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Chemical structure and immunobiological activity of lipid A from *Prevotella intermedia* ATCC 25611 lipopolysaccharide

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Abstract The novel chemical structure and immunobiological activities of *Prevotella intermedia* ATCC 25611 lipid A were investigated. A lipopolysaccharide (LPS) preparation of *P. intermedia* was extracted using a phenol–chloroform–petroleum ether method, after which its purified lipid A was prepared by weak acid hydrolysis followed by chromatographic separations. The lipid A structure was determined by mass spectrometry and nuclear magnetic resonance to be a diglucosamine backbone with a phosphate at the 4-position of the non-reducing side sugar, as well as five fatty acids containing branched long chains. It was similar to that of *Bacteroides fragilis* and *Porphyromonas gingivalis*, except for the phosphorylation site. *P. intermedia* lipid A induced weaker cytokine production and NF-κB activation in murine cells via Toll-like receptor (TLR) 4 as compared to *Escherichia coli* synthetic lipid A (compound 506). Our results indicate that *P. intermedia* lipid A activates cells through a TLR4-dependent pathway similar to *E. coli*-type lipid A, even though these have structural differences.

Key words: Lipid A; Branched fatty acid; Toll-like receptor 4; *Prevotella intermedia*

1. Introduction

Lipopolysaccharide (LPS) is an outer membrane component of Gram-negative bacteria, and exhibits powerful immunostimulatory and inflammatory activities. It is composed of a heterogeneous mixture of large molecular weight compounds composed of a polysaccharide part, O-antigen and core regions, and a lipid anchor called lipid A. Among these, lipid A moiety is known to be essential for the activity of LPS [1].

Recently, it has been shown that lipid A of *Escherichia coli* and its related species induces mammalian cell activation via an innate immunity receptor, Toll-like receptor (TLR) 4, and its accessory protein MD-2 complex [2]. On the other hand, LPS and lipid A preparations from *Bacteroides fragilis* and *Porphyromonas gingivalis* have been proposed to induce cell activation independently from TLR4 through TLR2-mediated signal transduction [3–6]. These different results have been shown to be responsible for the structural differences of lipid A moiety in LPS [7]. Lipid A from *E. coli* consists of a diglucosamine ($GlcN_2$) backbone carrying two phosphates at the 1-position of the reducing side and the 4-position of the non-reducing side of GlcN, which is a hexaacyl substituent. In contrast, *B. fragilis* and *P. gingivalis* lipid As are composed of the same backbone with a phosphate at the 1-position of the reducing side GlcN, and three to five branched long-chain fatty acids [8–10].

To confirm the activities of *P. gingivalis* lipid A, we recently prepared a highly purified version of its natural lipid A and a synthetic counterpart, and then examined their immunobiological activities [11,12]. *P. gingivalis* natural and synthetic lipid As, similar to *E. coli*-type lipid A, activated cells through a TLR4–MD-2-dependent pathway, but not via TLR2. These results suggest that the lipid A itself is recognized by the TLR4–MD-2 complex, regardless of its molecular structure, while the previous observation seems to have been affected by some contaminants, which activated cells in a TLR2-dependent manner in conventional-grade natural lipid A.

Prevotella intermedia, a Gram-negative black-pigmented bacterium, is dominant in the periodontal pockets of patients with gingivitis and has been implicated as a pathogen in periodontal diseases [13]. *P. intermedia* LPS has been reported to activate cells through a TLR4-independent pathway [4]. No information regarding the chemical structure of lipid A from *P. intermedia* or its immunobiological activity is available; therefore, we considered it necessary to determine whether our observation is suitable for *P. intermedia* lipid A. In this study, we elucidated the chemical structure of lipid A from *P. intermedia* and examined its immunobiological activities.

