Endotoxic properties of free lipid A from *Porphyromonas gingivalis*

Ken-ichi Tanamoto,¹ Satoko Azumi,¹ Yuji Haishima,¹ Hidefumi Kumada^{1,2} and Toshio Umemoto²

Author for correspondence: Ken-ichi Tanamoto. Tel: +81 3 3700 1141. Fax: +81 3 3707 6950.
e-mail: tanamoto@nihs.go.jp

¹ Division of Microbiology, National Institute of Health Sciences, Setagaya-ku, Tokyo 158, Japan

² Department of Oral Microbiology, Kanagawa Dental College, Yokosuka, Kanagawa 238, Japan

The relationship between chemical structure and biological activity of the lipid A from *Porphyromonas gingivalis*, which we recently isolated and whose complete chemical structure was determined [Kumada *et al.* (1995). *J Bacteriol* 177, 2098–2106], was studied. The lipid A exhibited endotoxic activity in all the assay systems tested: *Limulus* gelation activity, lethal toxicity in galactosamine-sensitized mice, mitogenicity in mouse spleen cells and induction of nitric oxide (NO) and tumour necrosis factor alpha (TNF) release from both mouse peritoneal macrophages and the J774-1 mouse macrophage-like cell line. The activity was, however, about 100-fold less than that of *Salmonella minnesota* LPS used as a control. The moderate activity of the lipid A may be partially explained by its unique fatty acid composition and the lack of a phosphate group in position 4'. In contrast, the lipid A as well as whole LPS of *P. gingivalis* unexpectedly exhibited an even stronger induction of TNF from the human monocytic THP-1 cell line than control LPS when measured by the minimum stimulatory dose. The difference in sensitivity of human and mouse cells to *P. gingivalis* lipid A suggests that the recognition mechanism, including that for the receptor for endotoxin, may be regulated in different ways in the two cells.

Keywords: *Porphyromonas gingivalis*, lipopolysaccharide, lipid A, endotoxin

INTRODUCTION

A high incidence of *Porphyromonas gingivalis* has been found in periodontal abscesses (Winkelhoff *et al.*, 1985) and the organism has been isolated from subgingival lesions and supragingival plaque in periodontal patients (Zambon *et al.*, 1981); hence it is assumed to be one of the bacteria involved in the pathogenicity of periodontal diseases (Slots & Genco, 1984; Slots & Gibbons, 1978; Winkelhoff *et al.*, 1993). The LPS from *P. gingivalis* expresses typical activities directly associated with the periodontal diseases of bone resorption, induction of various inflammatory cytokines (Hamada *et al.*, 1990; Iino & Hopes, 1984; Nair *et al.*, 1983; Sisney-Durrant & Hopps, 1991; Takada *et al.*, 1991), and induction of periodontal tissue alterations and destruction including attachment loss, collagen degradation and alveolar bone loss (Wilson, 1993). The LPS is, therefore, suspected to be one of the virulence factors of the bacterium, along

with others such as proteases, collagenase and adhesins (Birkedal-Hansen *et al.*, 1988; Fujimura & Nakamura, 1987; Slots & Genco, 1984). Some investigators have reported that the LPS of *P. gingivalis* has a far more moderate endotoxic activity than the LPS from enterobacteria (Nair *et al.*, 1983; Fujiwara *et al.*, 1990; Takada *et al.*, 1990). Since lipid A, the active centre of LPS, has not been isolated from LPS as the free form by conventional methods, all these data were obtained by using LPS, not lipid A, of structurally undefined preparations. Although the chemical composition of LPS has been analysed (Brondz *et al.*, 1989; Johnne *et al.*, 1988) and its low endotoxicity has been hypothesized to depend on its unique fatty acid composition, its chemical properties, such as the structure of the backbone, substitution of the phosphate groups and distribution of fatty acids, have remained unknown. Therefore the reason for its low endotoxicity has not been discussed on a structural basis. Recently we succeeded in isolating the lipid A and determining its complete chemical structure (Kumada *et al.*, 1995). The proposed chemical structure is shown in Fig. 1. The lipid A was characterized

Abbreviations: FCS, foetal calf serum; TNF, tumour necrosis factor alpha.

