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Chemical structure and immunobiological activity of *Porphyromonas gingivalis* lipid A

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TABLE OF CONTENTS

1. ABSTRACT

In 1933, Boivin *et al.* extracted an endotoxin from *Salmonella typhimurium* for the first time, after which a variety of chemical and biological studies on endotoxins have been performed. In 1952, the structural and functional properties of endotoxic lipopolysaccharide (LPS), extracted by a hot phenol and water method devised by Westphal *et al.*, were reported, which led to a number of studies of Gram-negative bacteria in regards to the host defense mechanism. Since 1960, the unique chemical structure and biological activity of *Bacteroides* species LPS have received a great deal of attention, and there is a long history of such studies. In addition, among oral bacterial strains that have received attention as causative periodontopathic bacteria, many have been classified as *Bacteroides* species. In particular, a number of researchers have investigated whether LPS of *Porphyromonas gingivalis* (formerly *Bacteroides gingivalis*), a black-pigmented oral anaerobic rod, is a virulent factor of the bacterium. The active center of the LPS of these *Bacteroides* species, the lipid A molecule, is known to be an active participant in endotoxic activation, though its other biological activities are weak, due to its unique chemical structure and action as an antagonist of LPS. On the other hand, many reports have noted that the LPS of those species activate cells in C3H/HeJ mice, which generally do not respond to LPS. We were the first to reveal the chemical structure of *P. gingivalis* lipid A and, together with other researchers, reported that *P. gingivalis* LPS and its lipid A have activities toward C3H/HeJ mice. Since that time, because of the popularity of Toll-like receptor (TLR) studies, a great deal of evidence has been reported indicating that *P. gingivalis* LPS and its lipid A are ligands that act on TLR2. In order to solve such problems as heterogeneity and contamination of the biologically active components of *P. gingivalis* lipid A, we produced a chemical synthesis counterpart of lipid A and test results indicated it to be a TLR4 agonist. Furthermore, in order to disprove the common belief that *P. gingivalis* LPS and its lipid A are TLR2 ligands, the TLR2-active component contained in a *P. gingivalis* LPS fraction was separated and purified, after which we showed its chemical structure to be a lipoprotein consisting of three fatty acid residues, thus answering a longstanding question regarding *Bacteroides* species LPS. In addition to the field of dentistry, many studies based on the misconception of "TLR2-active LPS/lipid A" still exist in the field of innate immunity. Based on the history of studies of ligands acting on TLR4, *Bacteroides* species LPS findings were reviewed and are presented here. In particular, we investigated *P. gingivalis* LPS and its lipid A.

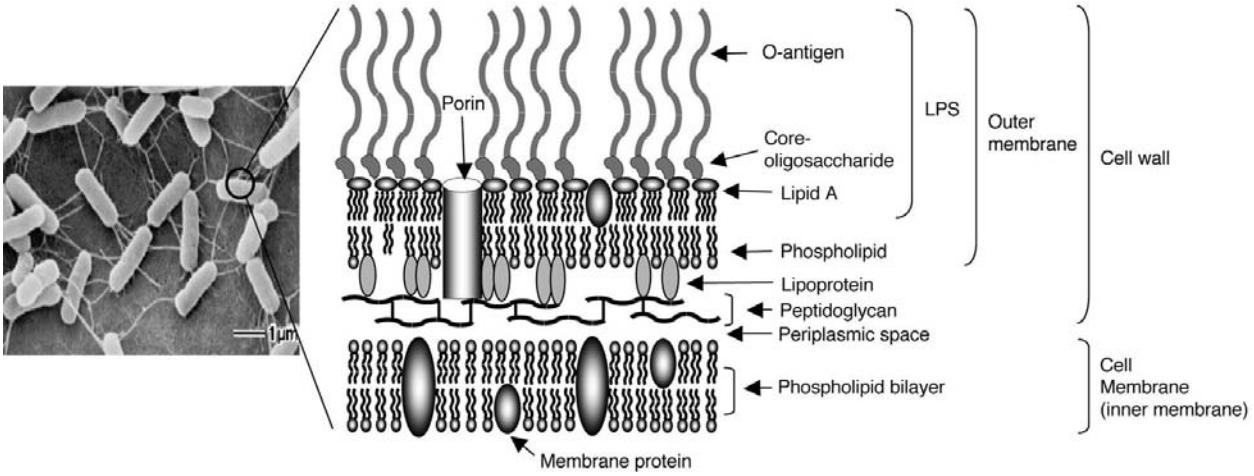

Figure 1. Cell surface structure of Gram-negative bacterium.

2. INTRODUCTION

It is well known that LPS is a glycoconjugate that exists in the cell walls of Gram-negative bacteria (Figure 1), and exhibits strong endotoxic activities in humans and animals, such as pyrogenicity, inflammation induction, and lethal toxicity, as well as beneficial effects including immunostimulatory activities (1). Analyses of synthesized lipid A specimens have shown that the lipid A part in LPS plays a main role in LPS biological activities (2-4).

It has recently been reported that TLR4 and its adaptor protein, MD-2, act as signal transduction receptors of LPS (5, 6). However, it has also been observed that the LPS and lipid A of *P. gingivalis*, which has received attention as a causative periodontopathic bacterium and been classified as *Bacteroides* species, act on host cells not through TLR4/MD-2, but rather through TLR2 (7). In addition, it was found that LPSs derived from non-enteric Gram-negative bacteria such as *Leptospira interrogans* induce signal transduction in cells dependent on TLR2 (8). Thus, it is considered that the existence of different receptors recognizing these LPSs is due to different chemical structures of the lipid A molecule (9).

Previously, we studied the highly purified *P. gingivalis* lipid A and its chemical synthesized counterpart, and found that the recognition receptor is not TLR2, but TLR4/MD-2 as well as enteric Gram-negative bacterium-derived LPS and lipid A (10, 11). The same results were obtained using a highly purified lipid A specimen derived from *Prevotella intermedia* (12). In addition, Coats *et al.* reported that *P. gingivalis* LPS inhibited cell activation via TLR4 of *Escherichia coli* LPS (13). These findings strongly indicate that LPSs derived from non-enteric Gram-negative bacteria, such as *P. gingivalis*, are recognized via TLR4/MD-2 and that *P. gingivalis* LPS fractions contain ligands that activate via TLR2. It is considered that TLR2 activates cells by recognizing peptidoglycan and lipoprotein, which are contained in nearly all bacterial cells (6). Furthermore, commercial enteric bacterium-derived LPS specimens are known to activate cells via both TLR4/MD-2 and TLR2. In those LPS specimens, TLR2-dependent cell active components can be removed by repeated extractions using a deoxycholate-phenol-water system and experiments have shown that the minor active component is lipoprotein (14, 15). On the other hand, the TLR2-active component in the *P. gingivalis* LPS fraction is very difficult to remove by repeated extractions (7, 16). Some parts of the TLR2 molecule remain unclear, however, we clarified its chemical structure by obtaining and purifying the active factor from the *P. gingivalis* LPS fraction (17). We have introduced our findings regarding the chemical structure and cell recognition capabilities of *Bacteroides* species LPS and their lipid As in the past, though studies of *P. gingivalis* LPS/lipid A are ongoing.

3. CHEMICAL STRUCTURE OF *BACTEROIDES* SPECIES LIPID A

Westphal *et al.* were successful in extracting LPS consisting of polysaccharide and lipid components by treating Gram-negative enteric bacteria with a hot phenol and water method (18) (Figure 2). In addition, by hydrating the LPS with acid, and separating it into a lipid component (lipid A) and soluble polysaccharide, they found that the lipid A contains amino sugar, nitrogen, phosphoric acid, and fatty acid, and also indicated that the lipid A part is an active center of endotoxic activation (19). Following that study, analyses of the chemical structures of *E. coli*- and *Salmonella*-derived lipid As, as well as synthetic lipid A synthetic studies, were actively performed. From those results, the chemical structures of lipid As were revealed, as shown in Figure 3, and synthesized lipid A compounds corresponding to those structures exhibited endotoxic activities equivalent to those of natural lipid A (2, 3). These synthesized lipid A compounds are considered to be important lipid A specimens for solving many problems regarding preparation methods, bacterial strains, and incubation conditions, associated with natural LPSs and their lipid As. In addition, composition analyses of Gram-negative obligate anaerobic bacteria were performed using LPS of *Bacteroides fragilis* found in indigenous microbiota in the human intestinum crassum (20). Tetradecanoic acid (myristic acid; 14:0) and 3-hydroxyl tetradecanoic acid (3-OH-14:0) were scarce in the fatty acid composition of *B.*

