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Response of human macrophage-like cells to stimulation by *Fusobacterium nucleatum* ssp. *nucleatum* lipopolysaccharide

D. Grenier, L. Grignon
Groupe de Recherche en Écologie Buccale, Faculté de Médecine Dentaire, Université Laval, Quebec City, Quebec, Canada

Grenier D, Grignon L. Response of human macrophage-like cells to stimulation by Fusobacterium nucleatum *ssp.* nucleatum *lipopolysaccharide.*
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Monocytes/macrophages are key members of the innate immune system and are present in higher numbers in active periodontal lesions than in inactive sites. The aim of this study was to characterize the response of human monocyte U937 cells, differentiated into adherent macrophages by treatment with phorbol-12-myristate 13-acetate, to stimulation by *Fusobacterium nucleatum* ssp. *nucleatum* lipopolysaccharide. Attachment of ^{3}H-lipopolysaccharide to macrophage-like cells was partially inhibited by anti-CD14 and anti-TLR4 polyclonal antibodies. Fusobacterial lipopolysaccharide did not cause cell apoptosis or block apoptosis induced by camptothecin. Lipopolysaccharide up-regulated the secretion of the pro-inflammatory cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α as well as the chemokine interleukin-8 by macrophage-like cells. In addition, it increased phospholipase C and D activities, which likely contributed to the high levels of prostaglandin E_2 detected in the cell culture supernatant. Lastly, the amount of matrix metalloproteinase-9 produced by macrophage-like cells was significantly increased by the lipopolysaccharide treatment. Interestingly, fusobacterial cells acquired matrix metalloproteinase-9 activity following incubation in the presence of the culture supernatant of lipopolysaccharide-stimulated macrophage-like cells. In summary, the lipopolysaccharide of *F. nucleatum* ssp. *nucleatum* has a large array of biological effects on macrophage-like cells. This monocytic responsiveness to lipopolysaccharide may be a key regulator of periodontitis.

Key words: cytokine; *Fusobacterium nucleatum*; lipopolysaccharide; macrophage; matrix metalloproteinase; periodontitis; prostaglandin E_2

D. Grenier, Groupe de Recherche en Écologie Buccale, Université Laval, Quebec City, Quebec, Canada, G1K 7P4
Tel.: +1 418 6567341;
fax: +1 418 6562861;
e-mail: daniel.grenier@greb.ulaval.ca
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Periodontitis, the major cause of adult tooth loss, is a mixed bacterial infection that develops when the equilibrium of the oral ecosystem tips toward pathogenic bacteria (11, 26). While bacteria are the primary factor in the etiology of periodontitis, tissue destruction is also a consequence of the excessive host response. Indeed, the continuous challenge of the host immune system by periodontopathogens and their products, more specifically lipopolysaccharides, initiates a number of host-mediated destructive processes (7). This complex inflammatory phenomenon results in the destruction of the tooth-supporting tissues, including the alveolar bone, and is mediated in part by host proteolytic enzymes (29).

The periodontium is composed of numerous cell types, including fibroblasts and epithelial cells, and inflammatory infiltrates of polymorphonuclear and mononuclear cells. Monocytes and macrophages are key members of the innate immune system and are present in higher numbers in active periodontal lesions than in inactive sites (32). Considering the multifunctional roles of monocytes and macrophages, they likely play an important role in the initiation and maintenance

of the inflammatory processes and alveolar bone loss observed in chronic periodontitis (7). These cells are thought to be an important source of pro-inflammatory cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), which stimulate the activation of cellular immunity, amplify the inflammatory cascade, and contribute to host tissue destruction (6). Lipopolysaccharides from gram-negative bacteria are potent inducers of pro-inflammatory mediators and can initiate a number of host-mediated destructive processes (12). Lipopolysaccharide stimulation of macrophages leading to the production of cytokines and matrix metalloproteinases (MMPs) involves the cell surface receptors CD14 and Toll-like receptor 4 (30).

