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Biological Activities of *Bacteroides forsythus* Lipoproteins and Their Possible Pathological Roles in Periodontal Disease

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Bacteroides forsythus **is a gram-negative, anaerobic, fusiform bacterium and is considered to be an etiological agent in periodontal disease. A lipoprotein fraction prepared from *B. forsythus* cells by Triton X-114 phase separation (BfLP) activated human gingival fibroblasts and a human monocytic cell line, THP-1, to induce interleukin-6 production and tumor necrosis factor alpha production. BfLP was found to be capable of inducing nuclear factor-κB translocation in human gingival fibroblasts and THP-1 cells. By using Chinese hamster ovary K1 cells transfected with Toll-like receptor genes together with a nuclear factor-κB-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 4. BfLP induced apoptotic cell death in human gingival fibroblasts, KB cells (an oral epithelial cell line), HL-60 cells (a human myeloid leukemia cell line), and THP-1 cells but not in MOLT4 cells (a T-cell 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 plays some etiological roles in oral infections, especially periodontal disease, by induction of cell activation or apoptosis.**

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). Many 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 its presence in subgingival flora has been significantly associated with severe periodontal disease (13, 14, 63). 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 a proteinous factor(s) from this bacterium is 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 (3, 5, 38, 39, 52). We have studied the 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 of 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

Chemicals. The mycoplasmal lipopeptide FSL-1, which is speculated to be the N-terminal lipopeptide moiety of an LP responsible for activating human gingival fibroblasts (GFh) purified from *Mycoplasma salivarium* cells, was synthesized with the structure *S*-(2,3-bispalmitoyloxypropyl)-cysteine-GDPKHSPKSF as described previously (52). *S*-(2,3-bispalmitoyloxypropyl)-*N*-palmitoyl-cysteine (Pam3-cysteine), which is the N-terminal structure of *E. coli* murein LP, 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.

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 of hemin per ml, 0.5 μg of vitamin K per ml, 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%

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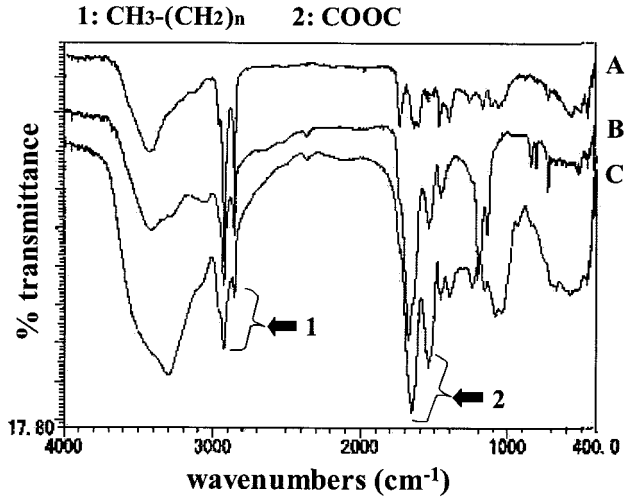

FIG. 1. IR spectra of Pam3-cysteine (A), FSL-1 (B), and BfLP (C). Arrows labeled 1 and 2 indicate signals showing the presence of fatty acid alkyl chains and typical ester bonds, respectively.

