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# Soluble CD14 Discriminates Slight Structural Differences between Lipid As That Lead to Distinct Host Cell Activation<sup>1</sup>

Yasuyuki Asai, Yutaka Makimura, Atsushi Kawabata, and Tomohiko Ogawa<sup>2</sup>

Soluble CD14 (sCD14) in serum is known to sensitize host cells to LPS. In the present study, the contributions of sCD14 and LPS-binding protein to a lipid A moiety from LPS preparations of periodontopathogenic *Fusobacterium nucleatum* sp. *nucleatum* were compared with that of *Escherichia coli*-type synthetic lipid A (compound 506). *F. nucleatum* lipid A was identified to be a hexa-acylated fatty acid composed of tetradecanoate (C<sub>14</sub>) and hexadecanoate (C<sub>16</sub>), similar to dodecanoate (C<sub>12</sub>) and C<sub>14</sub> in compound 506. The two lipid A specimens exhibited nearly the same reactivity in *Limulus* amoebocyte lysate assays, though *F. nucleatum* lipid A showed a weaker lethal toxicity. Both lipid A specimens showed nearly the same activities toward host cells in the absence of FBS, though compound 506 exhibited much stronger activity in the presence of FBS, sCD14, or sCD14 together with LPS-binding protein. Furthermore, native PAGE/Western immunoblot assays demonstrated that *F. nucleatum* lipid A had a weaker binding to sCD14 as compared with compound 506. These results suggest that sCD14 is able to discriminate the slight structural differences between these lipid As, which causes their distinct host cell activation activities. *The Journal of Immunology*, 2007, 179: 7674–7683.

**L**ipopolysaccharide is the principal constituent of the Gram-negative bacterial outer surface membrane and a causative agent of lethal endotoxin shock (1, 2). It is also known to predominantly activate monocytes/macrophages to secrete various cytokines, such as IL-1, IL-6, and TNF- $\alpha$ , which in turn activate endogenous inflammatory mediators. LPS consists of three covalently linked segments, including *O*-polysaccharide, a core oligosaccharide, and a lipid A moiety (3). *O*-polysaccharide has variable structures that differ among strains and is recognized by its adaptive immunity, which leads to the production of specific Abs (4). In contrast, the target constituent for innate immunity is lipid A, which is the bioactive center of LPS toxicity (5, 6).

TLRs play a pivotal role as innate immune receptors for recognition of pathogen-associated molecular patterns (7). Among the TLR family members, TLR4 is the major receptor for LPS and mediates intracellular signaling cascades, such as phosphorylation of MAPK and NF- $\kappa$ B activation (8). TLR4 is unique in that it coexists with an accessory molecule, MD-2, which is thought to be a secreted glycoprotein from the deduced amino acid sequences.

MD-2 has been demonstrated to be physically associated with TLR4 and participates in the recognition of ligands (9).

CD14 and LPS-binding protein (LBP)<sup>3</sup> have been demonstrated to play pivotal roles in host recognition (10). LBP in serum recognizes and forms a high-affinity complex with the lipid A moiety of LPS as free molecules or fragments, or when still bound to the outer membrane of intact bacteria (11). LBP pulls out LPS and transfers LPS monomers to the binding site of either a glycosylphosphatidylinositol-anchored membrane-bound CD14 (mCD14) expressed on the cell surface or soluble CD14 (sCD14) (12, 13). Then, CD14 forms a stable complex with monomeric LPS and transfers it to the signaling receptor complex TLR4/MD-2, which results in an increase in sensitivity to LPS (14).

*Fusobacterium nucleatum*, a Gram-negative obligate anaerobic spindle-shaped or fusiform bacillus, is a type species of the genus *Fusobacterium*, which belongs to the *Fusobacteriaceae* family (15, 16). *F. nucleatum* is the most encountered bacterium in humans and considered to be a key microorganism in periodontal diseases, as it is commonly present in dental plaque biofilms of adults and children, and plays a conclusive role in plaque development (17–19).

*F. nucleatum* LPS has been shown to possess biological activities comparable to those of LPS of *Escherichia coli* in terms of activation of *Limulus* amoebocyte lysate (LAL), local Shwartzman reaction, B cell mitogenicity, polyclonal B cell activation, induction of bone resorption, and cytokine production by macrophages (20, 21). Furthermore, Onoue et al. (22) demonstrated that most *F. nucleatum* LPS was extracted in the phenolic phase, but not in the aqueous phase, during which nearly all LPS is generally contained. *F. nucleatum* lipid A has also been reported to have a chemical composition similar to the classical structure of enterobacterial lipid A (23).

In the present study, we determined the chemical structure of lipid A from a phenolic phase LPS preparation of *F. nucleatum* sp. *nucleatum*. Thereafter, our experiments demonstrated that discrimination of the slight structural differences of the lipid A derived from *F. nucleatum* sp. *nucleatum* as compared with that from

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<sup>3</sup> Abbreviations used in this paper: LBP, LPS-binding protein; mCD14, membrane-bound CD14; sCD14, soluble CD14; LAL, *Limulus* amoebocyte lysate; LPL, lipoprotein lipase; MS, mass spectrometry; MS/MS, tandem MS; D-GalN, D-galactosamine hydrochloride; HGF, human gingival fibroblast; IP-10, IFN- $\gamma$ -inducible protein 10; MFI, mean fluorescence intensity; h, human; TRIF, Toll/IL-1R domain-containing adaptor inducing IFN- $\beta$ ; TICAM, Toll/IL-1R domain-containing adaptor molecule.

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*Escherichia coli* by sCD14, which contribute to their distinct host cell activation activities.