Fusobacterium nucleatum, which refers to a group of three subspecies (*nucleatum*, *vincentii*, and *polymorphum*), is a gram-negative anaerobic bacterium associated with gingivitis and chronic periodontitis (3). This periodontopathogen has also been implicated in a variety of nonoral infections such as pleuropulmonary infections, urinary tract infections, endocarditis, and intra-amniotic infections (2). Several components that allow this bacterium to colonize the host and that contribute to tissue destruction have been identified (2, 3). The aim of this study was to characterize the response of human monocyte U937 cells differentiated into adherent macrophages to stimulation by *F. nucleatum* ssp. *nucleatum* lipopolysaccharide. More specifically, the induction of apoptosis and the production of cytokines, prostaglandin E_2 (PGE_2), nitric oxide, matrix metalloproteinase 9 (MMP-9), and phospholipases were investigated.

Material and methods

Bacterial strain, growth conditions, and lipopolysaccharide preparation

F. nucleatum ssp. *nucleatum* ATCC 25586 was grown in Todd Hewitt Broth (BBL Microbiology Systems, Cockeysville, MD) supplemented with hemin (10 μg/ml) and vitamin K (1 μg/ml) at 37°C for 24 h under anaerobic conditions ($N_2/H_2/CO_2$, 75 : 10 : 15). Lipopolysaccharides were isolated using the method of Darveau & Hancock (5), which is based on protein digestion of a whole cell extract by proteinase K and successive solubilization and precipitation steps. The lipopolysaccharide preparation was freeze-dried, weighed, and kept at − 20°C. The presence of contaminating proteins in the preparation was evaluated using a protein assay kit (Bio-Rad Laboratories, Mississauga, ON, Canada). Lipopolysaccharides made up 4.5% of the total dry weight of *F. nucleatum* ssp. *nucleatum* ATCC 25586 cells. A 1 mg/ml lipopolysaccharide preparation contained less than 1 μg of contaminating proteins.

Radiolabeling of lipopolysaccharide

The method used to prepare ^{3}H-lipopolysaccharide was a modification of the procedure previously described by Rokita & Menzel (22). Briefly, a 1 mg/ml stock solution of lipopolysaccharide from *F. nucleatum* ssp. *nucleatum* ATCC 25586 was prepared in 100 mM carbonate buffer (pH 9) containing 100 mM NaCl. The lipopolysaccharide solution was vortexed in a glass tube for 5 min prior to adding a solution of ^{3}H-acetic anhydride in toluene (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada) to obtain a final concentration of 1.25 μCi/μl (2.4 μM). The lipopolysaccharide solution was further vortexed intensively for 5 min and incubated at room temperature for 1 h with gentle agitation. The lipopolysaccharide solution was then diluted 1 : 4 by adding three volumes of 100 mM phosphate-buffered saline (pH 7.2). Seven successive dialyses (10 h at 4°C) against phosphate-buffered saline (4 liters) were performed until less than 50 dpm/100 μl of buffer was detected in the dialysate. Radioactive counts and specific activity (μCi/mg lipopolysaccharide) of the ^{3}H-lipopolysaccharide preparation were determined using EcoLite scintillation liquid (ICN, Costa Mesa, CA) and a Multi-Purpose Scintillation Counter (Beckman Coulter, Fullerton, CA). Sodium azide (0.1%) was added to the ^{3}H-lipopolysaccharide preparation, which was then stored at − 20°C. The radiolabeling procedure produced a ^{3}H-lipopolysaccharide preparation with a specific activity of 4 μCi/mg. Radiolabeled lipopolysaccharide was analyzed by denaturing electrophoresis on a 12.5% polyacrylamide gel by the discontinuous method of Laemmli (16). The gel was treated with an Entensify solution (NEN Life Science Products, Boston, MA) and exposed on a Kodak BioMax MS Film at − 80°C for 1 week.

Monocyte cultures and differentiation into adherent macrophages

U937 cells, a monoblastic leukemia cell line, were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium supplemented with penicillin (100 μg/ml), streptomycin (50 μg/ml), and 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO_2 atmosphere. The cells were seeded at 2×10^5 cells/ml and split approximately three times per week to maintain the cell concentration between 1×10^5 and 2×10^6 monocytes/ml for optimal growth. The monocytes at an initial seeding of 2.5×10^5 monocytes/ml were differentiated into adherent macrophages by growing them for 48 h in the presence of 10 ng/ml of phorbol-12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO). This treatment has been reported to induce the appearance of the characteristics consistent with mature macrophages (23). The cells were incubated in PMA-free medium for 24 h prior to use.