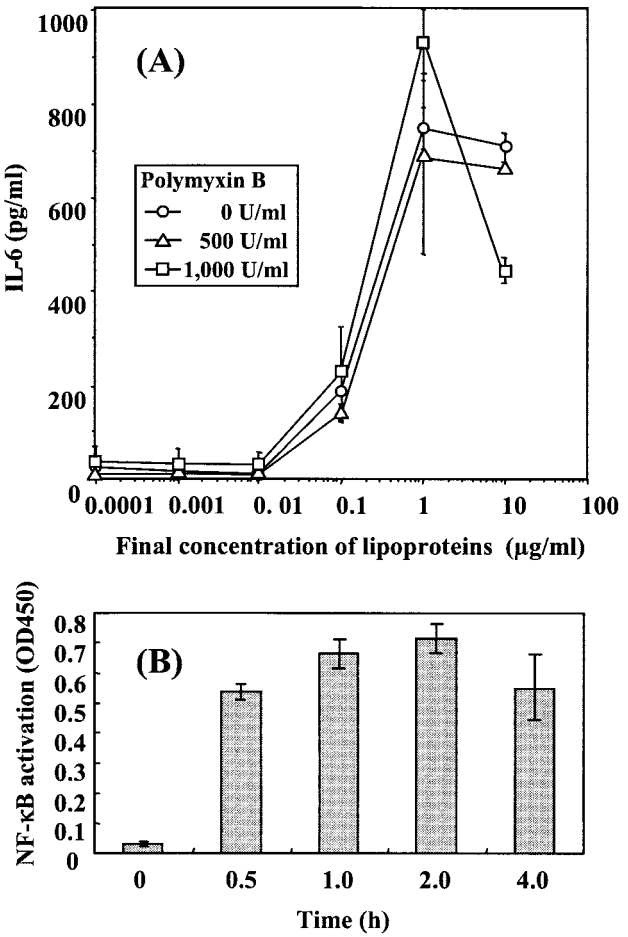

FIG. 2. (A) IL-6 production-inducing activity of BfLP toward GFh and the effect of polymyxin B on the activity. GFh at confluency in a 96-well flat-bottom plate were pretreated with polymyxin B and stimulated with BfLP for 15 h. IL-6 production in the culture supernatants was determined by ELISA. Results are expressed as the means and standard deviations of three determinations. (B) Detection of NF-κB activation in GFh stimulated with BfLP. GFh at confluency in a 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 the TransAM NF-κB p50 transcription factor assay kit as specified by the manufacturer, where activated NF-κB was detected by antibodies which detect only NF-κB p50 activated and bound to its target DNA containing the NF-κB consensus site. Results are expressed as the means and standard deviations of three determinations. OD450, optical density at 450 nm.

(vol/vol) fetal bovine serum (FBS; Cansera International Inc., Ontario, Canada). *B. forsythus* cells grown in the broth under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) were harvested by centrifugation at 8,000 × *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. GFh were prepared and cultured as described previously (9). An oral epithelial cell line, KB (ATCC CCL-17), was obtained from the 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 U/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 U/ml), and streptomycin (100 μg/ml).

Preparation of LP by TX-114 phase separation. The cell suspension was sonicated and treated with Triton X-114 (TX-114) to extract membrane LP by 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 × *g* at 4°C for 10 min 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 × *g* for 5 min. The upper, aqueous phase was discarded. To the TX-114 phase, 0.9 ml of TS buffer was added, and the mixture was treated twice in the same way as described above. LP were precipitated from the TX-114 phase by adding 9 volumes of methanol. The LP 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).

The protein concentration of BfLP was determined by the method of Dully and Grieve (10). The endotoxin concentration was determined by using Endospecy (Seikagaku Corp., Tokyo, Japan).

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

Cytokine assay. GFh were cultured in DME(+) in a 96-well flat-bottom plate, and the culture medium was exchanged with DME base medium when the cells reached confluency. Then the GFh were incubated for 6 h with various concentrations of BfLP. THP-1 cells (10^6) in RPMI 1640 medium were added to a triplicate set of wells of a 96-well round-bottom plate and incubated for 6 h with various concentrations of BfLP. Each of the cell culture supernatants was collected by centrifugation at 400 × *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 K1 (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 clonal line has been cotransfected with CD14 and a nuclear factor (NF)-κB-dependent reporter plasmid that drives the expression of surface CD25 antigen resulting from lipopolysaccharide (LPS)-, tumor necrosis factor alpha (TNF-α)-, or interleukin-1β (IL-1β)-induced NF-κB translocation. The cDNAs for human toll-like receptors 2 and 4 (TLR2 and TLR4) were gifts from Carsten Kirschning and Mike Rothe (Tularik, South San Francisco, Calif.) and were cloned into the vector pFLAG as described previously (24). Stable expression of TLRs was obtained by cotransfection of these epitope-tagged plasmids with pcDNA3 (Invitrogen, San Diego, Calif.) into CHO/CD14 reporter cells. After selection in G418 (1 mg/ml), clonal cell lines expressing high levels of human TLR2 or TLR4 were derived using fluorescence-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 10^5 cells per well. After overnight incubation, the cells were stimulated for 15 h with BfLP. They were detached from the surface

