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Comparison of inflammatory cytokine-inducing activity of lipopolysaccharides from major periodontal bacteria

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Porphyromonas gingivalis (Pg), *Aggregatibacter actinomycetemcomitans* (Aa), *Tannerella forsythia* (Tf), *Prevotella intermedia* (Pi), and *Fusobacterium nucleatum* (Fn) are major periodontal pathogens. Lipopolysaccharides (LPSs) from periodontal bacteria play an important role in periodontal pathogenesis by stimulating host cells to produce inflammatory cytokines. In this study, highly pure LPSs from the five major periodontopathogens were prepared, and their monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α)-inducing activities were compared in human umbilical vein endothelial cells (HUVECs) and THP-1 macrophagic cells, respectively. In HUVECs, LPSs from Aa and Fn were potent stimulators for MCP-1 induction; however, LPSs from Pg, Pi, and Tf were much weaker MCP-1 inducers. In THP-1 cells, LPSs from Pg, Aa, and Fn were relatively strong inducers of TNF-α, whereas LPSs from Pi and Tf produced little activity. The Toll-like receptor (TLR)2/TLR4 dependency of various LPSs was also determined by measuring NF-κB reporter activity in TLR2- or TLR4-expressing 293 cells. LPSs from Aa, Fn, and Tf stimulated only TLR4; however, LPSs from Pg and Pi stimulated both TLR2 and TLR4. These results suggest that LPSs from major periodontal bacteria differ considerably in their cell-stimulating activity.

Keywords: Periodontitis, Bacteria, Lipopolysaccharides, Cytokines, Toll-like receptors

Introduction

Periodontal disease is caused by infections with oral periodontitis-associated microorganisms. *Porphyromonas gingivalis* (Pg), *Aggregatibacter actinomycetemcomitans* (Aa), *Tannerella forsythia* (Tf), *Prevotella intermedia* (Pi), and *Fusobacterium nucleatum* (Fn) are major periodontal pathogens. Pg and Tf are well established periodontal pathogens, belonging to the red complex. Fn is one of the most common species in the human gingival sulcus and its prevalence increases with the severity of periodontal disease. Pi increases in numbers and proportions during periodontal disease. Aa plays a primary etiological role in localized aggressive periodontitis [1].

Lipopolysaccharide (LPS) of periodontal bacteria plays an important role in periodontal pathogenesis by stimulating host cells to produce inflammatory cytokines, including tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) [2,3]. The levels of TNF-α and MCP-1 have been shown to be elevated in the gingival crevicular fluid of chronic periodontitis patients [4]. LPS is present in the outer membrane of all gram-negative bacteria and consists of a lipid A moiety, a core polysaccharide, and a polysaccharide chain of repeating sugar subunits [5]. In humans, LPS is sensed primarily by Toll-like receptor (TLR) 4. However, many studies reported that Pg LPS is a TLR2 agonist. Therefore, the action of Pg LPS through TLR2 or TLR4 remains controversial [6-8].

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Ligand-stimulated TLR4 or TLR2 activates the transcription factor NF-κB that mediates gene transcription, leading to the production of proinflammatory cytokines.

As many of the periodontal pathogens are gram-negative, they may all be involved in initiating periodontal inflammation. However, the potency of LPS to trigger inflammation varies among different bacteria. Although many studies reported the effects of individual LPS on host cytokine responses, no studies compared the potency of LPSs from different periodontal pathogens in terms of host cell stimulation and cytokine response.

In the present study, we compared the potency of LPSs from 5 major periodontal bacteria by measuring the production of MCP-1 and TNF-α in human umbilical vein endothelial cells (HUVEC) and THP-1 cells, respectively. We also determined which type of TLRs, TLR2, or TLR4, is responsible for the cell stimulation by measuring NF-κB reporter activity in TLR2 or TLR4-expressing 293 cells.