Fig. 1. Proposed chemical structure of *P. gingivalis* lipid A. It consists of 1,6- β -linked glucosamine disaccharide which carries (*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', respectively, and phosphate groups at position 1 and partially at position 4'. The dotted line indicates partial substitution.

chemically by its unique components and substitution of branched fatty acids which are longer (15–17 carbon atoms) than found in enterobacterial LPS, which consists mainly of 3-hydroxytetradecanoic acid (Rietschel *et al.*, 1984, 1988). The isolation of lipid A from *P. gingivalis* LPS and its structural determination makes it possible to study the relationship between structure and biological activity at the native lipid A level and to verify the biological data obtained with the LPS.

In the present investigation, the endotoxic activity of the chemically well-defined lipid A isolated from *P. gingivalis* LPS was studied together with that of the LPS itself and was compared with that of *Salmonella minnesota* LPS. The relationship between biological activity and chemical structure of the lipid A, which has a unique chemical structure, is discussed.

METHODS

Materials. *P. gingivalis* SU 63, isolated from a periodontal pocket, was used for the lipid A preparation. Recombinant TNF standards and rabbit polyclonal antisera against murine TNF were obtained from Asahi Kasei Kogyo. *Escherichia coli* type synthetic lipid A (506) was purchased from Daiichi Pharmaceutical. THP-1 and J774-1 cell lines were obtained from the Japanese Cancer Research Resources Bank (JCRB). Rabbit IgG was obtained from ZYMED laboratories. RPMI 1640 medium with glutamine and Iscove's modified Dulbecco's medium were from Gibco. Phorbol myristate acetate (PMA), 1,25-dihydroxy vitamin D₃, and galactosamine were purchased from Sigma. Quantitative *Limulus* assay reagent (Endospecy) was obtained from Seikagaku Kogyo. Pyrogen-free water was a product of Hikari Seiyaku. Thioglycollate was purchased from Wako. LPS was extracted from *S. minnesota* by the aqueous phenol method (Westphal *et al.*, 1952).

Preparation of *P. gingivalis* lipid A. The method has been described by Kumada *et al.* (1995). LPS was extracted from

acetone-dried cells with hot phenol/water (Westphal *et al.*, 1952), digested with RNase A (Sigma), DNase I (Sigma) and proteinase K (Sigma) (Schifferle *et al.*, 1989), then purified by repeated ultracentrifugation (105 000 g, 12 h, 15 °C). The LPS was sonicated for 5 min in a mixture of phenol/chloroform/light petroleum (b.p. 30–60 °C) (PCP) (2:5:8, by vol.) and after centrifugation, the sediment was further washed twice with the PCP mixture and three times with acetone, then lyophilized. The purified LPS (yield; 4.2% from dried cells) contained 39.2% total carbohydrate, 20.1% total lipid, 19.7% amino sugar, 2.5% total phosphate and 2.2% protein. Lipid A was isolated and purified according to the methods described previously by Qureshi *et al.* (1985) and Qureshi & Takayama (1982), with some modification. The lipid A was liberated from the LPS (1.25 g) by heating at 100 °C in 1% (w/v) acetic acid for 90 min. Free lipid A was obtained from the hydrolysate by extracting with an equal volume of chloroform/methanol (1:1, v/v). The organic phase was washed three times with distilled water and evaporated under reduced pressure, then lyophilized to yield crude lipid A (434 mg). The sample was dissolved in PCP to eliminate possible contaminants. After evaporation, the remaining phenol phase was saturated with distilled water. Four volumes of cold acetone was added to the phenol phase, and the precipitate was collected by centrifugation followed by washing twice with acetone and twice with ethanol. The lipid A was dissolved in chloroform/methanol (3:1, v/v; 2 ml), passed through a small column (1 × 5 cm) of Dowex 50 (H⁺), and purified by gel permeation chromatography on Sephadex LH-20 (Pharmacia) in a column (2.5 × 100 cm) using chloroform/methanol (3:1, v/v) as the eluent (flow rate 24 ml h⁻¹). The purified lipid A contained about 1.8% protein as estimated by amino acid analysis, and no sugar according to the results of gas-liquid chromatography and gas-liquid chromatography/mass spectrometry, indicating no contamination by LPS. The proposed chemical structure is shown in Fig. 1. Since the lipid A was insoluble in water because of its extreme hydrophobicity, it was solubilized by converting it into a triethylamine form as follows. The lipid A was suspended in ice-cold 0.1 M HCl at a concentration of 2 mg ml⁻¹, sonicated and centrifuged in the

cold. It was washed twice with distilled water and solubilized in water by adding $0.2 \mu\text{l}$ triethylamine (mg lipid A^{-1}) at 0°C . The excess triethylamine in the solution was immediately removed by blowing N_2 to obtain a neutral pH (Tanamoto *et al.*, 1984b). The solubilized lipid A solution was used for biological assay.