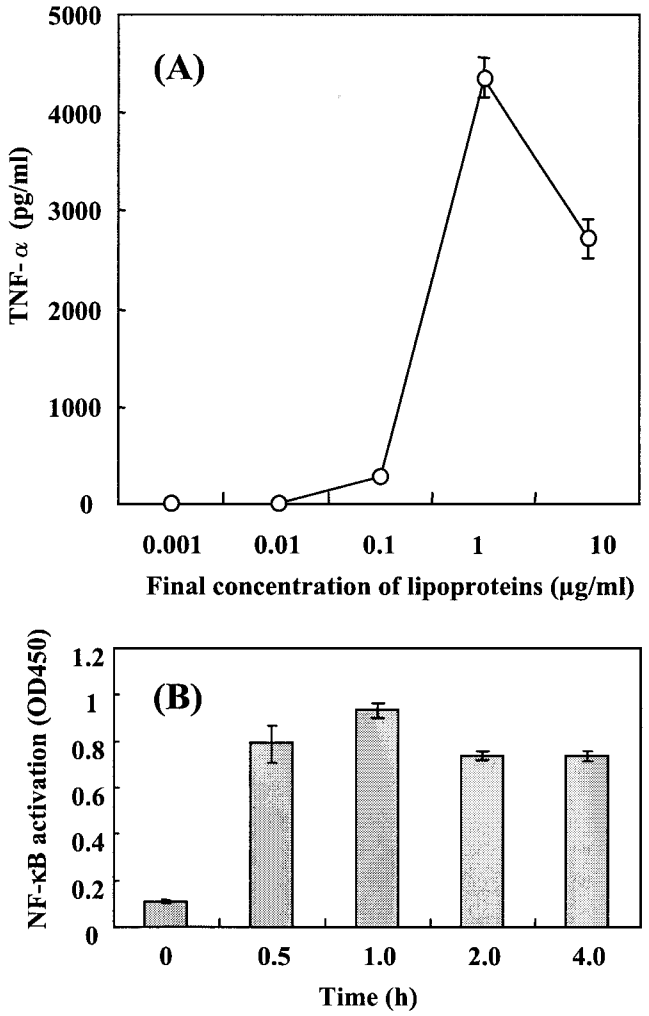

FIG. 3. (A) TNF-α production-inducing activity of BfLP toward THP-1 cells. A 200-μl volume of the cell suspension (5×10^6/ml) was added to a 96-well round-bottom plate, and the cells were stimulated with BfLP for 15 h. TNF-α production in the culture supernatants was determined by ELISA. Results are expressed as the means and standard deviations of three determinations. (B) Detection of NF-κB activation in THP-1 cells stimulated with BfLP. THP-1 cells in a six-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 the TransAM NF-κB p50 transcription factor assay kit as specified by the manufacturer, where activated NF-κB was detected by antibodies which detect only NF-κB p50 activated and bound to its target DNA containing the NF-κB consensus site. Results are expressed as the means and standard deviations of three determinations. OD450, optical density at 450 nm.

with trypsin-EDTA and assessed by flow microfluorometry for the presence of surface CD25 exactly as described previously (8).