Materials and Methods

1. Bacteria and growth conditions

The bacterial strains used in this study were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Pg ATCC 33277 and Pi ATCC 25611 was grown in tryptic soy broth (TSB) supplemented with yeast extract (1 mg/mL), hemin (5 μg/mL), and menadione (1 μg /mL). Fn ATCC 10953 was grown using brain heart infusion (BHI) broth containing yeast extract (5 mg/mL), hemin (5 μg/mL), and menadione (1 μg/mL). Tf ATCC 43037 was grown in BHI broth containing hemin (5 μg/ml), menadione (1 μg/mL), *N*-acetylmuramic acid (0.001%), and fetal bovine serum (FBS) (5%). Aa ATCC 33384 was grown in TSB supplemented with yeast extract (1 mg/mL) and horse serum (10%) at 37℃. All the five strains were incubated anaerobically (85% N_2, 10% H_2, and 5% CO_2) at 37℃.

2. Cell culture

HUVEC were purchased from Cascade Biologics (Portland, OR, USA) and cultured in medium 200 supplemented with 10% FBS and 50 μg/mL gentamicin at 37℃ in 5% CO_2. Human monocytic THP-1 cells were purchased from ATCC. They were differentiated with 100 ng/mL of phorbol myristate acetate (PMA) for 24 hours in polystyrene culture plates at 37℃ in a humidified atmosphere containing 5% CO_2. The culture medium consisted of RPMI 1640 medium supplemented with 10% FBS and 50 μg/ mL gentamicin.

3. Lipopolysaccharide purification

Bacteria were harvested at the end of the logarithmic phase of growth. LPS extraction was achieved by the hot phenol-water method [9]. Briefly, the bacterial cell pellet was suspended in pyrogen-free water, and then an equal volume of 90% phenol at 60℃ was added dropwise for 20 minutes and stirred constantly. The aqueous phase was separated by centrifugation at 7,000 × g for 15 minutes at 4℃ and collected. This process was repeated, and the aqueous phase was pooled and dialyzed against deionized water for 3 days at 4℃. The dialyzed LPS preparation was then centrifuged at 200,000 × g for 1.5 hours at 4℃ in a Beckman ultracentrifuge (Beckman Coulter, Atlanta, GA, USA). The precipitate was suspended with 30 mL of pyrogen-free water, dialyzed against distilled water for 3 days, lyophilized, and stored at 4℃. The protein content of the purified LPS, determined by the method of Markwell et al. [10], was less than 0.1%. LPS samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained for protein with Coomassie blue to confirm the purity of the LPS moieties.

4. Measurement of cytokines from culture supernatants

3×10^4 HUVEC were incubated with LPS in a final volume of 0.5 mL in 48-well plates for 18 hours. 5×10^5 PMA-differentiated THP-1 cells were incubated with LPS in a final volume of 0.5 mL in 48-well plates for 18 hours. The culture supernatants were sampled and centrifuged at 100 × g for 5 minutes for clarification of debris. The levels of cytokines were quantified using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's directions.

5. NF-κB luciferase reporter assay

To determine whether different LPSs stimulate TLR2 or TLR4, we measured NF-κB-stimulating luciferase activity in TLR2-expressing or TLR4-expressing 293 cells. 293-TLR2/CD14 and 293-TLR4/MD-2-CD14 cells were purchased from Invivogen (San Diego, CA, USA). 1×10^5 293 cells plated in 24-well plates were incubated for overnight. The 293 cells were transfected with 100 ng/well of pNFκB-luc and 25 ng/well of

pCMV-β-Gal using 5 μL/well of Lipofectamin 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Twenty four hours after transfection, the cells were treated with *Escherichia coli* (Ec) LPS (Invivogen, 0.1 μg/mL), prepared LPS (0.1 μg/mL) or Pam3Cys (EMC Microcollections, Tuebingen, Germany; 1 μg/mL) for 16 hours. The luciferase activities of the cell lysates were measured by a luminometer (Berthold Technologies, Bad Wildbad, Germany).

