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**Biological activities of *Bacteroides forsythus* lipoproteins and their possible pathological roles
in periodontal disease**

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Running title: Biological activities of *B. forsythus* lipoproteins

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ABSTRACT

Bacteroides forsythus is a Gram-negative, anaerobic, fusiform bacterium, and is considered to be an etiological agent in periodontal disease. Lipoprotein fraction prepared from *B. forsythus* cells by Triton X-114 phase separation (BfLP) activated human gingival fibroblasts and a human
5 monocytic cell line, THP-1, to induce interleukin-6 production and tumor-necrosis factor- α production. BfLP was found to be capable of inducing nuclear factor- κ B translocation in human gingival fibroblasts and THP-1. By using Chinese hamster ovary-K1 cells transfected with Toll-like receptor genes together with a nuclear factor κ B dependent CD25 reporter plasmid, it was found that signaling by BfLP was mediated by Toll-like receptor 2, but not by CD14 or Toll-like receptor
10 4. BfLP induced apoptotic cell death in human gingival fibroblasts, KB (an oral epithelial cell line), HL-60 (a human myeloid leukemia cell line) and THP-1 but not in MOLT4 (a T-leukemia cell line). Caspase-8, an initiator caspase in apoptosis, was found to be activated in these cells in response to BfLP stimulation.

Thus, this study suggested that BfLP play some etiological roles in oral infections, especially
15 periodontal disease, by induction of cell activation or apoptosis.

INTRODUCTION

Periodontal disease is generally accepted to be an infectious disease. It is a chronic disease characterized by the interaction between Gram-negative bacteria and host inflammatory response, which results in a destructive change that leads to the loss of bone and connective tissue attachment (41, 46, 49). A lot of oral bacterial species have been suspected to be associated with periodontal disease. To date, a few bacteria, including *Bacteroides forsythus*, have been considered to be key etiological agents of periodontal disease. *B. forsythus* is a Gram-negative, anaerobic, fusiform bacterium (60), and the presence of the bacterium in subgingival flora has been significantly associated with the severity of periodontal disease (13, 14, 62). However, only a few putative virulence factors have been identified in *B. forsythus* because of the fastidious nature of its growth and the difficulties in cultivating it from the human oral cavity. The virulence factors that have been identified so far are a trypsin-like protease (34, 60), a sialidase (21), N-benzoyl-Val-Gly-Arg-p-nitroanilide-specific protease encoded by the prtH gene (47), and a cell surface-associated protein of *B. forsythus* which is involved in adhesion to fibronectin and fibrinogen (22, 50). Recently, Arakawa *et al.* have reported that proteinous factor(s) from this bacterium are able to induce apoptosis in a human myeloid leukemia cell line, HL-60 (1).

Evidence has recently been accumulated that lipoproteins (LP) from *Borrelia burgdorferi*, *Escherichia coli*, *Mycobacterium tuberculosis* and some Mycoplasma species possess endotoxin-like activities (2, 5, 39, 52). We have studied biological activities of mycoplasmal LP (23, 51, 52). Therefore, we have a great interest in pathological roles of LP of periodontopathic bacteria in periodontal diseases, because there have been no reports on biological activities of LP from periodontopathic bacteria.

In this study, attempts were therefore made to determine the biological activities of *B. forsythus* lipoproteins (BfLP).

MATERIALS AND METHODS

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Chemicals. The mycoplasmal lipopeptide FSL-1, which is speculated to be the N-terminal lipopeptide moiety of a lipoprotein responsible for activating human gingival fibroblasts (GFh) purified from *Mycoplasma salivarium* cells, was synthesized with a structure [S-(2,3-bispalmitoyloxypropyl)-cysteine-GDPKHSPKSF] as described previously (52).

10 S-(2,3-bispalmitoyloxypropyl)-N-palmitoyl-cysteine (Pam3-cysteine), which is the N-terminal structure of *E. coli* murein lipoprotein, was purchased from Bachem AG (Bubendorf, Switzerland). Polymyxin B and staurosporine were purchased from Sigma-Aldrich (St. Louis, MO).

All of the other chemicals were obtained from commercial sources and were of analytical or reagent grade.

15 **Bacterial strains and culture conditions.** *B. forsythus* ATCC 43037 was grown in brain heart infusion broth (Eiken Chemical Co., Ltd., Tokyo, Japan) containing 0.5% (wt/vol) yeast extract, 5 µg/ml of hemin, 0.5 µg/ml of vitamin K, 0.001% (wt/vol) N-acetylneuraminic acid (Nacalai tesque, Inc., Kyoto, Japan), 0.1% (wt/vol) L-cysteine (Kanto Chemical Co., Inc., Tokyo, Japan), and 5% (vol/vol) fetal bovine serum (FBS; Cansera International Inc., Ontario, Canada). *B. forsythus* cells
20 grown in the broth under anaerobic conditions (85% N₂, 10% H₂, 5% CO₂) were harvested by centrifugation at 8,000 x g for 30 min and suspended in 10 mM Tris-HCl buffer (pH 7.4), containing 154 mM NaCl and a cocktail of protease inhibitors (TS buffer).

Cell lines. Human gingival fibroblasts (GFh) were prepared and cultured as described previously (9). An oral epithelial cell line, KB (ATCC CCL-17), was obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's (DME) medium (SIGMA-Aldrich) supplemented with 10% (vol/vol) FBS, penicillin G (100 units/ml) and streptomycin (100 µg/ml) [DME(+)]. A T-leukemia cell line, MOLT-4, and human monocytic cell lines, THP-1 and HL-60, were obtained from Health Science Research Resources Bank (Osaka, Japan) and cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FBS, penicillin G (100 units/ml) and streptomycin (100 µg/ml).

