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Migration of lactic acid, lactide and oligomers from polylactide food-contact materials

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Polylactide (PLA) is used for manufacturing lunch boxes and for packaging fresh food in Japan. PLA can be hydrolysed relatively easily to produce lactic acid, lactide and oligomers. Different types of PLA sheet were subjected to migration tests under various conditions and the lactic acid, lactide and oligomers contents of the migration solutions were determined using liquid chromatography/mass spectrometry (LC/MS). Furthermore, the change in molecular weight was determined by a migration test. PLA was stable at 40°C for 180 days; the total of lactic acid, lactide and oligomers migration levels were 0.28–15.00 $\mu g\,cm^{-2}$. PLA decomposed clearly at 60°C for only 10 days, the total migration levels were increased to 0.73–2840 $\mu g\,cm^{-2}$. PLA sheets with a high D-lactic acid content decomposed particularly rapidly. The amounts of alkali decomposition products, based on the conversion of lactide and oligomers to lactic acid by alkali hydrolysis, corresponded with the total migration levels.

Keywords: alkali decomposition products; lactic acid; lactide; molecular weight; oligomers; polylactide

Introduction

Polylactide (PLA) is used for manufacturing lunch boxes and for packaging fresh food in Japan. Therefore, PLA may be in contact with various type of food. It is produced by the polymerization of lactic acid or lactide (a cyclic dimer), which are mainly supplied from corn starch. PLA can be hydrolysed relatively easily to produce lactic acid, lactide and oligomers, which are subsequently decomposed into water and carbon dioxide by microorganisms (Tuominen 2002).

In the USA two types of PLA with D-lactic acid contents of 6% or less and between 6% and 16%, respectively, are permitted under the Food Contact Notification (FCN) system. In the European Union, lactic acid is registered on the list of monomers and other starting substances in European Directive 2002/72/EC (European Commission 2002).

Due to its biodegradability, there are concerns about the migration products of PLA, which might include lactic acid, lactoyl-lactic acid (a linear dimer), lactide and oligomers. With regard to lactic acid toxicity, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommended that neither D-lactic acid nor (DL)-lactic acid should be used in infant foods (World Health Organization (WHO) 1974). The toxicity of lactide was previously investigated by two- and 13-week studies on beagles. The findings revealed the presence of gross and microscopic lesions indicative of irritation, and suggested a no observed adverse effect level (NOAEL) of 100 $mg\,kg^{-1}$ body weight day^{-1} (Hebert et al. 1999). Based on these data, the Food Safety Commission of Japan settled on an acceptable daily intake (ADI) of lactide of 0.1 $mg\,kg^{-1}\,day^{-1}$.

In a previous paper we reported the basic properties of PLA which are relative viscosity, molecular weight, D-lactic acid contents and metal content. Furthermore, we performed overall migration tests on several types of PLA sheet, which gave results of less than 20 $\mu g\,cm^{-2}$, although the values increased under acidic or high-temperature conditions (Mutsuga et al. 2007). Lactic acid and lactide were not included in these residues because they evaporated under the test conditions during heating at 105°C.

Lactide is gradually decomposed to lactic acid under water-rich conditions, and oligomers also supply lactic acid through lactide. When the lactide migration level from PLA is examined, it is also necessary to measure simultaneously the lactic acid and oligomers in migration solution, and to consider them as these total amounts. The current study revealed the presence of lactic acid, lactide and oligomers in PLA migration solutions using liquid chromatography/mass spectrometry (LC/MS). In addition, the molecular weights of the PLA sheets were measured after the migration tests, and the degradation of the PLA was clarified.

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Materials and methods

Sample materials

Four types of PLA sheet (from two different manufacturers) were used in this study. Their properties reported in a previous paper (Mutsuga et al. 2007) are summarized in Table 1.

Reagents

L-lactic acid (98%) was purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Lactide (3,6-dimethyl 1,4-dioxane-2,5-dione) (98%) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Formic acid was purchased from Sigma-Aldrich Japan (Tokyo, Japan). High-performance liquid chromatography (HPLC)-grade acetonitrile was purchased from Merck Co., Inc. (Darmstadt, Germany). Water was purified by the Milli-Q SP system (Millipore Co., MA, USA). Sterilized water was prepared for the migration test by autoclaving.

Migration test

The PLA sheets were cut into 2×12.5 cm test samples. The test samples were placed into each glass tubes (24 mm i.d. $\times$ 220 mm) containing 100 ml of simulant (water, 4% acetic acid or 20% ethanol) and stopper the tubes. The sample tubes were replaced in the thermostatically controlled oven setting at each test temperature, and left for each test period. After their test periods each test sample was removed from simulant. The stimulant was analysed for lactic acid, lactide and oligomers by LC/MS. If the migrant level of lactic acid or lactide were under determination limit ($0.4\,\mu g\,cm^{-2}$), a ten-fold concentration of the migration solution was determined again, which was prepared by dissolving the freeze-dried residue of 10 ml migration solution in 1 ml water.

