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Chemical structure of lipid A from *Porphyromonas* (*Bacteroides*) *gingivalis* lipopolysaccharide

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The novel chemical structure of *Porphyromonas* (*Bacteroides*) *gingivalis* strain 381 lipid A was determined to be a glucosamine β-(1–6) disaccharide 1-monophosphate acylated by 3-hydroxy-15-methylhexadecanoic acid and 3-hexadecanoyloxy-15-methylhexadecanoic acid at the 2- and 2′-positions, respectively. The absence of ester-linked phosphate at the 4′-position and fatty acids at the 3- and 3′-positions, and the presence of fatty acids possessing 16–17 carbon atoms are unique features, differentiating the lipid A from enterobacterial and other lipid As. These structural features may be related to its low endotoxic activity. Furthermore, *P. gingivalis* lipid A as well as its LPS stimulated the splenocytes from C3H/HeN and C3H/HeJ mice.

Endotoxin; Lipid A; Purification; Structure; *Porphyromonas gingivalis*

1. INTRODUCTION

Lipopolysaccharides (LPS) are characteristic components found on the outer membrane of Gram-negative bacteria. They typically consist of the polysaccharide region covalently bound to the lipid region, termed lipid A. Although the lipid A being responsible for biological activity has been shown to have numerous beneficial biological properties, its properties have not been utilized clinically because of its characteristic high toxicity. Many efforts have been made to obtain nontoxic lipid A retaining the beneficial activities from chemically synthesized derivatives and from natural sources [1].

Porphyromonas (*Bacteroides*) *gingivalis* which is a Gram-negative, black-pigmented anaerobic rod is suspected of being a major periodontopathic organism in chronic periodontal diseases [2]. *P. gingivalis* lipopolysaccharides (LPS) has previously been studied immunobiologically. It was shown that their low endotoxic properties are quite different from that of enterobacterial LPS [3]. On the other hand, *P. gingivalis* LPS [4] similar to *Bacteroides fragilis* LPS [5] exhibit mitogenic effects on the splenocytes from C3H/HeJ as well as from C3H/HeN mice. It was also reported that the endotoxic potency of *B. fragilis* LPS was low compared with enterobacterial LPS, and that this low endotoxic activity may depend on the structure of its lipid A component defined chemically [6]. However, the chemical structure of *P. gingivalis* lipid A has still not been determined. In the present study, the novel chemical structure of the lipid A found in the LPS of *P. gingivalis* will be shown as compared with the enterobacterial and *B. fragilis* lipid As.

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Abbreviations: Ac, acetyl; COSY, correlation spectroscopy; FAB, fast atom bombardment; GLC; gas-liquid chromatography; HPLC, high performance liquid chromatography; LPS, lipopolysaccharides; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; TLC, thin-layer chromatography.

2. MATERIALS AND METHODS

2.1. *Bacteria and preparation of LPS*

P. gingivalis strain 381 was grown anaerobically in GAM broth (Nissui, Tokyo, Japan) supplemented with hemin and menadione at 37°C for 26 h. Bacterial cells were collected by centrifugation, washed three times with pyrogen-free water and lyophilized. LPS were extracted from lyophilized cells by the hot phenol/water method [7], and the purification of the crude extract was performed by repeated ultracentrifugation (100,000 × *g*, 3 h) followed by treatment with nuclease P1 (Yamasa Shoyu Co., Choshi, Japan) and finally lyophilized.