Preparation of lipoproteins by Triton X-114 phase separation. The cell suspension was sonicated and treated with Triton (TX)-114 to extract membrane lipoproteins according to the method described previously (51). Briefly, the cell suspension (0.9 ml) was mixed with 0.1 ml of 20% (vol/vol) TX-114 working stock solution. The tube containing the mixture was placed on a rotator at 4 °C for 2 h and then was centrifuged at $10,000 \times g$ for 10 min at 4 °C to remove insoluble materials. The supernatant was transferred into a new tube. The tube was incubated at 37 °C for 5 min for phase separation and then was centrifuged at $10,000 \times g$ for 5 min. The upper aqueous phase was discarded. To the TX-114 phase 0.9 ml of TS buffer were added and treated twice in the same way as described above. Lipoproteins were precipitated from the TX-114 phase by adding 9 volumes of methanol. The lipoprotein fraction obtained by the TX-114 phase separation was referred to as BfLP. BfLP was dissolved in phosphate-buffered saline containing 10 mM n-octyl-β-glucopyranoside (OG/PBS).

Protein concentration of BfLP was determined by the method of Dully and Grieve (10). Endotoxin concentration was determined by using Endospecky (Seikagaku Corp., Tokyo, Japan).

Infrared (IR) spectrometry. IR spectrometry was performed to confirm whether lipoproteins exist in BfLP. IR absorption spectrum of the dried fractions in KBr pellet was measured with Foulmer transform IR spectrometer (RT-210; Horiba, Kyoto, Japan). Pam3-cysteine and FSL-1 were used as standards.

5 **Cytokine assay.** GFh were cultured in DME (+) in a 96-well flat-bottomed plate and the culture medium was exchanged by DME base medium when the cells reached confluency. Then, GFh were incubated for 6 h with various concentrations of BfLP. THP-1 (1.0×10^6 cells) in RPMI 1640 medium were added to a triplicate set of wells of a 96-well round-bottomed plate and incubated for 6 h with various concentrations of BfLP. Each of the cells culture supernatants were collected by
10 centrifuging at $400 \times g$ for 10 min and cytokines produced in the cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (17).

CD25 reporter activity. Chinese hamster ovary (CHO)-K1 transfectants were established as follows. The engineering of the CD14-expressing CHO-K1 reporter fibroblast cell line CHO/CD14.elam.tac, also known as clone 3E10, has been previously described in detail (8). This
15 clonal line has been cotransfected with CD14 and a nuclear factor (NF)- κ B-dependent reporter plasmid that drives the expression of surface CD25 antigen resulting from lipopolysaccharide (LPS)-, tumor necrosis factor (TNF)- α -, or interleukin (IL)- 1β -induced NF- κ B translocation. The cDNAs for human toll-like receptors (TLRs) 2 and 4 were the gifts of Carsten Kirschning and Mike Rothe (Tularik, South San Francisco, CA), and were cloned into the vector pFLAG as described
20 (24). Stable expressions of TLRs were obtained by cotransfection of these epitope-tagged plasmids with pcDNA3 (Invitrogen, San Diego, CA) into CHO/CD14 reporter cells. After selection in G418 (1 mg/ml), clonal cell lines expressing high levels of human TLR2 or 4 were derived using

fluorescent-activated cell sorting combined with limiting dilution cloning.

Adherent monolayers of CHO transfectants were plated in 24-well tissue culture dishes at a density of 1×10^5 cells per well. After overnight incubation, the cells were stimulated for 15 h with BfLP. Cells were detached from the surface with trypsin/EDTA and assessed by flow
5 microfluorometry for the presence of surface CD25 exactly as described (8).

NF- κ B activation. The activation of NF- κ B was examined by using TransAM NF- κ B p50 transcription factor assay kit (Active Motif, Inc., Carlsbad, CA) as specified by the manufacturer. GFh and THP-1 were stimulated with BfLP at 37 °C for 0, 1, 2 and 4 h, and lysed in the buffer containing 20 mM HEPES, pH 7.5, 350 mM NaCl, 20% glycerol, 1% Igepal-CA630, 1 mM MgCl₂,
10 0.5 mM EDTA, 0.1 mM EGTA, 5 mM DTT and protease inhibitor cocktail to prepare the whole cell extracts. Then activated NF- κ B was detected by antibodies, which only detect NF- κ B p50 activated and bound to its target DNA containing NF- κ B consensus site.

Cytotoxicity assay. Cytotoxicity was assayed as described previously (22). A 100- μ l cell suspension of MOLT-4 (1×10^4 cells), THP-1 (1.5×10^4 cells) or HL-60 (2×10^4 cells) in RPMI
15 1640 medium was added to a triplicate set of wells of a 96-well round-bottomed plate and incubated with BfLP (6 or 12 μ g/ml) for 15 h. GFh or KB were cultured in DME (+) in a 96-well flat-bottomed plate. When the cells reached confluency, the culture medium was replaced by DME base medium and the cells were stimulated with BfLP (6 or 12 μ g/ml) for 15 h.

Each of these culture supernatants was collected by centrifuging at 400 \times g for 10 min. A
20 cytoplasmic enzyme, lactate dehydrogenase (LDH), released in the culture supernatant, was then colorimetrically measured by using a CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Co., Madison, WI.). Cytotoxicity (%) was expressed as $100 \times [(\text{experimental LDH release}) -$

(control LDH release)/(maximum LDH release) – (control LDH)], where values of control LDH release and maximum LDH release were obtained from nonstimulated target cells and complete lysis of the target cells by 0.9% (v/v) TX-100, respectively.

Apoptotic cell death in GFh, KB, THP-1 and HL-60 was determined by using an ApoStrand ELISA apoptosis detection kit AK-120 (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA) as specified by the manufacturer. Briefly, the cells prepared as described above were incubated with BfLP (6 or 12 µg/ml) for 15 h and treated with formamide, which denatures DNA in apoptotic cells but not in necrotic cells or in cells with DNA breaks in the absence of apoptosis (11). Then, the single-strand DNA in apoptotic cells was detected by the monoclonal antibody (mAb).