2. Materials and methods

2.1. Bacteria, LPS, and lipid A

P. intermedia ATCC 25611 organisms were grown anaerobically in Gifu anaerobic medium broth (Nissui, Tokyo, Japan) at 37°C for 24 h. Bacterial cells were collected by centrifugation, then washed three times with saline and lyophilized. LPS preparations were extracted from the lyophilized cells using a phenol–chloroform–petroleum ether method [14] and lipid A was prepared from the LPS preparations according to our method described previously [12]. Briefly, hydrophobic products obtained by weak acid hydrolysis of the LPS preparation were subjected to silica gel column chromatography to yield a lipid A fraction. The fraction was further subjected to two successive preparative silica gel thin-layer chromatography TLC) runs, using a solvent system consisting of chloroform–methanol–water (65/25/4, v/v/v) fol-

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Abbreviations: GlcN, glucosamine; IL, interleukin; LPS, lipopolysaccharide; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance; TLR, Toll-like receptor

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lowed by a chloroform–methanol–ammonia solution (65/25/5, v/v/v) to yield the purified lipid A. *E. coli*-type lipid A (compound 506) was chemically synthesized as described previously [12,15].

2.2. Analytical procedures

Sugar constituents were analyzed using the alditol acetate method [16]. The absolute configurations of sugar were determined using *R*-(+)-2-butanol [17], while fatty acids were analyzed according to the method of Ikemoto et al. [18]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 15% polyacrylamide gels according to the method of Laemmli [19]. The gel was partially oxidized with periodic acid and then visualized by the silver staining method [20]. Analytical TLC was performed on a TLC plate (No. 5715; Merck, Darmstadt, Germany) using a solvent system consisting of chloroform–methanol–water (65/25/4, v/v/v) and the spots were visualized with anisaldehyde-sulfuric acid reagent.

2.3. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS)

^{1}H, ^{13}C, and ^{31}P NMR spectra were measured at 500, 126, and 202 MHz, respectively, on a JMN-LA500 spectrometer (JEOL, Tokyo, Japan) equipped with an indirect detection gradient probe, IDG500-5VJ (Nanorac Cryogenics, Martinez, CA, USA). Spectra of lipid A were obtained at 303 or 310 K at a concentration of 2 mg ml^{-1} in $CDCl_3$–CD_3OD (4/1, v/v). The chemical shifts are expressed in δ values using chloroform ($\delta = 7.26$) as an internal standard for ^{1}H NMR spectra, methanol ($\delta = 49.0$) as an internal standard for ^{13}C NMR spectra. For ^{31}P NMR spectra, a capillary insert containing 85% phosphoric acid was used as an external standard ($\delta = 0$). The signals were assigned using DQF-COSY, NOESY, and ^{1}H–^{13}C heteronuclear multiple bond connectivity (HMBC) spectra. The coupling constants were determined by one-dimensional ^{1}H NMR in combination with DQF-COSY.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)-MS spectra were measured using an Ultraflex (Bruker Daltnics, Bremen, Germany) instrument. Samples were dissolved in chloroform–methanol (4/1, v/v) combined with 2,5-dihydroxybenzoic acid as a matrix, and then placed on a sample plate. Spectra were obtained in negative and positive ion reflector modes. Tandem MS (MS/MS) spectra were obtained in positive ion TOF/TOF mode.

2.4. Cytokine assays

C3H/HeN and C3H/HeJ mice were peritoneally injected with sterile Brewer's thioglycolate broth (Becton Dickinson, Franklin Lakes, NJ, USA). After 3 days, the peritoneal cavity of each animal was washed twice with phosphate-buffered saline (PBS; Sigma, St. Louis, MO, USA) and the cell suspensions thus obtained were washed three times with PBS by centrifugation. The peritoneal exudate cells were then suspended in RPMI1640 (Sigma) supplemented with 10% fetal bovine serum (FBS; Sigma) at 2×10^{5} cells/200 μl and distributed in each well of a 96-well microculture plate (Falcon 3072, Becton Dickinson), after which they were incubated for 2 h at 37°C in humidified air containing 5% CO_2. Each well was washed twice with PBS to remove non-adherent cells and cells attached to the culture plate served as peritoneal macrophages. The cells were incubated with the indicated doses of the test specimens for 24 h at 37°C in humidified air containing 5% CO_2. Interleukin 6 (IL-6) production was measured in the culture supernatants using a commercial ELISA kit system (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. The results were determined using a standard curve prepared for each assay.