The migration into heptane at 25°C for 1 h was performed. For the determination of lactic acid, a 10 ml migration solution was transferred to a separatory funnel and added 10 ml water. The water layer injected to LC/MS. Lactide was determined directly by gas chromatography-mass spectrometry (GC/MS) following with the condition of the previous report (Mutsuga et al. 2007).

Measurements of lactic acid, lactide and oligomers

The calibration curves for lactic acid and lactide determination were prepared each in a range of $0.2\text{–}5.0\,\mu g\,ml^{-1}$ ($0.4\text{–}20\,\mu g\,cm^{-2}$), each correlations were $R^2 > 0.99$. The levels of oligomers (from trimer to 13-mer) were estimated as lactide using the lactide calibration curve and they were then summed. The summation of lactic acid, lactide and oligomers was expressed as total migration level.

LC/MS conditions

The standard or test solution (20 µl) was injected into a LC/MS system (1100 series Agilent Technologies, Palo Alto, CA, USA) under the following conditions; LC column: TSKgel ODS-80TS QA (4.6 mm i.d. $\times$ 150 mm; Tosoh Co., Tokyo, Japan), column temperature: 40°C, flow rate: $0.3\,ml\,min^{-1}$, mobile phase: 0–30 min gradient from acetonitrile/0.1% formic acid (1 : 99) to acetonitrile and 30–40 min acetonitrile, MS electrospray capillary voltage: 3 kV, cone voltage: 60 V, drying gas temperature: 350°C, flow rate: $12\,l\,min^{-1}$ (N_2), ionization mode: electrospray ionization (negative), monitoring mode: selected ion monitoring (SIM), quantitative ion: m/z 89 (lactic acid), 161 (lactide) and $72n + 17$ (where oligomers $n = 3\text{–}13$).

Measurement of alkali decomposition products

The migration solution of water and 20% ethanol (1 ml) was saponified with 100 µl of 0.2 M sodium hydroxide, followed by heating for 15 min in a water bath at 60°C. After cooling, 100 µl of 0.2 M hydrochloride were added. The lactic acid in the solution was analysed by LC/MS. The calibration curve was produced by treating the lactic acid standard solutions in the same way. The 4% acetic acid migration solution (1 ml) was freeze-dried, and dissolved in 1 ml water and followed above. The amount of total lactic acid obtained by this operation was expressed as the alkali decomposition value.

Measurement of molecular weight

The test sheets were dried at room temperature. A 100-mg sample of each test sheet was dissolved

Table 1. Characterization of PLA sheets used in migration studies.

Sample	Manufacture	Thickness (mm)	Colour	Molecular weight (Mp)	Ratio of D-lactic acid (%)	Free lactide content ($\mu g\,g^{-1}$)
1	A	0.5	Colourless	1.8×10^5	<1.0	252
2	B	0.7	Colourless	1.7×10^5	1.3	1081
3	B	0.7	Colourless	1.7×10^5	4.1	1201
4	B	0.7	Colourless	1.9×10^5	11.3	1567

in 1 ml dichloromethane, and diluted to 25 ml with tetrahydrofuran (THF). Each solution was then subjected to gel-permeation chromatography (GPC). The relative molecular weight at peak top (Mp) were measured using the retention time by reference to a polystyrene standard from Mw 5×10^4 to 4.27×10^5 (TSK standard POLYSTYRENE, A-500, 1000, 2500, 5000, F-1, 2, 4, 10, 20, 40; Tosoh Co.). The GPC conditions were as follows: column: TSK-GEL G4000HXL (7.8 mm i.d. × 300 mm; Tosoh), column temperature: 40°C, mobile phase: THF, flow rate: 1 ml min^{-1}, injection volume: 50 µl, detection: ultraviolet (210 nm).

Results and discussion

Measurements of migrants

In a previous paper (Mutsuga et al. 2007), lactide was measured by GC/MS, but lactic acid and oligomers could not be measured. Therefore, lactic acid, lactide and oligomers in the migration solutions were determined simultaneously by LC/MS.

When the migration solution at 60°C for 5 days was measured in the SCAN mode, some peaks besides lactic acid and lactide were detected. They had m/z $72n+17$ (where $n=3$–13) as a base ion. When the alkali hydrolysis was performed, we only obtained lactic acid. Therefore, these peaks were identified with the oligomers. The chromatograms and mass spectra are shown in Figure 1.

The quantifications were performed in the SIM mode using m/z 89 $[M-H]^-$ for lactic acid, m/z 161 $[M+OH]^-$ for lactide and m/z $72n+17$ (where $n=$ 3–13) $[M-H]^-$ for oligomers. Oligomers could be detected from trimer to 13-mer. Each oligomer was determined using the lactide calibration curve, and the total quantities were calculated. The determination limit of each migrant was 0.04 µg cm^{-2} in ten-fold concentrated migration solution.

It is known that lactide and oligomers decompose easily to lactic acid, and this has been reported for the determination of migrants from PLA with the conversion from lactide and oligomers to lactic acid by alkali hydrolysis as the total amount of alkali decomposition products (Conn et al. 1995). The rate of decomposition of lactide solution (400 µg ml^{-1}) was 96.0%. In the migration solution shown in Figure 1a, the lactide and oligomers had completely decomposed to lactic acid (Figure 1b).

