thus FMLP can mimic this effect of bacterial extract (Fig. 2).

II. The effect of bacterial extract on H_2O_2 production induced by PMA. The effect of bacterial extract on H_2O_2 production induced by PMA is shown in Fig. 3. PMA significantly increased the production of H_2O_2 as compared to controls incubated in HBSS ($p < 0.001$). The optimal concentration of PMA was 20 μg/ml; this concentration induced a 17.5-fold increase in H_2O_2 production. Pre-incubation of the cells with bacterial extract (final concentration: 0.15 μg/ml or 15 μg/ml) induced 25–50% inhibition in H_2O_2 production induced by 20 μg/ml PMA ($p < 0.041$ and $p < 0.006$, respectively), although the effect with lower concentrations of PMA was not significant. Heated bacterial extract (15 μg/ml or 0.15 μg/ml) inhibited H_2O_2 production to the same extent as bacterial extract. In contrast, treatment of the bacterial extract with proteinase K or phenol extraction reversed the inhibitory activity; *i.e.* cells pre-incubated with BE-ProK or BE-LPS followed by stimulation with PMA (20 μg/ml) responded with H_2O_2 production comparable to cells stimulated with PMA alone. Pre-incubation of PMNs with FMLP (10^{-5} M, 10^{-7} M, 10^{-9} M) did not inhibit PMA stimulated H_2O_2 production, indicating that simple "priming" of the cells was not responsible for the effect of bacterial extract on the PMA pathway.

To determine if bacterial extract could competitively inhibit PMA-stimulated H_2O_2 production, cells were pre-incubated with 15 μg/ml bacterial extract followed by stimulation with increasing concentrations of PMA. Increasing the concentration of PMA by 530% (from 15 to 80 μg/ml), did not significantly change the magnitude of the inhibition (50% and 38%, respectively, Fig. 3); that is, bacterial extract inhibited H_2O_2 production by 38%, even when the cells were stimulated by excess concentration of PMA (80 μg/ml). To further characterize this inhibition of PMA-stimulated H_2O_2

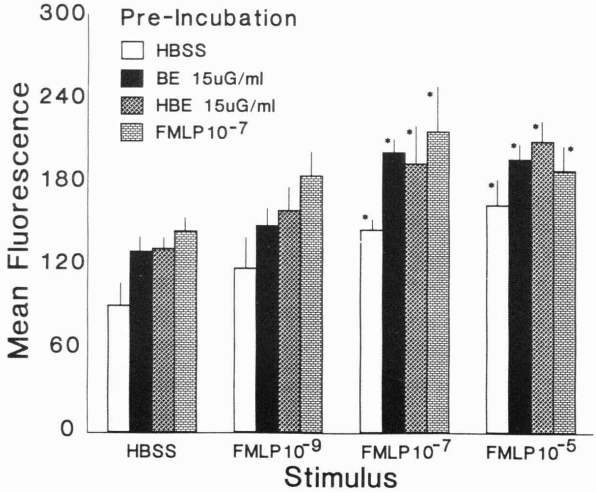


Fig. 2. FMLP-stimulated H_2O_2 production by PMNs pre-incubated with bacterial extract (BE) or FMLP. PMNs were pre-incubated with BE (4 experiments), heated bacterial extract (HBE, 2 experiments), or FMLP (10^{-7} M, 2 experiments) for 15 min, and then stimulated with FMLP. Controls were pre-incubated with HBSS (4 experiments). Results represent the mean fluorescence ± SEM of 2–4 experiments. No significant difference was found between bacterial extract and heated bacterial extract. Pre-incubation with FMLP mimicked the effect of bacterial extract in increasing the FMLP-stimulated H_2O_2 production (* = $p < 0.01$).