Binding of ^{3}H-lipopolysaccharide to macrophage-like cells

PMA-differentiated U937 cells were suspended in RPMI 1640–10% fetal bovine serum and 4 μg of ^{3}H-lipopolysaccharide was added to 5×10^6 cells in a volume of 200 μl. In preliminary assays, this amount of ^{3}H-lipopolysaccharide was found to be optimal. The mixture was incubated with shaking for 1 h at 37°C. The cells were then harvested by centrifugation ($300 \times g$ for 5 min) and washed twice in RPMI 1640. The amount of ^{3}H-lipopolysaccharide bound to the macrophage-like cells was estimated by counting and comparing the radioactivity to a standard curve with known quantities of radioactive lipopolysaccharide added to scintillation liquid. The assay was also carried out in the presence of sheep anti-CD14 or anti-TLR4 polyclonal antibodies (1 : 100 dilution; R & D Systems, Minneapolis, MN) and the percent inhibition of ^{3}H-lipopolysaccharide attachment was determined. Sheep IgG antibody was used as control. Independent experiments were performed in triplicate.

Stimulation of macrophage-like cells by lipopolysaccharide

PMA-differentiated U937 cells were adjusted to a final concentration of 5×10^6 cells/ml in RPMI 1640–10% fetal bovine serum. A volume of 200 μl of cells was treated with 1 μg of nonradioactive *F. nucleatum* ssp. *nucleatum* lipopolysaccharide for 24 h at 37°C in a 5% CO_2 atmosphere. Following stimulation, assay mixtures were centrifuged ($300 \times g$ for 5 min) and the supernatants were stored at − 20°C for

further analysis. Independent experiments were performed in triplicate.

Determination of cell apoptosis and viability

Following the treatment with fusobacterial lipopolysaccharide, apoptotic macrophage-like cells were detected by *in situ* TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP biotin nick end-labeling) using a TiterTACS kit from Trevigen (Gaithersburg, MD) according to the manufacturer's instructions. Treating the macrophage-like cells with the nuclease provided in the assay kit generated DNA fragments that were used as the positive apoptosis control. In this assay, the reactive DNA fragments generate a product that can be monitored by measuring the absorbance at 450 nm. The capacity of fusobacterial lipopolysaccharide to inhibit camptothecin-induced apoptosis was tested by including the inducing agent (2 μg/ml) during the stimulation of the cells by lipopolysaccharide. The viability of macrophage-like cells treated with fusobacterial lipopolysaccharide was determined using a Cell Proliferation Kit (Roche Diagnostics, Laval, QC, Canada), which measures the normal metabolic status of cells, particularly that of mitochondria. This assay is based on the cleavage of 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and the formation of a water-insoluble formazan dye, which is quantified by measuring the absorbance at 570 nm with a microplate reader.

Determination of cytokine, IL-1 β-converting enzyme, MMP-9, and prostaglandin E_2 production

The release of IL-1β, IL-6, IL-8, tumor necrosis factor-α, and MMP-9 into the culture supernatants of macrophage-like cells stimulated with lipopolysaccharide was quantified by indirect enzyme-linked immunosorbent assays (ELISA) using streptavidin horseradish-peroxidase as detection system. Commercial kits obtained from Diaclone Research (Besançon, France) (IL-1β), BioSource (Camarillo, CA) (IL-6, IL-8, and tumor necrosis factor-α), and R & D Systems (MMP-9) were used according to the manufacturers' instructions. The absorbance at 450 nm was read using a microplate reader with the wavelength correction set at 550 nm. The sensitivities of the commercial ELISA kits were 3.9 pg/ml for IL-1β, 9.3 pg/ml for IL-6, 31.2 pg/ml for IL-8, 15.6 pg/ml for tumor necrosis factor-α, and 310 pg/ml for MMP-9. A competitive enzyme immunoassay (EIA) was performed on the cell-free supernatant according to the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI) to determine PGE_2 levels. The absorbance at 415 nm was read using a microplate reader. The sensitivity of the commercial kit was 15 pg/ml. An ELISA kit purchased from R & D Systems with a detection level of 0.68 pg/ml was used to quantify IL-1β-converting enzyme (ICE) in lysates of lipopolysaccharide-stimulated and unstimulated macrophage-like cells. Lysates were prepared by incubating cells in 50 mM HEPES buffer (pH 7.0) containing 0.1% Triton X-100 for 30 min at 4°C. The lysates were then centrifuged to eliminate cell debris. The absorbance at 450 nm was recorded using a microplate reader with the wavelength correction set at 550 nm. All the above assays were performed in triplicate.

