Endotoxic properties of free lipid A from Porphyromonas gingivalis

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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; Fujisawa 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 (Bronzert et al., 1989; John 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.
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 (105000 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 x 5 cm) of Dowex 50 (H+), and purified by gel permeation chromatography on Sephadex LH-20 (Pharmacia) in a column (2.5 x 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 μl 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 units ml\(^{-1}\)) and streptomycin (100 μg ml\(^{-1}\)). The cells were washed three times with the medium and adjusted to 4 x 10\(^7\) 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 μl cell suspension in each well, for 48 h at 37 °C in a humidified environment in the presence of 5% (v/v) CO\(_2\), [\(^3\)H]Thymidine [0.2 μCi (7.4 kBq) per culture; Amersham] was added, and they were incubated for a further 2 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 intraperitonely 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 x 10\(^6\) 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 used 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 μM 2-mercaptoethanol, 5 mM HEPES, penicillin (100 units ml\(^{-1}\)) and streptomycin (100 μg 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 x 10\(^6\) cells ml\(^{-1}\) per well of 24-well dishes) were prepared for the experiments by adding 10 μl 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 μM 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 μM 2-mercaptoethanol, 5 mM HEPES, penicillin (100 units ml\(^{-1}\)) and streptomycin (100 μg ml\(^{-1}\)). Cells were detached with trypsin, washed, resuspended in medium at 4 x 10\(^6\) cells ml\(^{-1}\), and 100 μl aliquots were plated in 96-well flat-bottomed plates (Corning). After incubation for 3–5 h at 37 °C in 5% CO\(_2\), 50 μl actinomycin D (4 μg ml\(^{-1}\)) in RPMI medium was added to each well, and 50 μl test sample was then added to the wells (final volume 200 μl 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\(_{540}\) 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 μl test sample was mixed with the same volume of Griess reagent [0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride in H\(_2\)O/1% (w/v) sulfanilamide in 5% (v/v) H\(_2\)PO\(_4\), 1:1 (v/v)] in a 96-well plate, and the A\(_{540}\) 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 0.3 ml pyrogen-free PBS.

**Limulus amoebocyte gelation assay.** Activation of the 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.
Induction of TNF and NO release by \textit{P. gingivalis} lipid A from mouse peritoneal macrophages

TNF and NO released into the medium from mouse peritoneal macrophages after stimulation with \textit{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 \(\mu\)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 \textit{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 \(\mu\)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 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 \textit{S. minnesota} LPS ml\(^{-1}\). The ability of \textit{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 \(\mu\)g ml\(^{-1}\). This activity of
Biological activity of 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 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.
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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Route</th>
<th>No. of dead/tot. no. tested (μg per mouse)</th>
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<tr>
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<td>0.001</td>
<td>0.01</td>
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<tr>
<td>S. minnesota LPS</td>
<td>i.p.</td>
<td>3/8</td>
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<tr>
<td>E. coli synthetic lipid A (506)</td>
<td>i.v.</td>
<td>3/9</td>
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<tr>
<td>P. gingivalis LPS</td>
<td>i.p.</td>
<td>0/3</td>
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<td>i.v.</td>
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<tr>
<td>P. gingivalis lipid A</td>
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**Lethal toxicity of P. gingivalis lipid A**

The lethal toxicity of the samples was tested using galactosamine-sensitized C57BL/6 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-mononuclear 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<sub>A</sub> or 406) is an agonist in murine cells and exhibits strong lethality for mice (Galanos <i>et al.</i>, 1984; Kanegasaki <i>et al.</i>, 1984; Tanamoto, 1994b), while it expresses no endotoxicity in human cells and antagonizes LPS action (Golenbock <i>et al.</i>, 1991; Kovac <i>et al.</i>, 1990; Lopponnow <i>et al.</i>, 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 <i>et al.</i>, 1993; Wright <i>et al.</i>, 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 <i>et al.</i>, 1994; Weinstein <i>et al.</i>, 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 <i>P. gingivalis</i> LPS has also been found in human gingival fibroblasts for the induction of inflammatory mediators such as interleukin-1, -6 and -8 (Takada <i>et al.</i>, 1991; Tamura <i>et al.</i>, 1992; Yamaji <i>et al.</i>, 1995). The fact that the lipid A, as well as LPS, of <i>P. gingivalis</i> 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 <i>P. gingivalis</i> LPS is the stimulation of the LPS non-responsive mouse strain C3H/HeJ to induce splenocyte mitogenicity (Joiner <i>et al.</i>, 1982; Koga <i>et al.</i>, 1985). It is of great interest whether the free lipid A of <i>P. gingivalis</i> 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.

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