Stability of lactic acid and lactide in the simulants

Lactide is hydrolysed to lactic acid, which is then converted to water and carbon dioxide by microorganisms. Thus, it was important to consider the reduction of lactic acid and lactide during the migration test and the preservation before analysis. The stabilities of the compounds were confirmed using 1 µg ml^{-1} solution under the migration test conditions. Migration tests were performed with water, 4% acetic acid and

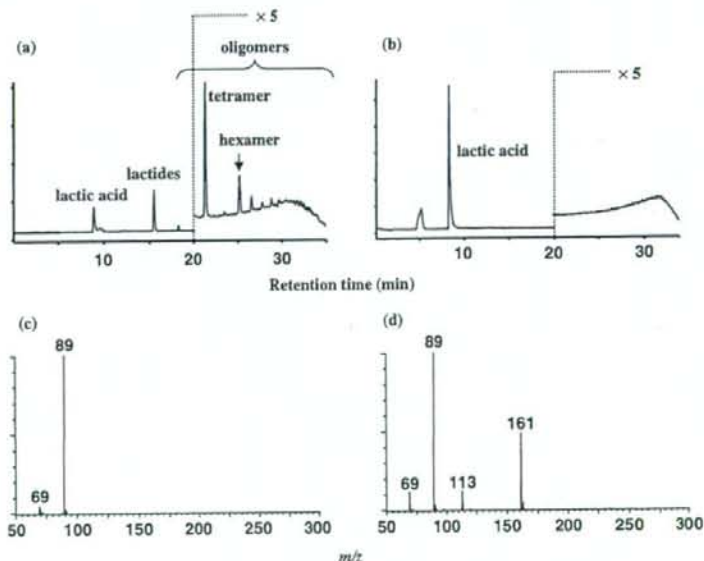

Figure 1. LC/MS/total ion chromatograms of migration solution from sample 3 at 60°C for 5 day into water (a) and after alkali hydrolysis (b), and mass spectra of lactic acid (c) and lactide (d).

20% ethanol as food simulants. Acetic acid is a simulant for acidic foods. Polylactide is polyester resin, and polyester is weak to alcohol. Therefore, 20% ethanol which is a simulant for liquor was also tested.

Lactic acid was stable under all of the test conditions, remaining at 86.6% even after incubation at 60°C for 10 days (Table 2). Lactide remained at 98.5–103.3% when incubated at 60°C for 30 min in each simulant, but the residual ratios were reduced to 73.0% and 21.0% after 1 and 10 days, respectively. By contrast, lactic acid was detected in these solutions at levels corresponding to the reduction of lactide. Thus, the lactide appeared to have been converted to lactic acid. This process also occurred on the oligomers which were converted to lactic acid via lactide. Therefore, it should be necessary to consider that migrants of lactide are as total of lactic acid, lactide and oligomers.

Effect of simulants and temperature in the short-term migration test

Migration tests were performed on four types of PLA sheet in water, 4% acetic acid and 20% ethanol at 60°C for 30 min. The migration levels of lactic acid, lactide, oligomers and alkali decomposition products were measured (Table 3). Lactic acid migration was detected at levels of 0.06–0.40 $\mu g\,cm^{-2}$ from all of the samples into water, 4% acetic acid and 20% ethanol. The highest level of lactic acid migration was observed in 4% acetic acid. The lactide migration was ranging from not determined (n.d.) to 2.4 $\mu g\,cm^{-2}$ and highest into 20% ethanol. Lactide migration was detected from samples 2–4, with sample 4 showing particularly high levels. By contrast, sample 1 showed little or n.d. lactide migration. Oligomers migration was detected only from samples 3 and 4 into 20% ethanol at 60°C for 30 min. However, sample 4 showed high levels of lactide migration into each type of simulant, particularly into 20% ethanol, which showed high migration levels and the presence of oligomers. With heptane at 25°C for 1 h, lactic acid and lactide were not detected from all of the samples.

The effect of the migration temperature was studied using sample 3 at 20, 40, 60, 80 and 95°C for 30 min (Table 4). The migration levels of lactic acid did not change at temperatures from 20°C to 80°C, and increased slightly at 95°C. While, the migration levels of lactide were increased to 0.24 $\mu g\,cm^{-2}$ at 60°C, to 0.64 $\mu g\,cm^{-2}$ at 80°C and to 4.12 $\mu g\,cm^{-2}$ at 95°C. Oligomers were detected at 1.98 $\mu g\,cm^{-2}$ only at 95°C. The sample became clouded after the migration test at 95°C.

Table 2. Stability of lactic and lactide under various conditions.

