Oral Microbiology Immunology 2008: 23: 60–69
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ORAL MICROBIOLOGY
AND IMMUNOLOGY

Biological properties of the native and synthetic lipid A of *Porphyromonas gingivalis* lipopolysaccharide

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Kumada H, Haishima Y, Watanabe K, Hasegawa C, Tsuchiya T, Tanamoto K, Umemoto T. Biological properties of the native and synthetic lipid A of Porphyromonas gingivalis *lipopolysaccharide.*
Oral Microbiol Immunol 2008: 23: 60–69. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

Introduction and methods: A pentaacyl and diphosphoryl lipid A molecule found in the lipid A isolated from *Porphyromonas gingivalis* lipopolysaccharide (LPS) was chemically synthesized, and its characteristics were evaluated to reconfirm its interesting bioactivities including low endotoxicity and activity against LPS-unresponsive C3H/HeJ mouse cells.
Results: The synthesized *P. gingivalis* lipid A (synthetic Pg-LA) exhibited strong activities almost equivalent to those of *Escherichia coli*-type synthetic lipid A (compound 506) in all assays on LPS-responsive mice, and cells. LPS and native lipid A of *P. gingivalis* displayed overall endotoxic activities, but its potency was reduced in comparison to the synthetic analogs. In the assays using C3H/HeJ mouse cells, the LPS and native lipid A significantly stimulated splenocytes to cause mitosis, and peritoneal macrophages to induce tumor necrosis factor-α and interleukin-6 production. However, synthetic Pg-LA and compound 506 showed no activity on the LPS-unresponsive cells. Inhibition assays using some inhibitors including anti-human Toll-like receptor 2 (TLR2) and TLR4/MD-2 complex monoclonal antibodies showed that the biological activity of synthetic Pg-LA was mediated only through the TLR4 signaling pathway, which might act as a receptor for LPS, whereas TLR2, possibly together with CD14, was associated with the signaling cascade for LPS and native lipid A of *P. gingivalis*, in addition to the TLR4 pathway.
Conclusion: These results suggested that the moderated and reduced biological activity of *P. gingivalis* LPS and native lipid A, including their activity on C3H/HeJ mouse cells via the TLR2-mediated pathway, may be mediated by bioactive contaminants or low acylated molecules present in the native preparations having multiple lipid A moieties.

Key words: biological properties; lipopolysaccharide; *Porphyromonas gingivalis;* synthetic lipid A

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Accepted for publication April 6, 2007

Porphyromonas gingivalis, an oral anaerobic gram-negative rod, is thought to be the most important mediator of the pathogenicity of periodontal disease (15, 47, 60). Many investigations have shown that the lipopolysaccharide (LPS) of *P. gingivalis* is a significant virulence factor, because it exhibits various activities, such as induction of inflammatory cytokines in human gingival fibroblast (HGF) cultures (12, 51) and bone resorption activity (18, 32), that are closely correlated with periodontal disease. *Porphyromonas gingivalis* LPS expresses a low level of endotoxic activity relative to enterobacterial LPS (29, 32). In addition, the LPS characteristically stimulates the splenocytes and macrophages from LPS-unresponsive C3H/HeJ mice

to cause mitosis or cytokine induction (8, 24, 59), in contrast to usual LPS, which do not exhibit any effects on these cells (43, 46).

The pathophysiological activity of LPS is dependent on the chemical structure of the hydrophobic portion, called lipid A, the biologically active center of LPS (16, 42). Recently, we found a characteristic structure of *P. gingivalis* lipid A containing branched and relatively longer fatty acids (15–17 carbon atoms) that are not present in enterobacterial lipid A molecules (26). In addition, we demonstrated, using LPS-antagonist and well-purified lipid A (although containing a small amount of protein), that the characteristic action of *P. gingivalis* lipid A against C3H/HeJ mice seems to be specifically mediated by the lipid A portion (54). These results suggested that the unique fatty acid components might be associated with the activity on C3H/HeJ mouse cells. This was also supported by studies of the chemical and biological properties of *Flavobacterium meningosepticum* lipid A, which has a structure very similar to *P. gingivalis* lipid A and also activates C3H/HeJ mouse cells (21, 56).

Toll, a *Drosophila* receptor molecule with extracellular leucine-rich repeats that currently has 10 published members [Toll-like receptors (TLRs) 1–10] in humans, has a role in triggering innate defenses against bacteria or fungi (1, 30, 52). Recent studies have suggested that TLR4, a member of the TLR family, might act as a receptor for LPS (4, 17, 39). TLR4 alone is not capable of sensing and signaling the presence of LPS, but another accessory molecule, MD-2, which is physically associated with TLR4, is required for LPS recognition through TLR4 (45). On the other hand, TLR2 has been proposed as a receptor for many microbial products and has been shown to signal the presence of peptidogylcan, lipoteichoic acid, lipoarabinomannan, lipoproteins and lipopeptides, as well as many whole gram-positive bacteria (4, 53). In addition, it has been reported that the co-dominant LPS^{d} allele of C3H/HeJ mice corresponds to a missense mutation in the third exon of the *TLR4* gene, which is predicted to result in replacement of proline with histidine at position 712 of the protein (39). Recently, we found that HGFs constitutively express *TLR2* and *TLR4*, and that their levels of expression are increased by stimulation with *P. gingivalis* LPS (50). These observations suggest that, in addition to TLR4, the biological action of *P. gingivalis* LPS may be mediated through the TLR2 pathway, which might not be correlated with LPS-mediated signaling.

In the present study, we chemically synthesized a pentaacyl and diphosphoryl lipid A analog corresponding to the lipid A species with the highest molecular mass found in *P. gingivalis* native lipid A in our previous study (26). The synthetic analog was subjected to biological assay to evaluate whether the interesting activity of LPS against C3H/HeJ mice is derived from the lipid A part.

Materials and methods

Reagents

RNase A, DNase I, and proteinase K were purchased from Sigma (St Louis, MO). (*R*,*S*)-3-hydroxy-13-methyltetradecanoic acid (3-OH-iC$_{15:0}$), (*R*,*S*)-3-hydroxy-15-methylhexadecanoic acid (3-OH-iC$_{17:0}$) and (*R*,*S*)-3-hydroxyhexadecanoic acid (3-OH-C$_{16:0}$) were purchased from Iatron-Biosupply Co. (Tokyo, Japan) and Wako Chemical Co. (Osaka, Japan). Quantitative *Limulus* amebocyte lysate (LAL) gelation assay reagent, Endospecy, was obtained from Seikagaku Kogyo (Tokyo, Japan). Iscove's modified Dulbecco and RPMI-1640 media were the obtained from Life Technologies (Grand Island, NY) and Gibco Laboratories (Grand Island, NY). [^{3}H]Thymidine was obtained from New England Nuclear (Boston, MA). Mono-Mac-6 (MM6) cells were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). HTA125, TL2.1, and MY4 clones, monoclonal antibodies (mAbs) to the human TLR4/MD-2 complex, and human TLR2 and CD14 molecules were purchased from MBL Medical & Biological Laboratories Co. (Nagoya, Japan), Cascade BioScience, (Winchester, MA) and Coulter Co. (Miami, FL), respectively.