2.2. *Preparation and purification of lipid A*

Purified LPS (100 mg) was hydrolyzed in 0.6% acetic acid at 105°C for 2.5 h, cooled and neutralized. H_2O was added to the hydrolysate to 100 ml, and added to 200 ml of $CHCl_3$/MeOH/H_2O/triethylamine (30:12:2:0.1). The lower phase was evaporated under reduced pressure. Subsequently, the lipid A fractions (30.5 mg) were separated by silica gel column chromatography (Silica Gel 60, 230–400 mesh: Nacalai Tesque Inc., Kyoto, Japan) with $CHCl_3$/MeOH/H_2O/triethylamine (30:12:1.5:0.1). The eluants were monitored by thin-layer chromatography (TLC) on silica gel 60 plate. The plates were developed with $CHCl_3$/MeOH/H_2O/triethylamine (30:12:2:0.1) and visualized by sulfuric acid, Dittmer-Lester reagent [8] and triphenyltetrazolium chloride (TTC) reagent [9]. The fast moving compound (**1**, the dephosphorylated *P. gingivalis* lipid A, R_f = 0.7) that was purified by silica gel column chromatography again with $CHCl_3$/MeOH/H_2O/Triethylamine (30:12:0.5:0.1) and the slow moving compound (**2**, *P. gingivalis* lipid A, R_f = 0.3) were both observed as single spots as the major components, respectively.

2.3. *Analysis of component sugars and fatty acids*

The dephosphorylated *P. gingivalis* lipid A (**1**) and *P. gingivalis* lipid A (**2**) were hydrolyzed with 6 N HCl at 105°C for 2.5 h. Sugars in the

H_2O layer were analyzed by high-performance liquid chromatography (HPLC) with glucosamine as a standard. The dephosphorylated *P. gingivalis* lipid A (**1**) was peracetylated by acetic anhydride in pyridine at room temperature for 18 h. The peracetate (**3**) was purified by silica gel chromatography ($CHCl_3$/ethyl acetate, 2:1). Total fatty acids were analyzed by GLC as methyl esters. Fatty acids were extracted into ethyl acetate and methylated by 5% methanolic HCl at 105°C for 3 h. The component of fatty acids were determined by GC-MS as methyl esters and MS-MS as free acids. Ester-linked fatty acids were analyzed by GLC as methyl esters after methanolysis with 1% methanolic KOH at room temperature for 45 min.

2.4. *Conditions for chromatography*

GLC was performed on a Shimadzu GC-9A (Shimadzu, Kyoto) instrument fitted with a glass column (3 mm × 2 m) packed with 5% Advance DS on 60/80 mesh Chromosorb W (Shimadzu). HPLC was carried out by a model 305 pump, a model 805 manomeric module, a model 231–401 intelligent auto-sample injector (MS Gilson, Osaka, Japan), and a SE-G1 RI detector (Shodex, Tokyo) on a guard column 4 × 50 mm and a column 4 × 250 mm in size filled with Asahipak NH2P-50 (Asahi Chemical Ind. Co., Kanagawa, Japan) using a solvent of 70% acetonitrile.

2.5. *Instrumental methods*

^{1}H NMR spectra were recorded at 300 MHz on a GN-300 spectrometer (General Electronic Co., Fremont, CA, USA). The dephosphorylated *P. gingivalis* lipid A (**1**) and *P. gingivalis* lipid A (**2**) were dissolved in $CDCl_3$/methanol-*d*4/D_2O (30:12:1). Chemical shifts were referenced to $CDCl_3$ (δ 7.26 ppm) or methanol-*d*4 (δ 3.30 ppm). Two-dimensional correlation spectroscopy (COSY) was carried out at 300 MHz on a GN-300 spectrometer. The peracetate of dephosphorylated *P. gingivalis* lipid A (**3**) was dissolved in $CDCl_3$. Chemical shifts were referenced to $CDCl_3$ (δ 7.26 ppm). FAB-MS and MS-MS analysis were carried out using a JMS-HX110/110 (JEOL, Akishima, Japan) operating at an accelerating voltage of 10 kV and a mass resolution of 1500. *m*-Nitrobenzyl alcohol-glycerol was employed as the supporting matrix for negative ion FAB analysis. A Xe atom beam of 6 kV was operated. Gas-liquid chromatography-mass spectrometry (GLC-MS) was done with a Hitachi M-80B spectrometer (Hitachi, Katsuta, Japan) fitted with a HP5710 gas chromatograph (Hewlett-Packerd, USA).

2.6. *Animals*

BALB/c, C3H/HeN and C3H/HeJ mice (male, 8 weeks old) were obtained from Japan SLC (Hamamatsu, Japan).