	Water			4% Acetic acid	20% Ethanol
	60°C, 30 min	60°C, 1 day	60°C, 10 day	60°C, 30 min	60°C, 30 min
Lactic acid	101.5 ± 2.0	94.1 ± 3.8	86.6 ± 4.0	116.5 ± 4.0	102.6 ± 1.8
Lactide	99.4 ± 1.7	73.0 ± 2.6	21.0 ± 6.0	98.7 ± 0.2	103.3 ± 1.5

Notes: Values are the mean (%) ± SD of three trials.
All trials each used 1 $\mu g\,ml^{-1}$ standard solution.

Table 3. Comparison of simulant in short-term test (60°C, 30 min).

		Level ($\mu g\,cm^{-2}$)				
Simulant	Sample	Lactic acid	Lactide	Oligomers	Total	Alkali decomposite
Water	1	0.12	n.d.	n.d.	0.12	0.18
	2	0.22	0.17	n.d.	0.43	0.45
	3	0.23	0.24	n.d.	0.53	0.68
	4	0.19	0.97	n.d.	1.40	1.63
4% Acetic acid	1	0.08	n.d.	n.d.	0.08	0.28
	2	0.34	0.19	n.d.	0.58	0.53
	3	0.35	0.30	n.d.	0.72	0.73
	4	0.40	1.22	n.d.	1.92	1.78
20% Ethanol	1	0.13	0.07	n.d.	0.22	0.33
	2	0.23	0.41	n.d.	0.74	0.93
	3	0.23	0.84	0.20	1.53	1.78
	4	0.24	2.41	1.16	4.70	5.18

Notes: Values are the mean of two trials.
n.d.: <0.04.
The oligomers (from trimer to 13mer) were measured as lactide.
Total = (lactic acid) + (lactide + oligomers) × 1.25, calculated as lactic acid.

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PLA hydrolysis has been reported to accelerate rapidly at temperatures above the glass-transition point of 55–60°C (Marcelo and James 1992; Conn et al. 1995). PLA decomposition appeared to have occurred at 60°C or more with the increasing of lactide levels (Table 4). However, the lactic acid levels did not alter from 20°C to 80°C. At 95°C, lactic acid was estimated to be produced from lactide generated by PLA decomposition.

Long-term migration into water at 40°C

The migration into water at 40°C was compared during 1, 3 and 6 months. The molecular weights of the tested samples were also measured. The levels of lactic acid, lactide, oligomers, alkali decomposition products and molecular weight are shown in Table 5.

Table 4. Effect of temperature on migrants from sample 3 into water.

	Level ($\mu g\,cm^{-2}$)			
Temperature (°C)	Lactic acid	Lactide	Oligomers	Total
20	0.24	n.d.	n.d.	0.24
40	0.24	n.d.	n.d.	0.24
60	0.22	0.24	n.d.	0.52
80	0.20	0.64	n.d.	1.00
95	0.36	4.12	1.98	7.99

Notes: Values are the mean of two trials.
n.d.:<0.04.
The oligomers (from trimer to 13mer) were measured as lactide.
Total = (lactic acid) + (lactide + oligomers) × 1.25, calculated as lactic acid.

Lactic acid was detected from all of the samples. However, the levels were generally less than $2\,\mu g\,cm^{-2}$ and the highest was $3.88\,\mu g\,cm^{-2}$ for sample 3 after 6 months. Lactide was detected from samples 2–4, and the levels were from n.d. to $0.24\,\mu g\,cm^{-2}$. Oligomers was detected only in samples 2–4 after 6 months. The total migrants were less than $1\,\mu g\,cm^{-2}$ after 6 months for most of the samples, though samples 3 and 4 were 9.00 and $14.66\,\mu g\,cm^{-2}$, which were similar to the short-term migration levels at 60°C for 30 min.

The main compound detected in the long-term tests was lactic acid. This was attributed to the fact that lactide and oligomers generated from PLA had decomposed to lactic acid during the test periods.

To clarify further the polymer decomposition, the molecular weights of the samples after the migration tests were measured. The Mp of the samples before the migration tests ranged from 1.7×10^5 to 1.9×10^5 (Table 1). The values after the migration tests are shown in Table 5. The molecular weights of samples 1–4 decreased to 1.1×10^5 to 1.6×10^5 after 3 months, and to 5.3×10^4 to 1.3×10^5 after 6 months. PLA was decomposed even below the glass-transition temperature in water for a long time. Since their Mp decreased by a small amount, it was speculated that decomposition of PLA had occurred by the removing of some lactide units from the terminal of polymer. The GPC chromatograms of samples 3 and 4 (Figure 2) revealed a clear decrease in Mp. Sample 4 showed a particularly notable decrease.

The most migrant levels were less than $1\,\mu g\,cm^{-2}$. PLA was comparatively stable when the PLA was in contact with water at 40°C. But, sample 3 and 4, which had a high D-lactic acid content, had clearly

Table 5. Migration levels into water at 40°C.