Microbes

P. gingivalis SU63, isolated from a periodontal pocket, was grown anaerobically at 37°C for 24 h in heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 0.0005% hemin, 0.0001% vitamin K1, 0.5% yeast extract, and 0.08% cysteine (25). The cells were heated (121°C for 15 min), harvested by centrifugation (7000 ***g***, 20 min), and washed successively with distilled water and acetone.

Animals

Japanese White rabbits were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Female C3H/HeN and C3H/HeJ mice aged 6 weeks were obtained from Clea Japan, Inc. (Tokyo, Japan), and used for the assay of splenic mitogenicity and the induction of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) by peritoneal macrophages.

Preparations of *P. gingivalis* LPS and lipid A

The procedures for the preparation of *P. gingivalis* LPS and lipid A were described previously (26). Briefly, the LPS was extracted from acetone-dried cells with phenol–water (58), digested with RNase A, DNase I, and proteinase K (44), and then purified by repeated ultracentrifugation (105,000 ***g***, 12 h, six times). The LPS was washed successively with phenol/chloroform/petroleum ether [2 : 5 : 8, volume/volume (V/V)] (10) and acetone and then lyophilized.

The free lipid A was recovered from hydrolysates (1% acetic acids, 100°C, 1.5 h) of LPS according to the methods of Qureshi et al. (40, 41). It was purified by passage through a Dowex 50 (H^{+}) column with chloroform/methanol (3 : 1, V/V) as the eluent and gel permeation chromatography with a Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column with the same solvent as the eluent (26).

Total synthesis of *P. gingivalis* SU63 lipid A

P. gingivalis lipid A analog (compound 1) (Fig. 1) was synthesized basically according to the procedure previously reported (26). As shown in Fig. 2, (*R*,*S*)-3-OH-iC$_{15:0}$, (*R*,*S*)-3-OH-iC$_{17:0}$ and (*R*,*S*)-3-OH-C$_{16:0}$ were selectively (*S*)-3-*O*-acetylated by lipase treatment (5) of the methyl esters. The non-acetylated methyl esters predominantly containing the (*R*)-forms were separated by silica-gel chromatography, and each free acid was fractionally crystallized from CH_3CN as the dibenzylamine salt to increase the percentage of enantiomorphic excess. The optically pure (*R*)-3-OH fatty acids (compounds 2–4) were converted to the phenacyl ester (compounds 5–7) and 3-*O*-acylated with C$_{16:0}$ or benzyloxycarbonyl chloride (Z-Cl) to obtain the phenacyl ester of (*R*)-3-*O*-Z-iC$_{15:0}$ (compound 8), (*R*)-3-*O*-Z-C$_{16:0}$ (compound 9), (*R*)-3-*O*-Z-iC$_{17:0}$ (coumpound 10) and (*R*)-3-*O*-(hexadecanoyl)-15-methylhexadecanoic acid [3-*O*-(C$_{16:0}$)-iC$_{17:0}$] (compound 11). After dephenacylation, each fatty acid (compounds 12–15) was purified by silica-gel chromatography, and the yields of

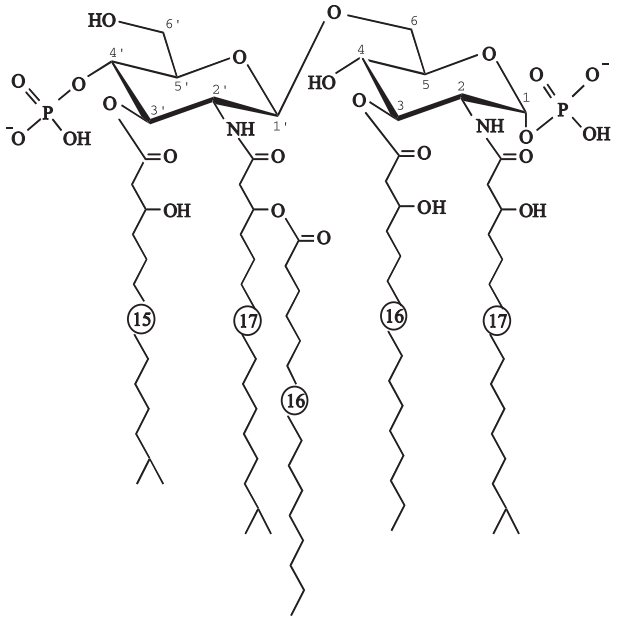

Fig. 1. Chemical structure of *Porphyromonas gingivalis* synthetic lipid A. In this study, we chemically synthesized a pentaacyl and diphosphoryl lipid A analog corresponding to the lipid A species with the highest molecular mass found in *P. gingivalis* native lipid A in our previous study (26). The synthetic Pg-LA consists of β(1-6)-linked D-glucosamine disaccharide 1,4′-bisphosphate backbone acylated with (*R*)-3-OH-$iC_{17:0}$, (*R*)-3-OH-$C_{16:0}$, (*R*)-3-OH-($C_{16:0}$)-$iC_{17:0}$ and (*R*)-3-OH-$iC_{15:0}$ at positions 2, 3, 2′ and 3′ of the hydrophilic backbone.

compounds 12–14 were 8.9%, 24.1%, and 9.7%, respectively.

The glycosyl donors were prepared from *N*-(2,2,2-trichloroethoxycarbonyl)-D-glucosamine (compound 16) as shown in FIG. 3. After allyl glycosidation, isopropylidenation of the glycoside followed by simple recrystallization afforded almost pure 4,6-*O*-isopropylidenated α-allyl glycoside, compound 17. This product was 3-*O*-acylated with (*R*)-3-*O*-*Z*-$iC_{15:0}$ to obtain compound 18, and the 4,6-*O*-protection was removed by mild acid hydrolysis to give product 19. Position 6 of the compound was protected with a carbobenzoxyl group to synthesize compound 20 followed by 4-*O*-diphenylphosphorylation (compound 21) and subsequent cleavage of the allyl group to make compound 22 (33, 38). This product was allowed to react with CCl_3CN in the presence of Cs_2CO_3 (57) as a catalyst to give glycosyl trichloroacetimidate, compound 23, to be used as the donor.