NF-κB activation. The activation of NF-κB was examined by using the TransAM NF-κB p50 transcription factor assay kit (Active Motif, Inc., Carlsbad, Calif.) as specified by the manufacturer. GFh and THP-1 cells were stimulated with BfLP at 37°C for 0, 1, 2, or 4 h and lysed in buffer containing 20 mM HEPES (pH 7.5), 350 mM NaCl, 20% glycerol, 1% Igepal-CA630, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 5 mM dithiothreitol, and protease inhibitor cocktail to prepare the whole-cell extracts. Then activated NF-κB was detected by antibodies which detect only NF-κB p50 activated and bound to its target DNA containing the NF-κB consensus site.

Cytotoxicity assay. Cytotoxicity was assayed as described previously (22). A 100-μl cell suspension of MOLT-4 (1×10^4), THP-1 (1.5×10^4), or HL-60 (2×10^4) cells in RPMI 1640 medium was added to a triplicate set of wells of a 96-well round-bottom plate and incubated with BfLP (6 or 12 μg/ml) for 15 h. GFh or KB cells were cultured in DME(+) in a 96-well flat-bottom 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 μg/ml) for 15 h.

Each of these culture supernatants was collected by centrifugation at $400 \times g$ for 10 min. A cytoplasmic enzyme, lactate dehydrogenase (LDH), released in the culture supernatant, was then colorimetrically measured by using a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega Co., Madison, Wis.). The percent cytotoxicity was expressed as 100 × (experimental LDH release − control LDH release)/(maximum LDH release − control LDH release), 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 cells was determined by using an ApoStrand AK-120 ELISA apoptosis detection kit (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-stranded 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 cells were incubated with BfLP as described above. Their cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrically transferred to nitrocellulose membranes. Capsase-8 activation was detected by an anti-caspase-8 MAb (Cell Signaling Technology Inc., Beverly, Mass.) followed by peroxidase-conjugated goat anti-mouse immunoglobulin G (Seikagaku Co., Tokyo, Japan). The MAb was able to detect both the 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, N.J.).

RESULTS

Characterization of BfLP. Hantke and Braun (16) reported that two ester-bound fatty acids attach to the cysteine residue at the N-terminal end of the polypeptide chain and the other fatty acid is bound as an amide to the N-terminal group in bacterial LP. Therefore, it was thought that fatty acid chains and typical ester bonds would be detected in BfLP. BfLP was subjected to IR spectroscopy to detect the characteristic signals of these moieties, and Pam3-cysteine was used as a standard. The IR spectra of BfLP, FSL-1, and Pam_3-cysteine exhibited signals at about 2,900 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-bound fatty acids.

We investigated the possibility that LPS with multiple biological activities was contaminating the 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 cells. Our previous study (9) demonstrated that mycoplasmal LP are capable of activating GFh to induce IL-6 and IL-8 production and of 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. 2A). However, it is possible that the activity is due to a small amount of LPS included in BfLP, as described above. To rule out this possibility, the effect of