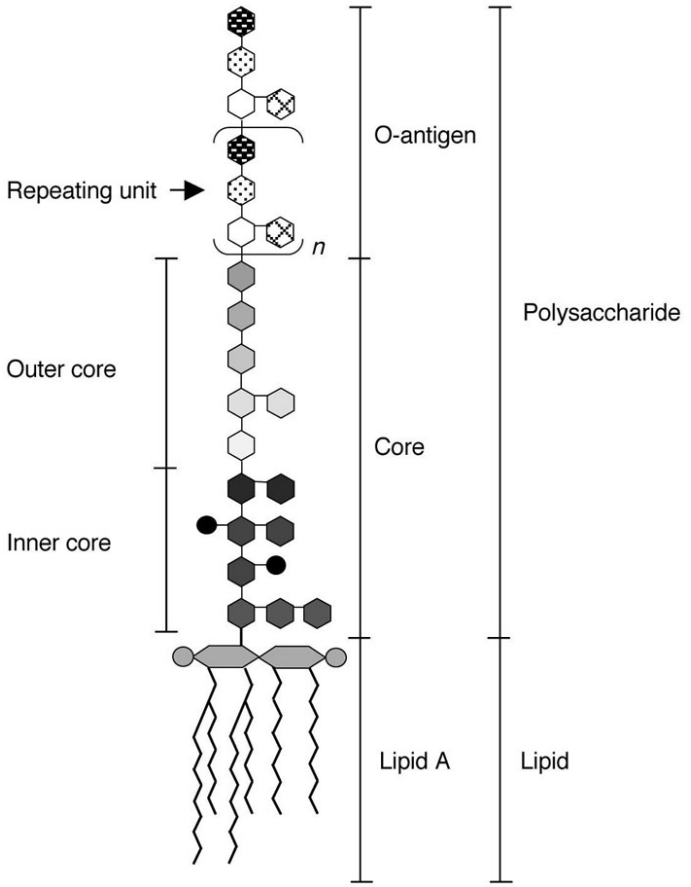

Figure 2. Schematic structure of LPS.

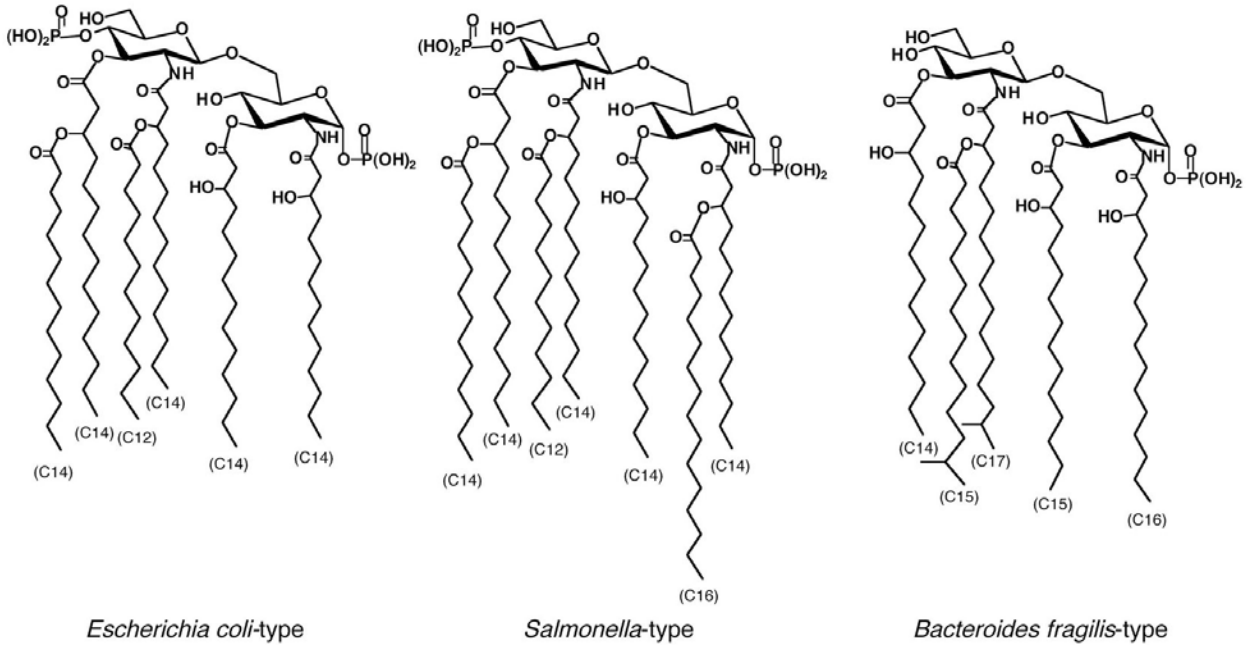

Figure 3. Proposed chemical structure of lipid A. *Escherichia coli*-type lipid A: compound 506 (2), *Salmonella*-type lipid A: compound 516 (3), and *Bacteroides fragilis*-type lipid A (21).

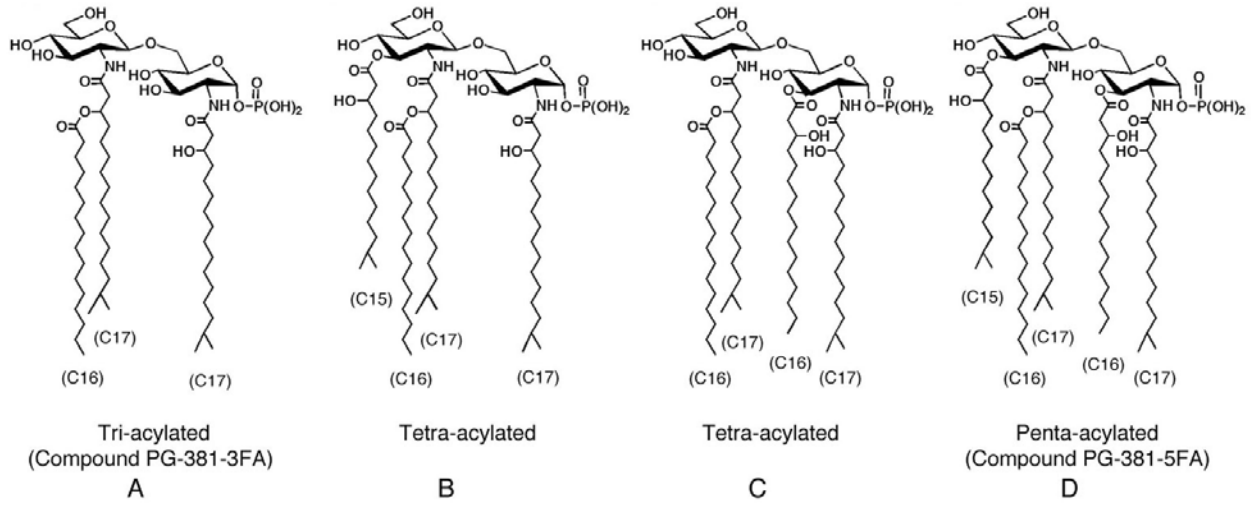

Figure 4. Proposed chemical structure of *Porphyromonas gingivalis* mono-phosphorylated lipid A species. A. Tri-acylated lipid A (compound PG-381-3FA) (23, 24, 78), B, C. tetra-acylated lipid A (24), and D. penta-acylated lipid A (compound PG-381-5FA) (23, 24, 78).

fragilis, whereas they exsited widely in the lipid As of enteric Gram-negative bacteria. Further, bifurcatio fatty acids such as 13-methyl-tetradecanoic acid (13-Me-14:0) and 3-hydroxyl-15-methyl-hexadecane acid (3-OH-15-Me-16:0) were detected at high rates. The chemical structure of the lipid A derived from *B. fragilis* NCTC 9343 was also determined (21) (Figure 3). When comparing *B. fragilis* lipid A with that of *E. coli*, the former has a single acyloxyacyl group consisting of a branched long chain fatty acid in the 2' position of the β(1-6) glucosamine disaccharide bone structure and a phosphate group in the 1 position, however, it is lacking a phosphate group in the 4' position. In addition, fatty acids with amidelinks in the 2 and 2' positions and esterlinks in the 3 and 3' positions are composed of long fatty acids, as illustrated in Figure 3.