2.5. Luciferase assays

Ba/F3 cells stably expressing p55IgκLuc, an NF-κB-dependent luciferase reporter construct (Ba/κB), murine TLR2 and the p55IgκLuc reporter construct (Ba/mTLR2), and murine TLR4/MD-2 and the p55IgκLuc reporter construct (Ba/mTLR4/mMD-2), were kindly provided by Dr. K. Miyake (Division of Infectious Genetics, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Japan), and maintained as described previously [12]. The cells were inoculated onto 96-well flat-bottomed microtiter plates at 1×10^{5} cells/well in 100 μl of RPMI1640 supplemented with 10% FBS, and stimulated with the indicated doses of the test specimens. After 4 h at 37°C, 100 μl of Bright-Glo™ luciferase assay reagent (Promega, Madison, WI, USA) was added to each well and luminescence was quantified with a luminometer (Turner Designs Luminometer Model TD-20/20; Promega). Results are shown as relative luciferase activity, which is the ratio of stimulated to non-stimulated activity in each cell line.

2.6. Statistical analysis

Data were analyzed by one-way ANOVA (analysis of variance), using the Bonferroni or Dunn method, and the results are presented as the mean ± S.E.M. When an individual result is presented, it is representative of at least three independent experiments.

3. Results and discussion

3.1. Isolation of lipid A from P. intermedia

Since LPS from *P. intermedia* ATCC 25611 was reported to be the rough form [21], the bacteria were subjected to PCP extraction [14] to obtain LPS preparations with a yield of 0.85%. An SDS–PAGE profile of the preparations proved them to be the rough form (data not shown). The preparations were subjected to weak acid hydrolysis to give hydro-

Table 1
Proton NMR data for *P. intermedia* lipid A

Residues	Chemical shifts (coupling constants)						
	H1 ($^{3}J_{1,2}$)	H2 ($^{3}J_{2,3}$)	H3 ($^{3}J_{3,4}$)	H4 ($^{3}J_{4,5}$)	H5	H6a	H6b
Sugars							
Major signals							
$GlcN^{I}$	4.89 (3.6)	3.88 (10.7)	4.97 (9.2)	3.27 (9.6)	3.85	3.55	3.85
$GlcN^{II}$	4.40 (8.3)	3.68 (10.4)	5.00 (9.2)	4.05	3.23	3.60	3.72
Minor signals							
$GlcN^{I}$	4.47 (8.6)	3.60 (10.6)	4.78 (8.6)	3.29	3.56	3.87	nd
$GlcN^{II}$	4.39	nd	nd	nd	nd	nd	
Fatty acids							
A	–	2.02	3.66	1.12			
		2.12		1.27			
B	–	2.18	3.78	1.25			
		2.28		1.30			
C	–	2.26	3.85	1.23			
				1.30			
D	–	2.14	4.90	1.36			
		2.27					
E	–	2.10	1.39				

Spectra were measured at 303 K. Chemical shifts are expressed as δ values and coupling constants are shown in parentheses.
nd: not determined.