Determination of nitric oxide production

Nitric oxide production by lipopolysaccharide-stimulated macrophage-like cells, in phenol red free medium, was determined by measuring the accumulation of nitrite NO_2^-, one of two primary, stable, nonvolatile breakdown products of nitric oxide, in the culture supernatant. The colorimetric test used the Griess reagent system (Promega Corporation, Madison, WI) and has a detection level of 2.5 mM. Samples were added to freshly prepared reagent in a 96-well plate, and the absorbance was read at 550 nm. The assays were run in triplicate and the nitrite concentrations were calculated using a sodium nitrite standard.

Acquisition of MMP-9 activity by *F. nucleatum* ssp. *nucleatum* cells

Equal volumes (200 μl) of *F. nucleatum* ssp. *nucleatum* ATCC 25586 cells (overnight culture) and culture supernatants of lipopolysaccharide-stimulated macrophage-like cells or recombinant human pro-MMP-9 (1 μg/200 μl; Oncogene, Cambridge, MA) were mixed. Following an incubation at 37°C for 2 h, the bacteria were harvested by centrifugation, washed twice in phosphate-buffered saline, and suspended in 200 μl of phosphate-buffered saline. *p*-Aminophenylmercuric acetate (APMA) was added at a final concentration of 0.03 mM to activate bound pro-MMP-9, and the suspension was incubated at 37°C for 1 h. The bacterial cells were washed twice in phosphate-buffered saline and the cell lysates were analyzed by zymography. Samples of cell suspensions were mixed with an equal volume of nonreducing electrophoresis buffer (0.2 M Tris-HCl, 20% glycerol, 6% sodium dodecyl sulfate, 0.05% bromophenol blue, pH 6.7) and separated at 120 V with cooling on a 10% polyacrylamide resolving gel containing 0.5% gelatin (Bio-Rad Laboratories). The gel was washed twice (20 min) in 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 2.5% Triton X-100 and then incubated for 16 h at 37°C in 50 mM Tris-HCl (pH 7.5) containing 0.1 mM NaCl and 10 mM $CaCl_2$. A zymogram was also performed in the presence of 1 mM EDTA, a general inhibitor of matrix metalloproteinases. Coomassie Blue R-250 staining of the gels revealed the presence of gelatinase activity as clear bands against a blue background.

Determination of phospholipase activity

Phospholipase activity in the culture supernatants of lipopolysaccharide-stimulated and unstimulated PMA-differentiated U937 cells was tested using ^{14}C-labeled phosphatidylcholine (0.025 μCi/ml; Amersham, Baie d'Urfé, QC, Canada). Briefly, 25 μl of culture supernatants were added to 30 μl of phosphate-buffered saline containing ^{14}C-labeled phosphatidylcholine (2 μl) and 6.5 ng of nonradioactive phosphatidylcholine. The mixtures were incubated for 48 h at 37°C with gentle agitation. A 20 μl aliquot of each assay mixture was applied to a silica gel (TLC) plate (Fisher, Nepean, ON, Canada) and the migration was carried out at room temperature in a solvent system composed of petroleum-ethanol-acetic acid (50 : 50 : 1; by vol.) until the solvent front was within 0.5 cm of the top of the plate. The plate was air-dried and exposed to a Kodak X-Omat S film for 6 days at − 80°C in presence of a transcreen (BioMAX, Kodak). Commercial phospholipases C and D (23.2 μg; Sigma-Aldrich) were used as controls to identify degradation products.