B. fragilis lives in the intestine. Although many Gram-negative obligate anaerobic bacteria producing blackpigments have been observed in the periodontal pockets of patients with periodontal diseases, *P. gingivalis* and *P. intermedia* in particular have received attention as causing periodontal diseases (22). These bacteria are classified into a new bacterial family and separated from *Bacteroides* species. LPSs derived from *P. gingivalis* and *P. intermedia* are considered to be important virulence factors in the mechanisms of periodontal diseases, and many studies of correlations of their chemical structures and biological activities have been performed.

We extracted lipid A from the LPS of *P. gingivalis*, which is regarded as the main bacterium causing chronic periodontitis and whose chemical structure was first determined in 1993 (23). As illustrated in Figure 4A, the proposal chemical structure of triacylated lipid A of *P. gingivalis* strain 381 is composed of glucosamine (2 molecules), hexadecanoic acid (1 molecule), 3-hydroxy-15-methyl-hexadecanoic acid (2 molecules), and a phosphate group (1 molecule). In the bone structure, glucosamines are combined by a ß(1-6) linkage and generation of a compound that lacks a phosphate group and obtains a reductive trait under a mild acid hydrolysis condition, with the phosphate group combining only at the first reducing terminal. Among the fatty acids, 3-hydroxy-15-methyl-hexadecanoic acids (2 molecules), which are not isolated by alkali-methanolysis, are amidelinked at the 2 and 2' positions. It has been revealed that hexadecanoic acid is esterified to the hydroxyl group at the 3 position of the 3-hydroxy-15-methyl hexadecanoic acid located at the 2’ position of the ß(1-6) glucosamine disaccharide bone structure, using fragment analyses of ^{1}H NMR and the negative ion FAB-MS-MS. A structure analysis of lipid A of *P. gingivalis* strain SU showed that it is heterogeneous and contains not only an acyl group amidelinked to the 2 and 2' positions of the ß(1-6) glucosamine disaccharide bone structure, as we previously reported, but also lipid A of the tetraacyl (Figure 4B, C) and pentaacyl (Figure 4D) forms, in which 3-hydroxyl-hexadecanoic acid and/or 3-hydroxy-15-methyl-tetradecanoic acid are esterlinked to the 3 and 3' positions (24). In addition, *P. gingivalis* ATCC 33277 LPS was reported to contain heterogenous lipid A mass ions at m/z 1,195, 1,435, and 1,450 (25). Thus, *P. gingivalis* LPS is recognized to contain tri-, tetra-, and penta-acylated components (24-26).

We have also analyzed the chemical structure of lipid A in *P. intermedia* (formerly *Bacteroides intermedius*) ATCC 25611 obtained from the periodontal pockets of patients with gingivitis. In contrast to lipid As derived from *B. fragilis* and *P. gingivalis*, a phosphate group is combined at the 4' position of the nonreducing terminal of the lipid A of *P. intermedia*, while acyl and acyloxyacyl groups are combined at the 2 and 2' positions, and an acyl group at the 3 and 3’ positions of the ß(1-6) glucosamine disaccharide bone structure. The chemical structure has been proposed to be penta-acylated (12).

4. BIOACTIVITY OF *BACTEROIDES* SPECIES LPS/LIPID A

LPS exhibits various endotoxic activities such as pyrogenicity, Shwartzman reaction, and lethal toxicity, as well as immunostimulatory and host defense actions, including adjuvant activity and induction of interferon production. The endotoxic activity of *Bacteroides* species LPS/lipid A is relatively weak when compared to that of enteric bacterium-derived LPS, however, it is known to activate cells of LPS-nonresponder C3H/HeJ mice. We intend to show that *Bacteroides* species LPS/lipid A has low toxicity, however, it is natural to think that the response of C3H/HeJ mice is due to the unique chemical structure of *Bacteroides* species LPS/lipid A. Many researchers including our group have studied the numerous biological effects of *Bacteroides* species LPS/lipid A by isolating and purifying specimens. Many studies have demonstrated that the endotoxic activities of *Bacteroides* species LPS/lipid A, such as pyrogenicity in rabbits, Shwartzman reactions in rabbit skin, and lethal toxicity in mice and chick embryos, are relatively weak in comparison to those of enteric bacterium-derived LPS (27-38). Those results were confirmed by studies of the structure-activity relationship using synthesized lipid A compounds. Thus, as shown in comparisons of the chemical structure of lipid A in Figures 3 and 4, the existence of a phosphate group, acyl group, hydroxyacyl group, and acyloxyacyl group combined with the glucosamine disaccharide bone structure of lipid A, as well as the carbon number of those acyl groups in lipid A, are very important for LPS and lipid A to demonstrate their biological activities. In comparison to *E. coli*-type lipid A, which has strong endotoxic activities and biological effects, *Bacteroides* species lipid A is a monophosphate type lacking a phosphate group in the 1 position (for *P. intermedia*) or the 4' position (for *B. fragilis* and *P. gingivalis*) that consists not of tetradecanoic acids (C14), but rather long-chain fatty acids made up of only acyloxyacyl groups. It is speculated that these structural characteristics produce its endotoxic activities and biological effects. In addition, by using monosaccharide-type synthetic lipid A compounds, we found that its bioactivity was dependent on acyl groups in combination with glucosamine, along with its carbon numbers and chemical structure (39). Furthermore, although the bacterial species is different, in *Helicobacter pylori* lipid A, long-chain fatty acids of 18 carbon numbers are linked to the glucosamine disaccharide bone structure to which a phosphate group is linked at the 1 position (40), and its endotoxic activities and biological effects are weaker than those of LPS of enteric Gram-negative bacteria (41).

It has been reported that *Bacteroides* species LPS and lipid A activate lymphocytes and macrophages of C3H/HeJ mice, which show no response to enteric bacterium-derived LPS. Joiner *et al.* were the first to report that *B. fragilis* LPS has mitogenic effects on spleen cells from C3H/HeJ mice (42). *B. fragilis* LPS is also reported to induce TNF production from C3H/HeJ peritoneal macrophages (43). Furthermore, *B. fragilis* showed a mitogenic effect, polyclonal B cell activation activity, and an antibody-producing effect toward LPS-responsive C3H/HeN mice, though each was weak in C3H/HeJ mice (38). On the other hand, purified lipid A isolated from *B. fragilis* LPS did not show mitogenic effects toward C3H/HeJ B cells (44).

There is some evidence that LPS and lipid A derived from different bacterial species similar to *B. fragilis*, such as *P. gingivalis* (formerly *B. gingivalis*), *P. intermedia* (formerly *B. intermedius*), *Prevotella oralis* (formerly *Bacteroides oralis*), *Prevotella loescheii* (formerly *Bacteroides loescheii*), and *Prevotella corporis* (formerly *Bacteroides corporis*), activate B lymphocytes and macrophages, and initiate lethal toxicity in C3H/HeJ mice (16, 37, 45-57). We previously demonstrated that *P. gingivalis* LPS and its lipid A have mitogenic effects and induce cytokine production by B lymphocytes from C3H/HeJ mice (27, 58-60). Many researchers, including our group, have the opinion that *Bacteroides* species LPS/lipid A demonstrate a variety of bioactivities in C3H/HeJ mice due to their unique chemical structures, which differ from traditionally recognized enteric bacterium-derived LPS and lipid A (see Figures 3 and 4).

5. TOLL-LIKE RECEPTOR (TLR) AS AN LPS/LIPID A RECEPTOR

In the mechanism of host defense against pathogenic agents, the recognition receptor molecule in *Drosophila* Toll was initially discovered, followed by Toll-like receptors (TLRs) in humans and mice, which have a similar structure to Toll. Thus far, 13 types of TLRs have been reported (61-63), among which TLR4 plays a role in inducing cell activation and recognizing LPS together with its accessory molecule (5). It has been reported that C3H/HeJ mice do not respond to LPS due to a point mutation of the *Tlr4* gene (64). We found that cell activation by *E. coli*-type synthesized lipid A (compound 506) did not occur in TLR4-knockout mice, while we also showed that LPS activates host cells via TLR4 (65). In addition, the cells of TLR2-knockout mice as well as those of wild-type mice are activated by compound 506 (66). Furthermore, a synthetic compound corresponding to *H. pylori* lipid A did not cause a response in C3H/HeJ mice and inhibited cytokine production in human peripheral blood mononuclear cells by anti-human TLR4 antibodies (11).