Results

1. Comparison of production of MCP-1 and TNF-α by LPSs from periodontal bacteria

All the LPSs from 5 periodontal bacteria induced the production of MCP-1 in HUVEC. However, there was a big difference in their potency among different bacteria. LPSs of Pg, Pi, and Tf only could induce MCP-1 production at a high concentra-

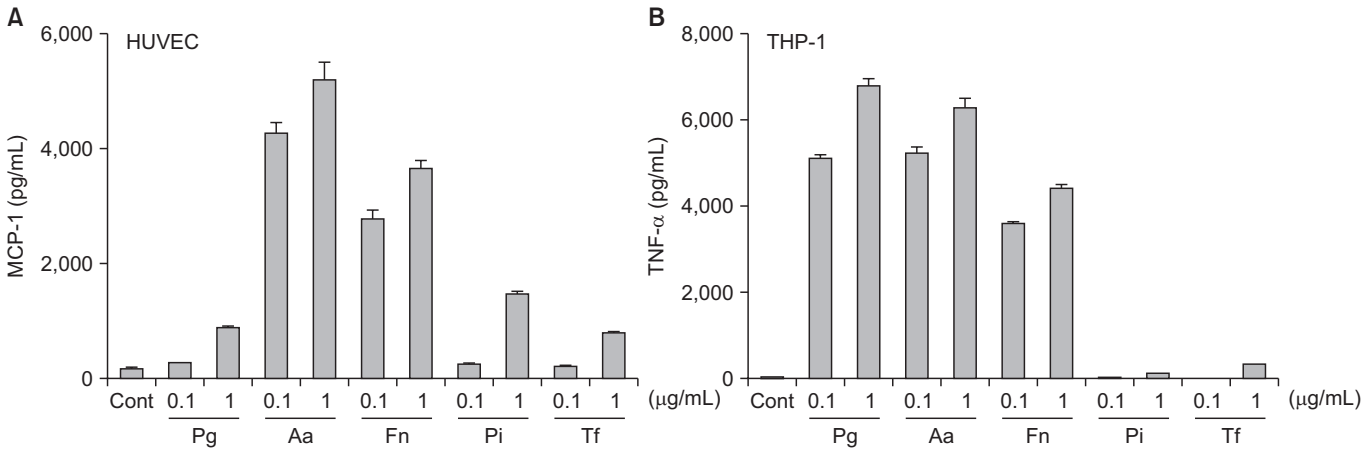

Fig. 1. Production of monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) by lipopolysaccharides (LPSs) from periodontal bacteria. (A) Human umbilical vein endothelial cell (HUVEC) was incubated with respective LPS from different periodontal bacteria at the concentrations of 0.1 and 1 μg/mL. (B) Phorbol myristate acetate-differentiated THP-1 cells were incubated with respective LPS from different periodontal bacteria at the concentrations of 0.1 and 1 μg/mL. After 18 hours, the amount of MCP-1 and TNF-α in the culture supernatants was measured by ELISA kits (R&D Systems). Data are the mean ± standard deviation of a representative experiment performed in triplicate. Similar results were obtained in two other experiments.
Cont, control; Pg, *Porphyromonas gingivalis*; Aa, *Aggregatibacter actinomycetemcomitans*; Fn, *Fusobacterium nucleatum*; Pi, *Prevotella intermedia*; Tf, *Tannerella forsythia*.