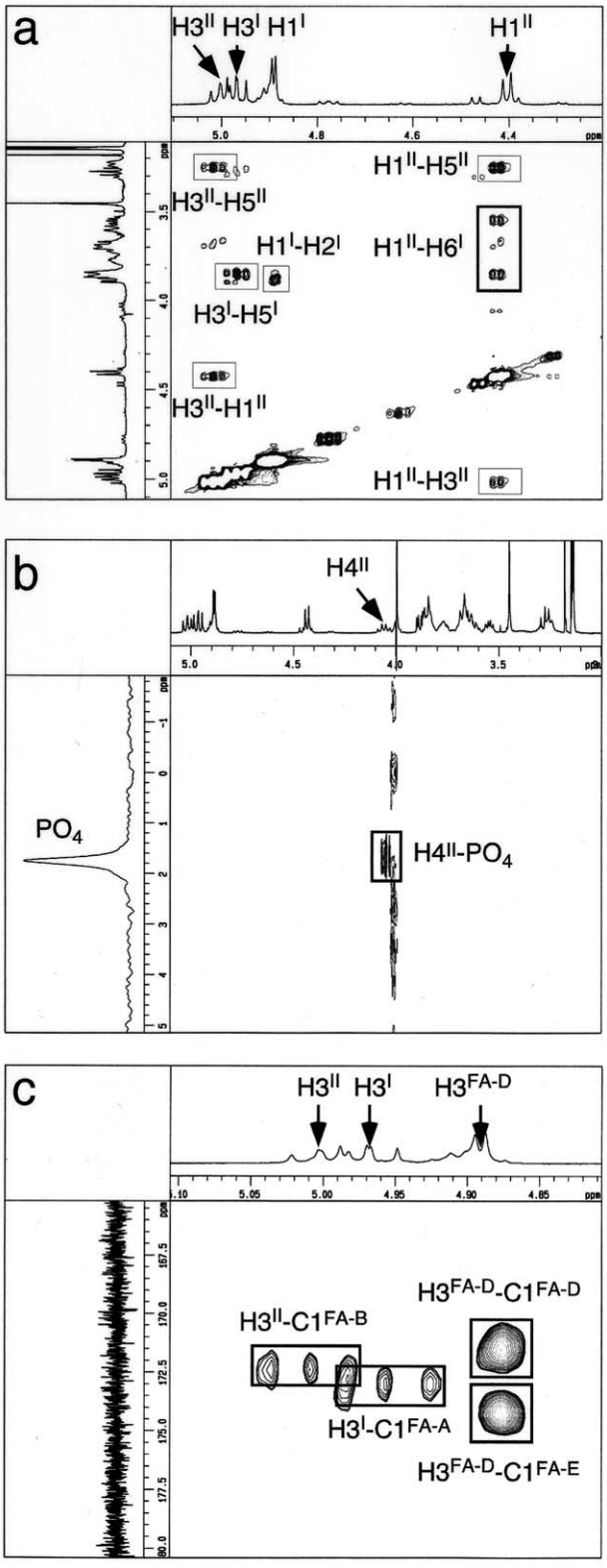

Fig. 1. NOESY spectra (a), ^{1}H–^{31}P HMBC spectra (b), and ^{1}H-^{13}C HMBC spectra (c) of *P. intermedia* lipid A. Spectra were measured at 303 K for panels a and c, and 310K for panel b.

phobic products. In the TLC analysis, one major ($R_f = 0.5$) and several minor spots were detected in the hydrophobic products. The major component was isolated by silica gel chromatography followed by two successive preparative TLC separations as described previously [12]. The yield of the component was 7%, based on the LPS preparation. This component was used as purified lipid A.

3.2. Structural elucidation of lipid A

The molecular mass of lipid A was measured by MALDI-TOF-MS in the negative ion mode. Ion peaks were observed at *m/z* 1688.4, 1702.4, and 1716.4 in a relative intensity ratio of 0.6:1.0:0.7, indicating lipid A structures that corresponded to the $GlcN_2$ backbone with a phosphate, four hydroxy fatty acids, and a non-hydroxy fatty acid. Fatty-acid analysis revealed that the component mainly contained 15-Me-16:0 (3-OH), 16:0 (3-OH), 12-Me-14:0, and 12-Me 13:0 in a molar ratio of 3.1:1.1:1.5:1.0, thus, the heterogeneity of the molecular weight was explained by the substitution of different combinations of fatty acids, e.g. *m/z* 1702.4 was considered to contain 13-Me-14:0, 16:0 (3-OH), 15-Me-16:0 (3-OH) in a molar ratio of 1:1:3.