As noted above, it is known that TLR4 is the receptor for recognizing LPS and lipid A. As a result, studies of TLR as the recognition receptor of *Bacteroides* species LPS and lipid A have been conducted. It was reported that when cytokine production and cell adhesion molecules from macrophages and endothelial cells stimulated with *E. coli* LPS and its synthetic lipid A (compound 506) are activated, LPSs and lipid As of *B. fragilis* and *P. gingivalis* act as antagonists (13, 67-70). Since *Bacteroides* species LPS and lipid A cause responses in C3H/HeJ mice, the existence of an LPS receptor that differs from enteric bacterium-derived LPS is possible. However, if enteric bacterium-derived LPS is an antagonist for *E. coli* LPS, it would be competitively antagonistic for the common receptor of these LPSs, TLR4.

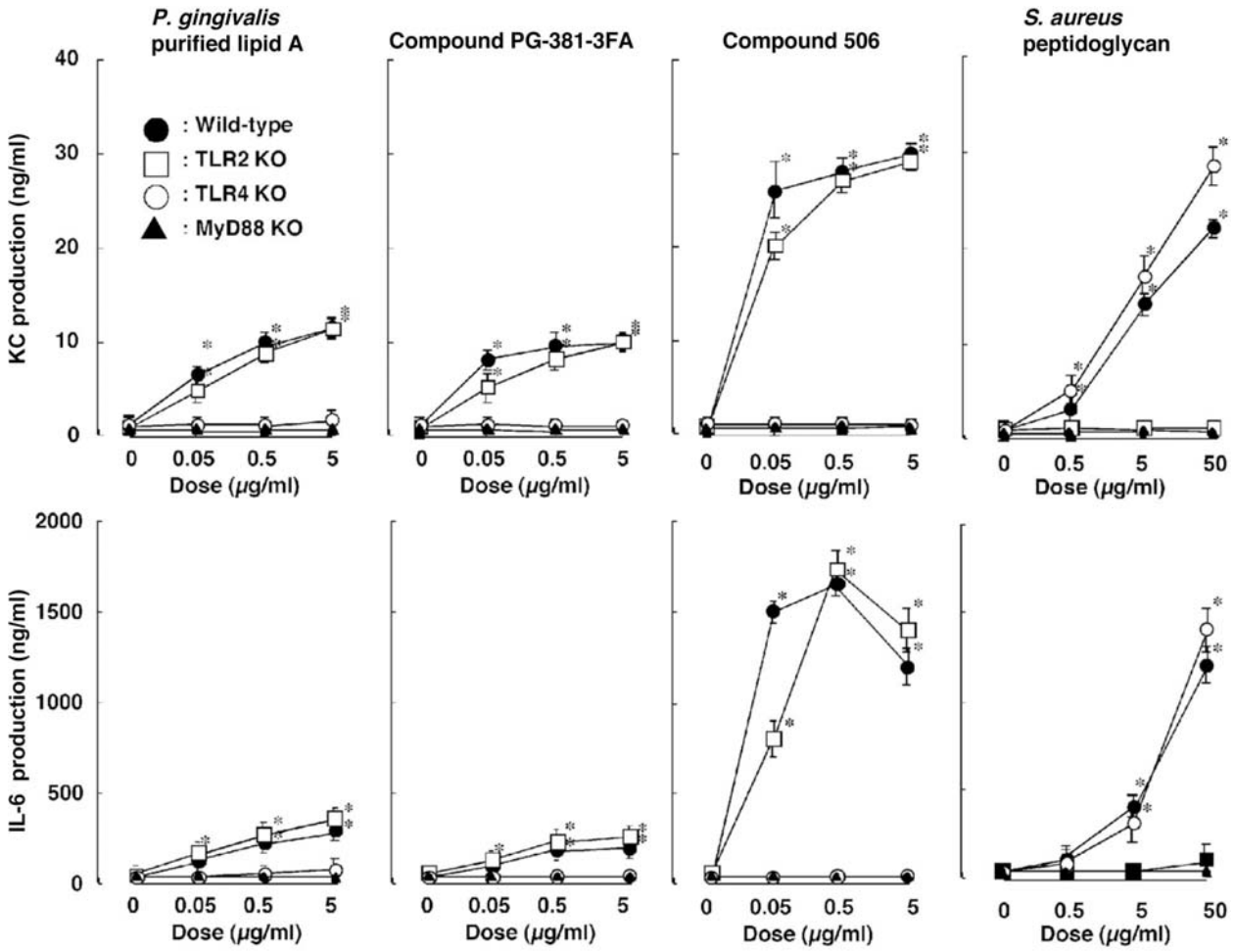

Figure 5. Cytokine production by gingival fibroblasts derived from TLR4 KO mice stimulated with *P. gingivalis* synthetic lipid A (compound PG-381-3FA). *$p < 0.01$ vs. None. Reproduced with permission from, ref 10.

Studies related to TLRs, in particular those that recognized *P. gingivalis* LPS and its lipid A, can be classified as follows. First, in addition to results showing that the receptor of *P. gingivalis* LPS and its lipid A cause responses in C3H/HeJ mice, it has been reported that they activate host cells not via TLR4, but rather TLR2 (7, 71-73). In addition, *P. gingivalis* LPS has been found to activate cells via TLR2 as an agonist, and also via TLR4 as an agonist or antagonist by heterogeneity, due to the fact that lipid As contain different numbers of acyl groups, while lipid A species with different chemical structures and conformation also exist (9, 25,74).

On the other hand, it has been suggested that there are TLR2-ligand contaminants as well as TLR4-ligand lipid As in lipid A fractions of *Bacteroides* species LPS. Based on our speculation that these structural characteristics of lipid A have effects on cell activation through TLR4, we investigated the recognition receptors for *P. gingivalis*- and *P. intermedia*-derived LPSs. *P. gingivalis* LPS fractions caused responses in C3H/HeJ mice and had mitogenic effects on their spleen cells. However, cytokine production from TLR4-knockout mouse-derived peritoneal macrophages was weaker than that of those from wild-type mice, and those biological activities were completely inhibited in mice without MyD88, a TLR/IL-1R family adaptor molecule (75). It was also reported that anti-TLR4 antibodies inhibited cytokine production from gingival fibroblasts in response to *P. gingivalis* LPS (76). We also examined the production of a synthetic counterpart corresponding to the chemical structure of *P. gingivalis* lipid A. A synthetic lipid A specimen (compound PG-381-3FA) (Figure 4A), composed of three acyl chains, caused cytokine-producing activities by peritoneal macrophages and gingival fibroblasts, however, those activities were not seen in C3H/HeJ and TLR4-knockout mouse-derived cells (10, 77) (Figure 5). In addition, *P. gingivalis* purified lipid A and its synthetic compound PG-381-3FA induced NF-κB activation in Ba/mTLR4/mMD-2 cells, as well as in murine TLR4 and MD-2 in Ba/F3 cells, however, no such activation was seen in murine TLR2-expressing Ba/F3 cells (10) (Figure 6). More recently, we synthesized *P. gingivalis*-type penta-acylated lipid A (compound PG-381-5FA) (Figure 4D) and demonstrated that both tri-acylated and penta-acylated lipid A compounds utilized TLR4/MD-2 but not TLR2 for cell activation (78). It was also shown