Mice. Female BALB/c and C3H/HeN mice (6–10 weeks old; Nihon SLC) were used for the assay of splenic mitogenicity and the induction of TNF release from peritoneal macrophages, respectively. For the lethality test we used female C57BL/6 mice, at least 10 weeks old, from Nihon SLC.

Mitogenicity assay. The method was originally described by Tanamoto (1984a). Spleen cells isolated from mice were mashed gently, passed through a wire grid and suspended in serum-free Iscove's modified Dulbecco's medium containing L-glutamine and HEPES buffer supplemented with penicillin ($100 \text{ units ml}^{-1}$) and streptomycin ($100 \mu\text{g ml}^{-1}$). The cells were washed three times with the medium and adjusted to $4 \times 10^6 \text{ cells (ml Iscove's medium)}^{-1}$. After the addition of various amounts of test samples they were cultured on 96-well microplates (Corning), containing $200 \mu\text{l}$ cell suspension in each well, for 48 h at 37°C in a humidified environment in the presence of 5% (v/v) CO_2 . [^3H]Thymidine [$0.2 \mu\text{Ci}$ (7.4 kBq) per culture; Amersham] was added, and they were incubated for a further 24 h. The cells were harvested on glass fibre filters, and the radioactivity incorporated into the cells was measured in toluene-based scintillation fluid (5 ml) by a scintillation counter. Data are expressed as mean c.p.m. of triplicate determinations.

Induction of TNF and NO release from mouse peritoneal macrophages. Mouse peritoneal macrophages were obtained by washing the peritoneal cavity with 5 ml Iscove's medium (Tanamoto, 1994b). For the NO induction test, macrophages from mice which had been injected intraperitoneally with 2 ml sterile 2.9% (w/v) thioglycollate broth 4 d earlier were used. Aliquots of a cell suspension (1 ml) adjusted to $2 \times 10^6 \text{ cells (ml Iscove's medium)}^{-1}$ were cultured in 24-well Costar plates at 37°C with 5% (v/v) CO_2 for 3 h, and the macrophages were allowed to adhere to the plate. After the cells had been washed three times with Dulbecco's PBS(–) (Nissui Pharmaceutical), 1 ml Iscove's medium was added to each well and the cells were incubated with the test sample for 6 h for TNF induction and 72 h for NO induction. The supernatant of each culture was transferred to a plastic tube, the cells were centrifuged at 2000 r.p.m. and the supernatant was stored at -80°C until use for the determination of TNF and NO.

Induction of TNF and NO release from the J774-1 macrophage cell line. J774-1 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS), $50 \mu\text{M}$ 2-mercaptoethanol, 5 mM HEPES, penicillin ($100 \text{ units ml}^{-1}$) and streptomycin ($100 \mu\text{g ml}^{-1}$) in a 5% CO_2 atmosphere at 37°C . The cells were harvested by scraping with a cell scraper (Costar) and suspended in fresh medium. The cells ($1 \times 10^6 \text{ cells ml}^{-1}$ per well of 24-well dishes) were allowed to adhere to the plastic for 3 h at 37°C , washed twice with medium and incubated with the stimulant for an additional 4 h for TNF induction and 72 h for NO induction. The supernatant of each culture was transferred to a plastic tube, the cells were centrifuged at 2000 r.p.m., and the supernatant was stored at -80°C until used to determine TNF and NO.

Induction of TNF release from THP-1 cells. THP-1 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, $50 \mu\text{M}$ 2-mercaptoethanol, 5 mM HEPES, penicillin ($100 \text{ units ml}^{-1}$) and streptomycin ($100 \mu\text{g ml}^{-1}$) in a 5% CO_2 atmosphere at 37°C . The cells ($2 \times 10^5 \text{ cells ml}^{-1}$ per

well of 24-well dishes) were prepared for the experiments by adding $100 \text{ ng PMA ml}^{-1}$, which had been stored as a 1 mg ml^{-1} solution in dimethyl sulfoxide and diluted with the medium immediately prior to use (Golenbock *et al.*, 1991), and $0.1 \mu\text{M}$ 1,25-dihydroxy vitamin D3, to induce adherence to plastic and expression of CD14 (Kitchens *et al.*, 1992), to cell suspensions in RPMI 1640 medium containing 10% FCS. The cell suspensions were allowed to differentiate and adhere to plastic for 72 h at 37°C . After washing twice with medium, the cells were incubated for an additional 24 h with the stimulant. The supernatant of each culture was transferred to a plastic tube, the cells were centrifuged at 2000 r.p.m., and the supernatant was stored at -80°C until used to determine TNF.