Caspase-8 activation assay by Western blotting. GFh, KB, THP-1 and HL-60 were incubated with BfLP as described above. Their cell lysates were separated by SDS-PAGE and electrically transferred to nitrocellulose membranes. Caspase-8 activation was detected by anti-caspase-8 mAb (Cell Signaling Technology Inc., Beverly, MA) and then peroxidase-conjugated goat anti-mouse IgG (Seikagaku Co., Tokyo, Japan). The mAb was able to detect both caspase-8 isoforms (zymogens: p55/p53) and the cleavage intermediates p43/p41. Immunoreactive proteins were detected by using enhanced chemiluminescence detection reagents (Amersham Biosciences Corp. Piscataway, NJ).

RESULTS

Characterization of BfLP. Hantke *et al.* (14) reported that two ester-bound fatty acids attach to cysteine residue at the N-terminal end of the polypeptide chain and the other fatty acid is bound as

amide to the N-terminal group in bacterial lipoprotein. Therefore, it was thought that fatty acid chains and typical ester bonds would be detected in BfLP. BfLP was applied to IR spectrometer to detect the characteristic signals of these moieties. In the spectrometry, Pam₃-cysteine was used as a standard. The IR spectra of BfLP, FSL-1 and Pam₃-cysteine exhibited signals about 2,900 cm⁻¹ and 1,700 cm⁻¹, which show the presence of fatty acid alkyl chains and typical ester bonds, respectively (Fig. 1). That is, this result clearly indicated that BfLP contains ester-bounded fatty acids.

We thought the possibility that LPS with multiple biological activities was contaminated in BfLP, because LPS is a major complex glycolipid found in the outer membrane of Gram-negative bacteria. Therefore, we determined whether BfLP is contaminated with LPS, and found that 1 µg of protein of BfLP is contaminated with 0.004 ng of LPS.

Activation of GFh and THP-1. Our previous study (9) demonstrated that mycoplasmal lipoproteins are capable of activating GFh to induce IL-6 and IL-8 production and activating human monocytes/macrophages to induce IL-1β and TNF-α production. Therefore, experiments were carried out to determine whether BfLP activated GFh and THP-1 to induce cytokine production. BfLP induced IL-6 production by GFh in a dose-dependent manner (Fig. 2 A). However, there is a possibility that the activity is attributed to a small amount of LPS included in BfLP as described above. To rule out the possibility, the effect of polymyxin B on the IL-6 production-inducing activity of BfLP was investigated, because polymyxin B is known to inhibit biological activities of LPS (35). Polymyxin B had no effect on its activity (Fig. 2 A). In addition, the effect of lipoprotein lipase on the IL-6 production-inducing activity of BfLP was also investigated, since lipoprotein lipase abrogated the activity of mycoplasmal lipoproteins to activate GFh (9). Lipoprotein lipase treatment significantly reduced the IL-6 production-inducing activity of BfLP (data not shown).

These data suggested that BfLP activated GFh and the activity of BfLP was attributed to lipoproteins in BfLP, but not to LPS. In response to 10 μ g/ml of BfLP, the amount of IL-6 produced appears diminished in the presence of 1000 U/ml of polymyxin B compared with 500 U/ml or in its absence (Fig. 2A). One possible explanation for this reduction might be that in the presence of 1000 U/ml of polymyxin B, the toxic effect of BfLP on cells as described below is augmented and thus the total number of IL-6-producing cells is reduced under these conditions (4, 7, 62), although some of them reported that polymyxin B had no effects on keratinocytes until 10000 U/ml (7).

Activation of NF- κ B in GFh by BfLP was also examined because mycoplasmal lipoproteins are known to activate NF- κ B in GFh (37), which regulates the transcription of several genes implicated in inflammatory responses. BfLP was found to be capable of inducing NF- κ B translocation in GFh (Fig. 2 B).

It is well known that mycoplasmal lipoproteins are capable of activating human monocytes/macrophages to induce IL-1 β and TNF- α production (9). Therefore, the activation of THP-1 by BfLP stimulation was also investigated. BfLP was able to activate THP-1 to induce TNF- α production in a dose-dependent manner, and NF- κ B translocation was also induced in THP-1 (Fig. 3 A, B). These data indicated that BfLP induced both NF- κ B activation and cytokine production.

Recognition of BfLP by TLR2. The innate immune system has evolved as the first line of defense against invading microorganisms. Medzhitov *et al.* (33) has first reported that TLRs play important roles in innate immune system. TLRs recognize pathogen-associated molecular patterns (PAMPs) that distinguish the infectious agents from self, and in addition, discriminate among

pathogens. Ten members of the TLR family have been identified in humans, and some of which are shown to recognize specific microbial products (29, 32, 55). TLR4, CD14, MD-2 and LPS-binding protein (LBP) are involved in the signaling by LPS (6, 37, 43, 44, 53). TLR2 has been reported to recognize microbial lipoproteins, peptidoglycan, lipoteichoic acid (48, 56, 64) and some other components. Heine *et al.* (19) demonstrated that TLR4 plays an important role in LPS-induced signaling by using CHO/TLR transfectants. In this study, experiments using the CHO transfectants were also carried out to determine whether signaling by BfLP was mediated by TLR2. BfLP induced CD25 expression on the surfaces of CHO/CD14/TLR2 cells in a dose-dependent manner, whereas BfLP did not induce it on the surfaces of both CHO/CD14 and CHO/CD14/TLR4 cells (Fig. 4). These results demonstrated that signaling by BfLP was mediated by TLR2. That is, TLR2, but not TLR4, is suggested to function as a receptor for BfLP in GFh and THP-1.