On the other hand, glycosyl acceptor was prepared through compound 17 (Fig. 4). The compound was 3-*O*-acylated with (*R*)-3-*O*-*Z*-$C_{16:0}$ to obtain compound 24. After removing the Troc group, the product 25 was *N*-acylated with (*R*)-3-*O*-*Z*-$iC_{17:0}$ to give compound 26 followed by cleavage of the 4,6-*O*-protection to yield glycosyl acceptor 27. Coupling reaction of compound 23 with 27 was performed using trimethylsilyl triflate (TMSOTf) in 1,2-dichloroethane to obtain disaccharide 28, which gave the desired β(1 → 6) linkage in a higher yield than the Königs–Knorr and oxazoline methods (9, 19). After removing the Troc group of the disaccharide compound, the 2'-amino group of product 29 was *N*-acylated with (*R*)-3-*O*-($C_{16:0}$)-$iC_{17:0}$ to prepare compound 30. Compound 31 with a free 1-hydroxyl group was prepared by cleavage of the allyl group, and then 1α-*O*-phosphorylation to yield the protected 1,4'-bisphosphate compound 32 was performed by 1-*O*-lithiation with buthyllithium (BuLi) and subsequent treatment with tetrabenzyl diphosphate (9, 19, 20). The product purified using silica-gel chromatography was deprotected by two-step hydrogenolysis (8 kg/cm^2 of H_2) with Pd-black in THF and subsequent platinum

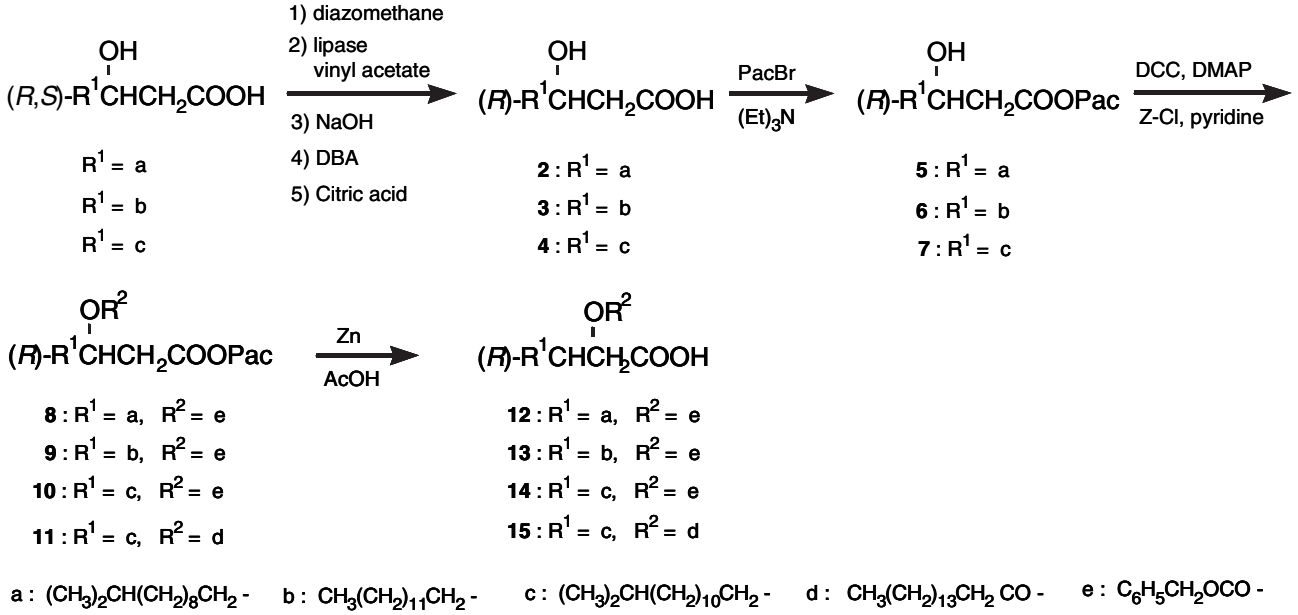

Fig. 2. Synthesis of (*R*)-3-hydroxy fatty acids; (*R*)-3-OH-$iC_{15:0}$, (*R*)-3-OH-$C_{16:0}$, (*R*)-3-OH-$iC_{17:0}$ and (*R*)-3-OH-($C_{16:0}$)-$iC_{17:0}$. Synthetic procedures of each fatty acid are described in the Materials and methods.

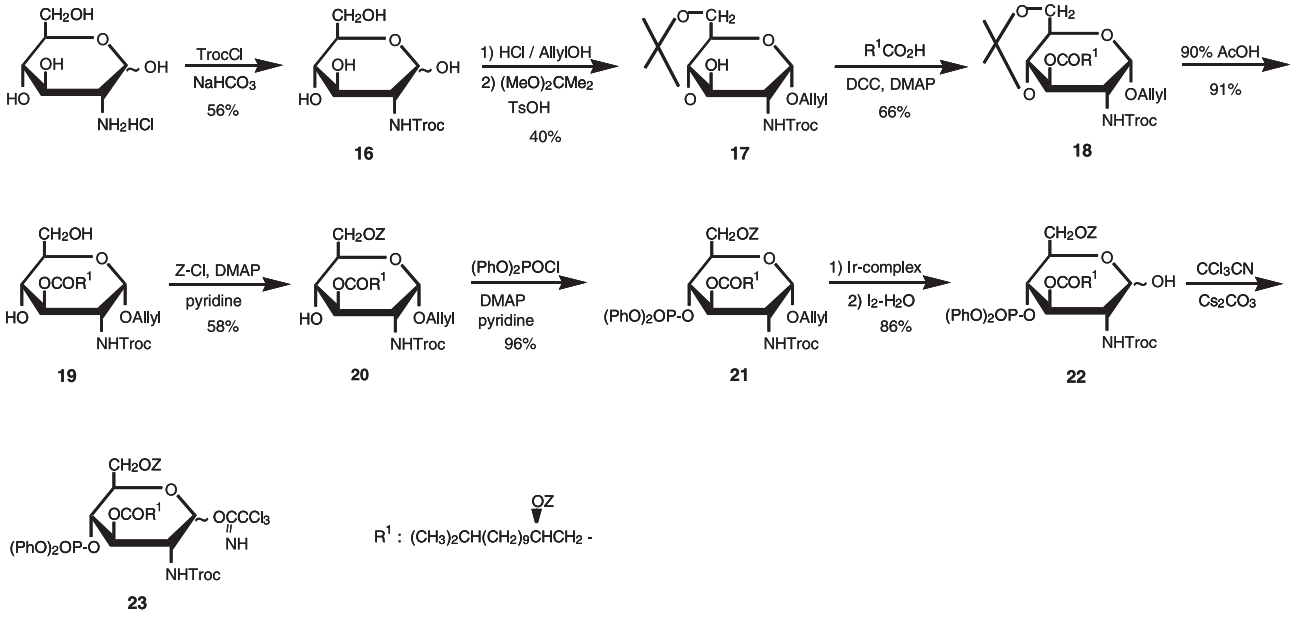

Fig. 3. Total synthesis of *Porphyromonas gingivalis* lipid A (step 1). Step 1 was performed for the preparation of a glycosyl donor corresponding to the terminal residue. Synthetic procedures of step 1 are described in the Materials and methods.

