Immunobiological activities of chemically defined lipid A from lipopolysaccharides of *Porphyromonas gingivalis*

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Immunobiological activities of chemically defined lipid A from lipopolysaccharides (LPS) of *Porphyromonas gingivalis* strain 381, which possesses β -(1 $\rightarrow$ 6)-linked glucosamine disaccharide 1-monophosphate, with 3-hydroxy-15-methylhexadecanoyl and 3-hexadecanoyloxy-15-methylhexadecanoyl groups at the 2- and 2'-positions, respectively, were compared with those of synthetic *Escherichia coli*-type lipid A (compound 506) and *Salmonella*-type lipid A (compound 516). *P. gingivalis* lipid A and its LPS induced stronger or comparable production of the cytokines interleukin-1 receptor antagonist (IL-1ra), IL-6, IL-8, granulocyte-macrophage colony-stimulating factor and interferon- γ as compared with compounds 506 and 516 in the culture supernatants of human peripheral blood monocytes or mononuclear cells. However, the *P. gingivalis* preparations showed low activity in inducing the production of IL-1 β and tumour necrosis factor- α . Clear antagonistic effects of *P. gingivalis* lipid A and its LPS against IL-1 β production induced by *E. coli* LPS or compound 506 were seen. Furthermore, *P. gingivalis* lipid A and its LPS had marked immunopharmacological activities, i.e. antitumour, natural killer cell and antiviral activities. Its monophosphorylation pattern and the presence and position of fatty acids possessing acyl chains of considerable length are unique to *P. gingivalis* lipid A, differing from enterobacterial lipid As. Its good balance between agonistic and antagonistic effects, making it a possible candidate for use as an immunomodulatory drug, may be attributable to these unique features.

Keywords: *Porphyromonas gingivalis*, lipopolysaccharide, lipid A, cytokine induction, immunopharmacological activity

INTRODUCTION

Lipopolysaccharides (LPS) are a major component of the outer membrane of Gram-negative bacteria. LPS stimulate the host cells in experimental animals and humans, and have a variety of pathophysiological effects (Morrison & Ryan, 1979). Among the host cells, the mononuclear phagocytes are important as primary targets of LPS. The LPS-stimulated monocytes/macrophages produce pro-inflammatory and anti-inflammatory cytokines, which

play an important role in mediating the endotoxic and beneficial effects of endotoxin (Vogel & Hogan, 1990).

Lipid A is generally regarded as the endotoxic principle and bioactive centre of LPS. The generalized structure of the lipid A component, which represents the lipophilic portion of LPS, consists of a β -(1 $\rightarrow$ 6)-linked disaccharide which carries a glycosidic and a nonglycosidic phosphoryl group, and (*R*)-3-hydroxy fatty acids in ester and amide linkage, some of which are acylated at their 3-hydroxyl group. The lipid A structures corresponding to preparations of *Escherichia coli*-, *Salmonella*- and other enterobacterial-type lipid As have been chemically defined and synthesized (Takayama & Qureshi, 1992; Kusumoto, 1992; Takada & Kotani, 1992). The lipid As, like the LPS, produce a variety of immunobiological effects in animals and humans (Takada & Kotani, 1992).

Abbreviations: FBS, foetal bovine serum; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN- γ , interferon- γ ; IL-1, interleukin-1; IL-1ra, interleukin-1 receptor antagonist; IL-6, interleukin-6; IL-8, interleukin-8; NK, natural killer; PBMC, peripheral blood mononuclear cells; TCID₅₀, 50% tissue culture infective dose; TNF- α , tumour necrosis factor.

Fig. 1. Chemical structure of *P. gingivalis* lipid A, and of synthetic *E. coli*-type and *Salmonella*-type lipid A compounds 506 and 516. Abbreviations: P, PO(OH)₂; C₁₄-OH, (R)-3-hydroxytetradecanoyl; C₁₄-O-(C₁₂), (R)-3-dodecanoyloxytetradecanoyl; C₁₄-O-(C₁₄), (R)-3-tetradecanoyloxytetradecanoyl; C₁₄-O-(C₁₆), (R)-3-hexadecanoyloxytetradecanoyl; iso-C₁₇-OH, 3-hydroxy-15-methylhexadecanoyl; iso-C₁₇-O-(C₁₆), 3-hexadecanoyloxy-15-methylhexadecanoyl.