Statistical analysis

Student's *t*-test for paired values was used and data were considered significant at $P < 0.05$.

Results

Analysis of the ^3H-lipopolysaccharide preparation by SDS-PAGE and autofluorography revealed a major band with a molecular mass of approximately 7 kDa

(Fig. 1), suggesting a rough morphotype lipopolysaccharide for this bacterial strain. This radiolabeled preparation was used to assess the binding of *F. nucleatum* ssp. *nucleatum* lipopolysaccharide to PMA-differentiated U937 cells. Under our assay conditions, 50.5 ± 2.2 ng of ^{3}H-lipopolysaccharide was attached by 5×10^{6} cells. Incorporating anti-CD14 polyclonal antibodies reduced the binding of lipopolysaccharide by 49%, whereas anti-TLR4 polyclonal antibodies reduced binding by 20%. No inhibition was observed with IgG control antibody.

The toxic effect of fusobacterial lipopolysaccharide on PMA-differentiated U937 cells was assessed using an MTT viability assay. A 24 h treatment at an lipopolysaccharide concentration of 1 μg/ml had no toxic effect. Since toxic effects were only seen at higher concentrations of lipopolysaccharide and since preliminary experiments showed that macrophage-like cells responded optimally at a concentration of 1 μg/ml, this concentration was used for the stimulation experiments. An *in situ* TUNEL assay, which measures DNA fragmentation, was performed to investigate a possible apoptotic effect of the lipopolysaccharide treatment. As seen in Fig. 2, no significant difference ($P < 0.05$) was observed between DNA fragmentation in control and lipopolysaccharide-stimulated macrophage-like cells. In addition, fusobacterial lipopolysaccharide did not block cell apoptosis induced by camptothecin ($P < 0.05$).

F. nucleatum ssp. *nucleatum* lipopolysaccharide significantly increased the secretion of the pro-inflammatory cytokines IL-1β, IL-6, and tumor necrosis factor-α, chemokine IL-8, and PGE_2 by PMA-differentiated U937 cells (Table 1). The most striking increase was observed for IL-8. However, lipopolysaccharide did not up-regulate the production of ICE, a cellular enzyme involved in the maturation of IL-1β. No nitric oxide was produced by macrophage-like cells.

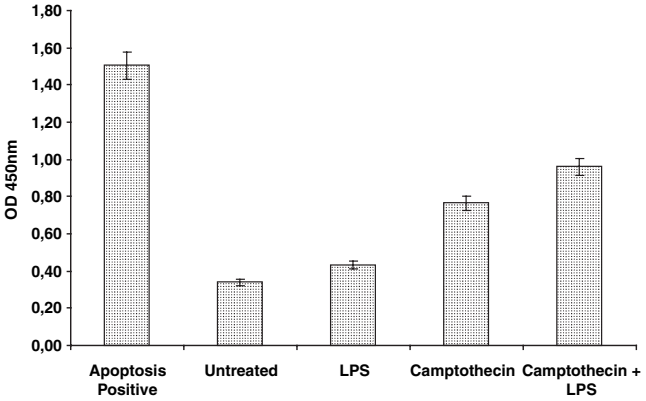

Fig. 2. Effect of *F. nucleatum* ssp. *nucleatum* lipopolysaccharide (1 μg/ml) on the apoptosis of PMA-differentiated U937 cells. Apoptosis was evaluated by an *in situ* TUNEL assay using a TiterTACS kit, which measures DNA fragmentation. The reactive DNA fragments generates a product that can be monitored by measuring the absorbance at 450 nm. The data are the means ± standard deviations of triplicate assays. There was no significant difference between lipopolysaccharide-treated and untreated cells ($P < 0.05$).