## Materials and Methods

### Bacteria, LPS, and lipid A

*F. nucleatum* sp. *nucleatum* JCM 8532 (originally ATCC 25586, type strain; American Type Culture Collection) was obtained from Riken Biore-source Center (Saitama, Japan) and grown anaerobically in Gifu anaerobic medium broth (Nissui), supplemented with 5  $\mu\text{g/ml}$  hemin and 1  $\mu\text{g/ml}$  vitamin K<sub>3</sub> at 37°C. Bacterial cells were collected by centrifugation, then washed three times with saline, and lyophilized. The lyophilized cells were subjected to a hot-phenol water method, with the phenolic phase recovered to obtain a smooth-type LPS preparation, as previously described (22, 24). Lipid A was then prepared from the LPS preparation according to a method described previously (25). Briefly, the LPS preparation was subjected to 0.6% acetic acid hydrolysis at 105°C for 2.5 h and hydrophobic products were obtained by chloroform-methanol water extraction. The products were then subjected to silica gel column chromatography to yield a lipid A fraction, which was further separated by two successive preparative silica gel thin-layer chromatography runs, using a solvent system consisting of chloroform-methanol-water (65/25/4, v/v/v) followed by a chloroform-methanol-ammonia solution (65/25/5, v/v/v) to yield purified lipid A. *E. coli* O55:B5 LPS was obtained from Sigma-Aldrich, and further purified as described by Manthey and Vogel (26). *E. coli*-type synthetic lipid A (compound 506) was chemically synthesized as described previously (27). In some experiments, 50  $\mu\text{g/ml}$  *F. nucleatum* LPS preparation or *E. coli* LPS were treated with 50  $\mu\text{g/ml}$  lipoprotein lipase (LPL) from *Pseudomonas* sp. (Sigma-Aldrich) for 16 h at 37°C as described previously, with a slight modification (28). Bacterial synthetic lipopeptide Pam<sub>3</sub>CSK<sub>4</sub> was purchased from EMC Microcollections.

### Analytical procedures

MALDI-TOF-mass spectrometry (MS) spectra were measured using a QSTAR XL (Applied Biosystems) instrument. Lipid A specimens were dissolved in chloroform-methanol (4/1, v/v) combined with 2,5-dihydroxybenzoic acid as a matrix, then placed on a sample plate. Spectra were obtained in negative ion reflector mode. Tandem MS (MS/MS) spectra were obtained in positive ion TOF/TOF mode.

### Animals

C57BL/6, C3H/HeN, and C3H/HeJ mice (males, 8-wk old) were obtained from Japan SLC. The animals received humane care in accordance with our institutional guidelines and the legal requirements of Japan.

### LAL assay

Various doses of test specimens were mixed separately with LAL reagent, then incubated at 37°C for 30 min, after which the activities were determined using a quantitative chromogenic assay (Seikagaku Kogyo).

### Lethal toxicity in galactosamine-sensitized mice

C57BL/6 mice were injected i.p. with 16 mg of D-galactosamine hydrochloride (D-GalN; Wako Pure Chemicals) in 0.5 ml of PBS (Sigma-Aldrich), followed immediately by an i.p. injection of various doses of the test specimens in 0.2 ml of PBS. Death of the mice due to intoxication was observed over a 1-wk period. The LD<sub>50</sub> for each group was calculated by the method of Kärber (29).

### Mouse peritoneal macrophages

Elicited peritoneal macrophages were obtained from C3H/HeN and C3H/HeJ mice 72 h after i.p. inoculation with 1.0 ml of 3% sterile Brewer modified thioglycolate medium (BD Biosciences). The peritoneal exudate cells were centrifuged and suspended in RPMI 1640 (Sigma-Aldrich) supplemented with 5% FBS (Sigma-Aldrich), 50 U/ml penicillin, and 50  $\mu\text{g/ml}$  streptomycin (Invitrogen Life Technologies) at  $1 \times 10^6$  cells/ml. These cells were distributed to each well of a 96-well flat-bottom plate at  $2 \times 10^5$  cells per 200  $\mu\text{l}$ , after which they were incubated for 2 h at 37°C in a humidified 5% CO<sub>2</sub> incubator. Each well was then washed with PBS to remove nonadherent cells and those attached to the culture plate served as peritoneal macrophages.

### Human monocytes

Blood samples were collected after receiving written informed consent under a protocol approved by the Institutional Review Board of Asahi

University. Heparinized venous blood samples drawn from healthy human donors were subjected to fractionation using a Histopaque-1077 (Sigma-Aldrich) to obtain human PBMC. These cells were suspended at a cell density of  $2 \times 10^6$  cells/ml in RPMI 1640 supplemented with 5% FBS, 50 U/ml penicillin, and 50  $\mu\text{g/ml}$  streptomycin at  $1 \times 10^6$  cells/ml. The cells were distributed to each well of a 96-well flat-bottom plate at  $2 \times 10^5$  cells/200  $\mu\text{l}$ , after which they were incubated for 2 h at 37°C in a humidified 5% CO<sub>2</sub> incubator. Each well was then washed with PBS to remove nonadherent cells and those attached to the culture plate served as human monocytes.

### Human gingival fibroblasts (HGF)

HGF were prepared from clinically normal gingival tissues according to a method similar to that described previously (25). A tissue sample was collected after receiving written informed consent under a protocol approved by the Institutional Review Board of Asahi University. HGF were cultured in  $\alpha$ -MEM (Sigma-Aldrich) containing 10% FBS, 50  $\mu\text{g/ml}$  gentamicin (Invitrogen Life Technologies), and 50 ng/ml amphotericin B (Sigma-Aldrich) at 37°C in a 5% (v/v) CO<sub>2</sub> atmosphere, then used for the assays at the sixth passage. The cells were distributed to each well of a 96-well flat-bottom plate at  $1 \times 10^4$  cells/200  $\mu\text{l}$ , after which they were cultured for 16 h at 37°C in a humidified 5% CO<sub>2</sub> incubator.