Porphyromonas (Bacteroides) gingivalis, a black-pigmented anaerobic rod, is suspected to be a periodontopathic organism (Duerden *et al.*, 1987; Mayrand & Holt, 1988; Slots & Listgarten, 1988). This organism possesses various cell surface components such as LPS, capsules, fimbriae and outer-membrane proteins, and extracellular vesicles (Mayrand & Holt, 1988; Holt & Bramanti, 1991; Ogawa *et al.*, 1991). *P. gingivalis* LPS have different chemobiological properties from the classical enterobacterial LPS (Hamada *et al.*, 1990). The endotoxic ability of LPS from *P. gingivalis* is very low as compared with enterobacterial preparations (Joiner *et al.*, 1982; Nair *et al.*, 1983; Lindberg *et al.*, 1990; Ogawa, 1994).

Ogawa (1993) purified lipid A from *P. gingivalis* strain 381 and characterized its chemical structure, showing it to consist of β-(1→6)-linked glucosamine disaccharide 1-monophosphate with one hydroxyacyl group and one acyloxyacyl group, i.e. 3-hydroxy-15-methylhexadecanoyl and 3-hexadecanoyloxy-15-methylhexadecanoyl groups at the 2- and 2'-positions, respectively. *P. gingivalis* lipid A exhibited no or very low endotoxic activities and beneficial immunobiological effects (Ogawa, 1994).

In the present study, we examined the cytokine-inducing activities of *P. gingivalis* lipid A in human peripheral blood monocyte/macrophage or mononuclear cell culture and studied its immunopharmacological effects such as antagonistic, antitumour, natural killer and antiviral activities. The unique and different structure-function relationships of *P. gingivalis* lipid A and its LPS as compared with those of synthetic *E. coli*-type and *Salmonella* type lipid A (compounds 506 and 516) are discussed.

METHODS

Bacteria and preparation of LPS. *P. gingivalis* strain 381 was grown anaerobically in GAM broth (Nissui, Tokyo), supplemented with haemin and menadione, at 37 °C for 26 h. Bacterial cells were collected by centrifugation, washed three times with pyrogen-free water and lyophilized. LPS were extracted from lyophilized cells by the hot phenol/water method (Westphal & Jann, 1965), and the crude extract was purified by repeated ultracentrifugation (100 000 g, 3 h) followed by treat-

ment with nuclease P₁ (Yamasa Shoyu, Choshi) and finally lyophilized (Ogawa, 1993).

Preparation and purification of lipid A. Purified LPS (100 mg) were hydrolysed in 0.6% (v/v) acetic acid at 105 °C for 2.5 h, cooled and neutralized. The hydrolysate was made up to 100 ml with H₂O and added to 200 ml CHCl₃/MeOH/H₂O/triethylamine (30:12:2:0.1, by vol.). The lower phase was evaporated under reduced pressure. Subsequently, the lipid A fractions (30.5 mg) were separated by silica gel column chromatography (Silica Gel 60, 230–400 mesh; Nacalai Tesque, Kyoto) with CHCl₃/MeOH/H₂O/triethylamine (30:12:1.5:0.1, by vol.). The eluates were monitored by thin-layer chromatography on silica gel 60 plates. The plates were developed with CHCl₃/MeOH/H₂O/triethylamine (30:12:2:0.1, by vol.) and visualized by sulfuric acid, Dittmer–Lester reagent (Dittmer & Lester, 1964) and triphenyltetrazolium chloride (TTC) reagent (Fischer & Dörfel, 1954). A slow-moving compound (R_F 0.3) was detected as a single spot as the major component (Ogawa, 1993). The chemical structure of lipid A from LPS of *P. gingivalis* strain 381 (Fig. 1), was characterized by conventional chemical procedures, gas-liquid chromatography, nuclear magnetic resonance spectroscopy and mass spectrometry (Ogawa, 1993).

Reference natural and synthetic products. *E. coli* O55:B5 LPS were obtained from List. *E. coli*- and *Salmonella*-type synthetic lipid A (compounds 506 and 516) were purchased from Daiichi Pure Chemicals; their chemical structures are indicated in Fig. 1 (Kusumoto, 1992). Both bacterial and synthetic products were dissolved at a concentration of 2 mg ml⁻¹ in 0.1% (v/v) triethylamine aqueous solution. The stock solution was kept at 4 °C and appropriately diluted with pyrogen-free phosphate buffered saline (PBS; Biken), or cell culture medium before use for assay.