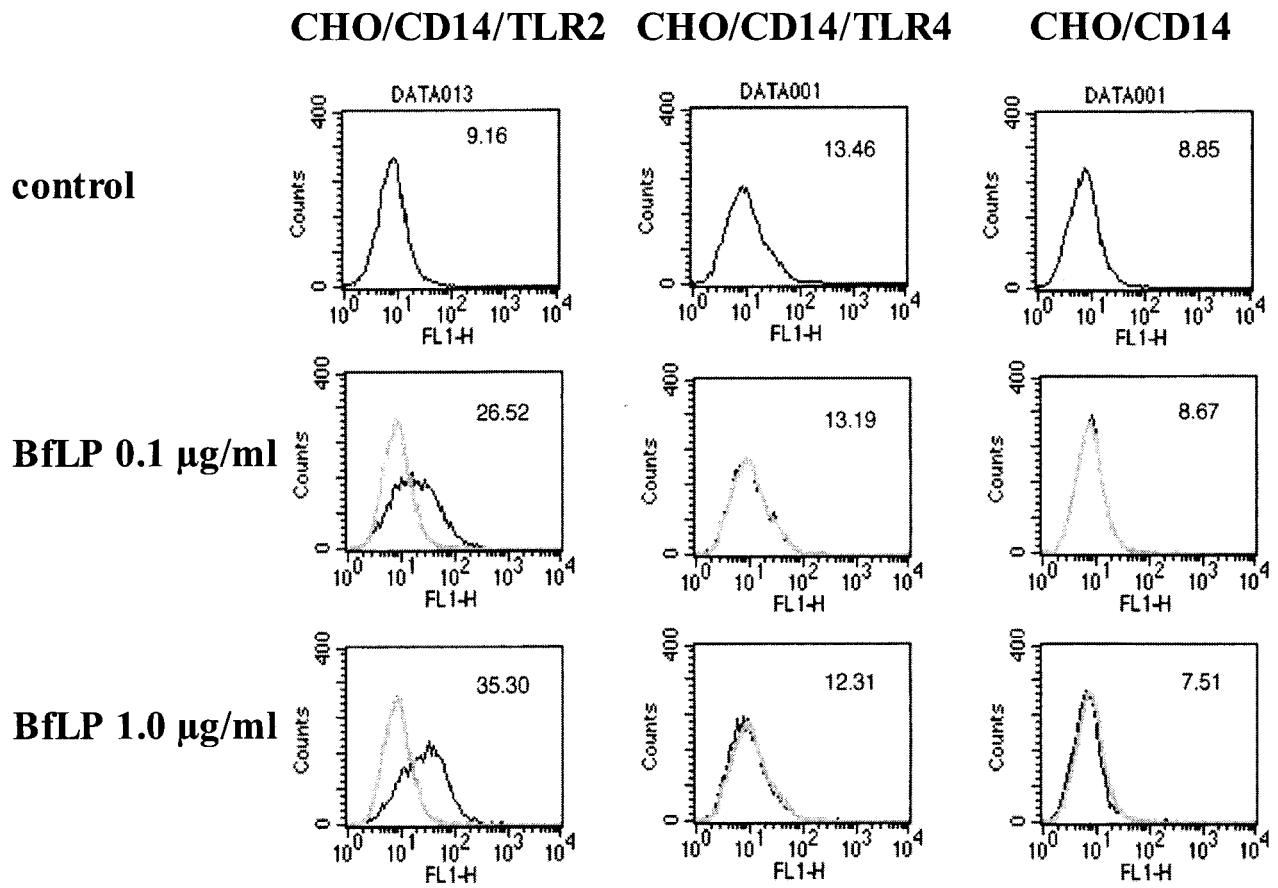

FIG. 4. Flow cytometric analysis of CD25 expression on the cell surface of CHO transfectants stimulated with BfLP. CHO/CD14/TLR2, CHO/CD14/TLR4, and CHO/CD14 reporter cell lines were stimulated with BfLP for 15 h. Each of the reporter line contains a stably transfected ELAM-CD25 reporter gene and expresses human CD25 on its surface as a consequence of NF-κB activation. Stimulated cells were stained with a phycoerythrin-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.

polymyxin B on the IL-6 production-inducing activity of BfLP was investigated, because polymyxin B is known to inhibit the biological activities of LPS (35). Polymyxin B had no effect on the activity of BfLP (Fig. 2A). In addition, the effect of LP lipase on the IL-6 production-inducing activity of BfLP was also investigated, since LP lipase abrogated the ability of mycoplasmal LP to activate GFh (9). LP 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 LP in BfLP, but not to LPS. In response to 10 μg of BfLP per ml, the amount of IL-6 produced appears diminished in the presence of 1,000 U of polymyxin B per ml compared with that in the presence of 500 U of polymyxin B or in its absence (Fig. 2A). One possible explanation for this reduction might be that in the presence of 1,000 U of polymyxin B per ml, 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 (2, 7, 62); however, some authors reported that polymyxin B had no effects on keratinocytes until its concentration reached 10,000 U/ml (7).