### Cytokine assay

Cells were incubated in a 96-well flat-bottom microtiter plate with the test specimens together with various doses of FBS for 24 h at 37°C in a humidified 5% CO<sub>2</sub> incubator. After incubation, the supernatants were collected and stored at -80°C until assays for cytokine production. Mouse IL-6 and human TNF- $\alpha$  were analyzed using ELISA kit systems (eBioscience). Human IL-8 and mouse IFN- $\gamma$ -inducible protein 10 (IP-10)/CXCL10 ELISA kits were purchased from R&D Systems. The assays were performed according to the manufacturer's instructions, with data determined using a standard curve prepared for each assay.

### Flow cytometry

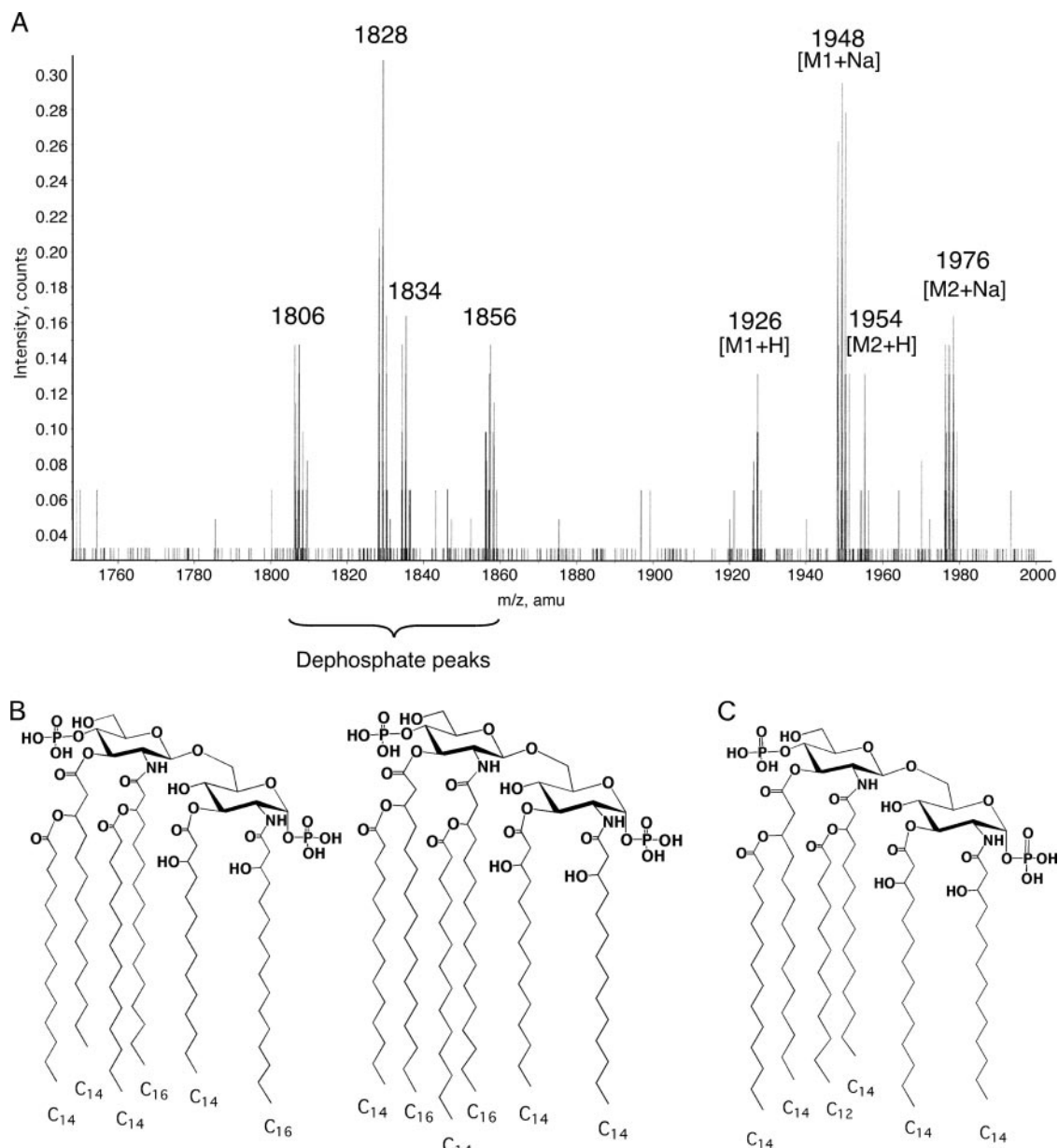
Peritoneal macrophages ( $4 \times 10^5$  cells) were stimulated with various doses of the lipid A specimens for 24 h at 37°C, then the cells were suspended in cold PBS containing 3% FBS and 0.1% NaN<sub>3</sub> (FACS buffer), and kept on ice with low light exposure during the procedure. The cells were stained with 100  $\mu\text{l}$ /tube of FACS buffer containing fluorochrome-conjugated Abs at 10  $\mu\text{g/ml}$  and incubated for 30 min. Thereafter, the cells were washed twice with FACS buffer and finally suspended at 500  $\mu\text{l}$ /tube in FACS buffer for flow cytometric analysis with a FACSCalibur (BD Biosciences), which was based on counting 10,000 cells. The following Abs used for staining were purchased from eBioscience: FITC-conjugated anti-mouse CD80 (B7-1), clone 16-10A1 (hamster IgG); PE-conjugated anti-mouse CD86 (B7-2), clone GL1 (rat IgG2a); and isotype controls for FITC-conjugated Armenian hamster IgG and PE-conjugated rat IgG2a. Histograms were drawn and mean fluorescence intensity (MFI) values were determined using CellQuest software (BD Biosciences).