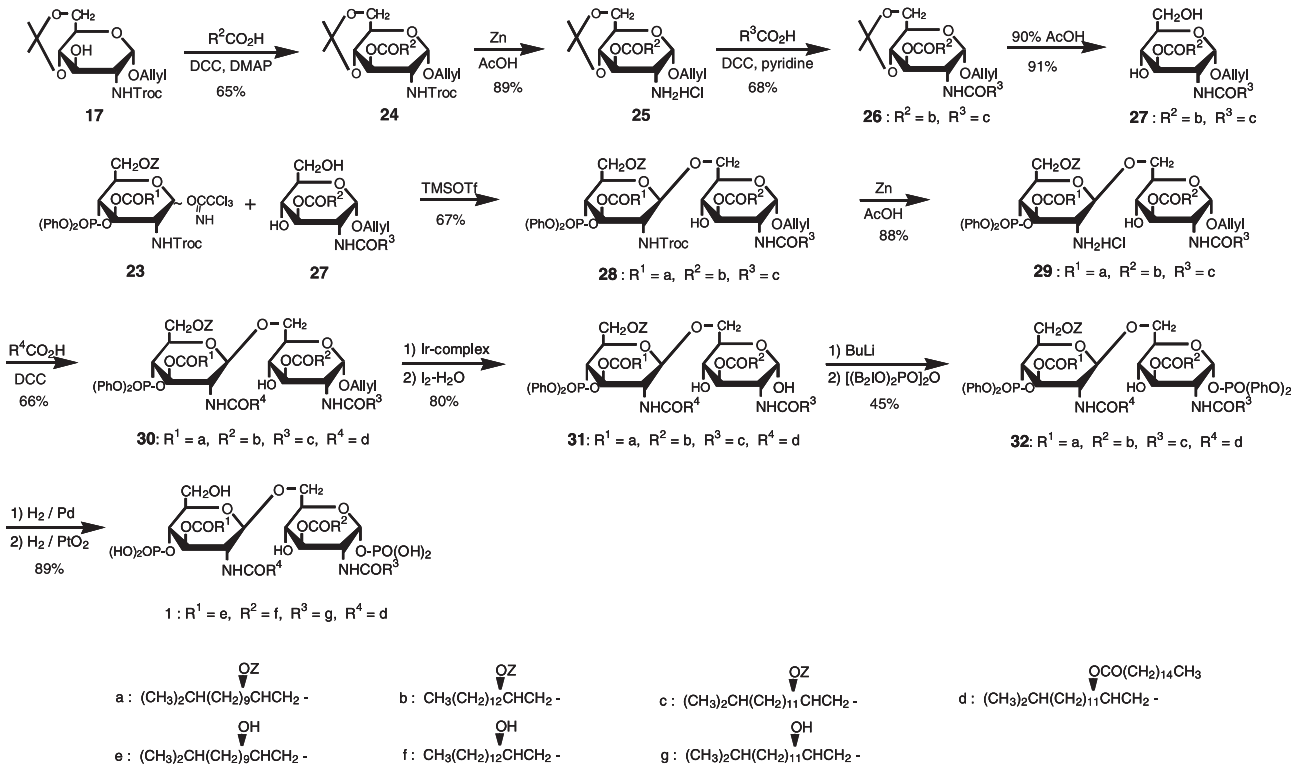

Fig. 4. Total synthesis of *Porphyromonas gingivalis* lipid A (step 2). Step 2 involved the preparation of a glycosyl acceptor corresponding to a non-terminal residue and coupling reaction of each unit. Synthetic procedures of step 2 are described in the Materials and methods.

oxide (PtO_2) in THF-H_2O (20 : 1) to give a good yield of *P. gingivalis* lipid A analog (compound 1), synthetic *P. gingivalis* lipid A (Pg-LA). Finally, the analog was effectively purified by centrifugal partition chromatography (49) using $CHCl_3$-MeOH-iPrOH-H_2O-Et_3N = 20 : 20 : 2.5 : 22.5 : 0.01 as a two-phase eluate on a Model LLB-M instrument (Sanki Engineering Ltd., Kyoto, Japan). The structure was confirmed by liquid secondary ion-mass spectrometry (m/z 1768.2 $[M–H]^-$) in negative ion mode and by nuclear magnetic resonance spectroscopy, which demonstrated

β-configuration of the glycosidic linkage, linkage positions of phosphate groups (1 and 4'), and α-configuration of the phosphorylated position 1.

Liquid secondary ion-mass spectrometry and nuclear magnetic resonance spectroscopy

Both liquid secondary ion-mass spectrometry and nuclear magnetic resonance spectroscopy were performed according to the methods reported previously (26).

LAL gelation assay

LAL gelation activity was measured by the chromogenic endotoxin-specific assay, Endospecy, using recombinant *Limulus* coagulation enzyme from horseshoe crab (34). Aliquots of 50-μl samples were incubated with the same volume of lysate containing chromogenic substrate in 96-well flat microplates at 37°C for 30 min. The absorbance was measured with a microplate reader (Wellreader SK-601, Seikagaku Kogyo) at 405 and 492 nm simultaneously, the latter as a reference. The data were expressed as the Δ absorbance (405–492 nm) per minute [ΔAbs/min (405–492 nm)].

Schwartzman assay

As described previously (27), the dermal Schwartzman assay was performed by injecting three male Japanese White rabbits (1.5–2.0 kg) intradermally into the shaved abdomen with 1, 10, or 100 μg of samples in 0.1 ml Dulbecco's phosphate buffered saline (PBS) (Nissui Pharmaceutical Co., Tokyo, Japan), followed 24 h later by a challenge intravenous injection of 100 μg *Salmonella typhimurium* LPS (Sigma) in 0.1 ml Dulbecco's PBS. The injection sites were examined for hemorrhagic necrosis 5 h after injection of the challenge dose. The results were expressed as the minimum dose of each sample to cause a hemorrhagic necrosis spot over 0.5 mm in diameter at the injection site.