The effect of fusobacterial lipopolysaccharide on MMP-9 production by PMA-differentiated U937 cells was tested. The amount of MMP-9 detected by ELISA was twofold higher following the lipopolysaccharide treatment, compared to that produced by untreated cells (Table 1). Given the reported capacity of *F. nucleatum* ssp. *nucleatum* to bind recombinant human MMP-9 to its cell surface (9), fusobacterial cells were incubated in a culture supernatant of lipopolysaccharide-stimulated macrophage-like cells and then tested for cell-associated MMP-9 activity by zymography. As shown in Fig. 3, fusobacterial cells acquired MMP-9 activity after a 2 h incubation. Gelatinolytic bands corresponding to latent and active forms of MMP-9 were detected, suggesting that both forms were bound by *F. nucleatum* ssp. *nucleatum*. When the zymogram was developed in the presence of EDTA, which inhibits MMPs, no gelatinase bands were detected (data not shown). Recombinant human MMP-9 was also bound by fusobacteria (Fig. 3).

Lastly, the capacity of fusobacterial lipopolysaccharide to induce the production of phospholipase activity by PMA-differentiated U937 cells was investigated. As shown in Fig. 4, the lipopolysaccharide strongly induced the secretion of phospholipases into the supernatant that degraded the ^{14}C-labeled phosphatidylcholine. Based on the major hydrolysis products generated (diacylglycerol and phosphatidic acid), the production of both phospholipases C and D appeared to be strongly up-regulated.

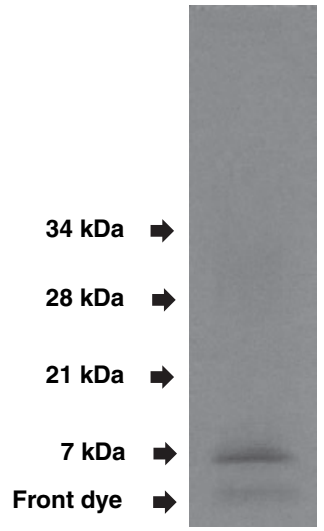

Fig. 1. Analysis of the ^{3}H-lipopolysaccharide (LPS) preparation from *F. nucleatum* ssp. *nucleatum* by SDS-PAGE and autoradiography.

Table 1. Effect of *F. nucleatum* ssp. *nucleatum* lipopolysaccharide on the production of potential deleterious mediators by PMA-differentiated U937 cells

	Amount secreted	
Compound	No treatment	Treatment with lipopolysaccharide (1 μg)
IL-1β	< 3.9 pg/ml	100.2 ± 1.2 pg/ml [a]
IL-6	< 9.3 pg/ml	140 ± 60 pg/ml [a]
IL-8	20 ± 1 pg/ml	630 ± 60 pg/ml [a]
TNF-α	< 15.6 pg/ml	50 ± 10 pg/ml [a]
ICE	195.0 ± 11.3 pg/ml	214.5 ± 15.8 pg/ml
Nitric oxide	< 2.5 mM	< 2.5 mM
PGE_2	50.5 ± 2.4 pg/ml	163.4 ± 18.1 pg/ml [a]
MMP-9	61.2 ± 2.6 ng/ml	120.1 ± 6.0 ng/ml [a]

[a] Significant differences between lipopolysaccharide-treated and untreated cells at $P < 0.05$.
ICE, IL-1β-converting enzyme; IL, interleukin; MMP-9, matrix metalloproteinase-9; PGE_2, prostaglandin E_2; PMA, phorbol-12-myristate 13-acetate; TNF-α; tumor necrosis factor-α.

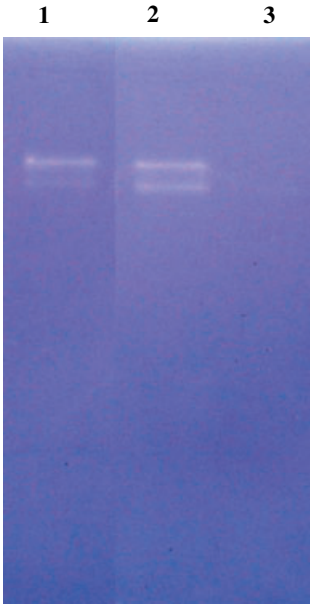

Fig. 3. Determination of *F. nucleatum* ssp. *nucleatum*-associated MMP-9 activity by zymography analysis using gelatin as a substrate. Bacteria were incubated in the presence of recombinant MMP-9 (1 μg/ml) (lane 1) or in the culture supernatant of lipopolysaccharide-treated PMA-differentiated U937 cells (lane 2). Bacteria incubated in fresh culture medium served as negative control (lane 3).