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

It is well known that mycoplasmal LP are capable of activating human monocytes/macrophages to induce IL-1β and TNF-α production (9). Therefore, the activation of THP-1 cells by BfLP stimulation was also investigated. BfLP was able to activate THP-1 cells to induce TNF-α production in a dose-dependent manner, and NF-κB translocation was also induced in THP-1 cells (Fig. 3). These data indicated that BfLP induced both NF-κB 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) first reported that TLRs play important roles the in innate immune system. TLRs recognize pathogen-associated molecular patterns 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 these recognize specific microbial products (29, 32, 55). TLR4, CD14, MD-2, and LPS-binding protein are involved in signaling by LPS (6, 37, 43, 44, 53). TLR2 has been reported to recognize microbial LP, peptidoglycan, lipoteichoic acid (48, 56, 64), and some other com-

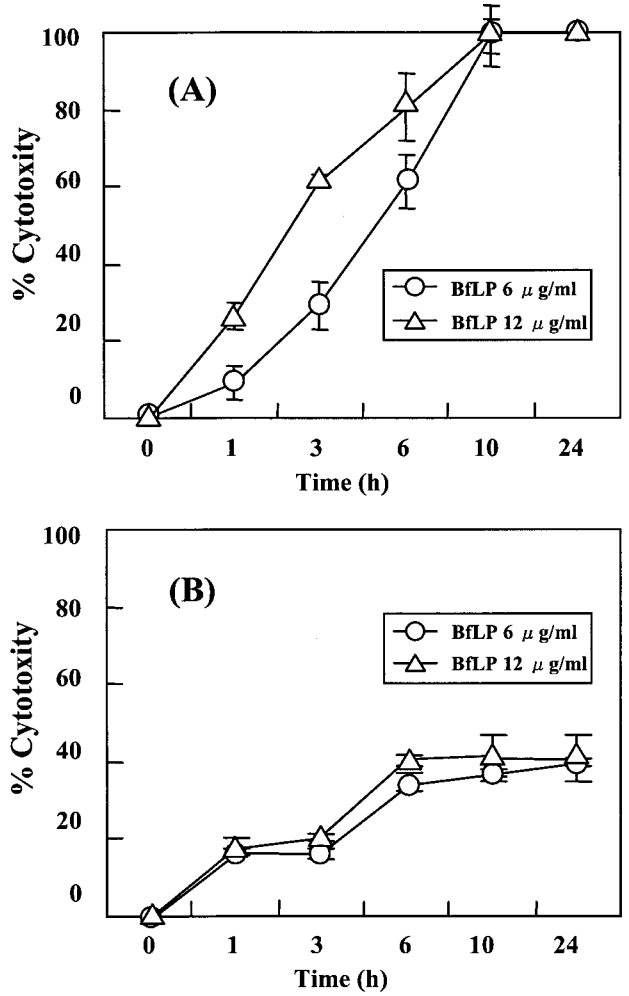

FIG. 5. Time course of BfLP cytotoxicity to GFh (A) and THP-1 (B) cells. GFh and THP-1 cells were stimulated with BfLP, LDH release in the culture supernatant was measured, and the percent cytotoxicity was calculated. Results are expressed as the means and standard deviations of three determinations.

ponents. 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.

TABLE 1. Cytotoxicity of BfLP to various cells[a]

Cell line	% Cytotoxicity to 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

[a] The cells were stimulated with BfLP for 15 h. LDH release in the culture supernatant was measured, and the percent cytotoxicity was calculated. Results are expressed as the means ± standard deviations of three determinations.