		Level ($\mu g\,cm^{-2}$)					
Testing time	Sample	Lactic acid	Lactide	Oligomers	Total	Alkali decomposite	Molecular weight (Mp)
1 Month	1	0.05	n.d.	n.d.	0.05	0.18	1.8×10^5
	2	0.12	n.d.	n.d.	0.12	0.23	1.6×10^5
	3	0.07	0.06	n.d.	0.15	0.43	1.6×10^5
	4	0.57	0.24	n.d.	0.87	0.93	1.7×10^5
3 Month	1	0.10	n.d.	n.d.	0.10	0.25	1.6×10^5
	2	0.23	0.05	n.d.	0.29	0.55	1.3×10^5
	3	0.20	n.d	n.d.	0.20	0.33	1.2×10^5
	4	1.04	0.11	n.d.	1.19	1.28	1.1×10^5
6 Month	1	0.13	n.d.	n.d.	0.13	0.28	1.3×10^5
	2	0.41	0.05	0.22	0.75	0.88	8.9×10^4
	3	3.88	0.08	4.02	9.00	9.93	6.7×10^4
	4	2.41	0.20	9.60	14.66	15.00	5.3×10^4

Notes: Values are the mean of three or four trials.
n.d.:<0.04.
The oligomers (from trimer to 13mer) were measured as lactide.
Total = (lactic acid) + (lactide + oligomers) × 1.25, calculated as lactic acid.

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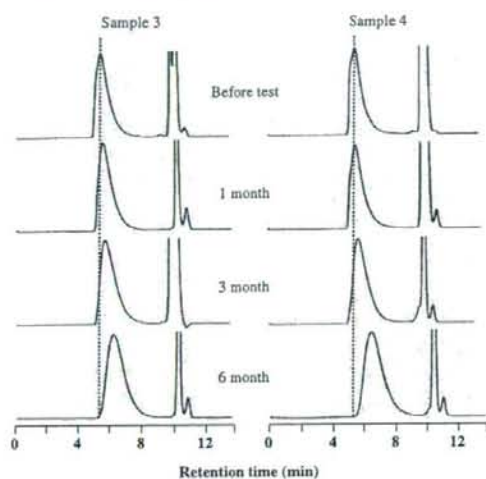

Figure 2. GPC chromatograms of sample 3 and 4 after migration test at 40°C.

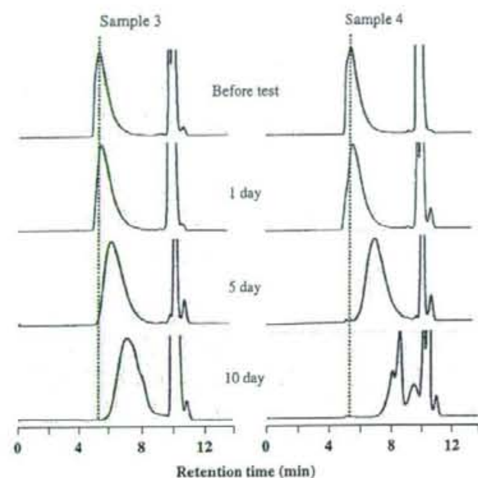

Figure 3. GPC chromatograms of sample 3 and 4 after migration test at 60°C.

Table 6. Migration test into water at 60°C.

Testing time	Sample	Level ($\mu g\,cm^{-2}$)					Molecular weight (Mp)
		Lactic acid	Lactide	Oligomers	Total	Alkali decomposite	
1 Day	1	0.20	0.08	n.d.	0.30	0.30	1.6×10^5
	2	0.13	0.44	n.d.	0.68	0.60	1.5×10^5
	3	0.28	1.08	n.d.	1.63	1.60	1.4×10^5
	4	0.85	5.30	0.46	8.05	8.38	1.4×10^5
5 Day	1	0.29	0.10	n.d.	0.41	0.63	1.4×10^5
	2	0.49	0.22	0.52	1.41	1.60	9.5×10^4
	3	1.70	0.75	1.00	3.89	4.38	6.0×10^4
	4	9.84	6.08	5.74	24.62	27.70	1.9×10^4
10 Day	1	0.60	0.08	n.d.	0.70	0.73	1.0×10^5
	2	2.65	0.22	0.04	2.98	3.55	4.2×10^4
	3	8.78	1.20	0.74	11.20	14.28	1.8×10^4
	4	420	398	1358	2615	2840	4.7×10^3

Notes: Values are the mean of two trials.
n.d.:<0.04.
The oligomers (from trimer to 13mer) were measured as lactide.
Total = (lactic acid) + (lactide + oligomers) × 1.25, calculated as lactic acid.

decomposed by the 6-month migrant test. The decomposition of polymer was correlated with the increase in the migrant levels.

Migration into water at 60°C

The migration test was carried out at 60°C for 30 min, 1, 5, and 10 days in consideration of the case where PLA lunch boxes are kept warm in a hot vendor. The levels of lactic acid, lactide, oligomers and molecular weight are shown in Table 6.

Advanced decomposition was detected far earlier at 60°C than at 40°C, and the migrant levels at 60°C after 1 day were similar to those at 40°C after 6 months. For sample 1, oligomers were not detected even after 10 days, and the total migrants were less than $1\,\mu g\,cm^{-2}$. However, the molecular weights had clearly decreased after 10 days, which suggested that decomposition had progressed slowly. For samples 2 and 3 the levels of lactic acid were notably increased, and oligomers migration was detected after 5 days.