### Luciferase assay

IL-3-dependent mouse Ba/F3 pro-B cells stably expressing the p55I $\gamma$ Luc reporter construct (Ba/ $\kappa$ B), p55I $\gamma$ Luc and mouse TLR4/MD-2 (Ba/mTLR4/mMD-2), p55I $\gamma$ Luc and mouse TLR2 (Ba/mTLR2), and p55I $\gamma$ Luc and human TLR4/MD-2 (Ba/hTLR4/hMD-2) were used to detect NF- $\kappa$ B-dependent luciferase activity, as described previously (30). Briefly, cells ( $1 \times 10^5$  cells) were stimulated with the test specimens together with various concentrations of FBS. In some experiments, Ba/human (h) TLR4/hMD-2 cells were stimulated with the test specimens in the presence or absence of 500 ng/ml sCD14 and 50 ng/ml LBP. After 4 h at 37°C, 100  $\mu\text{l}$  of Bright-Glo luciferase assay reagent (Promega) 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 was the ratio of stimulated to nonstimulated activity, for each cell line.

### RT-PCR

Peritoneal macrophages ( $2 \times 10^6$  cells) were stimulated with the indicated doses of lipid A specimens for 3 h at 37°C in the presence or absence of 5% FBS. Total RNA was isolated from macrophage cultures and RT-PCR was performed, as described previously (30). PCR was performed under the following conditions. The primers for detection of mRNA for mouse IFN- $\beta$  and  $\beta$ -actin have been published previously (31, 32) and their annealing temperature was 58°C. The number of cycles for amplification of each gene product was as follows: IFN- $\beta$ , 35; and  $\beta$ -actin, 30.



**FIGURE 1.** MS spectrum for *F. nucleatum* lipid A (A), and proposed chemical structures of *F. nucleatum* lipid A (B) and *E. coli*-type synthetic lipid A (compound 506) (C) (27).

### Binding of lipid A to sCD14

The binding of lipid A specimens to sCD14 was assessed as described previously, with a slight modification (33). Briefly, 50  $\mu\text{g/ml}$  sCD14 was mixed with various doses of lipid A specimens in the presence of 1.5  $\mu\text{g/ml}$  LBP for 1 h at 37°C. Reaction mixes were loaded onto 5–15% Ready Gel J (Bio-Rad) and separated by native PAGE. sCD14 was detected after incubation in mouse anti-human mCD14 Ab (clone MY-4; Beckman Coulter) for 1 h, followed by HRP-conjugated anti-mouse IgG Ab (KPL) for 1 h. Development was performed using a 3,3',5,5'-tetramethylbenzidine substrate solution (Promega).

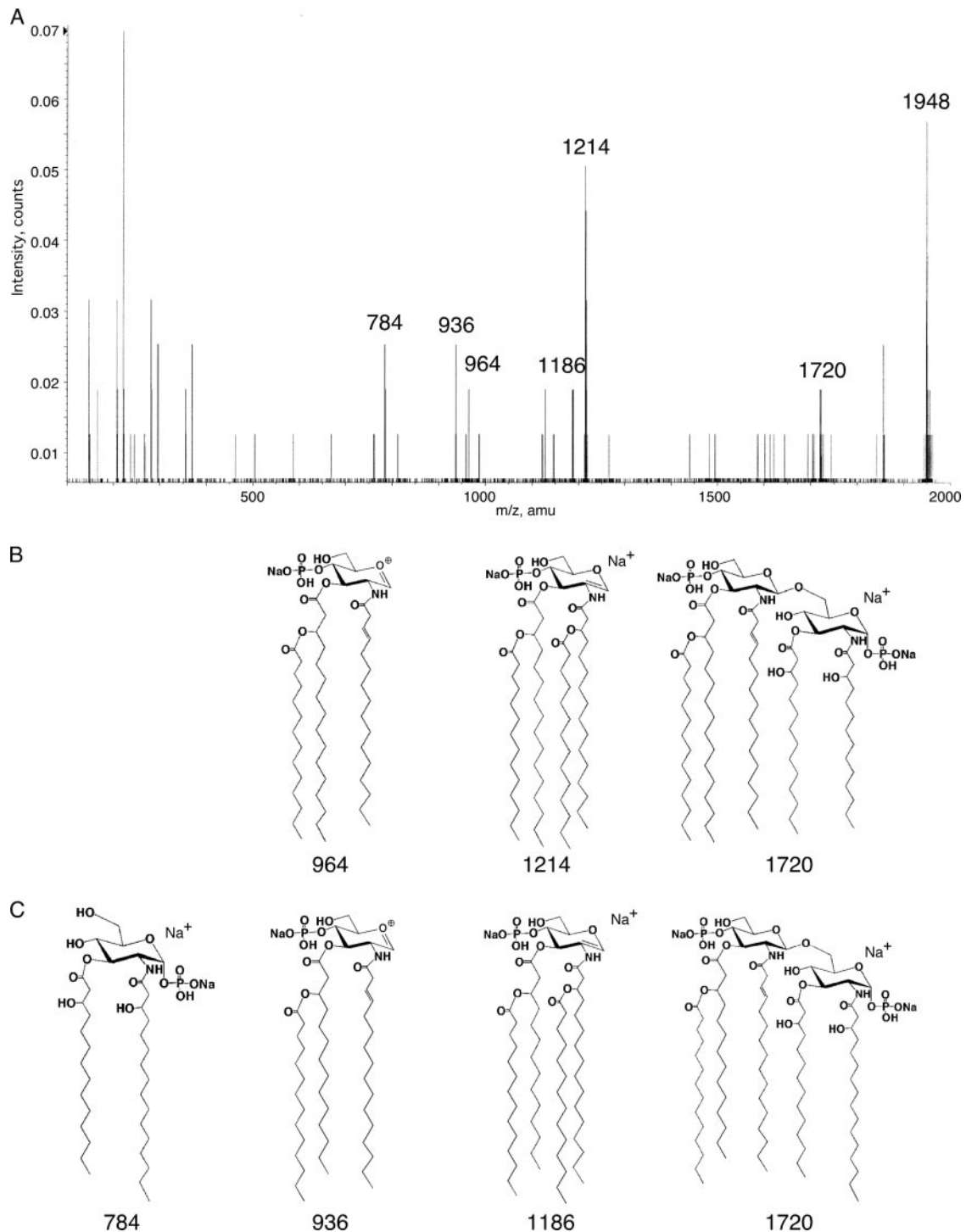
### Statistical analysis

The significance of cytokine production was assessed with a one-way ANOVA, using the Bonferroni or Dunn method, and the results are presented as the mean  $\pm$  SD. When an individual experiment is shown, it is representative of at least three independent experiments.