Cytotoxicity of BfLP. We have reported that mycoplasmal LP exhibit cytotoxicity to THP-1, MOLT-4, HL-60, and Raji cells (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 cells increased almost in parallel with the incubation time up to 10 h (Fig. 5). After a 10-h incubation, BfLP induced 100% cell death in GFh (Fig. 5A) and approximately 40% cell death in THP-1 cells (Fig. 5B). The cytotoxicity of BfLP to KB, HL-60, and MOLT-4 cells was also tested. BfLP did not induce cell death in MOLT-4 cells but induced cell death in GFh, KB, THP-1, and HL-60 cells in a dose-dependent manner (Table 1). Cell death is known to be classified into two forms, apoptosis and necrosis, on the basis of 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 cells, the single-stranded DNA present in apoptotic cells was detected by ELISA in these cells (Fig. 6A). It was shown that BfLP induced apoptosis in these cells (Fig. 6A). 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. 6B, 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 nonstimulated cells. These results showed that BfLP induced apoptosis mediated by caspase-8 activation in these cells.

DISCUSSION

Evidence has recently been accumulated that LP and lipopeptides have various biological activities. It has been demonstrated that mycoplasmal LP activate lymphocytes, monocytes/macrophages, and fibroblasts and that the activity resides in the N-terminal lipopeptide moieties (36, 52). We reported that mycoplasmal LP induced TLR2- and caspase-mediated cell death (23). *E. coli* LP induce an LPS-like endotoxic response from primary human endothelial cells (39), and *Borrelia burgdorferi* LP induce pro- and anti-inflammatory cytokine production by monocytes (12). However, less evidence has been obtained about biological activities of periodontopathic bacterial LP, 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. 2A). IL-6 is one of the proinflammatory cytokines, and NF-κB is one of the transcription factors that regulates its gene expression (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, and Ig induction in B cells (25). IL-6 also induces bone resorption by osteoclast formation with soluble IL-6 receptor (27), which is