Mitogenicity assay

Mitogenic activity was examined by the incorporation of [^{3}H]thymidine into spleen cells from C3H/HeN and C3H/HeJ mice as described (54). Mouse spleen cells were suspended in serum-free Iscove's modified Dulbecco's medium and washed with the same medium. The cells (8×10^5 cells/0.2 ml/well) were cultured in 96-well microplates containing various amounts of samples for 72 h at 37°C in a humidified 5% CO_2 atmosphere. During the final 24 h, 0.5 mCi (18.5 kBq) of [^{3}H]thymidine (18.2 Ci/mmol) per well was added and the incorporation of [^{3}H]thymidine by the cultured cells was measured with a liquid scintillation counter. The results were expressed as mean counts per minute (c.p.m.) of triplicate determinations.

Stimulation of murine macrophages and HGFs

Mouse peritoneal macrophages were obtained from C3H/HeN and C3H/HeJ mice injected intraperitoneally with 3.0 ml thioglycollate medium. The peritoneal cells (1×10^6 cells/ml), suspended in serum-free RPMI medium, were incubated for 2 h at 37°C in a humidified 5% CO_2 atmosphere. After incubation, adherent cells were stimulated for 47 h with samples to induce TNF-α and IL-6 production, and then the cell-free supernatants, passed through 0.22-μm Millex filters (Millipore Co., Bedford, MA), were stored at –20°C until used for the assays.

Normal HGFs obtained from patients were established by the explant growth method from clinically healthy gingival tissues as described elsewhere (61). The HGFs from passage 5 to 12 were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co.) containing 10% fetal calf serum (Gibco), penicillin (100 U/ml), and streptomycin (100 μg/ml) under 5% CO_2. After incubation for 4 days, the fibroblast layers were washed twice with Dulbecco's modified Eagle's medium and then incubated with 1 μg/ml of each sample without fetal calf serum for 47 h. The cell-free supernatants were harvested and stored at –20°C until used for the assays.

Cytokine assays

TNF-α and IL-6 activity in murine–macrophage culture supernatants were determined in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (Genzyme Co., Cambridge, MA), respectively.

TNF-α production was assayed using clone MM6-CA8 derived from Mono-Mac-6 (MM6) cells, a human monocytoid cell line with high sensitivity to LPS stimulation (48). MM6-CA8 cells exhibit a superior response to low concentrations of endotoxin and peptidogylcan in producing proinflammatory cytokines. MM6-CA8 cells were cultured in RPMI-1640 medium containing fetal bovine serum (10%), glutamine (2 mM), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM) and bovine insulin (9 μg/ml). After cell priming (72 h) with calcitriol (1,25-dihydroxy-vitamin D3), the cells (1×10^6 cells ; 0.9 ml/well) were seeded in 24-well plates, and various dilutions (0.1 ml) of sample were added. After incubation for 17 h, TNF-α released into the culture supernatants was immunoenzymatically measured using commercial ELISA kits as described above.

Inhibition assays

To examine the effects of polymyxin B and mAb to CD14 on the production of IL-8, 100 U/ml of polymyxin B sulfate (Sigma) and 2.5 μg/ml anti-CD14 (MY4, Coulter Co., Miami, FL) was added simultaneously or after pretreatment for 2 h, respectively, to the HGF cultures stimulated with 1 μg/ml of samples for 47 h. The production of IL-8 from HGFs was determined in duplicate using a human IL-8 ELISA kit (Amersham, Piscataway, NJ).

For the inhibition assay of TNF-α from MM6-CA8 cells, 5 μg/ml anti-human TLR2 and TLR4/MD-2 complex mAbs, and 10 μg/ml anti-human CD14 were added to MM6-CA8 cell suspension in 24-well plates, and after 1 h, each sample (10 ng/ml native lipid A, and 1 ng/ml synthetic Pg-LA and 506) was added to the cell suspension. After incubation, TNF-α production by the cells was measured.

Results

LAL gelation activity

The LAL gelation activity of each sample was estimated by the kinetic-chromogenic assay using LPS-specific reagent. As shown in Fig. 5, the activity increased in a dose-dependent manner over the range of concentrations tested (1 pg/ml to 1 μg/ml). Synthetic Pg-LA exhibited strong LAL gelation activity equivalent to that of compound 506, which was used as a control. On the other hand, LAL activities of *P. gingivalis* LPS and native lipid A, reported previously as a weakly toxic endotoxin (55), were approximately 10,000-fold or 100-fold weaker than that of compound 506, respectively.

Schwartzman reaction

Localized Schwartzman activity in rabbits was measured, and the results are shown in Table 1. *S. typhimurium* LPS and compound 506, used as positive controls, exhibited strong activity, and the minimum doses to induce a Schwartzman reaction of

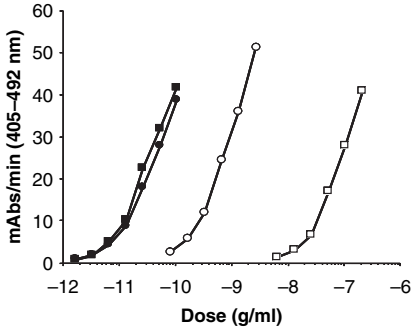

Fig. 5. LAL gelation activity of *Porphyromonas gingivalis* synthetic lipid A. LAL gelation activity was estimated by the kinetic–chromogenic assay using the LPS-specific reagent, Endospecy. Fifty-microliter aliquots of samples were incubated with the same volume of lysate at 37°C for 30 min. The data are expressed as the Δ absorbance (405–492 nm) per minute [ΔAbs/min (405–492 nm)]. ●, synthetic Pg-LA; ○, native lipid A; □, LPS; ■, compound 506.

each sample were 5 and 10 μg/site, respectively. Schwartzman activity of synthetic Pg-LA was similar to that of these positive controls, and the minimum inducing dose was 10 μg/site. However, minimum inducing doses of *P. gingivalis* LPS and native lipid A were 100 and 50 μg/site, respectively.