## Results

### Chemical structure of *F. nucleatum* sp. *nucleatum* (*F. nucleatum*) lipid A

The molecular mass of *F. nucleatum* lipid A was measured by MALDI-TOF-MS in the negative ion mode. Ion peaks were observed at  $m/z$  1926 (M1+H) and 1948 (M1+Na), 1954 (M2+H), and 1976 (M2+Na), and their dephosphate peaks were 1806, 1828, 1834, and 1856, respectively, in a relative intensity ratio of 1.0 (M1):0.5 (M2) (Fig. 1A), indicating that the lipid A structures corresponded to the GlcN2 backbone with diphosphates, four hydroxyl fatty acids, and two nonhydroxyl fatty acids. The fatty acid analysis also revealed that those components contained 16:0 (3-OH), 14:0 (3-OH), and 14:0 in a molar ratio of 1:1:1 based on MS/MS spectra pattern ( $m/z$  1948), as shown in Fig. 2A. Thus, two candidate structures of *F. nucleatum* lipid A at the main peak ( $m/z$

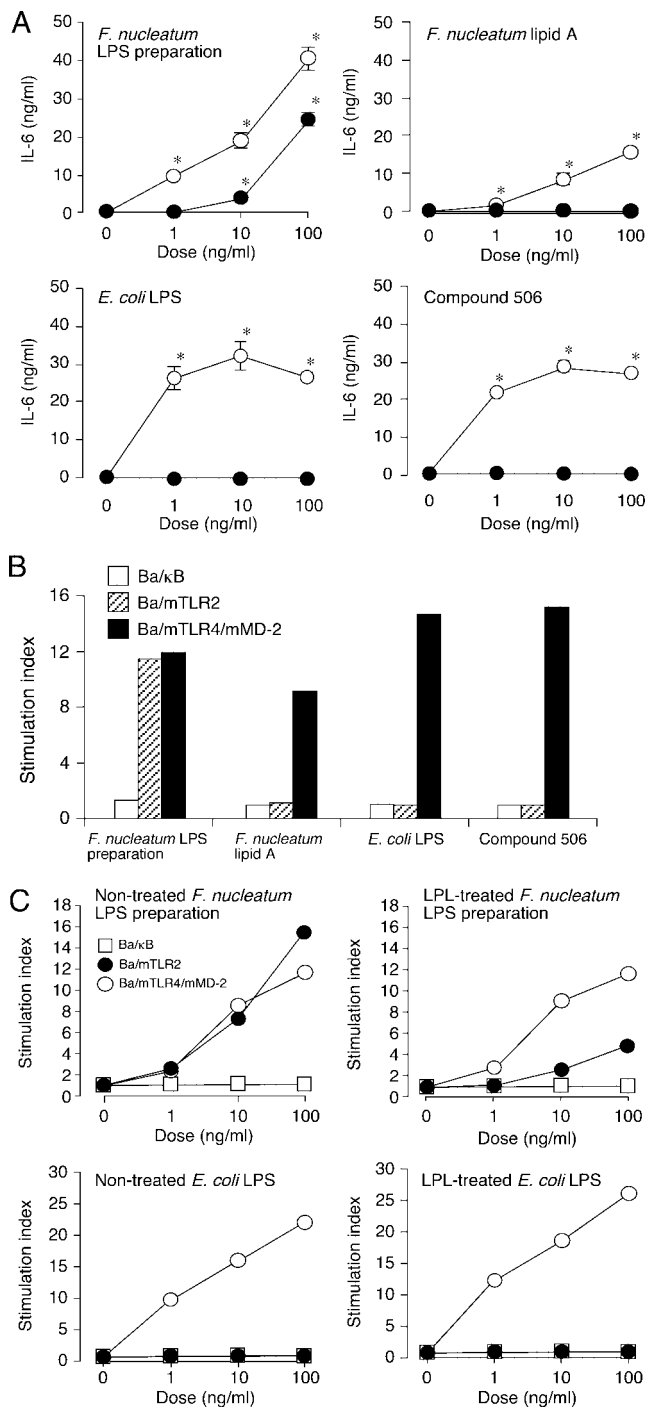


**FIGURE 2.** MS/MS spectra at  $m/z$  1948 of *F. nucleatum* lipid A (A) and two corresponding proposed structural patterns (B and C).

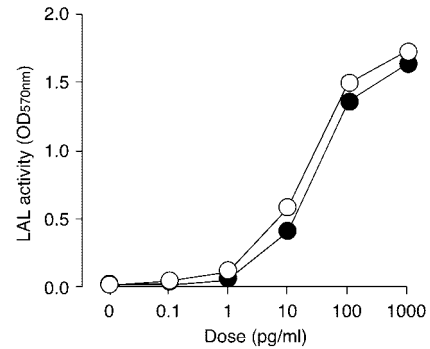
1926) were identified from MS/MS spectra patterns (Fig. 2, B and C), one of which is composed of two 3-hydroxyl hexadecanoate ( $C_{16}$ ) molecules in the nonreducing terminal, and the other of 3-hydroxyl  $C_{16}$  in the 2 position at the reducing terminal and 2' position in the nonreducing terminal of each (Fig. 1B). Furthermore, the chemical structure of the minor peak ( $m/z$  1954) indicated that one of the tetradecanoate ( $C_{14}$ ) molecules in lipid A ( $m/z$  1926) appeared to be substituted for  $C_{16}$  (Fig. 1A). These results indicate that the chemical structure of *F. nucleatum* lipid A is nearly identical with that of compound 506 (Fig. 1C), except for fatty acid length.