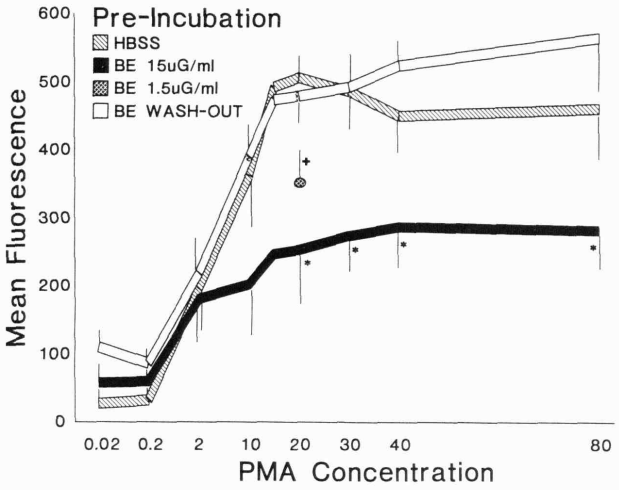


Fig. 3. Effect of pre-incubation with bacterial extract (BE) on PMA-stimulated H_2O_2 production by PMNs. PMNs were pre-incubated with BE (15 μg/ml) or HBSS and stimulated with PMA. To determine the need for continued exposure to BE, cells pre-incubated with BE were washed twice and then stimulated with PMA. The results represent the mean fluorescence ± SEM of 3 experiments. Bacterial extract at 15 μg/ml or 1.5 μg/ml significantly inhibited the H_2O_2 production following PMA stimulation (* = $p < 0.006$ and + = $p < 0.04$). The effect was not reversed competitively by increased levels of PMA, and required continued exposure during stimulation.

production, cells pre-incubated with bacterial extract (15 μg/ml) were washed twice, and then were stimulated with increasing excess concentrations of PMA. In this assay, H_2O_2 production in PMNs that were pre-incubated with bacterial extract and then washed was comparable to that of PMNs pre-incubated in HBSS (Fig. 3; $p > 0.05$).

Pre-incubation of the cells with bacterial extract, followed by stimulation with PMA or FMLP, did not significantly change the percentage of cells producing H_2O_2 following stimulation (89–100%; $p > 0.05$).

Phagocytic activity

The effect of bacterial extract on phagocytosis of zymosan is shown in Table 1. Pre-incubation of cells with bacterial extract increased H_2O_2 production following phagocytosis of zymosan in a dose-dependent fashion (Table 1). For example, bacterial extract at a concentration of 0.15 μg/ml, 1.5 μg/ml or 15 μg/ml increased H_2O_2 production following phagocytosis of zymosan (2 mg/ml opsonized with 100% serum) by 38%, 151% and 170%, respectively ($p > 0.05$, $p > 0.05$, $p < 0.02$). The effect of bacterial extract was more prominet when zymosan concentrations were sub-optimal (2 μg/ml, and 1 μg/ml). Bacterial extract (15 μg/ml) increased phagocytosis of 1 mg/ml, 2 mg/ml and 20 mg/ml of zymosan by 142%, 180%, and 152%, respectively. Similar trends were obtained with zymosan opsonized with serum concentrations of 10% and 100% and with unopsonized zymosan.

Pre-incubation of PMNs with heated bacterial extract increased the H_2O_2 production following phagocytosis of zymosan in a dose-dependent manner similar to non-heated bacterial extract (Fig. 4). Phagocytosis induced by heated bacterial extract was not statistically different from non-treated bacterial extract. Pre-incubation of PMNs with BE-ProK or BE-LPS induced similar H_2O_2 production following phagocytosis of zymosan as bacterial extract.

Pre-incubation of the cells with FMLP (final concentration: 10^{-5} M, 10^{-7} M, or 10^{-9} M), followed by stimulation with zymosan, increased phagocytosis in a dose-dependent manner (Table 1). FMLP increased phagocytosis of zymosan under various opsonization conditions and concentrations by an average of 34%, 116% and 138%, for FMLP at 10^{-9} M, 10^{-7} M or 10^{-5} M, respectively. Thus FMLP could mimic the increased phagocytosis obtained with pre-incubation with bacterial extract.

Pre-incubating the cells with bacterial extract and FMLP had an additive effect on the phagocytosis compared to pre-incubating the cells with FMLP or bacterial extract alone. The extent of the additive effect was higher when the concentration of the zymosan was sub-optimal (2 mg/ml and 1 mg/ml). Similar results were obtained with zymosan opsonized with a serum concentration of 100%, 10% or with unopsonized zymosan (Table 1).