2.7. *Mitogenic activity*

Splenocytes (5×10^5 cells) of mice were cultured with various concentrations of *P. gingivalis* LPS and its lipid A, *Escherichia coli* O55:B5 LPS (List Biological Laboratories, Campbell, CA) and *E. coli* bisphosphate type synthetic lipid A (compound 506; Daiichi Pure Chemicals, Tokyo) in 0.2 ml of RPMI1640 medium (Biken, Osaka, Japan) supplemented with 5% fetal bovine serum in a 96-well flat bottom Falcon 3040 microtiter plate for 48 h in 5% CO_2 and 95% air. During the final 16 h of cultivation, the cultures were pulsed with 1.0 μCi of [^{3}H]thymidine (74 GBq/mmol; Biomedicals, Inc., Costa Mesa, CA) per well and were harvested onto glass fiber filter strips. Thymidine uptake was measured in an LKB model 1215 liquid scintillation counter (Turku, Finland).

3. RESULTS

The less polar compound (**1**, $R_f = 0.7$) which increased during weak acid hydrolysis for lipid A preparation was visualized by TTC reagent for the detection of reducing groups, but not by Dittmer-Lester reagent for the detection of phosphate esters on TLC plate. The polar compound (**2**, $R_f = 0.3$) was visualized by Dittmer-Lester reagent, but not by TTC reagent. Therefore, compound **1** corresponded to the dephosphorylated lipid A, and the phosphate esters in *P. gingivalis* lipid A (**2**) linked to the reducing end of the dephosphorylated *P. gingivalis* lipid A (**1**).

P. gingivalis lipid A (**2**) contained only glucosamine (1.91 μmol/mg) by HPLC analysis of the H_2O layer of hydrolysate (6 N HCl, 105°C, 2.5 h). The ^{1}H NMR spectrum of *P. gingivalis* lipid A (**2**) in $CDCl_3$/methanol-*d*4/D_2O (30:12:1) showed two anomeric protons (δ 4.46 ppm: $J_{1,2} = 8.0$ Hz, δ 5.44 ppm: broad peak), i.e. *P. gingivalis* lipid A (**2**) contains two glucosamine, but because of the tendency to aggregate, it gave poorly resolved ^{1}H NMR spectra. As expected, the ^{1}H NMR spectrum of the dephosphorylated *P. gingivalis* lipid A (**1**) in $CDCl_3$/methanol-*d*4/D_2O (30:12:1) also gave two anomeric protons (δ 4.30 ppm: $J_{1,2} = 7.8$ Hz, δ 4.93 ppm: $J_{1,2} = 3.5$ Hz). The peracetate (**3**) of the dephosphorylated *P. gingivalis* lipid A (**1**) in $CDCl_3$ gave an improved spectrum in which the presence of seven acetyl groups were demonstrated. In this spectrum, the high-field anomeric proton (δ 4.69 ppm: $J_{1,2} = 8.4$ Hz) was characteristic of a β-linked glucosamine residue. The down-field shifted anomeric proton (δ 6.07 ppm: $J_{1,2} = 3.6$ Hz) suggested an *a*-glucosamine. As shown in Fig. 1, assignment of the proton signals of glucosamine was performed by the two-dimensional COSY spectra of the peracetate (**3**). From chemical shift values and *J* values it can be concluded that the peracetate (**3**) consisted of β-(1–6)-linked diglucosamine disaccharide backbone and its hydroxyl groups at the 1-, 3-, 4-, 3′-, 4′- and 6′-positions were acylated, and two amino groups at the 2- and 2′-positions were also acylated. Consequently, from the result of the TLC analysis given above, *P. gingivalis* lipid A (**2**) contains 1-phosphorylated β-(1–6)-diglucosamine structure.