TNF assay. The TNF produced was measured by cytotoxicity assay against L929 murine fibroblast cells. L929 cells were grown in tissue culture flasks of RPMI 1640 medium supplemented with 10% FCS, $50 \mu\text{M}$ 2-mercaptoethanol, 5 mM HEPES, penicillin ($100 \text{ units ml}^{-1}$) and streptomycin ($100 \mu\text{g ml}^{-1}$). Cells were detached with trypsin, washed, resuspended in medium at $4 \times 10^5 \text{ cells ml}^{-1}$, and $100 \mu\text{l}$ aliquots were plated in 96-well flat-bottomed plates (Corning). After incubation for 3–5 h at 37°C in 5% CO_2 , $50 \mu\text{l}$ actinomycin D ($4 \mu\text{g ml}^{-1}$) in RPMI medium was added to each well, and $50 \mu\text{l}$ test sample was then added to the wells (final volume $200 \mu\text{l}$ per well). After 20 h incubation, the cells were washed twice with Hanks' Balanced Salt Solution. The cells were then stained with crystal violet, and the cytotoxicity was measured by reading the A_{550} of the extract with ethanol solution. The results were expressed as the means of triplicate determinations.

NO assay. NO produced in the supernatant was measured as a stable form of nitrite (NO_2^-) by using Griess reagent (Green *et al.*, 1982). Briefly, $100 \mu\text{l}$ test sample was mixed with the same volume of Griess reagent [0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride in H_2O /1% (w/v) sulfanilamide in 5% (v/v) H_3PO_4 ; 1:1 (v/v)] in a 96-well plate, and the A_{550} was read with a microplate reader (Molecular Devices).

Lethal toxicity test. The lethality test was performed according to the method described by Galanos *et al.* (1979). Test samples in 0.1 ml pyrogen-free water were injected intravenously immediately after intraperitoneal administration of 12 mg D-galactosamine in 0.5 ml pyrogen-free PBS.

Limulus amoebocyte gelation assay. Activation of the pro-clotting enzyme of the horseshoe crab, *Limulus polyphemus*, was estimated colorimetrically by measuring the absorbance of *p*-nitroaniline released from a synthetic substrate in a quantitative assay (Endospecy). The assay was performed in 96-well Costar flat plates at 37°C for 30 min, and the chromogen was measured at 405 nm using a microplate reader. Pyrogen-free water was used to dilute the test samples.

RESULTS

Mitogenicity of *P. gingivalis* lipid A

The mitogenic activities of *P. gingivalis* LPS and lipid A were tested on murine splenic cells of LPS-responsive BALB/c mice. As shown in Fig. 2, *P. gingivalis* LPS and lipid A exhibited comparable mitogenicity. The activity was, however, about 100 times less than that of *S. minnesota* LPS when the minimum stimulation dose was compared.

Fig. 2. Mitogenic responses of murine spleen cells to *P. gingivalis* LPS and lipid A. Spleen cells from BALB/c mice were suspended in serum-free Iscove's medium at 4×10^6 cells ml^{-1} , 200 μl aliquots were plated in 96-well tissue culture dishes, and reciprocally diluted mitogen, in 10 μl water was added. After culturing the cells for 48 h, [^3H]thymidine [0.2 μCi (7.4 kBq) per well] was added. After additional culture for 24 h, the cells were harvested and the incorporated radioactivity was measured. The results are expressed as mean c.p.m. in triplicate experiments $\pm$ SD. The background c.p.m. with no lipid A was 845 ± 40 (mean $\pm$ SD). ■, *S. minnesota* LPS; ●, *P. gingivalis* LPS; ○, *P. gingivalis* lipid A.