Preparation of culture supernatants. Heparinized venous blood drawn from healthy donors was fractionated by using Histopaque (Sigma) to obtain peripheral blood mononuclear cells (PBMC) in which monocytes were enriched (Böyum, 1968). PBMC were washed with PBS and suspended at 1.5 × 10⁶ cells ml⁻¹ in RPMI 1640 medium (Biken) supplemented with 10% (v/v) foetal bovine serum (FBS, HyClone). In addition, PBMC (1.5 × 10⁵ per well) were incubated at 37 °C for 2 h in humidified air containing 5% (v/v) CO₂ in a 96-well culture plate (Becton Dickinson) followed by removal of nonadherent cells to perform monocyte/macrophage culture.

The indicated concentrations of test specimens were added to PBMC for interferon-γ (IFN-γ) assay or to monocytes/macrophages for other cytokine assays as described below; the

cells were incubated at 37 °C in air containing 5% (v/v) CO₂, for 72 h in PBMC culture or for 24 h in monocyte/macrophage culture.

In some experiments the antagonistic effects of *P. gingivalis* LPS and its lipid A against *E. coli* LPS- or compound 506-induced interleukin-1 β (IL-1 β) production were examined as follows. Monocytes/macrophages were cultured at 37 °C for 24 h in RPMI 1640 medium containing 10% (v/v) FBS with 0.5 μ g ml⁻¹ of *E. coli* LPS or compound 506 with or without the indicated doses of *P. gingivalis* LPS or its lipid A. After incubation, supernatants were collected and used for cytokine assays as described below.

Cytokine assay. The production and amount of the cytokines IL-1 β (Otsuka Pharmaceuticals) IL-6, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor- α (TNF- α) (Amersham), IFN- γ (Genzyme) and IL-1 receptor antagonist (IL-1ra; R&D) were measured in culture supernatants by means of a commercial ELISA kit system. These assays were performed according to the manufacturer's instructions, and the data were determined by standard curves prepared for each assay.

Preparation of splenic macrophages. Splenic macrophages were obtained from Balb/c mice (Japan SLC, Shizuoka) as described by Cowing *et al.* (1978). Briefly, spleen cells were washed twice with RPMI 1640 medium and suspended to 3 $\times$ 10⁷ cells ml⁻¹ in 0.01 M potassium-bicarbonate-buffered ammonium chloride (0.155 M, pH 7.2) containing 2% (v/v) FBS at 4 °C, to lyse erythrocytes. The cells were washed twice with RPMI 1640 supplemented with 10% (v/v) FBS and 5 ml aliquots of this suspension (1 $\times$ 10⁷ cells ml⁻¹) were added to 100 mm plastic Petri dishes. The cells were incubated at 37 °C for 2 h to permit adherence, and then the dishes were washed with warm RPMI 1640 medium and reincubated for 18 h. After incubation, the dishes were washed three times with warm medium, and treated with 5 ml 0.6 mM EDTA (Wako) at 37 °C for 20 min. The adherent cells were removed from the plates, washed and resuspended to 2 $\times$ 10⁶ cells ml⁻¹ in RPMI 1640 medium.

Cytostatic assay. Cytostasis of Meth A (methyl-cholanthrene-induced fibrosarcoma from a Balb/c mouse) as a target cell was assessed by a tritium-labelled thymidine ([³H]dThd) uptake inhibition assay described by Krahenbuhl *et al.* (1976). Briefly, splenic macrophages as described above were suspended at 2 $\times$ 10⁶ cells ml⁻¹ in RPMI 1640 medium containing 10% (v/v) FBS, and a 0.1 ml aliquot of the cell suspension was added to each well containing Meth A (2 $\times$ 10⁴ cells in 0.1 ml) together with, or without, test specimens at the indicated concentrations. After incubation for 18 h at 37 °C in a humidified atmosphere of air containing 5% (v/v) CO₂, groups of wells were pulsed with [³H]dThd (37 kBq in 0.01 ml, specific activity 74 GBq mmol⁻¹; ICN Biomedicals) and further incubated for 6 h; target cells were then harvested on glass filter paper. [³H]dThd uptake was measured by liquid scintillation counting (LKB-Wallac Rack-Beta 1215). The cytostatic activity of the macrophages was calculated as follows: cytostatic activity (%) = 100 $\times$ [(c.p.m. in cultures containing macrophages alone) - (c.p.m. in cultures containing macrophages with test specimen)] / (c.p.m. in cultures containing macrophages alone).