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Table 7. Migration levels into water at 95°C.

Testing time	Level ($\mu g\,cm^{-2}$)					
	Sample	Lactic acid	Lactide	Oligomers	Total	Alkali decomposite
30 min	1	0.08	0.45	n.d.	0.64	0.85
	2	0.10	2.49	0.76	4.16	4.88
	3	0.37	4.13	1.98	8.00	8.88
	4	1.49	11.81	5.96	23.69	25.28
60 min	1	0.28	0.94	0.10	1.58	1.38
	2	0.88	3.20	0.36	5.33	7.25
	3	1.52	8.08	0.76	12.57	12.70
	4	2.00	15.78	1.24	23.28	32.50
120 min	1	0.44	1.02	0.26	2.04	1.68
	2	1.68	6.56	0.74	10.81	11.73
	3	2.38	10.36	1.18	16.81	19.83
	4	9.88	29.94	1.86	49.63	51.75

Notes: Values are the mean of two trials.
n.d.:<0.04.
The oligomers (from trimer to 13mer) were measured as lactide.
Total = (lactic acid) + (lactide + oligomers) × 1.25, calculated as lactic acid.

The GPC chromatograms of samples 3 and 4 are shown in Figure 3. In particular, the polymer in sample 4 had broken down completely after 10 days, and the dried test sample had lost its elasticity and collapsed easily into a powder form.

Based on these findings, PLA is not suitable for use at temperatures over 60°C for periods of several days, particularly if it contains high D-lactic acid levels.

Migration into water at 95°C

The migration test at 95°C for 30, 60, and 120 min was carried out to mimic the use of lunch boxes in a microwave oven or over 100°C. Both lactic acid and lactide migration were detected from all of the samples, and the levels increased with the testing period (Table 7). Oligomers were detected from samples 2–4 after 30 min, and from sample 1 after 60 min. The migration levels, according to the sum of migrants at 95°C after 30 min, were same as those at 40°C after 6 months and at 60°C after 5 days. The lactide levels were particularly high. Sample 4 showed more migration than the other samples, but it was at a smaller level than at 60°C for 10 days.

Comparison between the summation of the migrants and alkali decomposition product values

The amount of alkali decomposition products were almost similar to total of lactic acid, lactide and oligomers. The quantities of alkali decomposition products produced were usually higher than the total migration. This difference was attributed to three factors: the fact that the oligomers were determined by a lactide calibration curve, the presence of oligomers at levels less than the determination limit, and the presence of oligomers larger than 14-mers.

Conclusions

The main migrants from PLA were lactic acid, lactide and oligomers. It is necessary for the stability and food safety of PLA to measure the total lactic acid, lactide and oligomers because PLA easily decomposes to oligomers, then to lactide and lactic acid. But these were not included in the overall migration test residues. In this paper the migrants were measured using two measuring methods: total migration and alkali decomposition products. We obtained almost the same results.

PLA was relatively stable at 40°C over 6 months. However, at 60°C or above the glass-transition temperature the polymer was decomposed and the migrant levels increased. This tendency was particularly notable in PLA that contained high D-lactic acid levels. Moreover, the PLA products should be chosen for their purpose according to the prevailing conditions.

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

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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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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

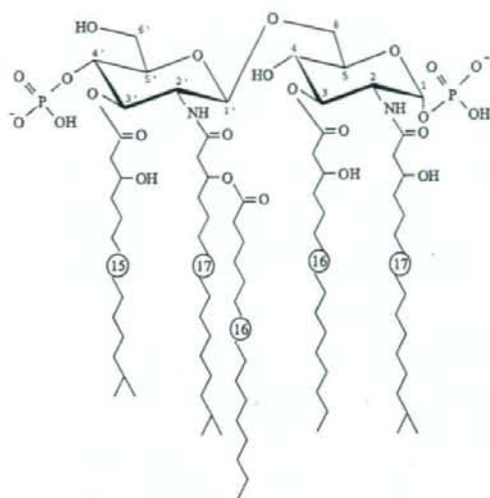

Fig. 1. Chemical structure of *Porphyromonas gingivalis* synthetic lipid A. In this study, we chemically synthesized a pentaacyl and diphosphoryl lipid A analog corresponding to the lipid A species with the highest molecular mass found in *P. gingivalis* native lipid A in our previous study (26). The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