The detailed structure of lipid A was established by NMR. The ^{1}H NMR of the purified lipid A was assigned using DQF-COSY, NOESY, and ^{1}H–^{13}C HMBC, and the data are summarized in Table 1. Two sets of sugar signals were mainly observed. The coupling constants and NOESY correlation (Fig. 1a) of the signals revealed a glucopyranosyl con-

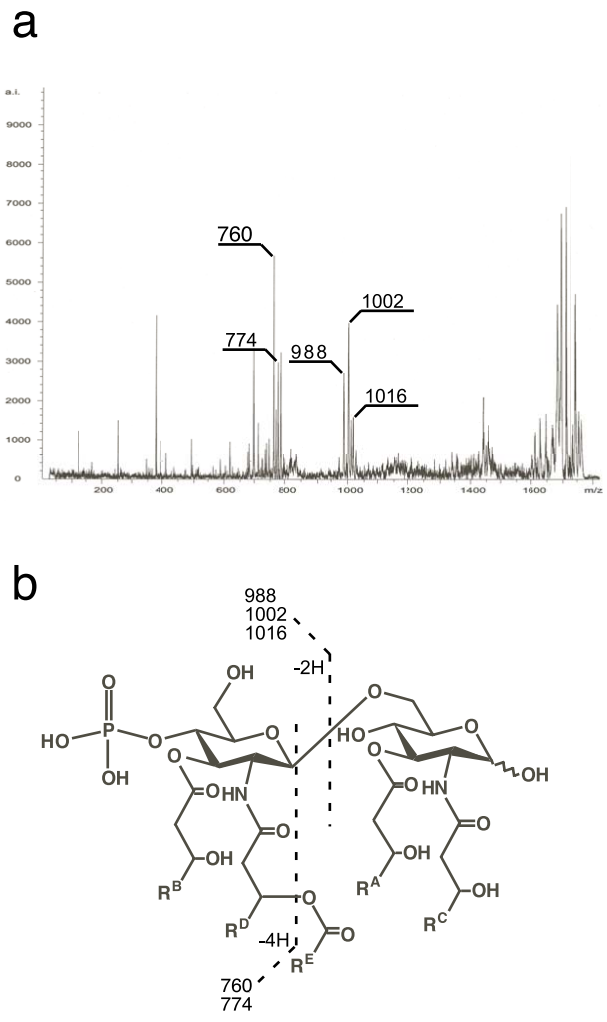

Fig. 2. a: MS/MS spectrum of the parent ion at *m/z* 1726.2. b: A proposed chemical structure of *P. intermedia* lipid A.

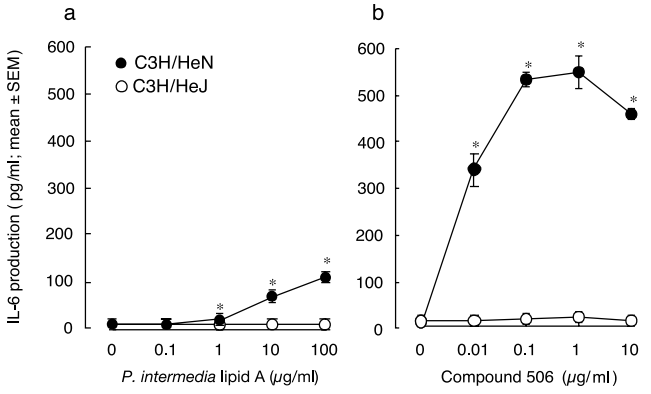

Fig. 3. IL-6 production in peritoneal exudate macrophages from C3H/HeN and C3H/HeJ mice in response to stimulation by *P. intermedia* lipid A (a) and compound 506 (b). Data are expressed as the mean ± S.E.M. Significant differences were seen between groups with and without the test specimens (*$P < 0.01$).