Induction of TNF and NO release by *P. gingivalis* lipid A from mouse peritoneal macrophages

TNF and NO released into the medium from mouse peritoneal macrophages after stimulation with *P. gingivalis* LPS and lipid A were measured. As shown in Fig. 3, the cells from C3H/HeN mice started to secrete TNF at a concentration of 10 ng ml^{-1} when stimulated with lipid A. The induction of TNF release from macrophages by lipid A increased dose-dependently, and maximum TNF production (22 ng ml^{-1}) was observed at the highest concentration tested, 10 $\mu\text{g ml}^{-1}$. Similar, but slightly higher, activity was induced by LPS of the same bacterium. However, the activity of these preparations was much lower, especially with regard to the minimum dose needed to stimulate TNF production, than that of *S. minnesota* LPS, which stimulated the cells at a concentration of 0.1 ng ml^{-1} and induced the maximum TNF production, 64 ng ml^{-1} , at a concentration of 10 $\mu\text{g ml}^{-1}$. In order to determine whether the cytotoxic activity against L929 cells was due to TNF, supernatants from a macrophage culture were incubated for 12 h with polyclonal rabbit antiserum to TNF, with nonspecific IgG used as the control. The polyclonal antibody to TNF completely abolished the cytotoxicity of the supernatants in all the samples tested. Although the resident peritoneal macrophages failed to produce a significant amount of NO, thioglycollate-induced

Fig. 3. Induction of TNF and NO release from murine peritoneal macrophages by *P. gingivalis* LPS and lipid A. Resident and thioglycollate-induced peritoneal macrophages from BALB/c mice were used for TNF- and NO-inducing assays, respectively. Macrophages, $2 \times 10^6 \text{ ml}^{-1}$, were incubated in serum-free Iscove's medium with various concentrations of LPS or lipid A. After 6 and 72 h incubation at 37 °C, the supernatants were examined for TNF and NO, respectively. The results are expressed as means $\pm$ SD of triplicate experiments. ■, *S. minnesota* LPS; ●, *P. gingivalis* LPS; ○, *P. gingivalis* lipid A.

macrophages responded to stimulation by LPS and produced NO. As shown in Fig. 3, however, the cells required a much higher concentration of LPS to induce NO than TNF, and they started to secrete NO at 10 ng ml^{-1} *S. minnesota* LPS ml^{-1} . The ability of *P. gingivalis* lipid A to induce NO was about 100 times lower than that of the control LPS, and the production of NO was first observed at a concentration of 1 $\mu\text{g ml}^{-1}$. This activity of

Fig. 4. Induction of TNF release from J774-1 cells by *P. gingivalis* LPS and lipid A. J774-1 cells (1×10^6 cells ml⁻¹ per well of 24-well dishes) were incubated in RPMI 1640 medium supplemented with 10% (v/v) FCS with the stimulant. After 4 and 72 h incubation at 37 °C, the supernatants were examined for TNF and NO, respectively. The results are expressed as means $\pm$ SD of triplicate experiments. ■, *S. minnesota* LPS; ●, *P. gingivalis* LPS; ○, *P. gingivalis* lipid A.

P. gingivalis LPS was significantly lower than that of the lipid A, in contrast to TNF induction.

Induction of TNF and NO release from J774-1 cells by *P. gingivalis* lipid A

The ability of *P. gingivalis* lipid A as well as LPS to induce TNF and NO release from mouse macrophage-like J774-1 cells was compared with that of *S. minnesota* LPS used as a control. The cells are very sensitive to

Fig. 5. Induction of TNF release from human THP-1 cells by *P. gingivalis* LPS and lipid A. Human THP-1 cells (2×10^5 cells ml⁻¹ per well) were incubated with 100 ng PMA ml⁻¹ and 0.1 μM 1,25-dihydroxy vitamin D3 for 3 d in RPMI medium containing 10% (v/v) FCS at 37 °C. After an additional 24 h incubation with 10 μl test sample, the supernatants were assayed for TNF. Values represent the mean concentration of TNF $\pm$ SD for triplicate experiments. ■, *S. minnesota* LPS; ●, *P. gingivalis* LPS; ○, *P. gingivalis* lipid A.

stimulation by the control LPS, and significant production of both TNF (1.3 ng ml⁻¹) and NO (1.2 μM) was observed at a concentration of 0.1 ng ml⁻¹ (Fig. 4). *P. gingivalis* lipid A started to induce secretion of both TNF and NO from J774-1 cells at a concentration of 10 ng ml⁻¹, showing that the activity is about 100 times lower than that of the control LPS. The activity of *P. gingivalis* LPS was a little higher than that of the lipid A in the case of TNF induction, but was about 10 times lower in the case of NO induction, giving results similar to those obtained with mouse peritoneal macrophages as described above.