The effect of bacterial extract, FMLP, zymosan concentration and different opsonization conditions on the percentage of activated cells

Pre-incubating the cells with FMLP (10^{-9} M, 10^{-7} M or 10^{-5} M), bacterial extract (15 μg/ml, 1.5 μg/ml or 0.15 μg/ml) or heated bacterial extract (15 μg/ml or 0.15 μg/ml) did not change the percentage of cells producing H_2O_2 following phagocytosis of zymosan (87%). Likewise the opsonization conditions (0%, 10%, or 100% serum) had no effect on the number of phagocytically active cells. In contrast, the zymosan concentration significantly changed the percentage of cells producing H_2O_2 following phagocytosis; at a concentration of 20 mg/ml zymosan, 62% of the population produced H_2O_2, while at lower concentrations of zymosan (2 and 1 mg/ml) 86% of the population produced H_2O_2 ($p < 0.0001$). Thus the only stimulus that significantly changed the percentage of cells producing H_2O_2 was the concentration of zymosan.

Table 1. H_2O_2 production induced by zymosan following pre-incubation with bacterial extract or FMLP

Zymosan Opsonization Conditions	Pre-incubation						
	HBSS	BE[a] 15 μg/ml	HBE 15 μg/ml	FMLP 10^{-5} M	FMLP 10^{-7} M	FMLP 10^{-9} M	BE 15 μg/ml +FMLP 10^{-7} M
No zymosan	52±11[b]	89±16	60±8	111±20*[c]	124±15*	62±10	171±8*
20 mg/ml 100% serum	308±17	389+33*	385±10*	519±26*	435±54*	377±22	457±51*
2 mg/ml 100% serum	146±16	396±50*	325±23*	393±47*	398±3*	215±29	517±12*
1 mg/ml 100% serum	108±15	228±56*	223±6*	289±36*	290±13*	177±25	461±14*
20 mg/ml 10% serum	208±23	415±44*	349±21*	466±24*	423±57*	236±17	465±54*
2 mg/ml 10% serum	119±22	317±40*	227±25*	304±41*	262±16*	164±32	402±15*
1 mg/ml 10% serum	96±17	257±34*	186±11*	227±23*	271±29*	137±29	352±4*
20 mg/ml 0% serum	106±10	256±17*	250±14*	289±8*	223±6*	127±10	264±5*
2 mg/ml 0% serum	82±15	230±24*	136±34*	270±24*	205±6*	122±18	339±5*
1 mg/ml 0% serum	80±15	202±23*	157±10*	233±27*	206±4*	121±25	357±71*

[a] BE = bacterial extract; HBE = heated bacterial extract. [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 of two experiments). [c] Different ($p < 0.05$) from cells pre-incubated with HBSS and exposed to the same stimulant.

Discussion

Infections with *A. actinomycetemcomitans* are seen frequently in patients with LJP. However, the relationship of this organism to the periodontal disease is unclear. Several studies have suggested that decreases in chemotaxis (15, 18–20, 34), phagocytosis (14, 26–28) and bactericidal activity (26, 31) by PMNs from patients with LJP play a major role in the pathogenesis of the disease. Although findings concerning the decrease in chemotaxis activity seen in patients with LJP are consistent, controversy exists concerning the ability of PMNs from LJP to function in phagocytotic processes and to produce superoxide. In this study we have evaluated the effect of a bacterial extract of *A. actinomycetemcomitans* on neutrophil phagocytosis and development of the respiratory burst following three different stimulants: FMLP, PMA and opsonized zymosan. It was demonstrated that pre-incubation of PMNs with bacterial extract increased H_2O_2 production following stimulation with FMLP and opsonized zymosan. Pre-incubation of the cells with FMLP mimicked this effect of bacterial extract. Pre-incubation of the cells with FMLP and bacterial extract had an additive effect in increasing the superoxide production following phagocytosis of zymosan. In contrast, pre-incubation of PMNs with bacterial extract inhibited PMA-induced H_2O_2 production. This inhibition was reversible and was a unique effect of the bacterial extract since FMLP could not mimic the inhibitory effect of bacterial extract on PMA stimulation. Thus, the inhibition obtained with bacterial extract seems to be related to a distinct effect of the bacterial extract component(s) on the pathway of activation stimulated by PMA.