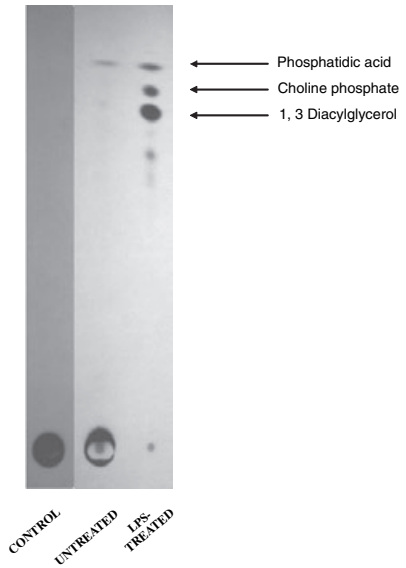

Fig. 4. Determination of phospholipase activity by thin layer chromatography and autoradiography using ^{14}C-phosphatidylcholine as a substrate. Samples incubated with the radioactive substrate were RPMI 1640–10% fetal bovine serum medium (control), a supernatant of untreated PMA-differentiated U937 cells, and a supernatant of lipopolysaccharide-treated PMA-differentiated U937 cells. LPS, lipopolysaccharide. PMA, phorbol-12-myristate 13-acetate.

Discussion

Cell surface components of periodontopathogens are thought to play a crucial role in the pathogenic process of periodontitis because they are involved in the colonization of subgingival sites, resistance to host defenses, induction of inflammatory processes, and destruction of periodontal tissue. Lipopolysaccharide consists of a carbohydrate-rich and a lipid-rich domain and is a major constituent of the outer membrane of gram-negative bacteria. While lipopolysaccharide molecules are only weakly toxic when anchored to the outer membrane, their release following cell lysis exposes the highly toxic moiety lipid A, which can initiate an inflammatory response in phagocytic, endothelial, and epithelial cells (30). Macrophages, which increase in numbers in periodontal sites during periodontitis (32), play a pivotal role in the cellular response to lipopolysaccharide by secreting a broad array of inflammatory mediators. In this study, we characterized the response of U937 cells differentiated into adherent macrophages to stimulation with lipopolysaccharide from *F. nucleatum* ssp. *nucleatum* ATCC 25586.

Apoptosis is an important biological process that allows multicellular organisms to eliminate damaged and infected cells. Jewett et al. (14) reported that whole cells of *F. nucleatum*, more specifically a cell surface heat labile component, induce the apoptosis of polymorphonuclear neutrophils. In our study, fusobacterial lipopolysaccharide did not induce apoptosis of PMA-differentiated U937 cells as determined with a TUNEL assay that measures DNA fragmentation. Lipopolysaccharide has also been reported to inhibit apoptosis of human macrophages through the induction of a cellular inhibitor of apoptosis protein-2 (4). In our study, fusobacterial lipopolysaccharide did not block camptothecin-induced apoptosis in macrophage-like cells, thus suggesting that lipopolysaccharide may behave differently according to its origin.

Little information is available on the inflammatory response of human cells stimulated by *F. nucleatum* ssp. *nucleatum* lipopolysaccharide, which has a classic chemical composition (20). Baqui et al. (1) showed that *F. nucleatum* lipopolysaccharide-stimulated monocytes isolated from HIV-1 infected patients exhibit increased production of IL-1β, IL-6, and tumor necrosis factor-α compared to those isolated from HIV-1 uninfected patients. *F. nucleatum* lipopolysaccharide also stimulates the release of IL-1β, tumor necrosis factor-α, and IL-8 by polymorphonuclear leukocytes isolated from healthy donors (31). We report here the capacity of *F. nucleatum* ssp. *nucleatum* lipopolysaccharide to significantly induce IL-1β and tumor necrosis factor-α secretion by macrophage-like cells. The release of these two pro-inflammatory cytokines is known to stimulate the production of secondary mediators, which amplifies the inflammatory response, the induction of connective tissue-degrading enzymes, and osteoclastic bone resorption (10).