Cytotoxicity of BfLP. We have reported that mycoplasmal lipoproteins exhibit cytotoxicity to THP-1, MOLT-4, HL-60 and Raji (23). Therefore, BfLP was also thought to exhibit cytotoxicity to these cells. Cytotoxicity was assayed by determining the amount of LDH released in cell culture supernatants stimulated with BfLP. The cytotoxicity of BfLP to GFh and THP-1 increased almost in parallel with incubation time up to 10 h (Fig. 5). After a 10-h incubation, BfLP induced 100 % cell death in GFh (Fig. 5 A), and approximately 40 % cell death in THP-1 (Fig. 5 B). The cytotoxicity of BfLP to KB, HL-60 and MOLT-4 were also tested. BfLP did not induce cell death in MOLT-4, but induced in GFh, KB, THP-1 and HL-60 in a dose-dependent manner (Table 1). Cell death is known to be classified into two forms, apoptosis and necrosis, by morphological and biochemical features. However, apoptosis and necrosis cannot be discriminated by the LDH release assay. To investigate whether BfLP induced apoptosis in GFh, KB, THP-1 and HL-60, single-stranded DNA

existed in apoptotic cells were detected by ELISA in these cells (**Fig. 6 A**). It was shown that BfLP induced apoptosis in these cells (**Fig. 6 A**). The activation of caspase-8 in these cells was analyzed because it is a major initiator that can activate downstream effector caspases (45). As shown in **Fig. 6 B**, the cleaved forms of caspase-8 were detected in these cells after a 15-h incubation. No cleaved forms of caspase-8 were found in non-stimulated cells. These results showed that BfLP induced apoptosis mediated by caspase-8 activation in these cells.

Discussion

Evidence has recently been accumulated that lipoproteins and lipopeptides have various biological activities. It has been demonstrated that mycoplasmal lipoproteins activate lymphocytes, monocytes/macrophages and fibroblasts, and the activity resides in the N-terminal lipopeptide moieties (36, 52). We reported that mycoplasmal lipoproteins induced TLR2- and caspases-mediated cell death (23). *E. coli* lipoproteins induce an LPS-like endotoxic response from primary human endothelial cells (39), and *B. burgdorferi* lipoproteins induce pro- and anti-inflammatory cytokine production by monocytes (12). However, less evidence has been obtained on biological activities of periodontopathic bacterial lipoproteins, although protein adhesions from *B. forsythus* were reported to induce proinflammatory cytokines through TLR2 and CD14-transmitted signaling pathways (15).

This study demonstrated that BfLP induced IL-6 production by GFh (**Fig. 2 A**). IL-6 is one of the proinflammatory cytokines, and NF- κ B is one of the transcription factors that regulates the gene expression of IL-6 (28). IL-6 is known to be a multifunctional cytokine that provides signals such as

induction of acute phase proteins in liver cells, cytotoxic T-cell differentiation, the growth of myeloma/plasmacytoma cells, Ig-induction in B cells, and so on (25). IL-6 also induces bone resorption by osteoclast formation with soluble IL-6 receptor (27), which is characteristic in periodontal disease. In addition, BfLP also induced TNF- α production by THP-1 (**Fig. 3 A**). As
5 TNF- α can trigger the release of enzymes that degrade the extracellular matrix (61), TNF- α might also be one of the important candidates as a causative mediator of tissue destruction. Okada *et al.* (40) demonstrated local accumulation of activated lymphocytes, macrophages and neutrophils in the inflamed gingival tissue. Therefore, it is considered that BfLP may be involved in the progress of periodontal disease by activating GFh and monocytes/macrophages to induce the production of
10 proinflammatory cytokines.

LPS is a representative endotoxin, which exists in the outer cell membrane of Gram-negative bacteria. The lipid A moiety of LPS is a powerful agonist for cells with appropriate receptors. Polymyxin B neutralizes many biological activities of LPS by binding to lipid A (35). Although it has not clearly been shown that polymyxin B abrogates the activities of *B. forsythus* LPS,
15 polymyxin B seems to be capable of reducing its activities for the reason as follows. Kobayashi *et al.* (26) reported that the interferon- γ production-inducing activity of *B. forsythus* cells, which might be attributed to LPS, was partially reduced by the addition of polymyxin B when peripheral blood mononuclear cells were stimulated with *B. forsythus* cells. Therefore, the finding that polymyxin B had no effect on the IL-6 production-inducing activity of BfLP toward GFh(**Fig. 2 A**) suggests that
20 LPS was not involved in the expression of activity of BfLP. TLRs are known to play important roles in innate immune defense recognizing PAMPs as described above. The response of TLR4 to LPS was strongly dependent on soluble CD14 and LPS-binding protein, which are included in serum

(37). In this study, GFh and THP-1 were stimulated with BfLP in a serum-free medium. BfLP was dissolved in PBS containing OG, which neutralizes the stimulatory effect of LPS on human macrophages (20). Henrich *et al.* (20) reported that the ability of LPS to activate macrophages were suppressed by OG at concentration range of 0.25 to 2.5 mM, and LPS at concentrations causing maximal stimulation of macrophages could be completely neutralized by non-toxic concentration of OG. In this study, the cells were incubated with various concentrations of BfLP containing 1 mM OG. Taken together, it is considered that the activity of BfLP was not attributed to a small amount of LPS included in BfLP.