figuration of the sugar. Since only D-GlcN was seen in the compositional analysis, the sugars were determined to be GlcN, and designated as $GlcN^{I}$ and $GlcN^{II}$ in order of the ^{1}H chemical shift of the anomeric proton. The coupling constant (3.6 Hz) for the anomeric proton of $GlcN^{I}$ ($H1^{I}$) at $\delta = 4.89$ showed an α configuration. The $H1^{I}$ signal did not shift to a lower field, confirming no phosphorylation at the anomeric position, i.e. $GlcN^{I}$ had a free anomeric hydroxy group. The coupling constant (8.3 Hz) for the anomeric proton of $GlcN^{II}$ ($H1^{II}$) at $\delta = 4.40$ showed a β configuration and interresidual NOESY coupling from $H1^{II}$ to $H6^{I}$ proved glycosylation at the 6-position of $GlcN^{I}$ (Fig. 1a). Therefore, $GlcN^{I}$ was categorized as a reducing side sugar and $GlcN^{II}$ as a non-reducing one. The downfield shift of the signal for $H4^{II}$ ($\delta = 4.05$) revealed a phosphate substitution at the 4-position of $GlcN^{II}$. Although the coupling constant for $J_{H,P}$ could not be determined at the $H4^{II}$ signal due to line broadening, the long-range HMBC coupling between $H4^{II}$ and the phosphate ($\delta = 1.75$) proved phosphorylation (Fig. 1b). $H3^{I}$ and $H3^{II}$ signals appeared at $\delta = 4.97$ and $\delta = 5.00$, respectively, and the downfield shift of the signals displayed the acyl substitution at each 3-position. ^{1}H–^{13}C HMBC and MS/MS spectra further revealed the acylation patterns. Couplings between $H3^{I}$ and the carbonyl carbon of fatty acid A ($C1^{FA-A}$), and $H3^{II}$ and $C1^{FA-B}$ were observed in the HMBC spectra, showing acylations of FA-A at the 3-position of $GlcN^{I}$ and FA-B at the 3-position of $GlcN^{II}$ (Fig. 1c). Coupling between $H3^{FA-D}$ and $C1^{FA-E}$ displayed that FA-E was attached at the 3-position of FA-D. The positions of FA-C and -D substitution were determined from positive ion MS/MS spectra (Fig. 2a). The fragmentation pattern of the parent ion at *m/z* 1726.2 $[M+Na]^{+}$ indicated that FA-D was linked at the 2-position of $GlcN^{II}$ (Fig. 2b).

In the NMR spectra, several minor signals, which had less than one-fifth the intensity of major signals, were observed and considered to be lipid A with a β anomeric configuration of $GlcN^{I}$. The existence of the opposite anomeric conformer was attributable to the free anomeric hydroxy group of lipid A. Thus, we proposed a structure for *P. intermedia* lipid A, as shown in Fig. 2b.

Lipid A from closely related bacteria, *B. fragilis* NCTC 9343 [8], *Bacteroides vulgatus* IMCJ 1204 [22], *P. gingivalis* 381 [9], and *P. gingivalis* SU63 [10], have been reported to possess fundamentally similar structures, e.g. a $GlcN_2$ backbone attached to a phosphate at the 1-position of the reducing side GlcN, and three to five branched long-chain fatty acids. The present results show that *P. intermedia* lipid A has a similar structure, including the acylation pattern and number of phosphate groups, as the above-mentioned bacteria, except for the phosphorylation position. Since some bacterial species have been reported to have lipid A with a phosphorylation only at the 4-position of the non-reducing side GlcN and no substituent at the 1-position of the reducing one [23–25], the lipid A in the present study was not an artifact created during the preparation steps. However, its structural significance remains unknown.