Analysis of fatty acids by GLC and GLC-MS revealed that the major fatty acids of *P. gingivalis* lipid A (**2**) were hexadecanoic acid (0.91 μmol/mg) and branched 3-hydroxyheptadecanoic acid (1.76 μmol/mg). The dephosphorylated *P. gingivalis* lipid A (**1**) also gave two of the same fatty acids in the same ratio as lipid A (**2**). According to the mol ratio of fatty acids to glucosamine, lipid A (**2**) contained 1 mol of hexadecanoic acid and 2 mol of branched 3-hydroxyheptadecanoic acid. By analysis of free branched 3-hydroxyheptadecanoic acid on negative MS-MS spectrum, absence of the fragment ion peak at $m/z = 255$ $(M\text{-}2H\text{-}CH_3CH_2)^-$ corresponded to 3-hydroxy-15-methylhexadecanoic acid. The ^{1}H NMR spectrum of these three compounds showed fifteen methyl protons in which a triplet and a doublet could be observed, which further confirmed the fact that *P. gingivalis* lipid A (**2**) contains hexadecanoic acid (1 mol) and 3-hydroxy-15-methylhexadecanoic acid (2 mol). In the alkaline methanolysate of *P. gingivalis* lipid A (**2**), only hexadecanoic acid

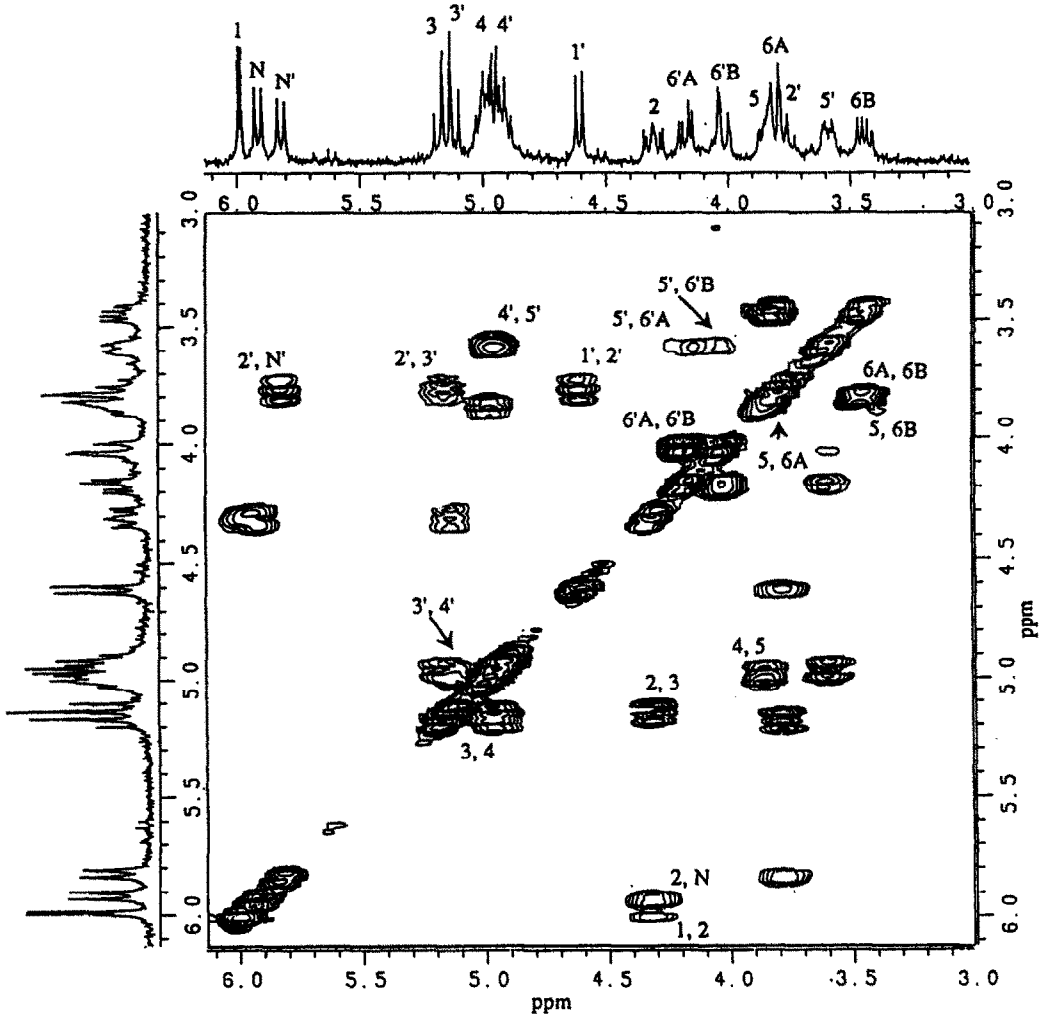

Fig. 1. Contour plot of the 2D COSY spectrum of the peracetate of dephosphorylated lipid A (3) from (300 MHz in $CDCl_3$).