Cytotoxicity assay. Cytolysis was measured as the release of ⁵¹Cr-labelled Meth A, which was used as a target cell (Taffer & Russell, 1981). Briefly, target cells in the exponential growth phase were incubated for 1 h in RPMI 1640 medium supplemented with ⁵¹Cr-labelled sodium chromate (3.7 MBq ml⁻¹; specific activity 10.5 GBq mg⁻¹; Radiomedicals). The cells were then washed three times with RPMI 1640 medium to remove

unbound radiolabel and resuspended in the same medium containing 10% (v/v) FBS at a concentration of 2 $\times$ 10⁵ cells ml⁻¹. A 0.1 ml aliquot of cell suspension was added to each well containing splenic macrophages (2 $\times$ 10⁵ cells in 0.1 ml) as described above with or without test specimens, and incubated for 18 h at 37 °C in a humidified atmosphere of air containing 5% (v/v) CO₂. After incubation, the supernatant was removed from each well and the radioactivity was measured with a gamma-counter (LKB-Wallac Compu Gamma 1282). Cytotoxicity was calculated as follows: cytolytic activity (%) = 100 $\times$ (experimental release - spontaneous release) / (total release - spontaneous release), in which spontaneous release was the amount of ⁵¹Cr released by target cells alone and total release was the amount of ⁵¹Cr released by target cells which had been lysed with 0.1 M NaOH.

Natural killer (NK) cell assay. NK cell activity was determined by a radioactive release assay (Brunner *et al.*, 1968). Balb/c mice were injected intravenously with the indicated doses of test specimens on days 0 and 7. The spleen cells (1 $\times$ 10⁶ in 0.1 ml) obtained from Balb/c mice 7 d after the final injection were then incubated with ⁵¹Cr-labelled YAC-1 lymphoma cell line (2 $\times$ 10⁴ cells in 0.1 ml), which is a moloney-virus-induced lymphoma of A/Sn origin, in an effector/target cell ratio of 50:1 for 4 h at 37 °C in a humidified atmosphere of air containing 5% (v/v) CO₂. After incubation, 0.1 ml supernatant was collected from each well and the radioactivity was counted with a gamma-counter. Cytotoxicity was calculated as follows: NK cell activity (%) = 100 $\times$ (experimental release - spontaneous release) / (total release - spontaneous release), in which experimental release was the mean c.p.m. released by target cells together with effector cells; spontaneous release was the mean c.p.m. released by target cells alone; and total release was the total amount of ⁵¹Cr incorporated into target cells which had been lysed with 0.1 M NaOH.

Antiviral activity against vesicular stomatitis virus. Antiviral activity was assessed by inhibition of the cytopathogenic effect of vesicular stomatitis virus, as an interferon-sensitive challenge virus (Armstrong, 1981). Briefly, murine fibroblastic L929 cells (4 $\times$ 10⁴ in 0.1 ml) were incubated with or without 0.1 ml of serial two-fold dilutions of each test specimen (maximum concentration 1 mg ml⁻¹) for 24 h at 37 °C in a humidified atmosphere of air containing 5% (v/v) CO₂. After removal of culture supernatant the cultures were exposed to vesicular stomatitis virus (100 TCID₅₀ in 0.1 ml) and incubated for 24 h at 37 °C; the L929 cells were then fixed in 5% (v/v) formaldehyde (Wako) followed by staining with 0.5% (w/v) crystal violet (Wako). After washing, the plates were dried in air. Since viable cells retained the dye, the A₅₉₅ of each well was read with a Titertek Multiskan MC photometer (Flow). Values obtained from triplicate cells were averaged to obtain the mean value. The 50% effective dose (ED₅₀) was calculated for each test sample by the method of Kärber (1931).

Statistics. Each assay was done in triplicate, and the means and standard errors were calculated. Comparisons between the test and control groups were done by Student's *t*-test for independent samples.

RESULTS

Induction of cytokine production

Table 1 summarizes the results of cytokine assays performed with *P. gingivalis* lipid A and its LPS as compared with *E. coli* LPS and synthetic *E. coli*- and *Salmonella*-type lipid A (compounds 506 and 516). *P. gingivalis* LPS and its lipid A induced considerably weaker IL-1 β and TNF- α

Table 1. Comparison of the production of various cytokines in human peripheral blood monocyte/macrophage or mononuclear cell culture induced by *P. gingivalis* LPS and its lipid A as compared with *E. coli* LPS and synthetic compounds

Cytokines produced were determined by an ELISA kit system. Test specimens were added to human peripheral blood mononuclear cell culture for IFN- γ assay and to human peripheral blood monocyte/macrophage culture for other cytokine assays. The data are expressed as means $\pm$ SE of three different cultures. ND, Not done.