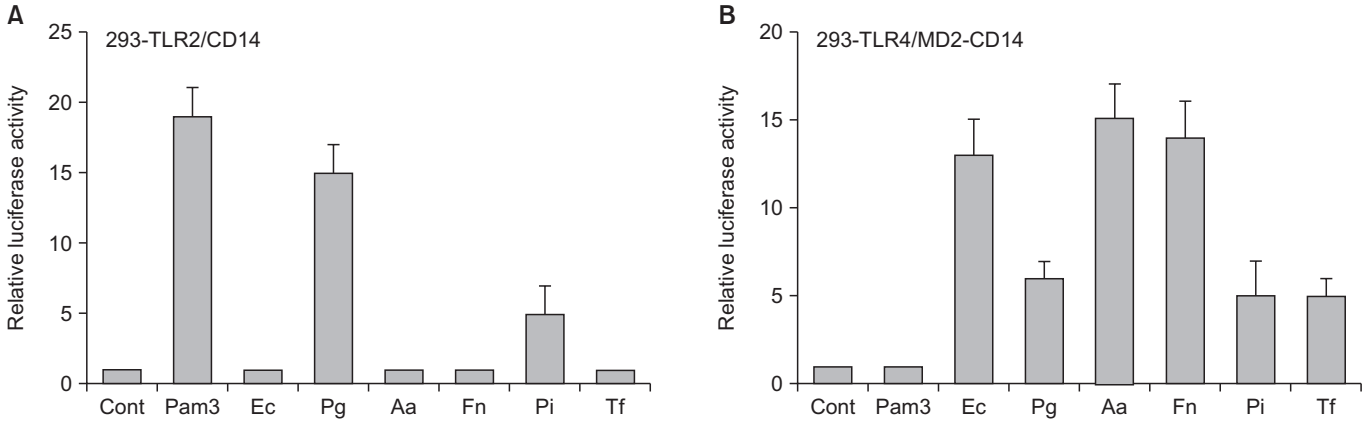

Fig. 2. Stimulation of Toll-like receptor (TLR)2 and TLR4 by lipopolysaccharides (LPSs) from periodontal bacteria. (A) 293-TLR2/CD14 were transfected with pNFκB-luc and pCMV-β-Gal. (B) 293-TLR4/MD-2-CD14 were transfected with pNFκB-luc and pCMV-β-Gal. Twenty four hours after transfection, the cells were treated with various LPSs and Pam3. After 16 hours, the luciferase activities of the cell lysates were measured by a luminometer. Data are the mean ± standard deviation of a representative experiment performed in triplicate. Similar results were obtained in two other experiments.
Cont, control; Pam3, Pam3CysSK4; Ec, *Escherichia coli*; Pg, *Porphyromonas gingivalis*; Aa, *Aggregatibacter actinomycetemcomitans*; Fn, *Fusobacterium nucleatum*; Pi, *Prevotella intermedia*; Tf, *Tannerella forsythia*.

tion of 1 μg/mL, while Aa LPS and Fn LPS were strong MCP-1 inducers even at the concentration of 0.1 μg/mL (Fig. 1A). In THP-1 macrophagic cells, LPSs from Pg, Aa, and Fn robustly induced the production of TNF-α at a low concentration of 0.1 μg/mL. However, LPSs of Pi and Tf hardly induced the production of TNF-α at both concentrations of 0.1 μg/mL and 1 μg/mL (Fig. 1B).

2. Determination of stimulation of TLR2 and TLR4 by LPSs from periodontal bacteria

TLR2-stimulating activity of LPSs of periodontal bacteria was evaluated by measuring NF-κB luciferase activity in HEK 293-TLR2/CD14 cells. Positive control Pam3CysSK4 (Pam3) strongly stimulated the luciferase activity while the negative control Ec LPS did not induce the luciferase activity. More than 10-fold increase of the luciferase activity was induced by Pg LPS, while Pi LPS achieved about 5-fold increase. Other LPSs of Aa, Fn, and Tf did not stimulate the NF-κB luciferase activity in TLR2-expressing cells (Fig. 2A). TLR4-stimulating activity of LPSs of periodontal bacteria was evaluated by measuring NF-κB luciferase activity in HEK 293-TLR4/MD2-CD14 cells. Positive control Ec LPS strongly stimulated the luciferase activity while the negative control Pam3 did not induce the luciferase activity. LPSs of Aa and Fn strongly stimulated TLR4-dependent NF-κB activity (more than 10-fold increase). LPSs of Pg, Pi, and Tf also stimulated the luciferase activity, leading to about 5-fold increase (Fig. 2B).