Mitogenicity

The mitogenic activities of samples were tested on murine splenic cells from LPS-responsive C3H/HeN and LPS-unresponsive C3H/HeJ mice. As shown in Fig. 6A, synthetic Pg-LA and compound 506 showed activity in response to splenic cells from C3H/HeN mice even at a dose of 1 μg/ml, and the activity increased in a dose-dependent manner over the dose range tested. *P. gingivalis* native lipid A also exhibited activity similar to those of both synthetic compounds. As shown in Fig. 6B, significant mitogenicity was observed in the splenic cells from LPS-unresponsive C3H/HeJ mice treated with

Table 1. Minimum dose of *Porphyromonas gingivalis* synthetic lipid A inducing a local Schwartzman reaction

Stimulants	Minimum inducing dose (μg/site)
P. gingivalis	
synthetic lipid A	10
native lipid A	50
LPS	100
Compound 506	
S. typhimurium	5

The minimum dose of samples for positive reaction was determined as the amount inducing a hemorrhagic spot more than 0.5 mm in diameter.

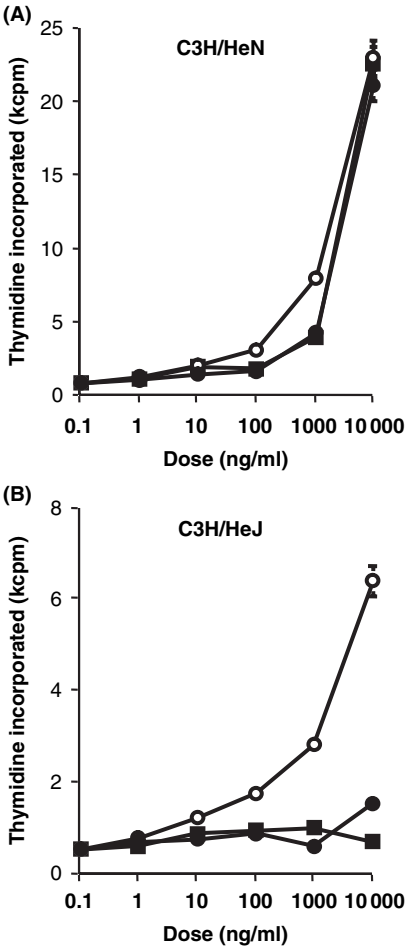

Fig. 6. Mitogenic responses of murine spleen cells from C3H/HeN and C3H/HeJ mice to *Porphyromonas gingivalis* synthetic lipid A. Spleen cells (8×10^5 cells/0.2 ml) were cultured in 96-well microplates containing various amounts of samples for 72 h. During the final 24 h, 0.5 mCi (18.5 kBq) of [^{3}H]thymidine (18.2 Ci/mmol) per well was added. The results are expressed as mean c.p.m. ± SD of triplicate experiments. ●, synthetic Pg-LA; ○, native lipid A; ■, compound 506.

P. gingivalis native lipid A, whereas synthetic Pg-LA and control compound 506 had no mitogenic activity even at a concentration of 10 μg/ml.

Induction of inflammatory cytokine release from various cells

Cytokine production by lipid A stimulation was assayed using HGFs, peritoneal macrophages from C3H/HeN and C3H/HeJ mice, and human MM6-CA8 cells. As shown in Table 2, native lipid A and synthetic Pg-LA exhibited activity for IL-8 induction activity in HGFs that was similar to that of compound 506 (1132.6, 1085 and 1056.9 pg/ml, respectively). The activity of synthetic analogs was significantly inhibited by polymyxin B and anti-CD14 mAb, whereas that of native lipid A was not inhibited by polymyxin B and anti-human CD14 mAb reduced the activity by only 50%.

Synthetic compound 506, used as a control, exhibited strong TNF-α and IL-6 induction in thioglycollate-elicited peritoneal macrophages from LPS-responsive C3H/HeN mice at doses of <10 ng/ml, as shown in Figs 7A and 8A. The activities of *P. gingivalis* synthetic and native lipid A were approximately 10- to 100-fold weaker than those of compound 506, respectively (Figs 7A and 8A). The *P. gingivalis* native lipid A significantly stimulated TNF-α and IL-6 production in peritoneal macrophages from LPS-unresponsive C3H/HeJ mice (Figs 7B and 8B). These LPS-unresponsive mice showed a similar minimum lipid A stimulatory dose and similar levels of cytokine production to C3H/HeN mice (Figs 7A and 8A). However, no induction of TNF-α or IL-6 release was observed with synthetic Pg-LA and compound 506, as shown in Figs 7B and 8B.

As shown in Table 3, synthetic Pg-LA and compound 506 stimulated TNF-α production in human monocytoid MM6-CA8 cells even at a low dose (each 1 ng/ml), whereas moderate activity in the cells was observed by native lipid A (10 ng/ml). Anti-human TLR4/MD-2 complex mAb significantly blocked the TNF-α production by synthetic Pg-LA and compound 506, whereas 7% inhibition and 6% inhibition were observed by anti-human TLR2 mAb (Table 3). On the other hand, both anti-human TLR2 and TLR4/MD-2 complex mAbs were essential to suppress cytokine production by *P. gingivalis* native lipid A (Table 3). In addition, anti-human CD14 mAb also completely inhibited TNF-α production induced by synthetic Pg-LA and compound 506, but approximately 70% inhibition was observed by native lipid A, as well as the results of the IL-8 production from HGF cells (Table 2).

Discussion

In the present study, we synthesized an analog of *P. gingivalis* lipid A according to the chemical structure proposed in our previous report (26), to reconfirm the biological data reported to date by some investigators using LPS or native lipid A (8, 24, 50, 54, 55, 59), including its action on C3H/HeJ mice. Some reports suggested that *P. gingivalis* LPS possesses lipid A structural heterogeneity, consisting of only a tri-acylated monophosphorylated form (37), and of a multiple heterogeneity

Table 2. Effects of anti-CD14 mAb on the production of IL-8 from human gingival fibroblasts stimulated with *Porphyromonas gingivalis* synthetic lipid A

	IL-8-producing activity		
		Treatment	
Stimulants		Polymyxin B	Anti-CD14
P. gingivalis			
synthetic lipid A	1085.0 ± 15.2	12.5 ± < 0.1	301.3 ± <0.1
native lipid A	1132.6 ± 2.9	1251.8 ± 80.5	640.8 ± 33.6
Compound 506	1056.9 ± 21.1	9.9 ± < 0.1	118.9 ± 14.1
None	12.2 ± <0.1	1.5 ± < 0.1	2.2 ± < 0.1

Human gingival fibroblasts were cultured with 1 μg/ml of each sample for 48 h. Polymyxin B (100 U/ml) was added simultaneously with stimulant cultivation and anti-CD14 mAb MY4 (2.5 mg/ml) was precultured for 2 h.