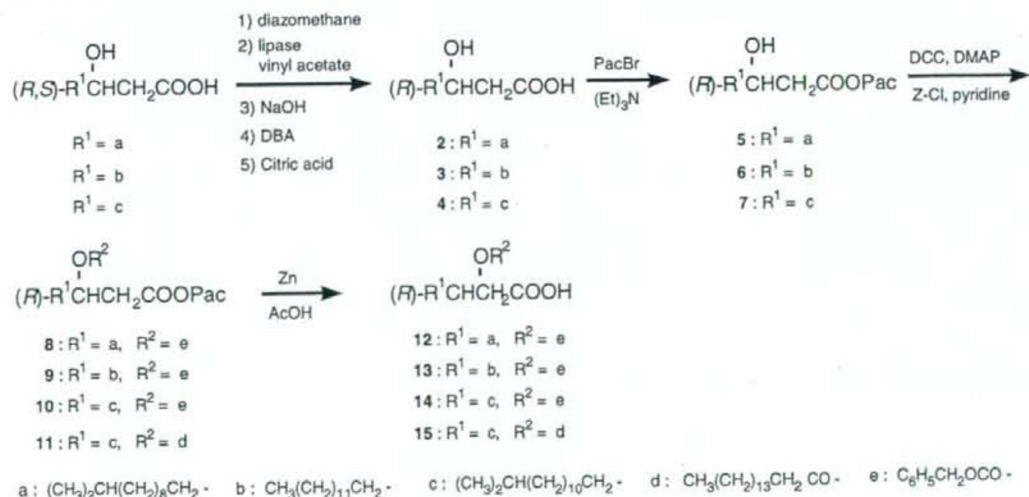

Fig. 2. Synthesis of (*R*)-3-hydroxy fatty acids; (*R*)-3-OH-i$C_{15:0}$, (*R*)-3-OH-$C_{16:0}$, (*R*)-3-OH-i$C_{17:0}$ and (*R*)-3-OH-($C_{16:0}$)-i$C_{17:0}$. Synthetic procedures of each fatty acid are described in the Materials and methods.

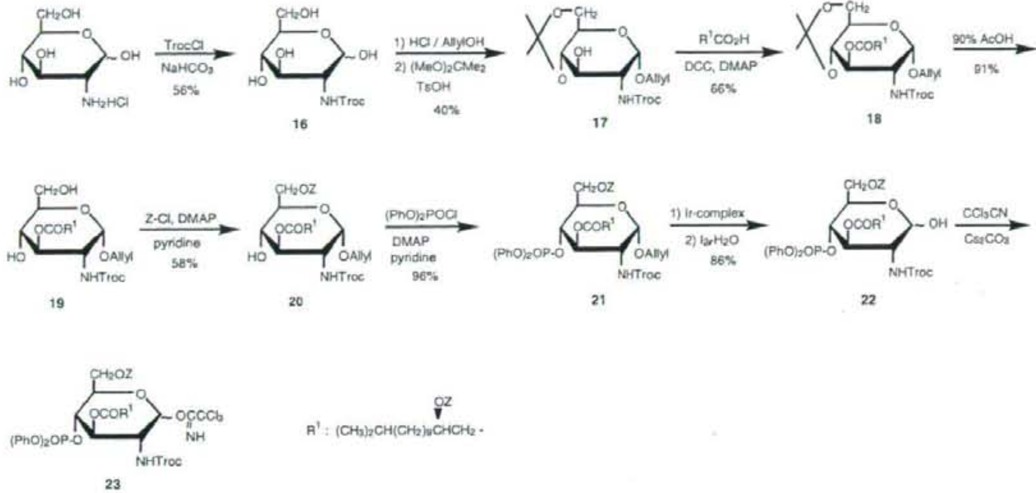

Fig. 3. Total synthesis of *Porphyromonas gingivalis* lipid A (step 1). Step 1 was performed for the preparation of a glycosyl donor corresponding to the terminal residue. Synthetic procedures of step 1 are described in the Materials and methods.