Test specimen	Dose ($\mu\text{g ml}^{-1}$)	Cytokine-producing activity (pg ml^{-1})						
		IL-1 β	TNF- α	IL-1ra	IL-6	IL-8	GM-CSF	IFN- γ
<i>P. gingivalis</i> 381 LPS	100	189 $\pm$ 23*	223 $\pm$ 12*	1886 $\pm$ 123*	2211 $\pm$ 256*	65825 $\pm$ 5534*	51 $\pm$ 4*	236 $\pm$ 21*
	50	196 $\pm$ 20*	263 $\pm$ 16*	1950 $\pm$ 125*	2411 $\pm$ 200*	75825 $\pm$ 4287*	72 $\pm$ 6*	655 $\pm$ 57*
	5	132 $\pm$ 12*	186 $\pm$ 19*	1879 $\pm$ 100*	1921 $\pm$ 172*	63675 $\pm$ 8312*	38 $\pm$ 4*	46 $\pm$ 5*
	0.5	98 $\pm$ 5*	142 $\pm$ 27*	1666 $\pm$ 183*	1620 $\pm$ 140*	44238 $\pm$ 2889*	21 $\pm$ 1*	28 $\pm$ 3*
<i>P. gingivalis</i> 381 lipid A	100	163 $\pm$ 17*	206 $\pm$ 17*	1711 $\pm$ 161*	1772 $\pm$ 101*	62775 $\pm$ 5144*	195 $\pm$ 20*	50 $\pm$ 5*
	50	180 $\pm$ 12*	252 $\pm$ 22*	1758 $\pm$ 103*	2286 $\pm$ 199*	69670 $\pm$ 4650*	240 $\pm$ 27*	52 $\pm$ 5*
	5	143 $\pm$ 22*	222 $\pm$ 26*	1265 $\pm$ 95*	1824 $\pm$ 167*	54279 $\pm$ 6464*	76 $\pm$ 6*	41 $\pm$ 4*
	0.5	49 $\pm$ 4*	178 $\pm$ 16*	1020 $\pm$ 121*	1574 $\pm$ 142*	37591 $\pm$ 3464*	31 $\pm$ 2*	28 $\pm$ 6*
<i>E. coli</i> O55:B5 LPS	100	1962 $\pm$ 101*	865 $\pm$ 72*	697 $\pm$ 54*	4930 $\pm$ 434*	52122 $\pm$ 6114*	199 $\pm$ 22*	47 $\pm$ 5*
	50	2280 $\pm$ 221*	932 $\pm$ 44*	897 $\pm$ 83*	5590 $\pm$ 485*	56122 $\pm$ 5440*	233 $\pm$ 14*	49 $\pm$ 5*
	5	2520 $\pm$ 183*	788 $\pm$ 55*	1042 $\pm$ 110*	5518 $\pm$ 376*	84453 $\pm$ 8851*	177 $\pm$ 18*	40 $\pm$ 4*
	0.5	1448 $\pm$ 98*	512 $\pm$ 47*	976 $\pm$ 68*	4339 $\pm$ 396*	56534 $\pm$ 3635*	143 $\pm$ 8*	33 $\pm$ 5*
Compound 506	100	881 $\pm$ 77*	556 $\pm$ 43*	1178 $\pm$ 98*	2885 $\pm$ 269*	34056 $\pm$ 3123*	58 $\pm$ 5*	34 $\pm$ 3*
	50	907 $\pm$ 71*	602 $\pm$ 71*	1326 $\pm$ 106*	3006 $\pm$ 213*	35601 $\pm$ 2754*	62 $\pm$ 5*	60 $\pm$ 5*
	5	853 $\pm$ 96*	371 $\pm$ 26*	1226 $\pm$ 108*	2282 $\pm$ 177*	44961 $\pm$ 3463*	48 $\pm$ 3*	38 $\pm$ 2*
	0.5	807 $\pm$ 41*	313 $\pm$ 20*	693 $\pm$ 55*	1756 $\pm$ 104*	41007 $\pm$ 4102*	27 $\pm$ 3*	28 $\pm$ 3*
Compound 516	100	698 $\pm$ 14*	220 $\pm$ 24*	ND	634 $\pm$ 31*	35506 $\pm$ 3122*	ND	46 $\pm$ 5*
	50	709 $\pm$ 15*	293 $\pm$ 42*	ND	652 $\pm$ 27*	36683 $\pm$ 2029*	ND	52 $\pm$ 5*
	5	680 $\pm$ 23*	232 $\pm$ 31*	ND	512 $\pm$ 41*	22746 $\pm$ 2723*	ND	44 $\pm$ 3*
	0.5	578 $\pm$ 61*	125 $\pm$ 12*	ND	289 $\pm$ 32*	11720 $\pm$ 1403*	ND	38 $\pm$ 3*
None		7 $\pm$ 1	3 $\pm$ 1	86 $\pm$ 5	12 $\pm$ 1	1639 $\pm$ 125	5 $\pm$ 1	5 $\pm$ 1