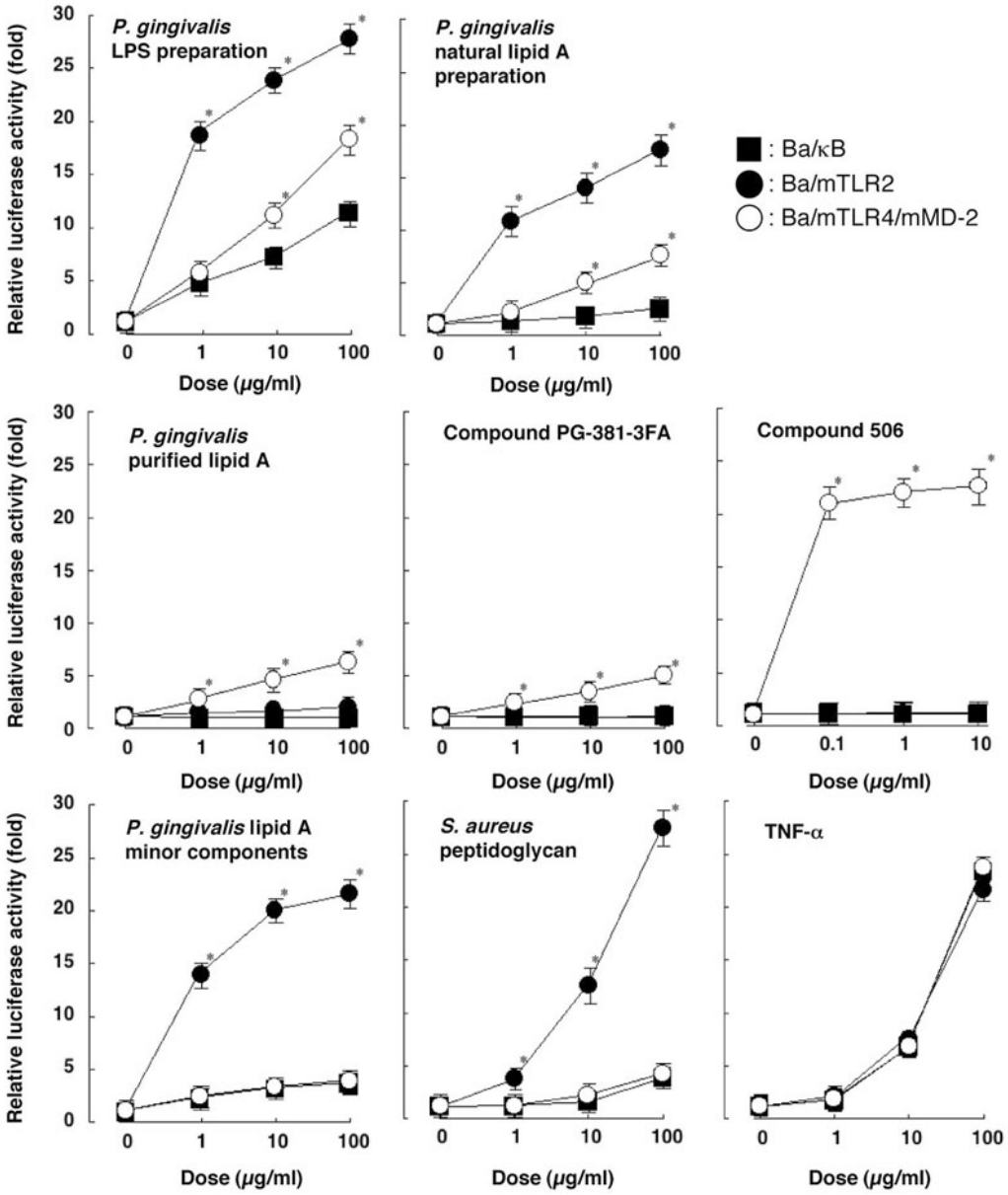

Figure 6. NF-κB activation in Ba/F3 cells stimulated with *P. gingivalis* phenol-water extract and synthetic lipid A (compound PG-381-3FA). Ba/F3 cells stably expressing an NF-κB reporter construct (Ba/κB) were transfected with murine TLR2 (Ba/mTLR2) or murine TLR4/MD-2 (Ba/mTLR4/mMD-2). *$p < 0.01$ vs. None. Reproduced with permission from, ref 10.

that *P. gingivalis* LPS activates a signal transduction system in cementoblasts and gingival fibroblasts through TLR4 (79, 80). Furthermore, we demonstrated that *P. intermedia* purified lipid A, composed of five acyl chains, activates cells via TLR4 (12). As noted above, many findings regarding receptors for *Bacteroides* species LPS and lipid A have been reported. However, our study using chemical synthetic counterparts showed that despite the structural heterogeneity of lipid A species in *P. gingivalis* LPS fractions, LPS and its lipid A activate cells through TLR4 in a manner similar to those derived from enteric bacteria.

6. CELL ACTIVATION MOLECULE IN *P. GINGIVALIS* LPS FRACTIONS VIA TLR2

From results of serial structural and functional studies using purified lipid As and their synthetic compounds corresponding to natural lipid As of *P. gingivalis* and *P. intermedia*, it is suggested that these contaminants, which activate cells via TLR2, contain *Bacteroides* species LPS and lipid A fractions. However, the minor TLR2 ligand in these fractions has not been found. As shown in Figure 7, we performed an analysis using silica gel thin-layer chromatography of *P. gingivalis*

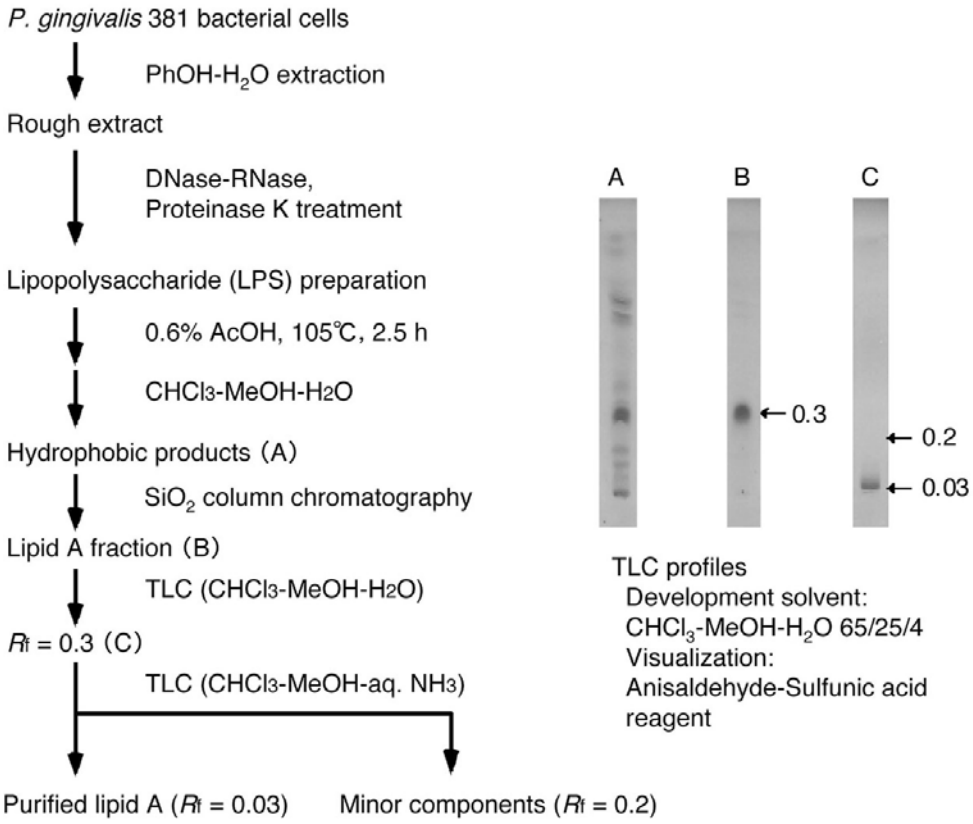

Figure 7. Purification of *P. gingivalis* lipid A. Reproduced with permission from, ref 10.

lipid A fractions. They were developed with a mixture of chloroform and methanol in water, with a rate of flow value (Rf) of 0.3 observed in a spot that was subsequently developed using chloroform, methanol, and 25% ammonia. As a result, two compounds in the main spot with an Rf of 0.03 and a minor spot with an Rf of 0.2 were detected. Those findings indicated that the component of the main spot was *P. gingivalis* lipid A as a TLR4 ligand, while that of the minor spot was a contaminant in the lipid A. As compared to the main spot TLR4 ligand, the minor spot TLR2 ligand showed very strong cell activation (10). These results strongly indicate that a TLR2 ligand with strong cell activation along with a TLR4 ligand in LPS and its lipid A fractions were extracted from *P. gingivalis* contaminants. As a result, we reviewed the TLR2-active component in *P. gingivalis* LPS fractions in terms of isolation, purification, and chemical structure analyses, in addition to its synthesis of structural analogs and cell activation ability (17, 81, 82).