Fusobacterial lipopolysaccharide also induced a significant increase in IL-8 secretion by macrophage-like cells. This points to the possibility that leukocytes accumulate at inflamed sites and thus amplify the host inflammatory response in periodontal tissues. No significant nitric oxide production was observed for macrophage-like cells treated with fusobacterial lipopolysaccharide. Shapira et al. (25) previously reported that lipopolysaccharide preparations from different strains of *Porphyromonas gingivalis* can affect human monocytes and mouse macrophages differently with regards to the release of nitric oxide. One should thus not exclude the possibility that lipopolysaccharide prepared from other strains of *F. nucleatum* ssp. *nucleatum* may induce a nitric oxide secretory response.

MMPs are involved in connective tissue remodeling and may play a significant role in the degradation of the collagenous structure of periodontal tissue, a critical outcome of periodontal disease. Periodontal tissue destruction has been associated with high levels of active MMP-9 in gingival crevicular fluid (28). This metalloproteinase is also highly expressed in the inflamed gingival tissues of patients with periodontitis (8, 27). In our study, the stimulation of macrophage-like cells by fusobacterial lipopolysaccharide induced a significant increase in MMP-9 secretion. The up-regulation of IL-1β and tumor necrosis factor-α secretion, which was observed in our study, was previously reported to enhance MMP-9 production by monocytes through a PGE_2-independent mechanism (33). In addition to degrading host tissue proteins, high levels of MMP-9 may regulate IL-1β activity by processing the human IL-1β precursor into the biologically active form, as shown by Schönbeck et al. (24). Since fusobacterial lipopolysaccharide did not increase the levels of ICE (an intracellular enzyme involved in the maturation of IL-1β) in macrophage-like cells, the overproduction

of MMP-9 might contribute to the activation of IL-1β.

We previously reported the capacity of *F. nucleatum* ssp. *nucleatum* whole cells to bind recombinant MMP-9 (9). In this study, we showed that this bacterium was able to acquire MMP-9 activity following incubation in a supernatant of PMA-differentiated U937 cells stimulated by lipopolysaccharide. The acquisition of MMP-9 activity may facilitate bacterial dissemination to surrounding periodontal tissues as we demonstrated that MMP-9-coated *F. nucleatum* ssp. *nucleatum* cells penetrate a reconstituted basement membrane model significantly more efficiently than uncoated cells (9).

Stimulation of macrophage-like cells with fusobacterial lipopolysaccharide resulted in an induction of both phospholipase C and D production. These enzymes may have an impact on macrophage membrane integrity as well as macrophage fragility. Their action on membrane phospholipids is associated with the production of diacylglycerol and phosphatidic acid, both of which are known as important signaling molecules leading to inflammatory processes (13). In a recent study, Lee et al. (17) showed that the secretion of MMP-9 by murine macrophages is stimulated by phosphatidic acid, a product of phospholipase D activity. Such a mechanism may contribute to the increased production of MMP-9 observed in our study.

PGE_2 is a potent stimulator of bone resorption (21) and is associated with attachment loss (18). High levels of PGE_2 have been detected in the gingival crevicular fluid of periodontitis sites (15, 19). These *in vivo* observations were supported by our results, which show that fusobacterial lipopolysaccharide induced an increase in PGE_2 secretion by the macrophage-like cells. The production of PGE_2 may be related to the observed induction of phospholipase C activity and the generation of diacylglycerol. Indeed, diacylglycerol can be enzymatically converted into arachidonic acid, which is a substrate for the arachidonic acid cascade leading to the formation of PGE_2.

In summary, our results support the view that lipopolysaccharide from *F. nucleatum* ssp. *nucleatum* possesses a large array of biological activities toward macrophage-like cells. This monocytic responsiveness and likely that of other host cells to fusobacterial lipopolysaccharide may be key regulators of periodontitis. Since periodontitis is a mixed infection, the inflammatory response induced by lipopolysaccharide of other periodontopathogens should also be considered.

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