* Statistically significant difference from the value in control cultures at $P \leq 0.01$.

Fig. 2. Antagonistic effects of *P. gingivalis* LPS and its lipid A against production of IL-1 β induced by *E. coli* LPS or its synthetic lipid A compound 506. Human peripheral blood monocytes/macrophages were cultured at 37 °C for 24 h in RPMI 1640 medium containing 10% (v/v) FBS with 0.5 $\mu\text{g ml}^{-1}$ of *E. coli* LPS (●) or compound 506 (■), with or without the indicated doses of *P. gingivalis* LPS or its lipid A (○). After incubation, the supernatants were collected, and the production and amount of IL-1 β were measured by using an ELISA kit system. Experiments were done at least three times, and representative results are presented. Each assay was done in triplicate, and the data were expressed as means $\pm$ SE. The difference between the groups with and without the test specimens added was significant by Student's *t*-test (* $P \leq 0.05$ or † $P \leq 0.01$).

production than did *E. coli* LPS and compounds 506 and 516 in human peripheral blood monocyte/macrophage cultures. In contrast, *P. gingivalis* LPS and its lipid A

induced higher IL-1ra production than did *E. coli* LPS and compound 506. Furthermore, *P. gingivalis* lipid A and its LPS stimulated human monocytes/macrophages, re-

Fig. 3. Antitumour activities of *P. gingivalis* LPS and its lipid A. Cytostasis of Meth A as a target cell was assessed by a [³H]dThd uptake inhibition assay (see Methods for details). The cytostatic activity was determined as the degree of inhibition of [³H]dThd uptake in the target cells and the data are expressed as means $\pm$ SE of three different cultures. Cytolytic activity was measured as release of ⁵¹Cr-labelled Meth A (see Methods). The data are expressed as means $\pm$ SE of three different cultures. The difference between the test samples and the negative control was significant by Student's *t*-test (**P* $\leq$ 0.05 or †*P* $\leq$ 0.01).

leasing very high levels of IL-6 and IL-8 into the culture supernatants in a dose-dependent manner. *P. gingivalis* lipid A and *E. coli* LPS also elicited GM-CSF production in cell culture supernatants when stimulated. IFN- γ production was considerably more strongly induced by *P. gingivalis* LPS than by the other test specimens (Table 1).

Antagonistic effects of *P. gingivalis* LPS and its lipid A against IL-1 β production induced by *E. coli* LPS or compound 506 were assessed in human monocyte/macrophage cultures. As shown in Fig. 2, the addition of *P. gingivalis* lipid A or its LPS significantly inhibited IL-1 β production induced by *E. coli* LPS or compound 506 in a dose-dependent manner, with IL-1 β production being almost completely inhibited at a dosage of 50 μ g ml⁻¹ of either *P. gingivalis* LPS or its lipid A.

Immunopharmacological activities

Antitumour activities of activated splenic macrophages of mice. Cytostasis and cytolysis of Meth A fibrosarcoma as a target cell by splenic macrophages of Balb/c mice activated by *P. gingivalis* LPS or its lipid A were assessed. As shown in Fig. 3, the proliferation of Meth A was significantly inhibited in a dose-dependent manner when target cells were cultured with splenic macrophages in the presence of the indicated doses of *P. gingivalis* LPS or its lipid A. *P. gingivalis* lipid A and its LPS reduced the proliferation of Meth A more effectively than *E. coli* LPS and compound 506. In the cytolytic assay, *P. gingivalis* LPS and its lipid A exhibited tumoricidal activities at concentrations ranging from 0.5 to 50 μ g ml⁻¹. *P. gingivalis* lipid A and compound 506 had weaker cytolytic activity than their respective parent LPS (Fig. 3).