was detected in the GLC. Under the condition of the methanolysis used here, ester-linked fatty acids were released, but amide-linked fatty acids still remained. Consequently, 2 mol of 3-hydroxy-15-methylhexadecanoic acid must be linked to each amino group of the two glucosamines. The two dimensional COSY spectrum yielded more information on the substitution of the 3-hydroxy-15-methylhexadecanoic acid residues in the dephosphorylated *P. gingivalis* lipid A (**1**). An α-methylene signal located at δ 2.13 ppm coupled with a proton at δ 3.75 ppm, due to a β-methines of 3-hydroxy acid, while a signal at δ 2.30 ppm coupled with a β-methines of 3-acyloxy acid at δ 5.00 ppm. Consequently, the lipid A component of *P. gingivalis* possesses 1-phosphorylated β-(1–6)-diglucosamine structure, and both amino groups of disaccharide are acylated by 3-hydroxy-15-methylhexadecanoic acid and 3-hexadecanoyloxy-15-methylhexadecanoic acid. The position of acyl residue was studied with *P. gingivalis* lipid A (**2**). The negative ion FAB-MAS on *P. gingivalis* lipid A (**2**) showed major molecular ions at m/z = 1193 $(M\text{-}H)^-$. The presence of minor molecular ions which differed in 14 mass units could be attributed to the presence of heterogeneity of length of fatty acids. The locations of fatty acids were deduced from the fragmentation observed in the negative FAB-MS-MS spectrum of *P. gingivalis* lipid A (**2**). Negative fragment ion peaks were observed at m/z = 554, 508 and 406 (Fig. 2). From these values it could be concluded that one hexadecanoic acid acylates to the amine of the reducing glucosamine unit and one 3-hexadecanoyloxy-15-methylhexadecanoic acid acylates to the amine of the nonreducing glucosamine unit.

P. gingivalis lipid A (**2**) as well as LPS exhibited strong mitogenic activities on splenocytes from BALB/c, C3H/HeN and C3H/HeJ mice (data not shown). *E. coli* O55:B5 LPS and synthetic lipid A compound 506 were found to be clearly mitogenic on splenocytes from BALB/c and C3H/HeN mice, but had no effect on C3H/HeJ mice. The mitogenicity of *P. gingivalis* lipid A (**2**)