Induction of TNF release from THP-1 cells by *P. gingivalis* lipid A

It is suggested that human cells respond to LPS in a different way from murine cells, as was seen in the response to the lipid A precursor. To determine the ability of *P. gingivalis* lipid A to activate human cells, the human monocyte-macrophage cell line THP-1 was examined for TNF production in response to *P. gingivalis* lipid A. As shown in Fig. 5, the cells were stimulated with *S. minnesota* LPS at a concentration of 10 ng ml⁻¹ and produced 4.9 ng TNF ml⁻¹. They unexpectedly produced a comparable amount of TNF in the medium after stimulation with the same concentration of *P. gingivalis* lipid A. More pronounced activity was expressed by *P. gingivalis* LPS, which induced 8.8 ng TNF ml⁻¹ even at a concentration of 1 ng ml⁻¹.

Table 1. Lethal toxicity of *P. gingivalis* LPS and lipid A for galactosamine-sensitized C57BL/6 mice

Test samples in 0.1 ml pyrogen-free water were injected either intraperitoneally (i.p.) or intravenously (i.v.) immediately after intraperitoneal administration of 12 mg D-galactosamine in 0.5 ml pyrogen-free PBS.

Sample	Route	No. of dead/total no. tested at the following dose (µg per mouse)				
		0.001	0.01	0.1	0.3	1
<i>S. minnesota</i> LPS	i.p.	3/8	6/6			
<i>E. coli</i> synthetic lipid A (506)	i.v.	3/9	6/6			
<i>P. gingivalis</i> LPS	i.p.			0/3		0/6
	i.v.			0/6		3/3
<i>P. gingivalis</i> lipid A	i.v.			0/6	3/6	3/3

Fig. 6. *Limulus* gelation activity of *P. gingivalis* LPS and lipid A. *Limulus* amoebocyte lysate was incubated with test samples for 30 min, and the chromogen released was measured. ■, *S. minnesota* LPS; ●, *P. gingivalis* LPS; ○, *P. gingivalis* lipid A.

Lethal toxicity of *P. gingivalis* lipid A

The lethal toxicity of the samples was tested using galactosamine-sensitized mice. The results are shown in Table 1. In this system, *S. minnesota* LPS exhibited almost 100% lethality at 10 ng per mouse, and moderate lethality was observed even at 1 ng per mouse. On the other hand, the lethality of *P. gingivalis* LPS was first observed at 1 µg per mouse when it was injected intravenously, and no lethality was observed at 0.1 µg per mouse, or at 1 µg per mouse injected intraperitoneally. The lipid A of *P. gingivalis* exhibited 100% lethality at 1 µg per mouse, and no lethality was seen at 0.1 µg per mouse.

Chromogenic *Limulus* amoebocyte lysate test of *P. gingivalis* lipid A

S. minnesota LPS activated the cascade of the clotting system of the horseshoe crab at a concentration of several pg ml⁻¹, and linear dose-dependency was

obtained up to 100 pg ml⁻¹. In contrast, both LPS and lipid A from *P. gingivalis* showed negligible activity. The same chromogenicity, A₄₀₅ 0.3, was obtained at 30 pg *S. minnesota* LPS ml⁻¹, 3.5 ng *P. gingivalis* lipid A ml⁻¹ and 5 ng *P. gingivalis* LPS ml⁻¹ (Fig. 6).

DISCUSSION

We examined the biological activity of the free form of lipid A from *P. gingivalis*, which was recently isolated and chemically characterized (Kumada *et al.*, 1995). Although some differences were observed between the lipid A and its original LPS in activating mouse peritoneal macrophages and J774-1 cells to induce TNF and NO, the endotoxic activity of the two preparations was found to be almost identical, and was generally much more moderate than that induced by the LPS of *S. minnesota*, which was used as a control. In most of the systems used in this study to measure endotoxic activity, the lipid A exhibited activity at least 100-fold less than the LPS of *S. minnesota*. The lipid A used in the present study contained a small amount (1.8%) of protein. The possibility of its participation in the activity, however, seems to be small considering that the protein content is low and that it is unlikely that the residual protein retains its intact form and function after proteinase treatment. Furthermore, the activity of the lipid A relative to that of *S. minnesota* LPS in B cell mitogenicity and activation of macrophage and other cells, which are possibly induced by the contaminated protein, is almost the same as that in lethal activity and *Limulus* gelation activity, which are caused entirely by lipid A, not by protein.