TLR2 is considered to be a receptor for various bacterial components, such as peptidoglycan, lipoprotein, and lipoarabinomannan (31, 48, 56, 64). Takeuchi *et al.* (57) have reported that co-expression of TLR2 and 6 was absolutely required to respond mycoplasmal 2-kDa macrophage-activating lipopeptide. The amino group of the N-terminal cysteine of mycoplasmal lipoproteins or lipopeptides is free, whereas those of lipoproteins of many bacteria are bound to some fatty acid. However, Bulut *et al.* (3) have also reported that there was a functional interaction between TLR2 and 6 in the cellular response to outer surface protein A lipoprotein of *B. burgdorferi*. In contrast to this, Takeuchi *et al.* (58) recently reported that TLR1 interacts with TLR2 to recognize the lipid configuration of microbial lipoproteins. In the present study, it was demonstrated that BfLP induced CD25 expression on the surfaces of CHO/CD14/TLR2 cells in a dose-dependent manner, but did not on the surfaces of CHO/CD14 and CHO/CD14/TLR4 cells (**Fig. 4**). These results suggested that TLR2, but not TLR4, functions as a receptor for BfLP. Since BfLP would be triacylated lipoprotein, endogenous TLR1 in CHO transfectant might associate with TLR2 and recognize BfLP. It still remains unknown that TLR1 or 6 is also involved in the signal transduction

of BfLP.

The present study indicated that BfLP induced apoptosis in oral epithelial cells, GFh and monocytes/macrophages but did not induce in T-leukemia cells. BfLP may play an important role in exacerbation of periodontal disease because of its cytotoxicity. Epithelial cells are known to be the initial site of host invasion by bacterial pathogens. In periodontal disease, some periodontopathic bacteria are known to be capable of invading oral epithelial cells (30). BfLP may help a further development of inflammation and bacterial invasion to periodontal tissue because of its ability to induce epithelial cell death.

It is known that exudation of monocytes/macrophages are one of the characteristic feature of periodontal disease (42). BfLP will exacerbate periodontal disease by killing monocytes/macrophages in gingival area, because they play important roles in antibacterial defense, immune response, wound healing and remodeling, etc.

For all the reasons stated above, it can be thought that BfLP may be involved in progress of periodontal disease as follows. As a result of inducing proinflammatory cytokines production by GFh and monocytes/macrophages, BfLP may indirectly destroy periodontal tissue by inducing bone resorption and release of enzymes that degrade the extracellular matrix. When BfLP accumulates in an inflamed area, then it would directly destroy periodontal tissue, help periodontopathic bacteria invading to host cells, and exacerbate periodontal disease by killing epithelial cells, monocytes/macrophages, and GFh.

Although it still remains to be elucidated that detailed mechanism of cytokine production and cell death induced by BfLP, the present findings may give an insight into etiological roles of *B. forsythus* in oral infections, especially periodontal disease. LPS of oral gram-negative bacteria,

suspected to be pathogens in periodontal diseases, are well known to induce proinflammatory cytokines such as IL-1, IL-6 and IL-8 (54, 59) and upregulate the expression of adhesion molecules in GFh (18). Thus, oral gram-negative bacteria possess the pro-inflammatory capacities of these two distinct membrane constituents, LPS and LP. That is, our findings also suggest the possibility that
5 LP and LPS reveal additive/synergistic influences on development of periodontal diseases.

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FIGURE LEGENDS

Fig. 1. IR spectra of Pam3-cysteine (A), FSL-1 (B), and BfLP (C). Arrows 1 and 2 are signals showing the presence of fatty acid alkyl chains and typical ester bonds, respectively.

5 Fig. 2. (A) IL-6 production-inducing activity of BfLP toward GFh and the effect of polymyxin B on the activity. GFh reached confluency in a 96-well flat-bottomed plate were pretreated with polymyxin B (○: 0 U/ml, △: 500 U/ml, □: 1,000 U/ml), and stimulated with BfLP for 15 h. IL-6 produced in the culture supernatants were determined by ELISA. Results are expressed as the means ± SD of three determinations. (B) Detection of NF-κB activation in GFh stimulated with
10 BfLP. GFh reached confluency in 6-cm dish were stimulated with BfLP (0.5 μg/ml) and whole cell extracts were prepared. The activation of NF-κB was examined by using TransAM NF-κB p50 transcription factor assay kit (Active Motif, Inc., Carlsbad, CA) as specified by the manufacturer, where activated NF-κB was detected by antibodies which only detect NF-κB p50 activated and bound to its target DNA containing NF-κB consensus site. Results are expressed as the means ± SD
15 of three determinations.

Fig. 3. (A) TNF-α production-inducing activity of BfLP toward THP-1. A 200-μl volume of the cell suspension (5×10^6 /ml) was added into 96-well round-bottomed plate, followed by the stimulation with BfLP for 15 h. TNF-α produced in the culture supernatants were determined by ELISA.
20 Results are expressed as the means ± SD of three determinations. (B) Detection of NF-κB activation in THP-1 stimulated with BfLP. THP-1 in 6-well plate (5×10^6 /well) were stimulated with BfLP (0.5 μg/ml) and whole cell extracts were prepared. The activation of NF-κB was examined by using

TransAM NF- κ B p50 transcription factor assay kit (Active Motif, Inc., Carlsbad, CA) as specified by the manufacturer, where activated NF- κ B was detected by antibodies which only detect NF- κ B p50 activated and bound to its target DNA containing NF- κ B consensus site. Results are expressed as the means \pm SD of three determinations.

5

Fig. 4. Flow cytometric analysis of CD25 expression on the cell surface of CHO transfectants stimulated with BfLP. CHO/CD14/TLR2, CHO/CD14/TLR4 and CHO/CD14 reporter cell lines were stimulated with the BfLP for 15 h. Each of the reporter line contains a stably transfected ELAM-CD25 reporter gene and expresses human CD25 on their surface as a consequence of
10 NF- κ B activation. Stimulated cells were stained with a PE-labeled anti-CD25 mAb and subjected to flow cytometry analysis to measure the expression of CD25. Cells stimulated with 10 mM OG/PBS were used as controls.