#### Recognition of *F. nucleatum* LPS/lipid A by TLR

To evaluate TLR recognition, we assessed IL-6 production by peritoneal exudate macrophages derived from LPS-responsive C3H/HeN and LPS-hyporesponsive C3H/HeJ mice in the presence of 5% FBS (Fig. 3A). The *F. nucleatum* LPS preparation induced IL-6 production by those from both mouse strains. In contrast, the *F. nucleatum* lipid A specimen induced IL-6 production by those from C3H/HeN mice but not from C3H/HeJ mice, similar to *E. coli* LPS and compound 506. Because C3H/HeJ mice possess a dominant-negative point mutation in the cytoplasmic domain of



**FIGURE 3.** TLR4/MD-2-specific responsiveness to *F. nucleatum* lipid A. **A**, Peritoneal macrophages from C3H/HeN (○) and C3H/HeJ (●) mice were stimulated with the indicated doses of test specimens for 24 h in the presence of 5% FBS. After incubation, the supernatants were collected and IL-6 production was determined by ELISA. Experiments were done at least three times, with representative results presented. Each assay was done in triplicate and the data are expressed as the mean  $\pm$  SD. The mean values were significantly different from medium alone. \*,  $p < 0.01$ . **B**, Ba/kB, Ba/mTLR2, and Ba/mTLR4/mMD-2 cells were stimulated with 10 ng/ml of the test specimens for 4 h in the presence of 5% FBS, after which luciferase activities were measured. Results are shown as relative luciferase activity, which was determined as the ratio of stimulated to nonstimulated activity. **C**, Ba/kB, Ba/mTLR2, and Ba/mTLR4/mMD-2 cells were stimulated with the indicated doses of the LPL- or nontreated test specimens for 4 h in the presence of 5% FBS, after which luciferase activities were measured.



**FIGURE 4.** LAL activity of *F. nucleatum* lipid A. The indicated doses of *F. nucleatum* lipid A (●) and compound 506 (○) were mixed with LAL reagent, after which LAL activity was determined using a quantitative chromogenic assay. Representative results from three independent experiments are presented.

TLR4 (34), these results demonstrate that TLR4 signaling is essential for cell activation by *F. nucleatum* lipid A. Next, we examined NF- $\kappa$ B activation in mouse TLR2- and TLR4/MD-2-transfected Ba/F3 cells in the presence of 5% FBS. As shown in Fig. 3B, the *F. nucleatum* LPS preparation induced NF- $\kappa$ B activation in both Ba/mTLR2 and Ba/mTLR4/mMD-2 cells, whereas NF- $\kappa$ B activation by *F. nucleatum* lipid A, as well as by *E. coli* LPS and compound 506, was observed in Ba/mTLR4/mMD-2, but not in Ba/mTLR2 cells. Furthermore, NF- $\kappa$ B activation in Ba/mTLR2 cells stimulated with *F. nucleatum* LPS preparation was abrogated by LPL treatment, whereas TLR4/MD-2-induced NF- $\kappa$ B activation by *F. nucleatum* LPS preparation as well as *E. coli* LPS was not changed regardless of the treatment (Fig. 3C). These results clearly indicate that *F. nucleatum* lipid A induced cell activation through TLR4/MD-2 and TLR2-activating components contained in the *F. nucleatum* LPS preparation appeared to be a lipoprotein.

#### Limulus activity of *F. nucleatum* lipid A

To address the endotoxic activity of *F. nucleatum* lipid A, we measured its LAL activity and compared it with that of compound 506. As shown in Fig. 4, both lipid A specimens showed activity in a concentration-dependent manner that was approximately the same as the LAL activity.

#### Lethal toxicity of *F. nucleatum* lipid A

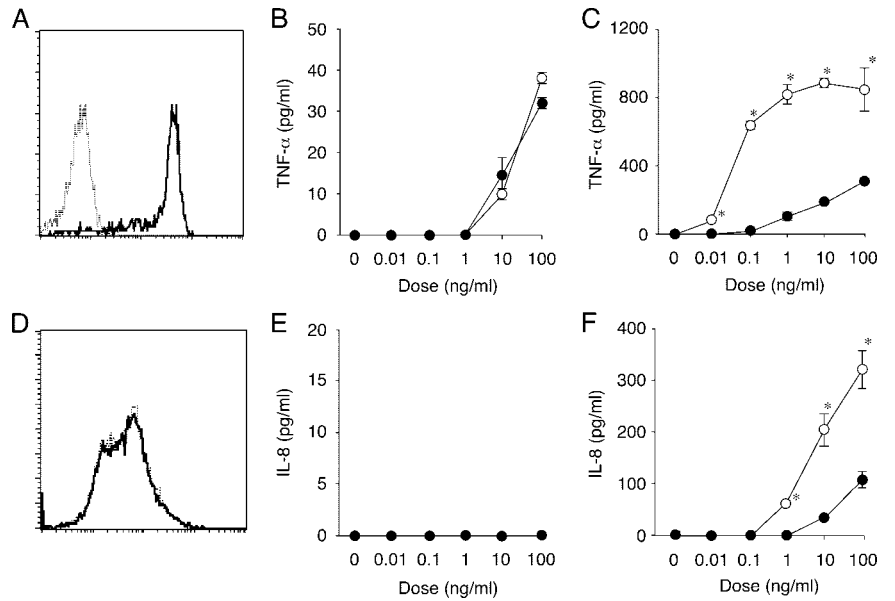
We also examined the lethal toxicity of *F. nucleatum* lipid A and compared it with that of compound 506 against D-GalN-sensitized mice. *F. nucleatum* lipid A exhibited lethal toxicity at a dose of up to 50 ng/mouse (Table I), with an LD<sub>50</sub> of 48.0 ng/mouse. In contrast, compound 506 was markedly toxic in D-GalN-sensitized

Table I. Lethal toxicity of *F. nucleatum* lipid A and compound 506 in D-GalN-sensitized mice

Dose of Lipid A (ng/mouse)	No. of Dead Mice/Total No. of Mice Tested	
	<i>F. nucleatum</i> lipid A	Compound 506
1	0/5	0/5
5	0/5	2/5
10	0/5	5/5
50	3/5	5/5
100	5/5	5/5
LD <sub>50</sub> (ng/mouse) <sup>a</sup>	48.0	5.7

<sup>a</sup> LD<sub>50</sub>, 50% lethal dose, calculated by the method of Kärber (29).