H_2O_2 production involves activation of an electron transport complex, the "NADPH oxidase" that is mediated by several distinct pathways; we have investigated three of them. Opsonized zymosan interacts with Fc and C3b receptors, with receptor activation involving phospholipase A_2 (44–45). FMLP binds to specific membrane receptors and activates NADPH oxidase through phosphoinositide breakdown (45–46). PMA binds to protein kinase C, activating the kinase which functions directly in phosphorylation and activation of cellular components including NADPH oxidase (47). The PMA pathway of activation appears uniquely separate from the other pathways of activation since treatment of PMNs with the protein kinase C inhibitor 1-(5-isoquinolinesulfonyl)-piperizine, results in inhibition of PMA-stimulated, but not FMLP-stimulated superoxide production (48-51). Moreover, NADPH oxidase can be activated independently of protein kinase C by arachidonic acid or SDS (52–53). Our results suggest that bacterial extract of *A. actinomycetemcomitans* has different effects on these two pathways, i.e., it "primes" the cells for events occurring through Fc, C3b and FMLP receptors, but inhibits the PMA-stimulated pathway involving activation of protein kinase C.

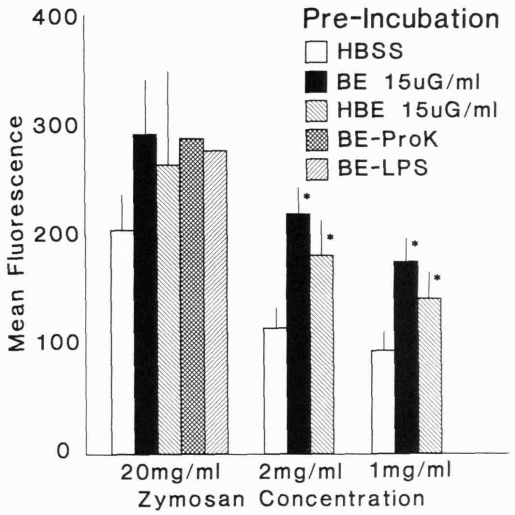


Fig. 4. Effect of pre-incubation with bacterial extract (BE) on H_2O_2 production by PMNs during zymosan phagocytosis. Cells were pre-incubated with bacterial extract (BE; 15 μg/ml), heated BE (HBE, 15 μg/ml), proteinase K treated BE (BE-ProK), or phenol extracted BE (BE-LPS) for 15 min, and then stimulated with zymosan. Results represent the mean fluorescence $\pm$ SEM of 4 experiments except BE-ProK and BE-LPS which each represent 2 experiments. Bacterial extract, regardless of treatment, significantly increased the H_2O_2 production following phagocytosis of zymosan only at reduced concentrations of zymosan (2 mg/ml and 1 mg/ml, * = $p < 0.05$).

Priming of neutrophils for stimulation of the respiratory burst by FMLP has been shown to be independent of the activation of protein kinase C (50–51, 54), which is consistent with our findings. The fact that protein kinase C inhibitors significantly reduce chemotaxis further supports our hypothesis for bacterial extract having a protein kinase C inhibitory activity since we have previously shown that bacterial extract inhibits chemotaxis (40).

The finding that PMNs pre-incubated with bacterial extract produced increased H_2O_2 following stimulation with FMLP and opsonized zymosan, but (as we have previously reported (40)), decreased chemotaxis toward FMLP, zymosan and toward bacterial extract itself, illustrates the differential effect of the bacterial extract on diverse PMN activities. Separation of oxidative burst development and chemotaxis toward a broad family of bacterial products has been established by several pharmacologic studies showing that two broad biologic responses (migration and superoxide production) are regulated differently. For example, it has been shown that certain anti-filament agents, such as the cytochalasins, inhibit chemotaxis but greatly enhance superoxide production and lysosyme secretion in response to chemoattractant (55). The antibiotic amphotericin B similarly inhibits chemotaxis but not other PMNs functions (56). Conversely, some aliphatic alcohols (e.g. butanol and pentanol) prevent stimulation of superoxide production but increase the rate of migration of human PMNs to FMLP (57).