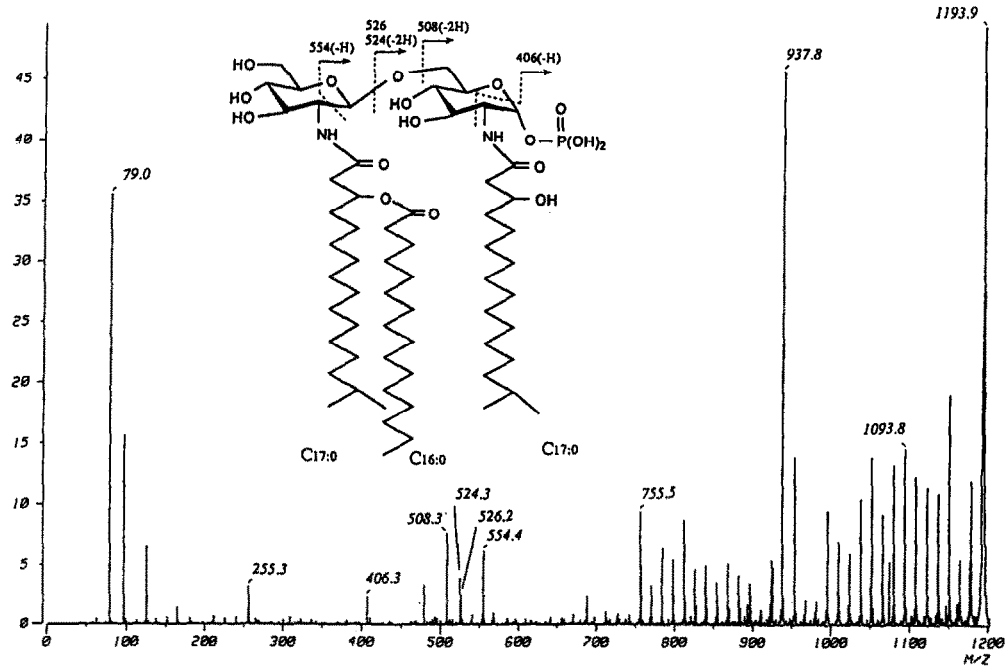

Fig. 2. Negative ion FAB-MS-MS spectrum of *P. gingivalis* lipid A (2).

was comparable to that of compound 506 in the splenocytes of BALB/c and C3H/HeN mice.

4. DISCUSSION

The present study clearly indicates that the lipid A of *P. gingivalis* strain 381 has been validly chemically characterized. The backbone of *P. gingivalis* lipid A (2) consists of a β-(1–6)-linked glucosamine disaccharide which is phosphorylated at the 1-position of the reducing sugar, but this lipid A structure lacks an ester-linked phosphate group which is bound to the hydroxy group at the 4′-position of the nonreducing sugar. It was reported that there is no 4′-*O*-phosphoryl group in the lipid A backbone of *B. fragilis* [6] and *Bacteroides intermedius* (*Prevotella intermedia*) [10]. It was also found that hydroxyl groups at the 3-, 3′-, 4-, 4′- and 6′-positions of *P. gingivalis* lipid A (2) are free (Fig. 3). It was previously described that hydroxyl groups in the 4- and 6′-positions of *E. coli*-, *Salmonella minnesota*- and *Pseudomonas aeruginosa*-type lipid As [1,11] and in position 4, 4′ and 6′ of *B. fragilis*-type lipid A [6] are free, respectively.

At the 2- and 2′-positions of the disaccharide backbone, *P. gingivalis* lipid A (2) possesses two acyl groups. That is, 3-hydroxy-15-methylhexadecanoic acid and 3-hexadecanoyloxy-15-methylhexadecanoic acid are amide linked at the 2- and 2′-positions, respectively (Fig. 3). *E. coli* lipid A expresses endotoxic activities such as pyrogenicity, lethal toxicity, Shwartzman reaction, and *Limulus* test, namely, activation of clotting enzyme cascade of *Tachypleus tridentatus* [12]. The lipid A of *E. coli*, *S. minnesota* and *P. aeruginosa* consist of a β-(1–6)-linked glucosamine disaccharide 1,4′-bisphosphate which is acylated at the 2-, 2′-, 3- and 3′-positions [1,11]. On the other hand, *B. fragilis* LPS exhibits low endotoxic potency which is probably related to the chemical structure of the lipid A portion, namely, a different phosphorylation and acylation pattern as compared with the lipid As of Enterobacteriaceae and *Pseudomonas* [6].

The lipid A (2) as well as LPS from *P. gingivalis* but not *E. coli* was mitogenic for splenocytes from endotoxin nonresponder C3H/HeJ mice. It was shown that LPS from *P. gingivalis* and *B. fragilis* were also mitogenic for splenocytes from the same murine strain