The difference between the chemical structure of the lipid A of *P. gingivalis* and that of enterobacteria was in the substitution of the phosphate group at position 4' of nonreducing glucosamine and the type of fatty acids and their binding sites. The lipid A of *P. gingivalis* consists of unique branched fatty acids with longer carbon-atom chains than those of *S. minnesota* and other enterobacteria, as is shown in Fig. 1. The accumulated data on the relationship between the chemical structure and biological activity of lipid A obtained by using natural and chemically synthesized lipid A preparations suggest that both of these components, phosphate and fatty acids, play a crucial role in the endotoxic activity of lipid A (Galanos *et al.*, 1984; Homma *et al.*, 1985; Kanegasaki *et al.*, 1984, 1986; Tanamoto *et al.*, 1984a, b; Tanamoto, 1994a). Thus, the reason for the low endotoxicity of the *P. gingivalis* lipid A is thought to be the unique fatty acid components and the partial defect in the phosphate group. In fact, *Bacteroides fragilis* lipid A, which chemically resembles that of *P. gingivalis*, was found to express low or no endotoxic activity (Weintraub *et al.*, 1989). However, the lipid A and, particularly, the LPS of *P. gingivalis* unexpectedly showed strong activity, even stronger than that of enterobacterial LPS with regard to the minimum stimulatory dose, in induction of TNF in the human pro-monocytic cell line THP-1. These results indicate that the chemical structure of the lipid A needed for the manifestation of endotoxic activity differs

in humans and mice. Alternatively the difference suggests that a different pathway may exist in the recognition mechanism of LPS including that for the receptor. A difference in behaviour of some lipid A analogues toward human and murine macrophages has also been reported, i.e. a lipid A precursor (lipid IV_A or 406) is an agonist in murine cells and exhibits strong lethality for mice (Galanos *et al.*, 1984; Kanegasaki *et al.*, 1984; Tanamoto, 1994b), while it expresses no endotoxicity in human cells and antagonizes LPS action (Golenbock *et al.*, 1991; Kovach *et al.*, 1990; Loppnow *et al.*, 1989). Recently the signal transducing pathway of LPS in macrophages has become well, but not completely, understood. The activation in both human and mouse cells is thought to occur by the mediation of LBP and CD14 (Tobias *et al.*, 1993; Wright *et al.*, 1990). However, the possibility still remains that there are molecules on the cells which directly accept the LPS molecule and transduce a signal which is independent of the serum factor and therefore CD14 (Kitchens & Munford, 1995; Nishijima *et al.*, 1994; Weinstein *et al.*, 1993). Although we still do not know what difference there is in the stimulation pathway in humans and mice, there may be a special point which discriminates the response of human and murine cells, and regulates the sensitivity depending on the chemical structure of the lipid A. The strong sensitivity of human cells to *P. gingivalis* LPS has also been found in human gingival fibroblasts for the induction of inflammatory mediators such as interleukin-1, -6 and -8 (Takada *et al.*, 1991; Tamura *et al.*, 1992; Yamaji *et al.*, 1995). The fact that the lipid A, as well as LPS, of *P. gingivalis* is a potent stimulator in human cells suggests that it may play a more important role as a pathogenic factor in periodontopathic diseases than is expected from the findings of its low endotoxicity in mice.

Another novel action of the *P. gingivalis* LPS is the stimulation of the LPS non-responsive mouse strain C3H/HeJ to induce splenocyte mitogenicity (Joiner *et al.*, 1982; Koga *et al.*, 1985). It is of great interest whether the free lipid A of *P. gingivalis* is an activator in the mouse. However the stimulation by lipid A of LPS non-responsive mice was not as strong as that of LPS responsive mice and therefore we cannot ignore the possible activity of contaminating protein in this case. Since our preparation still contains a small amount of protein, which may cause stimulation of the LPS non-responsive mouse, we cannot determine whether the lipid A part of the unique chemical structure is really responsible for the activity. We are trying to clarify this by using several strategies, including the chemical synthesis of the complete and partial structure of lipid A determined by us.

ACKNOWLEDGEMENTS

The skilful technical assistance of T. Iida is greatly appreciated. This work was supported by grants from the Ministry of Education, Science and Culture (06670313 to K.T., 06771618 to Y.H., 04671111 to H.K. and 06670313 to T.U.).

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Received 24 June 1996; accepted 27 August 1996.