It has been reported that enteric bacterium-derived LPS fractions are more highly purified by hydrophobic chromatography (83). Therefore, LPS fractions extracted from *P. gingivalis* lyophilized cells were fractionated using a hot phenol and water method, after which the isolation of TLR2-dependent cell-activating components was examined using TLR-expressing cells possessing an NF-κB luciferase reporter gene. An elution pattern of *P. gingivalis* LPS fractions used for determination of phosphorus and hexose contents was carried out, which showed two peaks (Figure 8A). Peak 1 represented unbound fractions, with most of the phosphorus and hexose eluted. It was also considered to represent components derived from nucleic acid and polysaccharides. Peak 2 also indicated components adsorbed to the column, which appeared in 104-140 fractions with a propanol concentration of 60%. The SDS-PAGE patterns also indicated that these fractions included *P. gingivalis* LPS (Figure 8B). On the other hand, 104-112 fractions activated NF-κB via TLR2 in peak 2 and were defined as Pg-AF (Figure 8C). To analyze the active components contained in Pg-AF, they were isolated by SDS-PAGE (Figure 8D). Bands of 10, 12, 14, 16, and 17 kDa as well as 18-40 kDa were observed by silver staining, and it was indicated that Pg-AF contains LPS. The 16-kDa band was also observed by CBB staining. Furthermore, a monocyte western blot analysis was conducted and NF-κB activation was observed in bands of 14-18 kDa via TLR2. On the other hand, the 16-kDa band seen with CBB staining disappeared in the SDS-PAGE assay of Pg-AF, which was treated with a proteolytic enzyme, though there was no change in the silver staining pattern (Figure 8E). NF-κB activation ability was not seen in bands of 14-18 kDa, though it was observed in the electrophoresis tip of the gel. These results indicate that a protein component migrating into the 16-kDa band plays a role in activation. In addition, this 16-kDa component could not be removed by re-extraction using a deoxycholic acid-phenol-hydrothermal system (7, 16). In addition, Pg-AF was isolated by preparative isolation SDS-

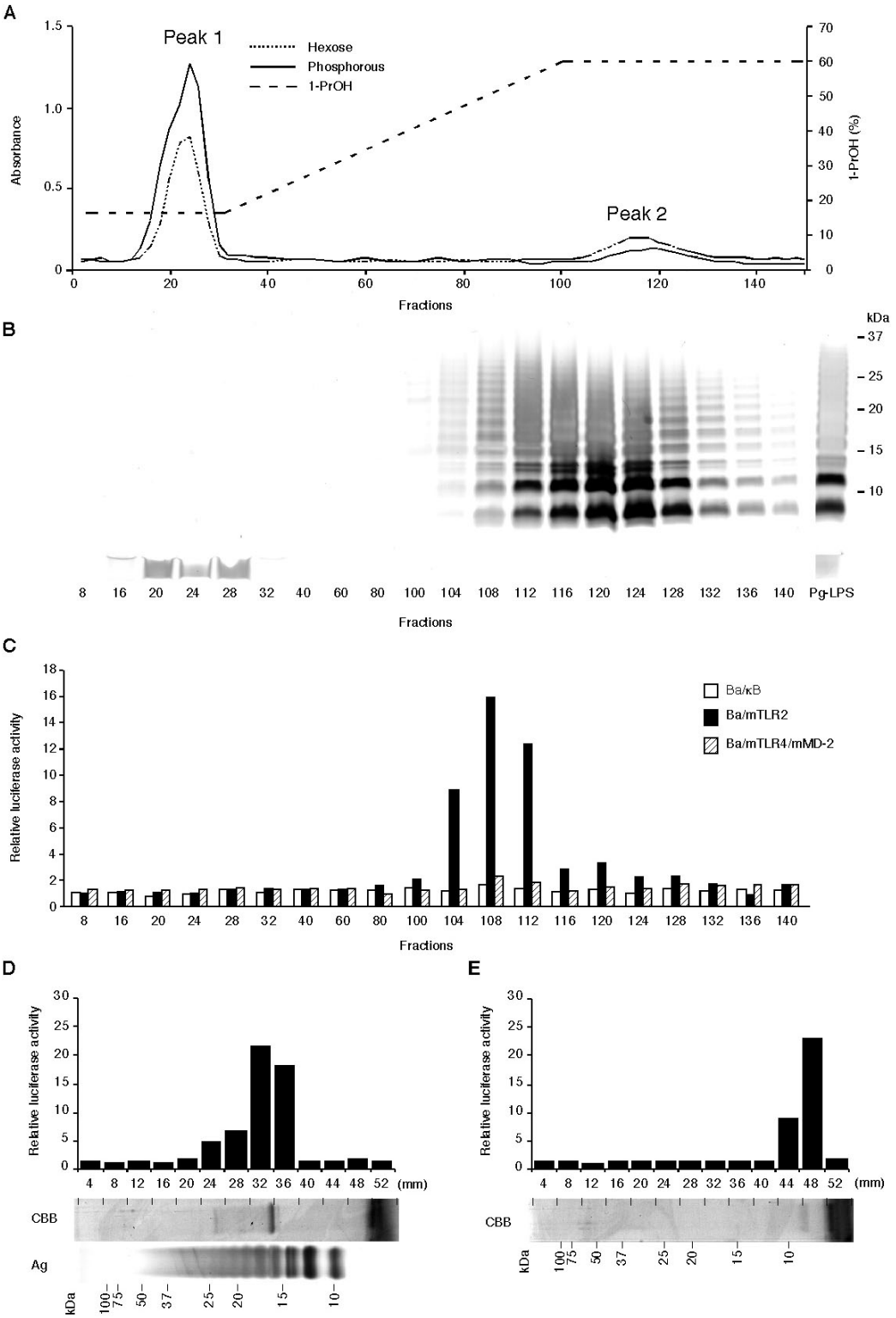

Figure 8. Fractionation of TLR2-activating component in a *P. gingivalis* LPS preparation. A. Elution profile of a *P. gingivalis* LPS preparation from an Octyl Sepharose CL-4B column. B. SDS-PAGE profile of a fraction from an Octyl Sepharose column. C. NF-κB activation of fractions from an Octyl Sepharose column using Ba/κB, Ba/mTLR2, and Ba/mTLR4/mMD-2 cells. D. TLR2-dependent NF-κB activation of a fraction of Pg-AF separated by SDS-PAGE. The membrane and gel were visualized by CBB and silver staining, respectively. E. TLR2-dependent NF-κB activation of a fraction of proteinase K-digested Pg-AF separated by SDS-PAGE. The membrane was visualized by CBB staining. Reproduced with permission from, ref 17.

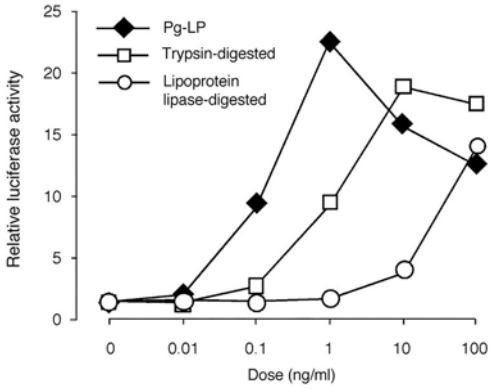

Figure 9. TLR2-dependent NF-κB activation of Pg-LP, trypsin-digested Pg-LP, and lipoprotein lipase-digested Pg-LP. Reproduced with permission from, ref 17.

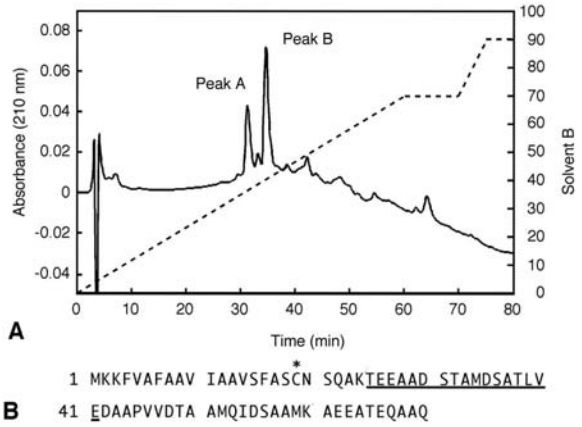

Figure 10. Identification of Pg-LP. A. Elution profile of a tryptic digest of Pg-LP using reverse phase HPLC. B. Deduced amino acid sequence of PG1828 in *P. gingivalis* 381. The underlined sequence was determined by internal peptide sequencing and the asterisk indicates the N-terminal lipidated cystein. Reproduced with permission from, ref 17.

PAGE and the 16-kDa component was treated with enzymes to analyze its properties. Following trypsin digestion of the component, activity in a luciferase reporter assay was reduced by one–tenth and almost disappeared following lipoprotein lipase digestion (Figure 9). These results indicate that the component is a lipoprotein and the 16-kDa band was determined to be Pg-LP.