Fig. 5. Time course of BfLP cytotoxicity to GFh (A) and THP-1 (B). GFh and THP-1 were
15 stimulated with BfLP (\circ : 6 μ g/ml, Δ : 12 μ g/ml). LDH release in the culture supernatant was measured and the cytotoxicity (%) was calculated. Results are expressed as the means \pm SD of three determinations.

Fig. 6. (A) Detection of single-stranded DNA existed in apoptotic cells by ELISA. Each of the cell
20 suspensions was prepared in 96-well flat-bottomed plate as specified by the manufacturer. Then the cells were stimulated with BfLP and ELISA was carried out. Staurosporine (1 μ M) was used as a control to induce apoptosis. Results are expressed as the means \pm SD of three determinations. (B)

Western blotting analysis of caspase-8. GFh and KB reached confluency, and 1×10^6 cells of THP-1 and HL-60 were stimulated with BfLP. Each of the cells was stimulated with 0, 6 and 12 $\mu\text{g/ml}$ of BfLP and staurosporine (1 μM).

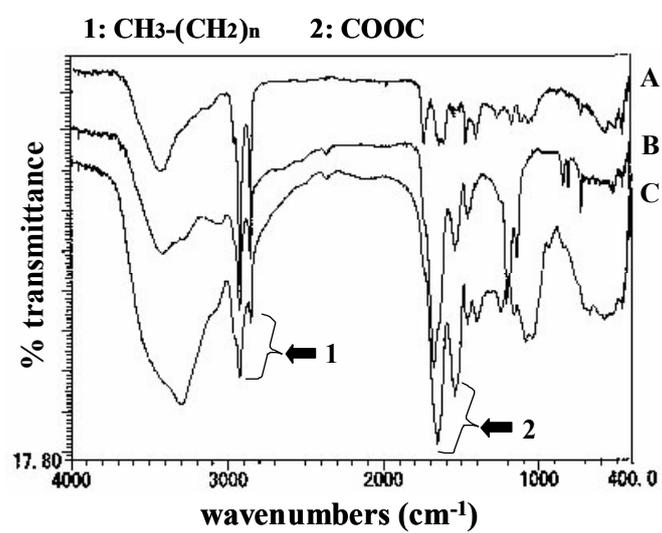


Fig. 1.

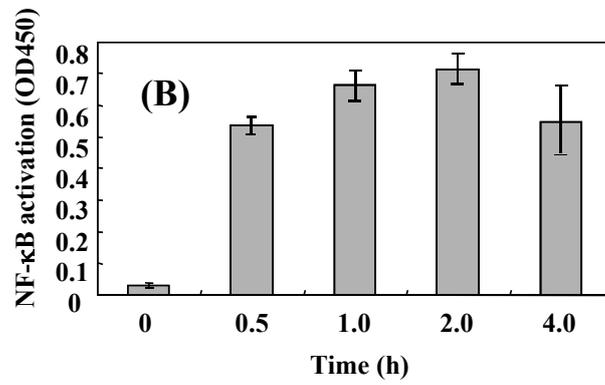
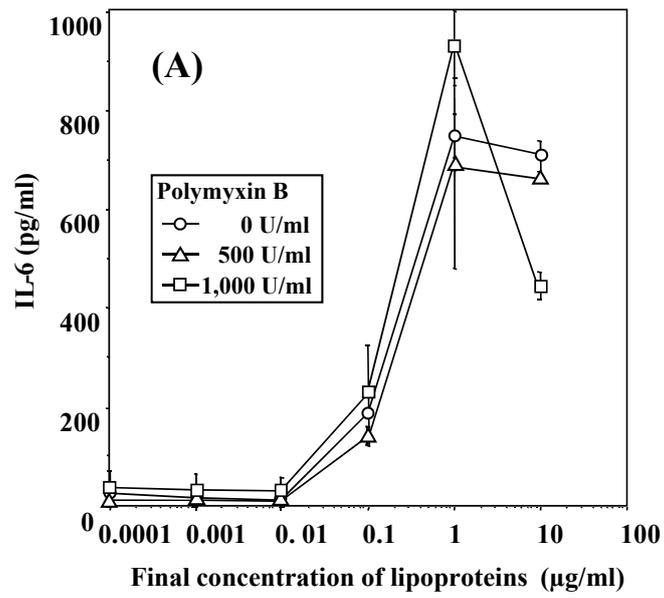


Fig. 2.

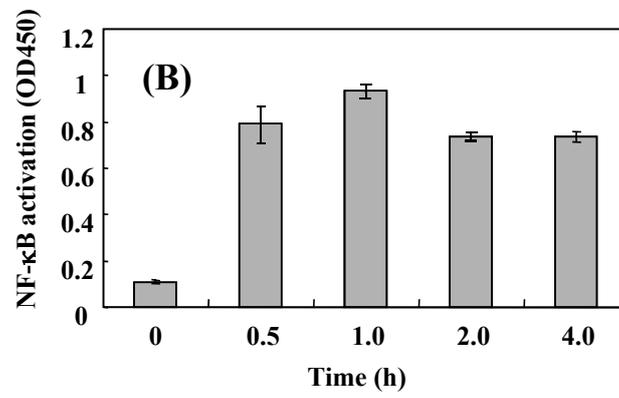
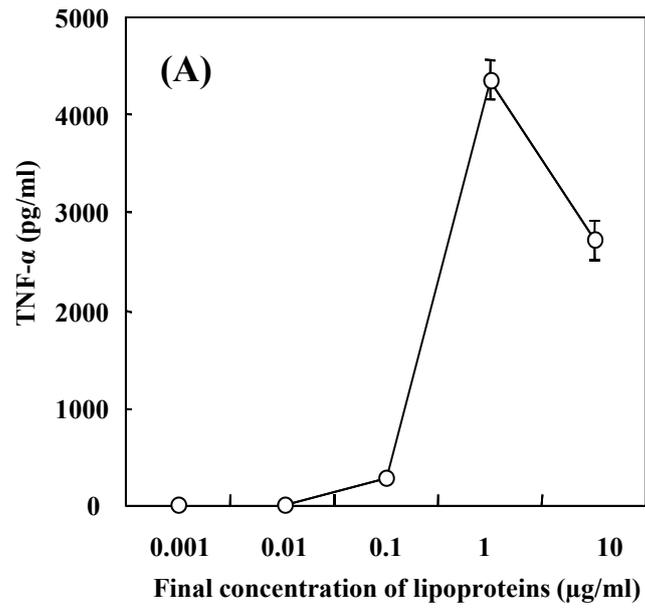


Fig. 3.