Discussion

LPS, also called endotoxin, activates the immune system and induces cytokines, causing a variety of biologic effects. LPS extracted from periodontal bacteria has shown to stimulate host cells to produce various cytokines. The produced cytokines are the main cause of tissue destruction and bone resorption in periodontal disease. Many studies reported the production of various cytokines by LPSs from periodontal bacteria in different cell types. The most studied cell type with which the LPS stimulation experiments were done was macrophages since macrophages are the typical LPS responsive cells. Fn LPS up-regulated the secretion of the pro-inflammatory cytokines interleukin (IL)-1β and TNF-α as well by macrophage-like cells [11]. Stimulation of human macrophages with Tf LPS resulted in the production of the proinflammatory cytokines IL-1, IL-6, and TNF-α in a dose-dependent manner [12]. Aa LPS induced the expression of chemokines in murine macrophages [13]. However, there have been no reports regarding the comparative potency of different LPSs from different periodontal pathogens.

In this study, we demonstrated LPSs from different periodontal bacteria have different potency in terms of stimulation of cytokine production. In HUVEC, LPSs of Aa and Fn were strong stimulators for MCP-1 production. 0.1 μg/mL of LPSs from Aa or Fn was stronger than 1 μg/mL of LPSs from Pg, Pi, or Tf. A previous report showed that Pg LPS was a weak stimulator for E-selectin expression in human endothelial cells [8]. Therefore, it is suggested that Aa LPS and Fn LPS, as cell wall-associated or free form, interact with endothelial cells to stimulate the production of inflammatory cytokines, which contributes to the periodontal pathogenesis. In macrophagic THP-1 cells, Pg LPS as well as Aa LPS and Fn LPS strongly induced the production of TNF-α. As in HUVEC, LPSs of Pi and Tf were very weak stimulator for TNF-α production. Therefore, it is suggested that different LPSs stimulate host cells with different potency, which is further affected according to types of responding cells. LPS is composed of a polysaccharide and lipid A which is responsible for the immunological activity of LPS. Different molecular species of lipid A are present in various gram-negative bacteria [5]. This explains the difference of the potency of LPS to trigger inflammation among different bacteria.

We also determined which type of TLRs, TLR2, or TLR4, is stimulated by measuring NF-κB reporter activity in TLR2 or TLR4-expressing 293 cells. In TLR2-expressing 293 cells, the positive control Pam3 strongly stimulated the NF-κB reporter activity, whereas the negative control Ec LPS did not stimulate the reporter activity. Conversely, the positive control, Ec LPS, but not the negative control Pam3 stimulated the NF-κB reporter activity in TLR4-expressing 293 cells. This demonstrated the validity of our experimental system for evaluating TLR2 or TLR4 stimulation. Previously, Aa LPS and Pi LPS activated cells through TLR4 [14,15]. Regarding Pg LPS, there has been controversy. Pg LPS was described as a TLR2 agonist, both TLR2/TLR4 agonist, or a TLR4 agonist [7]. In our study, all the five types of LPSs stimulated TLR4, and Pg LPS and Pi LPS also stimulated TLR2. The discrepancies may lie in the form of LPS presented to the cells. A recent study reported that TLR2 activity of Pg LPS is attributed to a contaminant lipoprotein [16]. It may be a reasonable thought that Pg LPS in its cell-wall associated form or free released form should be tightly associated with or covalently attached to protein. Therefore,

even though our LPS preparation may contain any protein, our results are meaningful and noteworthy. Moreover, the commercially available ultrapure Pg LPS (Invivogen) was also both TLR2/TLR4 agonists (data not shown).

To summarize, Aa LPS and Fn LPS were relatively strong inflammatory stimulators in endothelial cells. LPSs of Pg, Aa, and Fn were strong cell stimulators in macrophagic cells. All the five types of LPS were TLR4 agonists and Pg LPS and Pi LPS were also TLR2 agonists. These results indicate that LPSs of various periodontal bacteria considerably differ in their cell-stimulating activity.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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