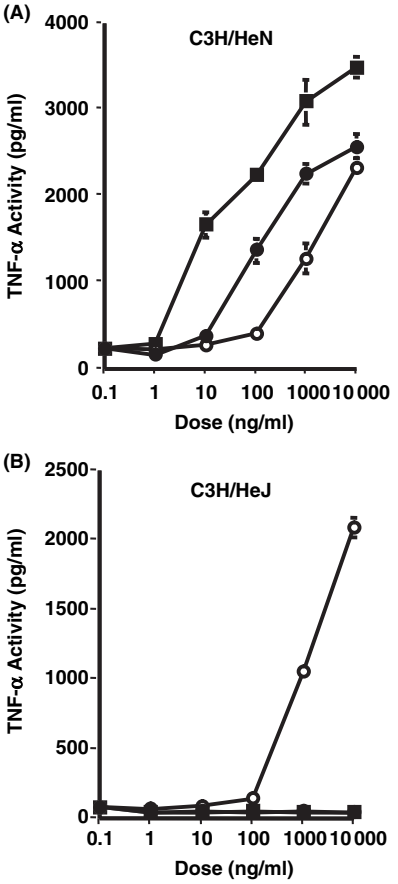

Fig. 7. Induction of TNF-α release from murine peritoneal macrophages from C3H/HeN and C3H/HeJ mice stimulated by *Porphyromonas gingivalis* synthetic lipid A. Thioglycollate-induced peritoneal macrophages (1×10^6 cells/ml), suspended in serum-free RPMI medium, were incubated for 2 h. After incubation, cells were stimulated for 47 h with various amounts of samples, and then the supernatants were examined for TNF-α. The results are expressed as means ± SD of duplicate experiments. ●, synthetic Pg-LA; ○, native lipid A; ■, compound 506.

regarding the degree of acylation and/or phosphorylation; tetra- and penta-acylated monophosphorylated species seem to be the predominant molecules (3, 7, 26). In particular, Darveau's group and others have reported that specific bacteria, such as *P. gingivalis*, *Yersinia pestis*, and *S. typhimurium*, possess the ability to alter or regulate these lipid A forms under specific environmental conditions, and these lipid A alterations might modify the innate host responses to each pathogenic bacterium (3, 6, 7, 11, 22). However, these lipid A species may not reflect the complete structure of *P. gingivalis* lipid A, because pentaacyl and diphosphoryl molecules were detected in the native lipid A complexes, although this is not the main species of *P. gingivalis* lipid A (26). The analog synthesized in this study consisted of a β(1–6)-linked D-glucosamine disaccharide 1,4′-bisphosphate backbone acylated with (*R*)-3-hydroxy-15-methylhexadecanoic acid, (*R*)-3-hydroxyhexadecanoic acid, (*R*)-3-*O*-(hexadecanoyl)-15-methylhexadecanoic acid and (*R*)-3-hydroxy-13-methyltetradecanoic acid at positions 2, 3, 2′, and 3′ of a hydrophilic backbone, as shown in Fig. 1. This analog represents a lipid A molecule with the highest molecular mass of all the species found in native lipid A complexes (26), and does not contain bioactive contaminants including the LPS protein (54) or lipopeptide (13, 28) that is present in native preparations. Nor does it have the heterogeneity that may lead to decreased endotoxicity based on the elimination of acyl and phosphoryl groups in the native lipid A complex (55), eliciting low endotoxic activities of synthetic *P. gingivalis* lipid A (compound PG-381), which consists of triacylated monophosphorylated lipid A molecule (35–37).

The endotoxic activities of *P. gingivalis* LPS or native lipid A were moderate relative to that of compound 506, which was used as a control, and significantly stimulated cells from LPS-unresponsive C3H/HeJ mice, as reported previously (8, 24, 54, 55, 59). However, *P. gingivalis* synthetic lipid A exhibited an activity similar to that of compound 506 in all the biological assays in this study, including the test using LPS-unresponsive C3H/HeJ mice. These results indicated that fully acylated and phosphorylated *P. gingivalis* lipid A is a strong agonist and, at the least, low toxicity and biological activity against LPS-unresponsive mice found in the LPS may not be dependent on the unique acyl residues, iso-form fatty acids consisting of 15–17 carbon atoms, that are characteristic components of *P. gingivalis* lipid A.

The relationship between the chemical structure of lipid A and its endotoxic activity has been studied using both natural and chemically synthesized lipid A analogs (36, 37, 54, 55). As a tentative conclusion, the two phosphates at positions 1 and 4′ in the lipid A molecule appear to influence the activity considerably, and the degree of acylation, binding sites and type seem to be critical determinants of the potency for endotoxic activity. Taking these findings into consideration, the moderate toxicity of *P. gingivalis* LPS may originate from the low levels of acylation and phosphorylation based on heterogeneity of the lipid A part, as described above.

Recent studies have indicated that TLR4 may play an important role in LPS-mediated immune responses (4, 17, 39), and TLR2 may be associated with cellular responses to numerous microbial products (4, 53). Many preparations of LPS contain low concentrations of highly bioactive contaminants described previously as LPS protein, suggesting that these contaminants could be responsible for the TLR2-mediated signaling observed upon LPS stimulation (2, 14, 53). TNF-α production by synthetic Pg-LA and compound 506 in human monocytoid MM6-CA8 cells was significantly suppressed by the anti-human TLR4/MD-2 complex mAb (HTA125) but not by the anti-human TLR2 mAb (TL2.1), indicating that these synthetic compounds act on the cells only through the TLR4 signaling pathway. However, TL2.1 in addition to HTA125 was essential to inhibit the TNF-α-producing activity of *P. gingivalis* native lipid A. These findings indicated that both TLR2 and TLR4 pathways may be associated with the action of the native preparations on MM6-CA8 cells, in contrast to the case of synthetic analogs. Furthermore, it was also suggested that the murine TLR2 signaling pathway is associated with mitogenicity and cytokine-inductive activity by *P. gingivalis* LPS in LPS-unresponsive C3H/HeJ mice having a missense mutation in the third exon of the *TLR4* gene (39). These results indicated that the unique biological activity of *P. gingivalis* native LPS or lipid A to