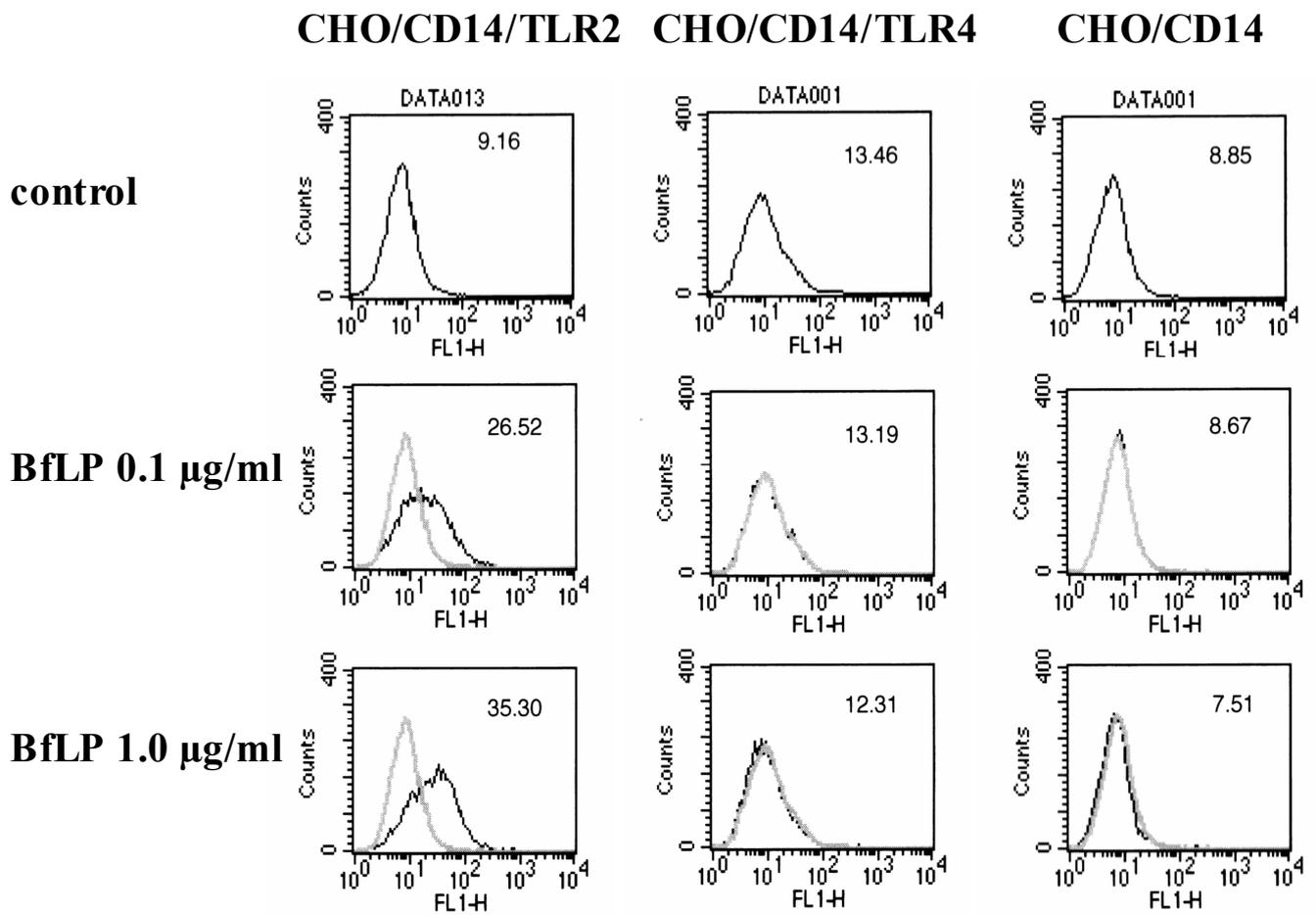


Fig. 4.