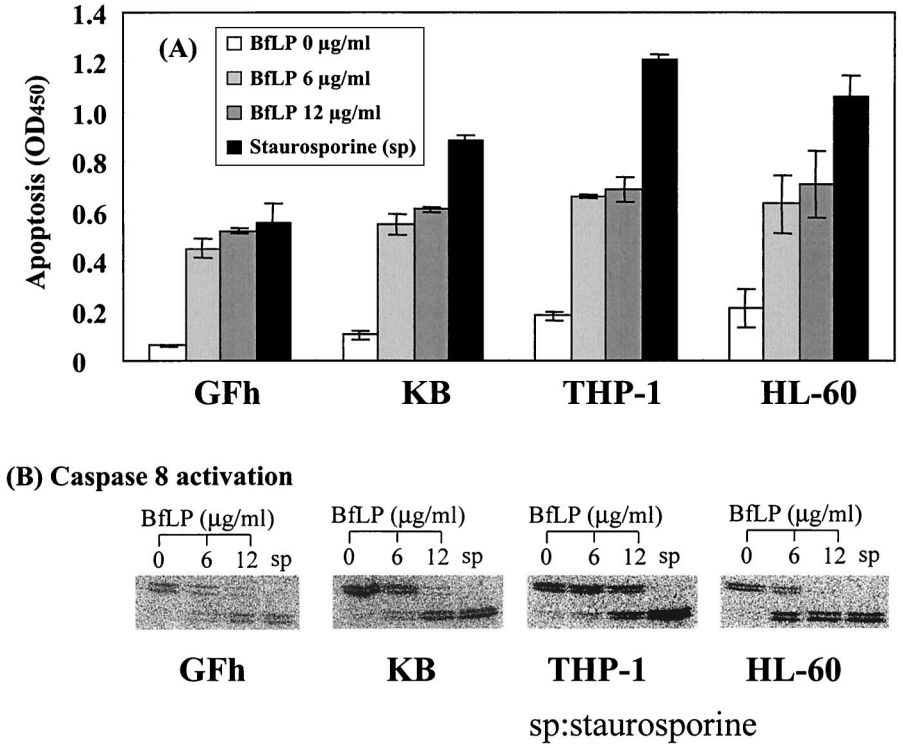

FIG. 6. (A) ELISA detection of single-stranded DNA present in apoptotic cells. Each of the cell suspensions was prepared in a 96-well flat-bottom plate as specified by the manufacturer. Then the cells were stimulated with BfLP, and ELISA was carried out. Staurosporine (1 μM) was used as a control to induce apoptosis. Results are expressed as the means and standard deviations of three determinations. (B) Western blot analysis of caspase-8. GFh and KB cells reached confluency, and 10^6 THP-1 and HL-60 cells were stimulated with BfLP. Each of the cells was stimulated with 0, 6, and 12 μg of BfLP per ml and staurosporine (1 μM). OD_{450}, optical density at 450 nm.

characteristic of periodontal disease. In addition, BfLP induces TNF-α production by THP-1 (Fig. 3A). Since 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 progression of periodontal disease by activating GFh and monocytes/macrophages to induce the production of 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, it seems to be capable of reducing the activities of LPS for the reason as follows. Kobayashi et al. (26) reported that the gamma interferon 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 in GFh (Fig. 2A) suggests that LPS was not involved in the expression of BfLP activity. TLRs are known to play important roles in innate immune defense-recognizing pathogen-associated molecular patterns 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 OG/PBS, which neutralizes the stimulatory effect of LPS on human macrophages (20). Henrich et al. (20) reported that the ability of LPS to activate macrophages was suppressed by OG in concentration range of 0.25 to 2.5 mM, and LPS at concentrations causing maximal stimulation of macrophages could be completely neutralized by nontoxic concentrations of OG. In this study, the cells were incubated with various concentrations of BfLP containing 1 mM OG. Taking these results 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, LP, and lipoarabinomannan (31, 48, 56, 64). Takeuchi et al. (57) have reported that coexpression of TLR2 and TLR6 was absolutely required to respond to mycoplasmal 2-kDa macrophage-activating LP. The amino group of the N-terminal cysteine of mycoplasmal LP or lipopeptides is free, whereas those of LP of many bacteria are bound to some fatty acid. However, Bulut et al. (4) have also reported that there was a functional interaction between TLR2 and TLR6 in the cellular response to outer surface protein A LP of *Borrelia burgdorferi.* In contrast, Takeuchi et al. (58) recently reported that TLR1 interacts with TLR2 to

recognize the lipid configuration of microbial LP. 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 do so 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 a triacylated LP, endogenous TLR1 in CHO transfectants might associate with TLR2 and recognize BfLP. It still remains unknown whether TLR1 or TLR6 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 apoptosis in T-cell leukemia cells. BfLP may play an important role in the exacerbation of periodontal disease because of its cytotoxicity. Epithelial cells are known to be the initial site of host invasion by bacterial pathogens. Some periodontopathic bacteria are known to be capable of invading oral epithelial cells during periodental disease (30). BfLP may encourage further development of inflammation and bacterial invasion of periodontal tissue because of its ability to induce epithelial cell death.

It is known that exudation of monocytes/macrophages is one of the characteristic features 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, remodeling, etc.

For all the reasons stated above, it may be concluded that BfLP may be involved in the progress of periodontal disease as follows. As a result of inducing proinflammatory cytokine 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, it would directly destroy periodontal tissue, help periodontopathic bacteria invade host cells, and exacerbate periodontal disease by killing epithelial cells, monocytes/macrophages, and GFh.

Although the detailed mechanism of cytokine production and cell death induced by BfLP still remains to be elucidated, the present findings may give an insight into the 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 proinflammatory capacities of these two distinct membrane constituents, LPS and LP. That is, our findings also suggest the possibility that LP and LPS have additive or synergistic influences on development of periodontal disease.

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