**FIGURE 5.** Contribution of FBS to lipid A-induced cytokine production. Cell surface expressions of mCD14 on human monocytes (A) and HGF (D) were determined with a specific Ab (solid line) or its isotype control (dotted line), as described in *Materials and Methods*. Human monocytes (B and C) and HGF (E and F) were stimulated with the indicated doses of *F. nucleatum* lipid A (●) or compound 506 (○) for 24 h in the absence (B and E) or presence (C and F) of 5% FBS. After incubation, the supernatants were collected, and TNF- $\alpha$  (B and C) and IL-8 (E and F) were determined by ELISA. Each assay was done in triplicate and the data are expressed as the mean  $\pm$  SD. The mean values were significantly different from *F. nucleatum* lipid A at each dose. \*,  $p < 0.01$ .

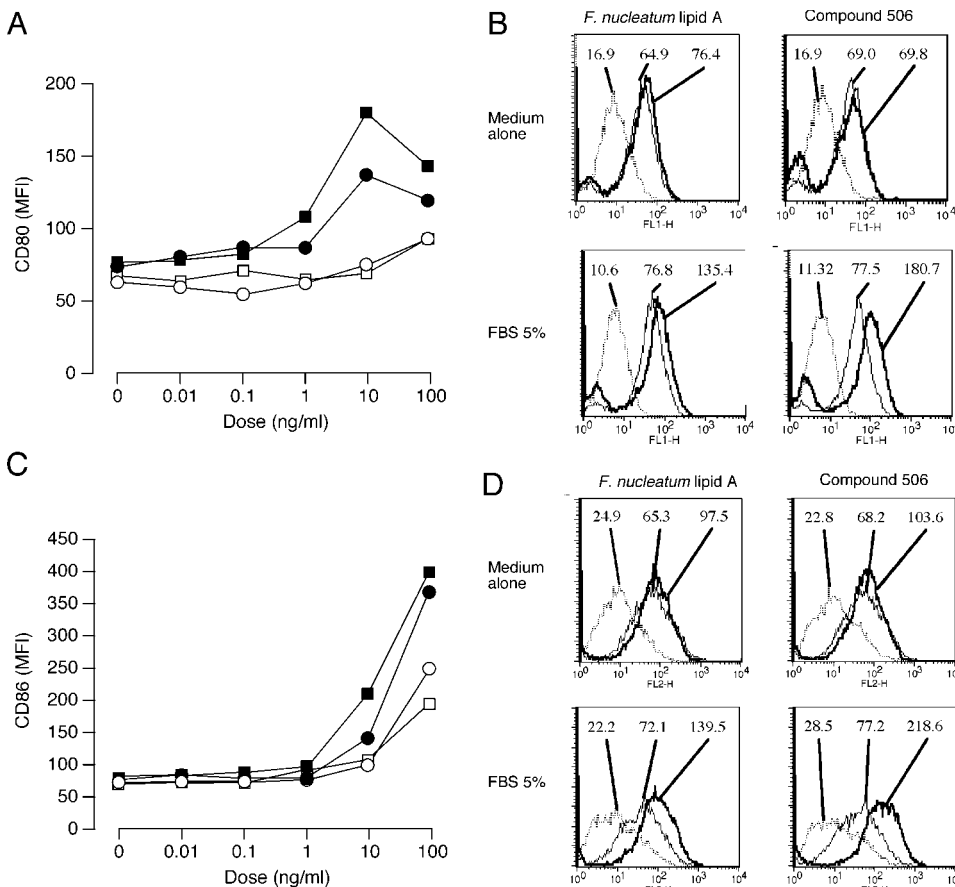


mice, with an LD<sub>50</sub> of 5.7 ng/mouse. In contrast to LAL activity, the lethal toxicity of *F. nucleatum* lipid A was weaker than that of compound 506.

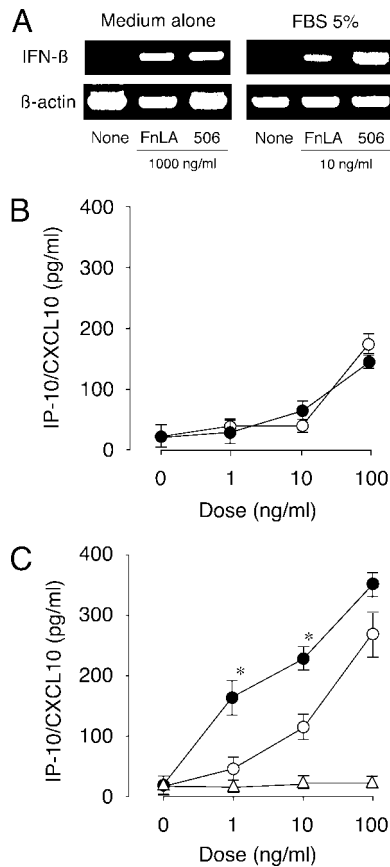
*Effect of serum on cell activation by lipid A*

mCD14 has been demonstrated to sensitize host cells for LPS recognition (35). To examine the effect of serum on lipid A-induced cell activation, we assessed cytokine production by high mCD14-expressing human monocytes (Fig. 5A) and non-

mCD14-expressing HGF (Fig. 5D) after 24 h of stimulation with *F. nucleatum* lipid A and compound 506 in the presence or absence of 5% FBS. In the serum starvation condition, human monocytes showed reductions of sensitivity and activating capacity to *F. nucleatum* lipid A and compound 506, and the lipid A specimens exhibited nearly the same amounts of TNF- $\alpha$  production (Fig. 5B). In the case of HGF, both lipid A specimens showed no IL-8 production up to 100 ng/ml in the absence of FBS (Fig. 5E). In contrast, *F. nucleatum* lipid A as well as