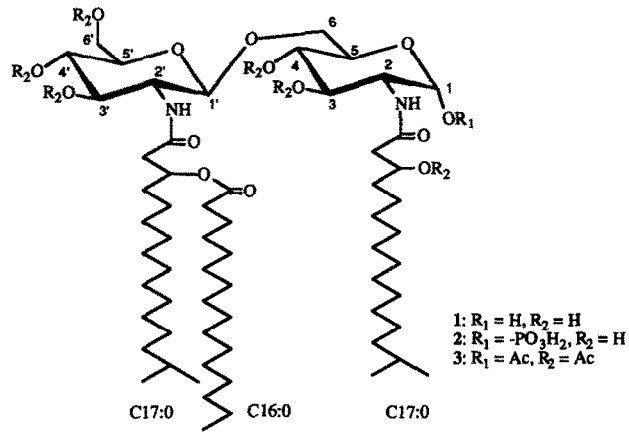

Fig. 3. Proposed structure of the dephosphorylated lipid A (1), the lipid A (2) and the peracetate of dephosphorylated lipid A (3) from *P. gingivalis*.

[4,5]. It was suggested that the stimulatory effect on splenocytes from C3H/HeJ mice is attributed to a unique structure of *P. gingivalis* lipid A (**2**). Furthermore, the LPS from *B. gingivalis* (*P. gingivalis*) and *B. fragilis* exhibit low endotoxic potency [3,13,14]. Once more, low endotoxic activities, such as pyrogenicity, Shwartzman reaction, lethal toxicity and *Limulus* test of the lipid A (**2**) as well as the LPS from *P. gingivalis* as compared with that of *E. coli* were seen (unpublished results). The low toxic activity is probably attributable to the structural differences, i.e. the absence of a 4′-*O*-phosphoryl group, the presence of only two fatty acids and the considerable length of acyl chains.

Taken together, it has been clearly demonstrated here that *P. gingivalis* lipid A possesses a unique structure which differs from enterobacterial and *B. fragilis* lipid As. Further work is in progress to investigate the biological and beneficial effects of *P. gingivalis* lipid A. In addition, I am currently attempting to synthesize lipid A, mimicking the native lipid A portion of LPS from *P. gingivalis* to unequivocally confirm its low endotoxic potency and immunobiological activity.

REFERENCES

[1] Takada, H. and S. Kotani. (1989) Crit. Rev. Microbiol. 16, 477–523.
[2] Slots, J. and Listgarten, M. A. (1988) J. Clin. Periodontal. 15, 85–93.
[3] Nair, B. C., Mayberry, W. R., Dziak, R., Chen, P. B., Levine, M. J. and Hausmann, E. (1983) J. Periodontal. Res. 18, 40–49.
[4] Hamada, S., Takada, H., Ogawa, T., Fujiwara, T. and Mihara, J. (1990) Int. Rev. Immunol. 6, 247–261.
[5] Joiner, K. A., Keith, P. W., McAdam, W. J. and Kasper, D. L. (1982) Infect. Immun. 36, 1139–1145.
[6] Weintraub, A., Zähringer, U., Wollenweber, H.-W., Seydel, U. and Ritschel, E. Th. (1989) Eur. J. Biochem. 183, 425–431.
[7] Westphal, O. and Jann, K. (1965) Methods Carbohydro. Chem. 5, 83–91.
[8] Dittmer, J. C. and Lester, R. L. (1964) J. Lipid Res. 5, 126–127.
[9] Fischer, F. G. and Dörfel, H. (1954) Hoppe-Seyler's Z. physiol. Chem. 297, 164–178.
[10] Johne, B., Olsen, I and Bryn, K. (1988) Oral Microbiol. Immunol. 3, 22–27.
[11] Karunaratne, D. N., Richards, J. C. and Hancock, R. E. W. (1992) Arch. Biochem. Biophys. 299, 368–376.
[12] Kotani, S., Takada, S., Tsujimoto, M., Ogawa, T., Takahashi, I., Ikeda, T., Otsuka, K., Shimauchi, H., Kasai, N., Mashimo, J., Nagao, S., Tanaka, A., Tanaka, S., Harada, K., Nagaki, K., Kitamura, H., Shiba, T., Kusumoto, S., Imoto, M. and Yoshimura, H. (1985) Infect. Immun. 49, 225–237.
[13] Kasper, D. L. (1976) J. Infect. Dis. 134, 59–65.
[14] Sveen, K. (1977) J. Periodontal Res. 12, 340–350.