NK cell activity. NK cell activities of *P. gingivalis* LPS and

Fig. 4. NK cell activity of *P. gingivalis* LPS and its lipid A, determined by a ⁵¹Cr release assay (see Methods). The data are expressed as means $\pm$ SE of three different cultures. The difference between the test samples and the negative control was significant by Student's *t*-test (**P* $\leq$ 0.05 or †*P* $\leq$ 0.01).

its lipid A were tested against YAC-1 lymphoma cell line in spleen cells treated with these stimuli. As shown in Fig. 4, the induction of NK cell activities was found in the spleen cells of Balb/c mice administered intravenously with the indicated doses of either *P. gingivalis* LPS or its lipid A. The NK cell activities were almost equivalent to those obtained with *E. coli* LPS and compound 506. The degrees of inhibitory effects were comparable to those of spleen cells obtained from Balb/c mice injected intravenously with 200 μ g of test specimens (data not shown).

Antiviral activity against vesicular stomatitis virus. Antiviral activities of *P. gingivalis* LPS and its lipid A were assessed by inhibition of the cytopathogenic effects of

vesicular stomatitis virus. *P. gingivalis* lipid A and its LPS exhibited clear antiviral activity at ED₅₀ values ranging from 83.3 to 107.1 µg ml⁻¹. The antiviral effect was stronger than that of *E. coli* LPS or compound 506, both of which had an ED₅₀ of 277.8 µg ml⁻¹.

DISCUSSION

Recently, the chemical structure of *P. gingivalis* strain 381 lipid A has been determined (Ogawa, 1993). The lipid A backbone consists of a β(1→6)-linked glucosamine disaccharide which is phosphorylated at the 1 position of the reducing sugar. However, this lipid A structure lacks an ester-linked phosphate group which is bound to the hydroxy group at the 4'-position of the nonreducing sugar (Fig. 1). The hydroxyl groups at the 3-, 3'-, 4-, 4'- and 6'-positions of *P. gingivalis* lipid A backbone are also free. The molecule possesses 3-hydroxy-15-methylhexadecanoic acid and 3-hexadecanoyloxy-15-methylhexadecanoic acid moieties amide-linked at the 2- and 2'-positions, respectively (Fig. 1).

The present study showed that *P. gingivalis* lipid A induces very weak production of proinflammatory cytokines such as IL-1β and TNF-α in human peripheral blood monocyte culture as compared with compounds 506 and 516 (Table 1). On the other hand, higher production of the anti-inflammatory cytokine, IL-1ra, was observed in culture supernatants of human peripheral blood monocytes/macrophages stimulated by *P. gingivalis* lipid A. Ulich *et al.* (1991) reported that IL-1ra inhibits LPS-induced acute inflammation. Among the proinflammatory cytokines, *P. gingivalis* lipid A and its LPS up-regulate IL-6 and IL-8 production in human monocyte/macrophage culture, whereas these molecules down-regulate IL-1β and TNF-α production (Table 1). Our results show that *P. gingivalis* lipid A possesses clear immunopharmacological effects such as antagonistic, antitumour, NK cell and antiviral activities. *P. gingivalis* lipid A exhibits stronger immunobiological effects – such as mitogenic responses in C3H/HeJ and C3H/HeN mouse spleen cell cultures, polyclonal B-cell activation of Balb/c mouse spleen cells, and immunoadjuvant effects – as compared with compound 506 (Ogawa, 1994). The up- or down-regulation of cytokine production may influence not only its non-toxicity but also the strong beneficial effects of *P. gingivalis* lipid A.

Flad *et al.* (1993) reported that maximal production of cytokines such as IL-1, IL-6 and TNF from human peripheral mononuclear cells may be associated with the bisphosphorylated and hexa-acylated lipid A (compound 506) corresponding to *E. coli*-type lipid A, while hepta-, penta-, tetra- or biacyl lipid A is less active. In the present study *P. gingivalis*-type triacylated lipid A induced lower levels of IL-1β and TNF-α production than did compounds 506 (hexa-acylated) and 516 (hepta-acylated) (Table 1). However, higher or comparable production of other cytokines such as IL-1ra, IL-6, IL-8, GM-CSF and IFN-γ by *P. gingivalis* lipid A was seen as compared with compound(s) 506 and/or 516. It is generally considered that an increased number of 3-acyloxyacyl groups is