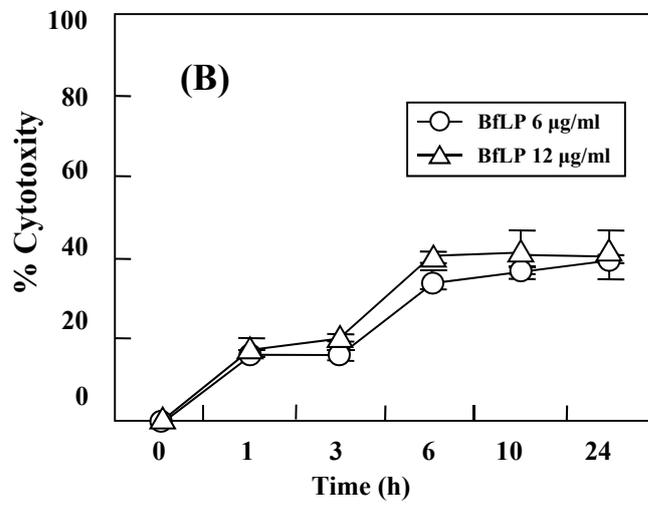
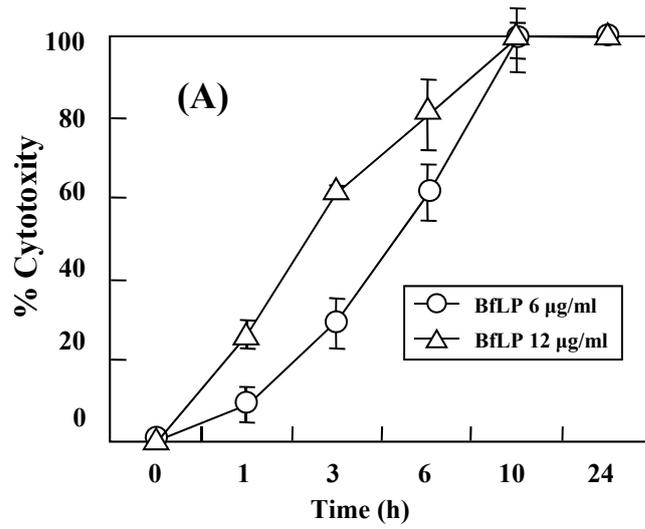
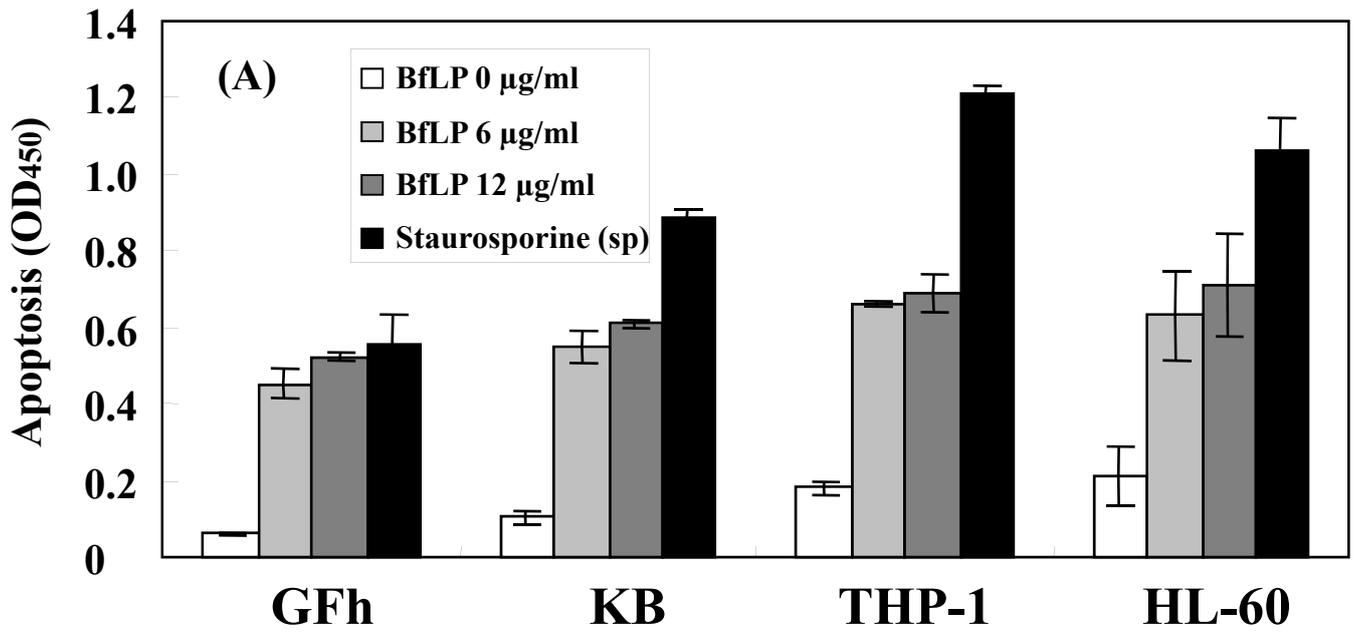


Fig. 5.

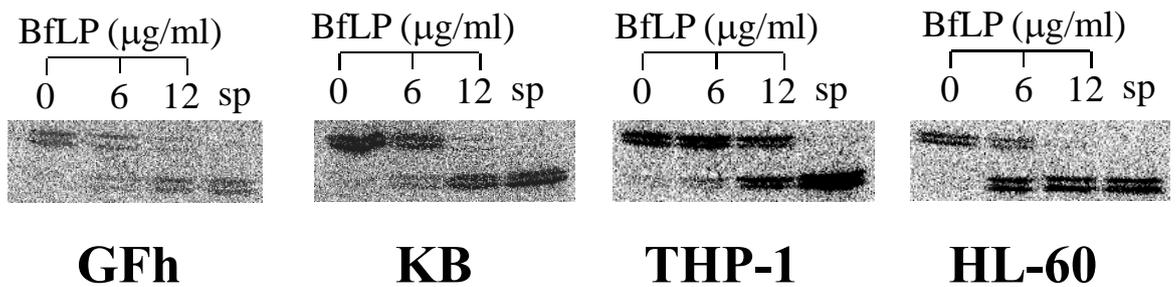
Table 1. The cytotoxicity of BfLP to various cells

Cells	% cytotoxicity to the cells incubated with BfLP at:	
	6 µg/ml	12 µg/ml
GFh	100	100
KB	80.6 ± 1.9	84.8 ± 1.0
THP-1	39.8 ± 1.1	46.1 ± 3.9
HL-60	28.5 ± 3.4	52.4 ± 0.7
MOLT-4	1.5 ± 1.2	1.6 ± 1.4

The cells were stimulated with BfLP for 15 h. LDH release in the culture supernatant was measured and the cytotoxicity (%) was calculated. Results are expressed as the means ± SD of three determinations.



(B) Caspase 8 activation



sp:staurosporine

Fig. 6.