In order to analyze the chemical structure of the obtained Pg-LP, N-terminal amino acid analysis was carried out. As a result, an N-terminal amino acid was not detected by Edman degradation of Pg-LP and it was speculated that the amino group of the N-terminal was modified. In order to analyze the internal amino acid sequences, a tryptic digest of Pg-LP was isolated by reverse phase HPLC (Figure 10A). The isolated peptide was degraded using the Edman degradation method, and it was clarified that the amino acid sequence at peak A was TEEAADXTAMDXATLVE and the sequence at peak B was TEEAADXTAMDXATLV (X indicates a non-identified amino acid). We searched for the component in the FASTA database and found that it was highly homologous to estimated lipoprotein PG1828 (Accession No. NP_905926), which is coded in the *P. gingivalis* W83 genome (84). We also determined the DNA sequence of *P. gingivalis* 381-derived PG1828 and its sequence (Accession No. AB164391), and found that the estimated amino acid sequence (Figure 10B) was the same as the Pg-LP sequence. The open reading frame of PG1828 codes for 70 amino acid residues and a lipoprotein signal peptide of a discriminative prokaryotic organism was observed in the sequence. From these observations, it can be assumed that the N-terminal part was modified by fatty acids, after the portion around it was cut off. As a result, the maturation protein is composed of 52 amino acid residues along with a fatty acid residue, and the molecular weight is estimated to be *ca.* 6 kDa. Matured PG1828 is an acidic protein with a pI of 3.7 and electrophoresis behavior was observed in SDS-PAGE, because of a decrease in the amount of SDS bonding (85).

It is known that the bacteria-derived lipoprotein has an *S*-(2,3-dihydroxyl propyl) cysteine at the N-terminal (86). Next, we examined the N-terminal structure of Pg-LP. After isolating the trypsin-digested product by normal phase HPLC, a product that activated NF-κB through TLR2 was fractionated into 8-10 fractions (Figure 11A). When these activated fractions were analyzed by MALDI-TOF-MS, they reached peaks of 1411.0 and 1425.0 m/z. In addition, the low peaks were 1397.0 and 1439.0 m/z. MS/MS was measured at 1425.0, m/z and y-ion fragments of the C-terminal were observed at 547.3, 433.2, 346.2, 218.1, and 147.1 m/z (Figures 11B, C). These ions were shown to be equivalent to the amino acid sequence NSQAK. In addition, a b-ion fragment of the N-terminal was observed at 878.7 m/z, which corresponds to *S*-(2,3-dihydroxyl propyl) cysteine with three fatty acid residues. A compatible fragment with monoacyl dihydroalanyl NSQAK produced by ß desorption of the 2,3-diacyl propyl group was observed at 854.6 and 840.5 m/z. The same fragments were observed at another peak of MS/MS and produced by the same amino acid sequence in combination with a different fatty acid. The amino acid sequence of this lipopeptide coincided with the N-terminal sequence of the estimated mature structure of Pg-LP and it was determined that Pg-LP is a lipoprotein. In addition, since a lipopeptide with three fatty acid residues is capable of TLR2-dependent cell activation (87), the component of Pg-LP was identified as a lipopeptide. Pg-LP induced not only NF-κB activation, but also cytokine production from human gingival fibroblasts (Figure 12). Pg-LP also had a higher induction of cytokine production, as compared with the *P. gingivalis* LPS fraction. Furthermore, *P. gingivalis* lipid A showed very weak cytokine-inducing activity as compared to the *P. gingivalis* LPS fraction. These results indicate that the main component of cell activation is not an LPS molecule, but rather Pg-LP in a *P. gingivalis* LPS fraction (Figure 13). We recently produced a triacyl-type lipopeptide derivative (PGTP) corresponding to the Pg-LP structure (82), and showed that the steric configuration of the fatty acid residue at the glycerol part in the PGTP structure is very

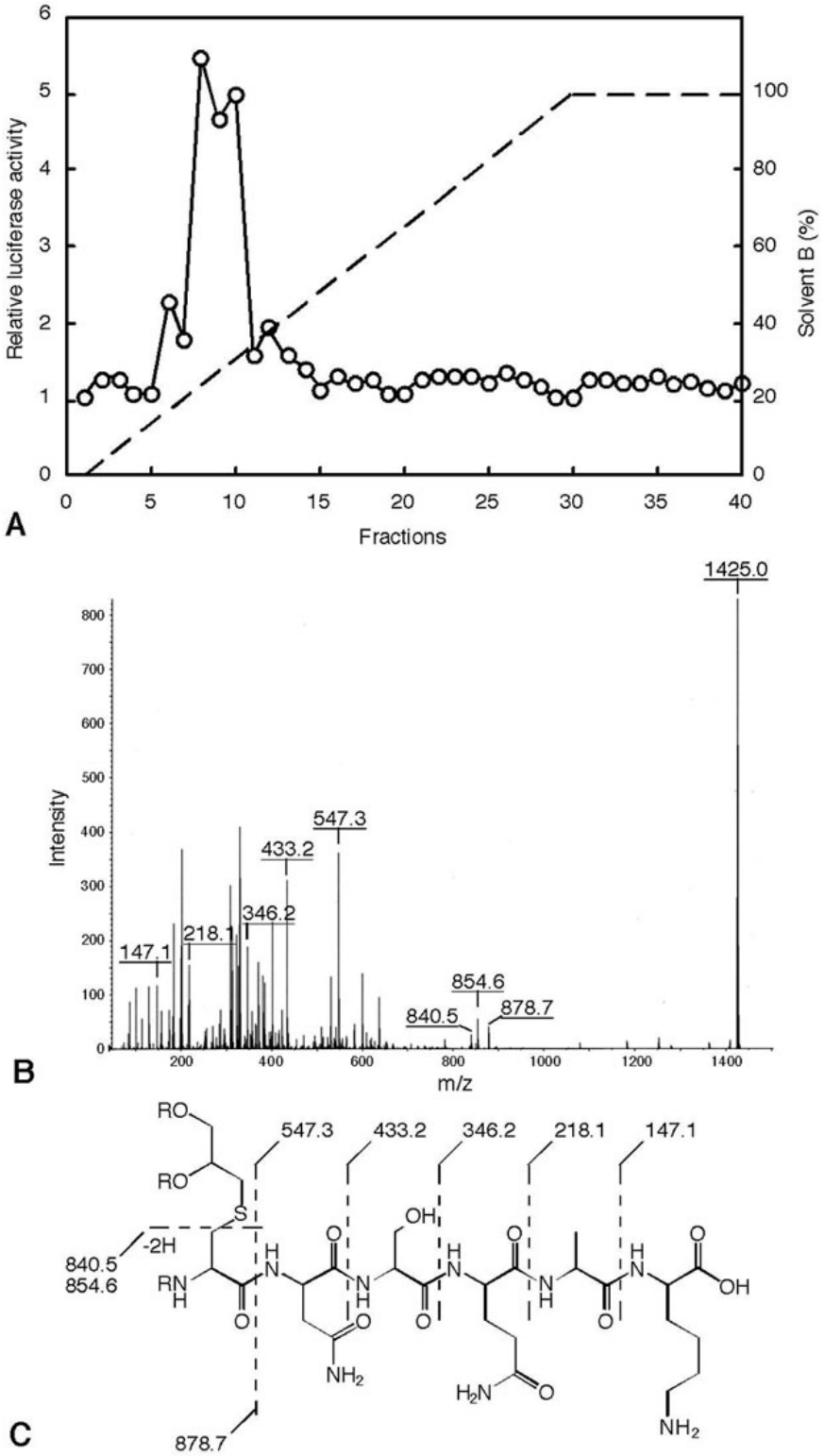

Figure 11. Characterization of N-terminal lipopeptide of Pg-LP. A. Elution profile of tryptic digest of Pg-LP using normal phase HPLC. NF-κB activation of the fraction was determined using Ba/mTLR2 cells. B. MS/MS spectra of the lipopeptide. The procedure ion at 1425.0 m/z was decomposed using CID mode. C. Structure of the lipopeptide. Reproduced with permission from, ref 17.