related to a reduction in the bioactivity of the disaccharide molecule (Takada & Kotani, 1992). *Salmonella*- and *Proteus mirabilis*-type lipid A were less active than *E. coli*-type lipid A in most bioassays (Kotani *et al.*, 1986; Shimizu *et al.*, 1987). Aschauer *et al.* (1990) also reported that the disaccharide 1-phosphate, acylated at positions 2, 2', 3, but not the corresponding isomer 2, 3, 2', was a potent inducer of TNF release. *P. gingivalis* lipid A, having only one 3-acyloxyacyl group, exerted strong immunobiological activities. The different chemical structure due to phosphorylation and acylation patterns of hexosamine disaccharide may be a key factor in the up- or down-regulation by lipid A of the various cytokine-producing activities of immunocytes. The existence of a suitable balance between agonistic and antagonistic effects of *P. gingivalis* lipid A in the induction of cytokines, followed by a complex regulation of the cytokine network, may result in the diminution of endotoxic activities, while eliciting definite immunopharmacological effects.

P. gingivalis LPS and its lipid A inhibit *E. coli* LPS- or compound 506-induced IL-1β production in human peripheral blood monocytes/macrophages (Fig. 2). It was previously reported that non-toxic LPS derived from *Bacteroides fragilis*, *Rhodobacter capsulatus* and *Rhodopseudomonas sphaeroides* inhibited LPS-induced proinflammatory cytokine (Loppnow *et al.*, 1990; Kirkland *et al.*, 1991; Kirikae *et al.*, 1991). Flad *et al.* (1993) reported that tetra-acylated synthetic compound of *E. coli* precursor Ia(VI_A) (compound 406) and its α-oxyethyl analogue PE-4, but not a monosaccharide lipid A precursor, lipid X, inhibited LPS-induced IL-1β, TNF and IL-6 production by human mononuclear cells or monocytes. *P. gingivalis* lipid A exhibits no or very low endotoxic activities such as lethal toxicity in galactosamine-loaded mice, preparative ability for local Shwartzman reaction, pyrogenicity, and activity in the *Limulus* test (Ogawa, 1994). This can be explained by the fact that *P. gingivalis* lipid A possesses antagonistic effects for LPS- or lipid A-induced IL-1β production, and not only weak IL-1β- but also high IL-1ra-inducing activities.

The defence mechanisms against bacterial infection or tumour cells induced by LPS are mainly macrophage activation (Cohn, 1962; Adams & Nathan, 1983). *P. gingivalis* LPS and its lipid A exhibit clear macrophage activation activities such as antitumour activities and NK cell activity in Balb/c mice (Figs 3 and 4). GM-CSF and IFN-γ have been regarded as important factors in host defence mechanisms, and are well known to be associated with activation of macrophages and NK cells (Vogel & Hogan, 1990). *P. gingivalis* lipid A can also induce higher or comparable production of GM-CSF and IFN-γ as compared with compound 506 (Table 1). *P. gingivalis* LPS elicited IFN-γ production more effectively than its lipid A (Table 1), and this effect may thus be due to other component(s) within the LPS molecule. There is high interest in the induction of IFN-γ by *P. gingivalis* LPS, so work is in progress to characterize the LPS components. It was recently reported that a synthetic compound GLA-60, which is a 4-O-phosphono-D-glucosamine derivative carrying 3-O-3-tetradecanoyl-oxytetradecanoyl and N-3-

hydroxytetradecanoyl groups, exhibits NK cell activity, antiviral activity against murine cytomegalovirus and phagocytic activity (Ikeda *et al.*, 1993). Our study also demonstrated that *P. gingivalis* lipid A and its LPS exhibit a stronger antiviral activity against vesicular stomatitis virus than either *E. coli* LPS or compound 506. These molecules may directly affect L929 cells and/or vesicular stomatitis virus, resulting in protection of the cells against the virus, or induce the release of cytokines from L929 cells.

In conclusion, we have demonstrated that *P. gingivalis* lipid A has different immunobiological activities from those of enterobacterial lipid A. *P. gingivalis* lipid A may be considered as a potent immunomodulator molecule for possible clinical use as a non- or low-toxic and immunobiologically active drug. Further studies are needed to investigate the immunopharmacological effects of *P. gingivalis* lipid A and to obtain new compounds; we are attempting to synthesize the lipid A and analogues with a better balance between beneficial and toxic activities.

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