3.3. Immunobiological activity of lipid A

Cytokine production in peritoneal exudate macrophages from LPS-responsive C3H/HeN and LPS-hyporesponsive C3H/HeJ mice was investigated. *P. intermedia* lipid A induced IL-6 production in peritoneal exudate macrophages from C3H/HeN mice, whereas IL-6 production in those from C3H/HeJ mice was negligible, similar to *E. coli*-type lipid A, compound 506 (Fig. 3). Since C3H/HeJ mice have a dominant negative point mutation in the cytoplasmic domain of TLR4 [26], these results indicate that cell activation by *P. intermedia* lipid A is mediated by TLR4. Furthermore, the TLR4-mediated signaling of *P. intermedia* was confirmed using murine TLR-transfected Ba/F3 cells (Fig. 4). NF-κB activation by *P. intermedia* lipid A, as well as by compound 506, was observed in Ba/mTLR4/mMD-2, but not in Ba/mTLR2 or Ba/κB cells, indicating that *P. intermedia* lipid A stimulates cells via a TLR4-dependent pathway, but not by TLR2. These findings are consistent with the results of our previous study of *P. gingivalis* [12], which found that its lipid A activated cells through TLR4 in a similar manner as compound 506.

Although the signaling pathway of *P. intermedia* lipid A is similar to *E. coli*-type lipid A, their activating intensity is quite different. The activity of *P. intermedia* lipid A is more than four orders weaker than compound 506 (Figs. 3 and 4). The present results are also highly comparable with those for *P. gingivalis* lipid A [12]. Furthermore, the weaker activities of *B. fragilis* lipid A appear to be caused by differences in their chemical structure [27]. Compound 506 is composed of $GlcN_2$ backbone, six fatty acids and two phosphates, and *P. intermedia* lipid A similar to *P. gingivalis* and *B. fragilis*

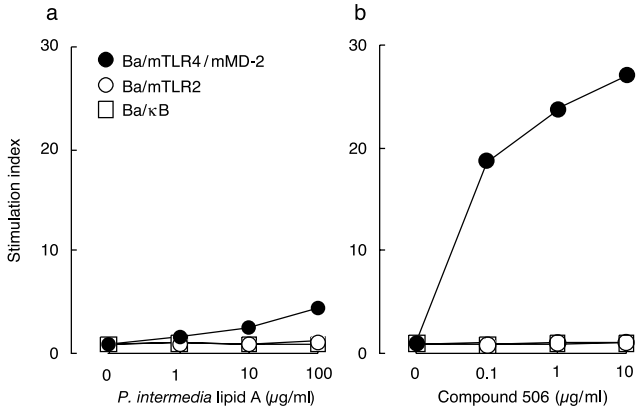

Fig. 4. NF-κB activation in Ba/F3 cells in response to stimulation by *P. intermedia* lipid A (a) and compound 506 (b). Data are shown as relative luciferase activity.

lipid A contains fewer fatty acids and phosphate as well as longer fatty-acid chain lengths. Absence of a phosphate on reducing or non-reducing GlcN of compound 506 reduced endotoxic activities [28]. The activities of compound 406, which lacks two of six fatty acids in compound 506, were also reduced. A longer fatty-acid chain length may be responsible for the reduction of activities [29]. Thus the weak activity of *P. intermedia* lipid A is likely to be explained on the basis of the structural differences.

Taken together, these results clearly demonstrate the structure of lipid A from *P. intermedia* ATCC 25611 to be composed of a $GlcN_2$ backbone with five fatty acids and a phosphate. We also found that the lipid A activates murine cells through a TLR4-mediated signaling pathway. Our findings indicate that *P. intermedia* lipid A as well as that of *E. coli* stimulate cell activation via TLR4, regardless of the substitution pattern of fatty acids and phosphate, or the kind of fatty acids.

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