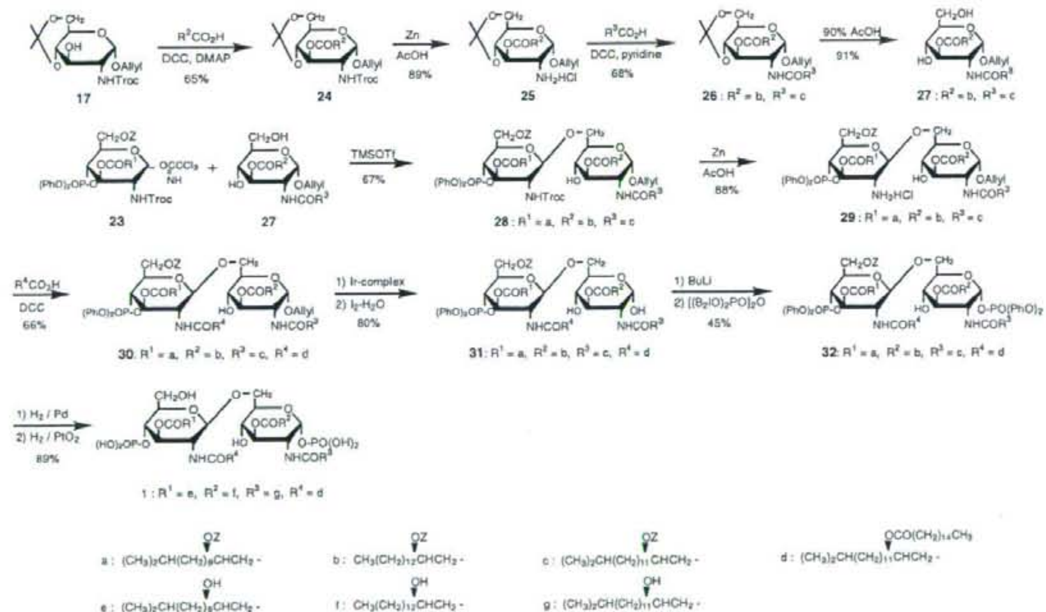

Fig. 4. Total synthesis of *Porphyromonas gingivalis* lipid A (step 2). Step 2 involved the preparation of a glycosyl acceptor corresponding to a non-terminal residue and coupling reaction of each unit. Synthetic procedures of step 2 are described in the Materials and methods.

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

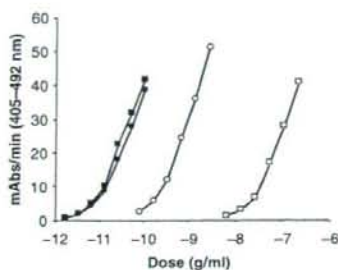

Fig. 5. LAL gelation activity of *Porphyromonas gingivalis* synthetic lipid A. LAL gelation activity was estimated by the kinetic–chromogenic assay using the LPS-specific reagent, Endospecy. Fifty-microliter aliquots of samples were incubated with the same volume of lysate at 37°C for 30 min. The data 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.

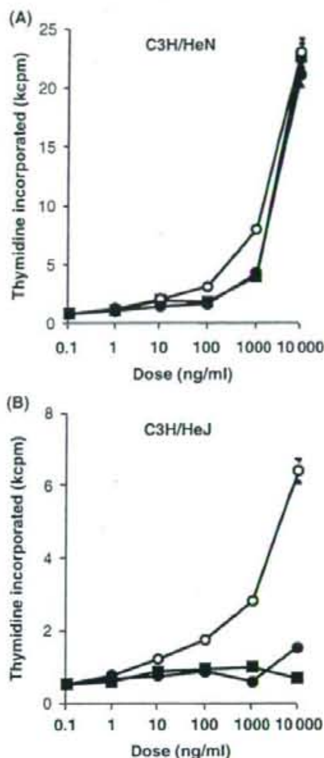

Fig. 6. Mitogenic responses of murine spleen cells from C3H/HeN and C3H/HeJ mice to *Porphyromonas gingivalis* synthetic lipid A. Spleen cells (8×10^5 cells/0.2 ml) were cultured in 96-well microplates containing various amounts of samples for 72 h. During the final 24 h, 0.5 mCi (18.5 kBq) of [^{3}H]thymidine (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

Stimulants	IL-8-producing activity	Treatment	
		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.

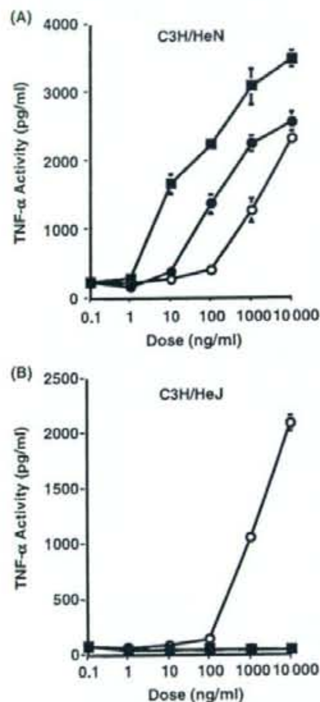

Fig. 7. Induction of TNF-α release from murine peritoneal macrophages from C3H/HeN and C3H/HeJ mice stimulated by *Porphyromonas gingivalis* synthetic lipid A. Thioglycollate-induced peritoneal macrophages (1×10^6 cells/ml), suspended in serum-free RPMI medium, were incubated for 2 h. After incubation, 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

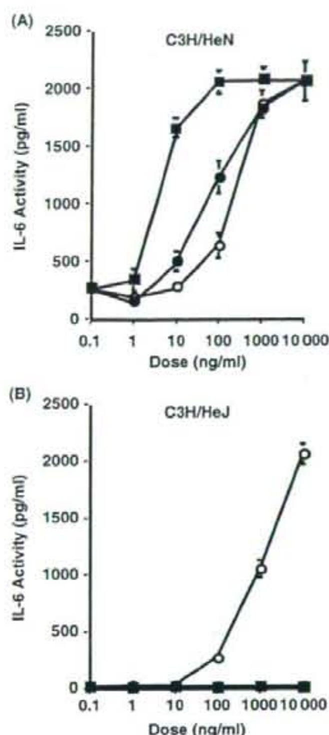

Fig. 8. Induction of IL-6 release from murine peritoneal macrophages from C3H/HeN and C3H/HeJ mice stimulated by *Porphyromonas gingivalis* synthetic lipid A. Stimulation of peritoneal macrophages was performed as well as that of TNF-α, and IL-6 activity in the supernatants was determined by an ELISA 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.

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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.

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