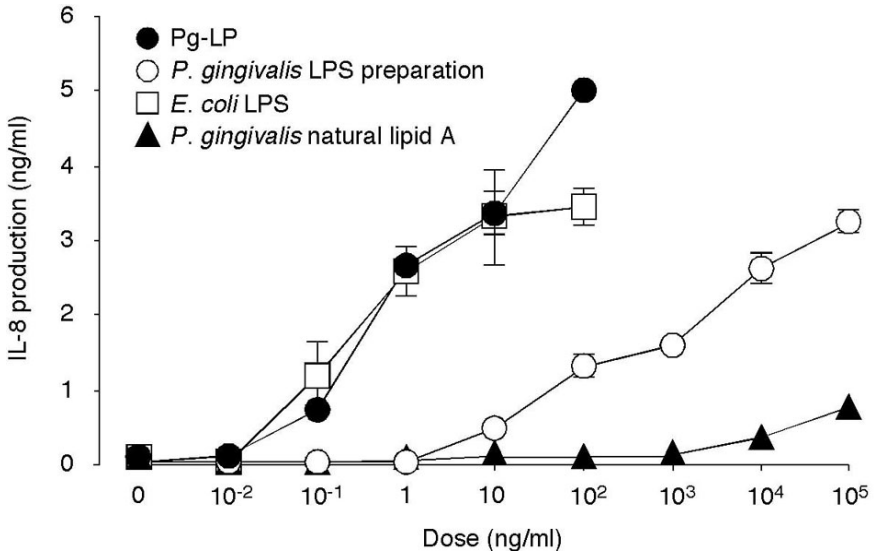

Figure 12. IL-8 production by human gingival fibroblasts stimulated with Pg-LP. Reproduced with permission from, ref 17.

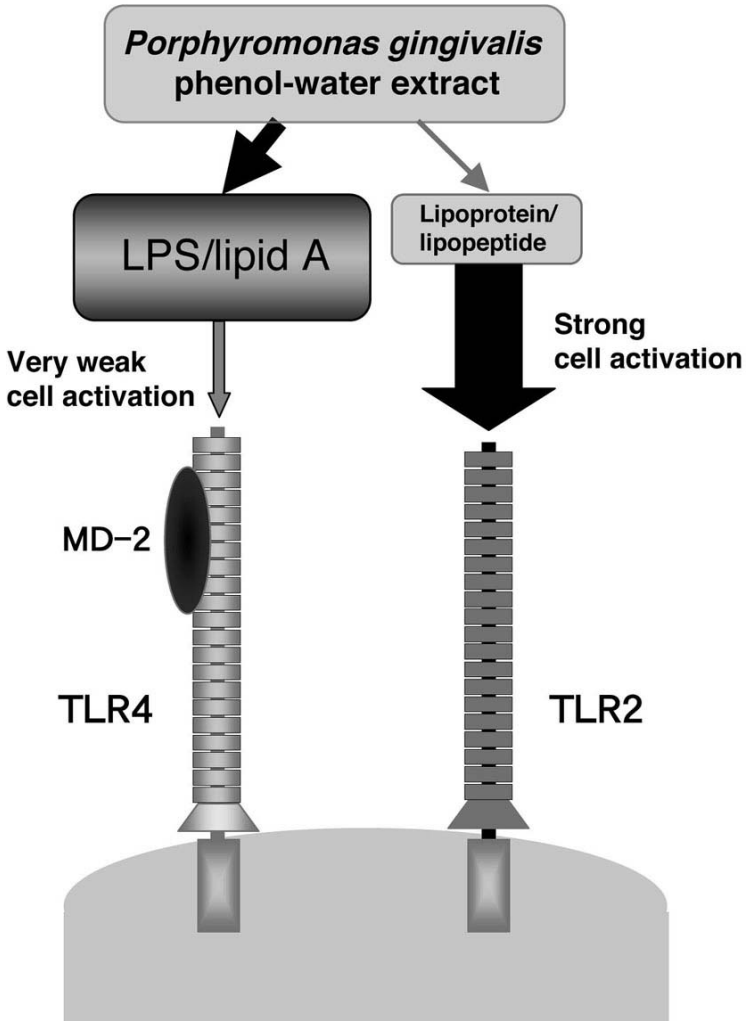

Figure 13. *P. gingivalis* phenol-water extract-induced cell activation through TLRs.

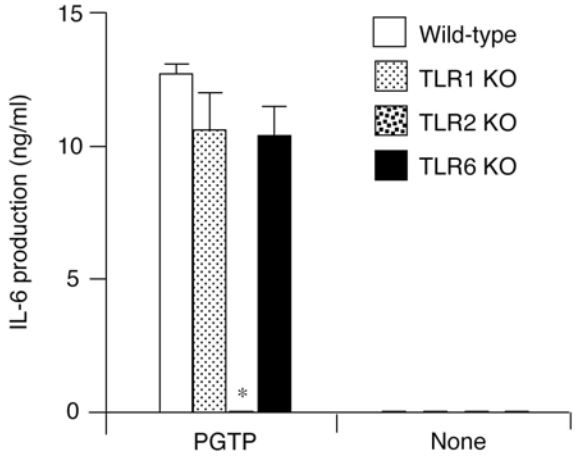

Figure 14. TLR2-dependent IL-6 production by peritoneal exudated macrophages in response to *P. gingivalis* synthetic lipopeptide derivatives. $*P < 0.01$ vs. wild-type mice.

important for cell activation, which was found to be TLR2-dependent and TLR1/TLR6-independent (Figure 14). On the other hand, it was demonstrated that *P. gingivalis* LPS preparation elicited TLR1/TLR2-dependent cell activation (74). We are not able to conclude the possibilities that other lipoprotein contaminant except for Pg-LP induced TLR1/TLR2 activity. This discrepancy should be clarified in the near future.

7. CONCLUSION

Structural and functional studies of LPS and its lipid A of *P. gingivalis*, which has been conventionally classified as an oral *Bacteroides* species, were outlined in this review. Along with the remarkable advances in studies of host defense mechanism, interest in the interactions between pathogenic bacteria and their bacterial components, as well as the receptors that recognize them, has been expressed. In conducting our studies on the structure-activity relationship of LPS, the fact that a unique LPS that exhibits activity in C3H/HeJ mice, which do not respond to LPS, exists in oral bacterial species, the subjects of our field of study, served as additional motivation to conduct this investigation. We assumed that the structure of the acyl group is deeply related to activation in C3H/HeJ mice, if the structure of *P. gingivalis* lipid A can be clarified, especially the structural characteristic in which an acyl group binding to glucosamine disaccharide bone structure consists of a branched long-chain fatty acid in a manner different from enteric bacterium-derived lipid As. We determined the chemical structure of *P. gingivalis* lipid A, however, the lipid A specimens that induced various biological activities were so-called natural lipid As. Heterogeneity of lipid A species and contamination of biologically active components due to different bacterial species and strains, cultural conditions, preparation methods of the specimen, other causes, are important issues. Further, studies of enteric bacterium-derived LPS and lipid A were active as far back as the 1980's. However, we are concerned that even researchers who have studied endotoxins for a long period might have failed to recognize these issues in some cases. In our investigations of the recognition mechanism of innate immunity against infectious diseases, we have become keenly aware of the importance of the homogenization of ligands under examination. We produced a synthetic compound corresponding to the chemical structure of *P. gingivalis* lipid A, and demonstrated that the chemical synthetic counterpart was non-responsive in C3H/HeJ mice as well as enteric bacterium-derived lipid As, and activated cells via TLR4. Thus, we consider that the issues in the study on *Bacteroides* species LPS so far have been finalized. Although some researchers have claimed that the difference in recognition by structurally different lipid A species in LPS fractions (so called heterogeneity) is a hypothesis, we consider that these aspects can be solved by synthesizing the ligands under examination. Furthermore, it is not easy to remove the mixed-in lipoprotein in the LPS fraction of *Bacteroides* species and these contaminants exert a strong biological activity via TLR2. On the other hand, the endotoxic activity and biological effect of lipid A are very weak, as is clear from the results of structural studies. The LPS and lipid A fractions of *Bacteroides* species that contain contaminants are considered to be TLR2 agonists and TLR4 antagonists. The TLR2 agonist contained in the *P. gingivalis* lipid A fraction that showed strong activity was a lipoprotein composed of *S*-(2,3-dihydroxypropyl) cysteine with three fatty acid residues (17), and the lipopeptide synthetic compound derivatives acted as a TLR2 ligand (82). LPS extracted from the *P. gingivalis* lipoprotein-defective strain caused a decrease in cell-activating capacity via TLR2 in comparison to the LPS of the wild-strain (81). In the near future, studies on ascribable virulent factors of infectious diseases, as well as the recognition and signal transduction in host cells will be conducted. Through our studies of *P. gingivalis* LPS and its lipid A, we have recognized how important it is to be careful with research materials when performing tests. In conclusion, further evaluations of probes presented in previous studies should be conducted, while exchanges among researchers presently investigating the interaction of infectious diseases with innate immunity should be further developed.

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Abbreviations: LPS: lipopolysaccharide, TLR: Toll-like receptor

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