Our findings that bacterial extract increased H_2O_2 production following stimulation with FMLP and zymosan, are compatible with several clinical findings that the superoxide production of PMNs from LJP patients is normal or elevated when the stimulant is opsonized bacteria or zymosan (32, 34, 35, 38). These results are consistent with "priming" by the bacterial extract through a protein kinase C-independent pathway. The observation that pre-incubation with heated bacterial extract resulted in assay results that were equivalent or higher than non-treated bacterial extract is also consistent with a "priming" effect. The results can be due to heat aggregation and/or partial proteolysis with an increase in the concentration of bacterial peptides. Increased up-regulation of cell surface receptors by the heated bacterial extract as reported in the previous paper (40) would favor an increase in phagocytic activity by the treated cells.

Microorganisms elaborate a variety of products which may affect neutrophil function, including proteolytic enzymes, endotoxin and exotoxin. In this study, the active component(s) that affected H_2O_2 production following FMLP, PMA or zymosan phagocytosis may be partially related to endotoxin-like materials. In contrast, the component(s) which decrease H_2O_2 production following PMA stimulation is a protein(s) resistant to 56°C, and is not endotoxin. The argument that the inhibitory component is not endotoxin is supported by studies showing that endotoxins have been found to stimulate platelet protein kinase C (58), and that blood PMNs from patients with acute bacteremias had elevated PMA-stimulated oxidative product formation in comparison to healthy adults (59). These studies suggest that endotoxin present in the bacterial factor would have produced *elevated* PMA-stimulated H_2O_2 production. An exotoxin produced by *A. actinomycetemcomitans* – leukocidin – kills neutrophils and inhibits neutrophil chemotaxis (36). Since leukocidin has been shown to be heat-labile, it appears not to play an important role in modulation of PMN function by the bacterial extract.

Collectively, our data support the hypothesis that *A. actinomycetemcomitans* produces a protein, not sensitive to 56°C, with a molecular weight greater than 10 kDa (non-dialyzable), which inhibits two PKC-dependent events in PMNs; chemotaxis and PMA-stimulated H_2O_2 production. Proteins have been identified which inhibit PKC activity. Toker *et al.* (60) have identified protein kinase C inhibitor proteins in sheep brains. These inhibitors are 29-33 kDa proteins. Activity is abol-

ished by heating at 100°C for 5 min, but is totally unaffected by heating at 70°C for 5 min. Half-maximal inhibition of PKC is obtained at 1.7 μM of inhibitor. Balazovich *et al.* (61) have identified a heat-sensitive (100°C) protein associated with neutrophil-specific granules which functions as an endogenous inhibitor of PKC. The possibility exists that *A. actinomycetemcomitans* may produce a PKC inhibitor similar to that found in the brain or PMN. Alternatively, a protein produced by *A. actinomycetemcomitans* may "activate" the endogenous PKC inhibitor in PMNs with a resultant inhibition of PKC-related events.

Infections with *A. actinomycetemcomitans* are frequently seen in patients with LJP. Our finding that bacterial extract of *A. actinomycetemcomitans* significantly inhibits hydrogen peroxide production following PMA stimulation, but increases hydrogen peroxide production following stimulation with FMLP and zymosan, supports the hypothesis that infection with *A. actinomycetemcomitans* may lead to altered PMNs function. This may explain the conflicting results obtained in clinical studies concerning the bactericidal capacity of PMNs from patients with LJP following exposure to different stimulating agents. Elucidation of the mechanisms involved and the chemical nature of the inhibitory factor(s) may result in a better understanding and management of LJP. Further investigation of modulating factors of host responses may be relevant to patients infected with *A. actinomycetemcomitans* with LJP as well as to patients with other types of periodontal disease.

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Address:

David K. Dennison, Ph.D., D.D.S.
Department of Periodontics, Dental Branch
University of Texas Health Science Center-Houston
P.O. Box 20068
Houston, TX. 77225-0068
U.S.A.