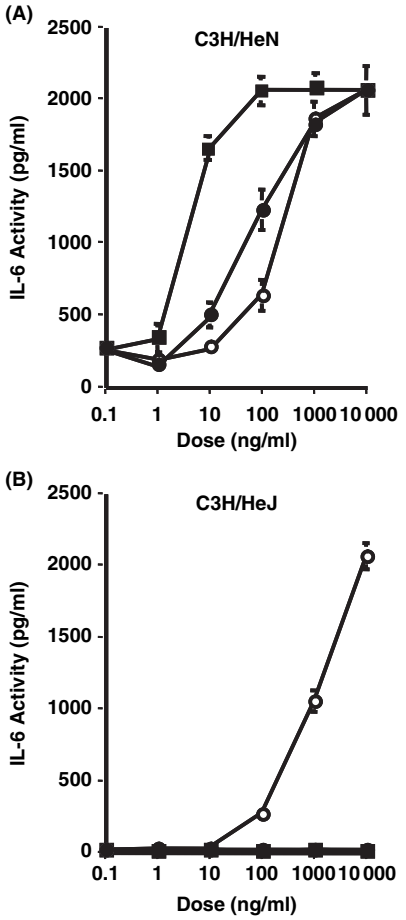

Fig. 8. Induction of IL-6 release from murine peritoneal macrophages from C3H/HeN and C3H/HeJ mice stimulated by *Porphyromonas gingivalis* synthetic lipid A. Stimulation of peritoneal macrophages was performed as well as that of TNF-α, and IL-6 activity in the supernatants was determined by an ELISA kit. The results are expressed as means ± SD of duplicate experiments. ●, synthetic Pg-LA; ○, native lipid A; ■, compound 506.

LPS-unresponsive mice appears to be induced by bioactive contaminants such as LPS protein (54) and other microbial components present in the native preparations. Ogawa's group recently suggested that *P. gingivalis* purified natural lipid A and compound 381 lacked the ability to activate gingival fibroblasts from C3H/HeJ, TLR4 knockout and myeloid differentiation factor 88 knockout mice (35), and also a triacylated lipoprotein, consisting of two palmitoyl groups and one pentadecanoyl group at the N-terminal of glycerocysteine from *P. gingivalis* lipid A, is a principal component for TLR2-mediated cell activation (13, 28). On the other hand, Darveau's group seem to have considered the possibility that multiple lipid A species of *P. gingivalis* may functionally interact with both TLR2 and TLR4, such as the under acylated lipid A moiety activate cells through TLR2 (6, 7).

IL-8 induction activity of synthetic Pg-LA to HGFs was significantly inhibited by polymyxin B in this study. However, no inhibitory effects of polymyxin B were observed on the immune responses of native *P. gingivalis* lipid A. The results were similar to those of other reports that the effect of polymyxin B on *P. gingivalis* LPS was relatively low (23). Polymyxin B may neutralize the endotoxicity of active-type molecules present in *P. gingivalis* LPS by binding to phosphate groups in the lipid A part (31), but may not inhibit the immune responses induced by the other contaminating bacterial components that act through the TLR2 pathway. This may be one of the reasons for the low inhibitory potency of polymyxin B to *P. gingivalis* LPS, in addition to the factors affecting the LPS-neutralization potency such as the absence of a phosphate group at position 4′ and presence of a polar head group in the native lipid A (26).

Anti-human CD14 mAb inhibited both IL-8 production from HGFs and TNF-α production from human monocytoid MM6-CA8 cells by synthetic Pg-LA as described in this study. On the other hand, it has been reported that anti-murine CD14 mAb could block *P. gingivalis* LPS-mediated immune responses (50) that may be mediated through both TLR2 and TLR4. These findings suggested that CD14 associates closely with not only the TLR4 but also the TLR2 signaling pathway mediated by *P. gingivalis* LPS. This was also supported by our previous report (54) that the LPS-antagonist, succinylated lipid A precursor (succinylated 406), inhibited TNF-α induction activity of *P. gingivalis* native lipid A in peritoneal macrophages from C3H/HeN and C3H/HeJ mice. In addition B464, a low-toxicity lipid A analog (62), significantly inhibited TNF-α production from human monocytoid MM6-CA8 cells induced by *P. gingivalis* native and synthetic lipid A (data not shown). Succinylated 406 and B464 competitively inhibit LPS action at the same stages in the LPS signaling pathway involving LBP, CD14 and TLRs. Taking these points into consideration, both LPS inhibitors appear to suppress the immune responses through CD14 and the TLR family including TLR2, by blocking the function of CD14 in the signaling cascade.

In conclusion, these findings suggested that the moderated and reduced biological activity of *P. gingivalis* LPS and native lipid A, including the activity on C3H/HeJ mouse cells via the TLR2-mediated pathway, may be mediated by bioactive contaminants or low acylated molecules present in the native preparations having high heterogeneity in lipid A moiety. To elucidate these problems, we are now attempting to evaluate the biological characterizations of tetra-acylated monophosphorylated or diphosphorylated species with the predominant molecules found in *P. gingivalis* native lipid A, using each chemically synthesized analog.

Acknowledgments

We would like to thank Dr Shoichi Kusumoto for helpful advice in the synthesis process of lipid A. We would also like to thank Drs Toshimi Murai and Yukari Nakagawa for helpful advice on the culture method of MM6-CA8 cells and Daiichi Pharmaceutical Co. for providing *Escherichia coli*-type synthetic lipid A (compound 506). This work was supported in part by grants from the Ministry of Education,

Table 3. Effects of mAbs on the production of TNF-α by human monocytoid MM6-CA cells stimulated with *P. gingivalis* synthetic lipid A

	Stimulants *P. gingivalis* lipid A					
	Native		Synthetic		Compound 506	
mAbs	TNF-α release[1]	% control	TNF-α release[1]	% control	TNF-α release[1]	% control
Non	118.0 ± 2.1	100.0	428.6 ± 45.6	100.0	420.8 ± 48.9	100.0
Anti-TLR2	65.0 ± 3.0	55.1	398.2 ± 89.5	92.9	396.0 ± 22.2	94.1
Anti-TLR4	67.0 ± 0.2	56.8	5.7 ± 3.1	1.3	11.5 ± 3.6	2.7
Anti-TLR2 + TLR4	35.0 ± 3.6	29.7	18.2 ± 7.8	4.2	40.3 ± 5.6	9.6
Anti-CD14	38.0 ± 2.1	32.2	3.8 ± 1.2	0.9	3.4 ± 1.1	0.8

[1]TNF-α release in pg/ml. Dose of each antibody: anti-human TLR2 mAb TL2.1, 5 μg/ml; anti-human TLR4/MD-2 complex mAb HTA125, 5 μg/ml; anti-human CD14 mAb MY4, 10 μg/ml. Dose of each lipid: *Porphyromonas gingivalis* native lipid A, 10 ng/ml; *P. gingivalis* synthetic lipid A, 1 ng/ml; compound 506, 1 ng/ml.

Science and Culture (17592170 to H.K. and 04671111 to Y.H.)

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