**FIGURE 6.** Contribution of FBS to lipid A-induced up-regulation of costimulatory molecule. Murine peritoneal exudate cells were stimulated with the indicated doses of *F. nucleatum* lipid A (circle) or compound 506 (square) for 24 h in the absence (open symbol) or presence (closed symbol) of 5% FBS. After incubation, the cells were stained with Abs specific for CD80 and CD86, or an isotype-matched control. Values in the histograms represent the MFI of cells stained with each Ab, and the graph is representative of results from three experiments. Representative results for the expressions of CD80 (B) and CD86 (D) on cells stimulated with (bold line) or without (thin line) 10 ng/ml of the test specimens are shown. Dotted lines indicate cells stained with the isotype control Ab. Values indicated in the histograms represent the MFI of cells stained with each Ab.

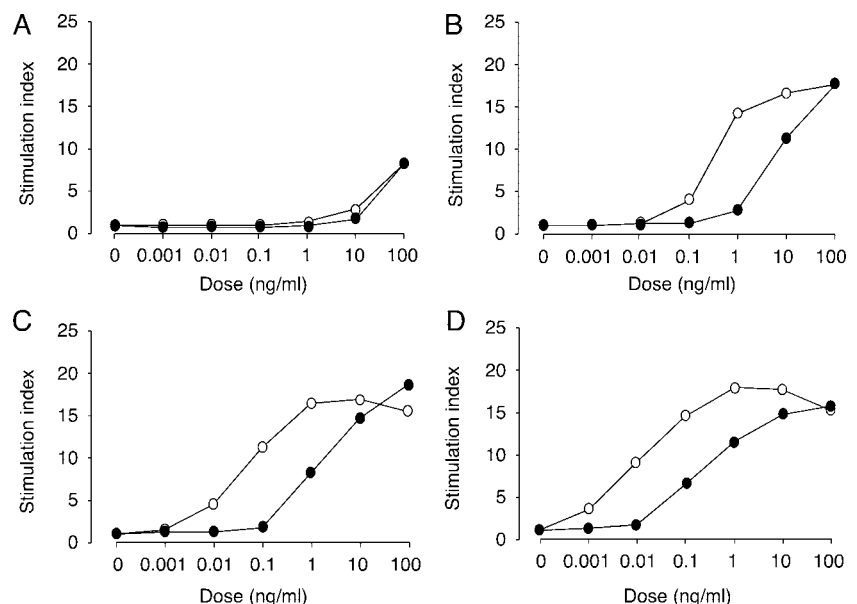


**FIGURE 7.** Contribution of FBS to lipid A-induced TRIF/TICAM-1-dependent cell activation. **A**, IFN- $\beta$  mRNA expression in murine peritoneal macrophages stimulated with the indicated doses of *F. nucleatum* lipid A (FnLA) or compound 506 (506) for 3 h in the absence or presence of 5% FBS. **B** and **C**, Peritoneal macrophages were stimulated with the indicated doses of FnLA (●), compound 506 (○), or Pam<sub>3</sub>CSK<sub>4</sub> (△) for 24 h in the absence (**B**) or presence (**C**) of 5% FBS. After incubation, the supernatants were collected and IP-10/CXCL10 production was determined by ELISA. Experiments were done at least three times, with representative results presented. Each assay was done in triplicate and the data are expressed as the mean  $\pm$  SD. The mean values were significantly different from FnLA at each dose. \*,  $p < 0.01$ .

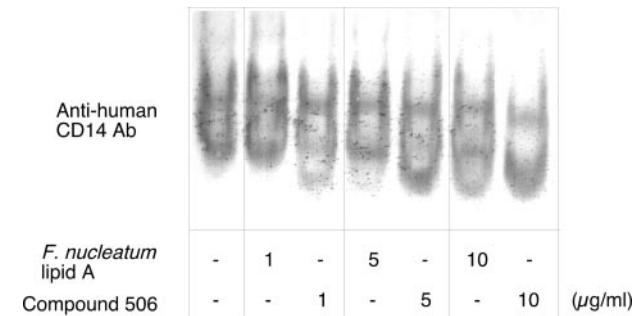
compound 506 induced definite cytokine production by human monocytes and HGF at lower concentrations in the presence of 5% FBS, while compound 506 exhibited much stronger cytokine production than *F. nucleatum* lipid A (Fig. 5, C and F). A possibility of the weak cell activation by *F. nucleatum* lipid A would be caused by its degradation in the experiments. However, it seems to be ruled out because the lipid A exhibited a comparable *Limulus* activity to compound 506 (Fig. 4).

TLR4 signaling is well-known to activate host cells through both MyD88- and Toll/IL-1R domain-containing adaptor inducing IFN- $\beta$  (TRIF)/Toll/IL-1R homology domain-containing adaptor molecule (TICAM)-1-dependent pathways (36, 37). The MyD88 signaling in response to TLR ligands was demonstrated to be important for the production of proinflammatory cytokines (8), whereas the TRIF/TICAM-1 cascade was shown to activate IFN-regulatory factor-3, followed by an induction of IFN- $\beta$  in addition to proinflammatory signals (37, 38). After that, IFN- $\beta$  binds to the type I IFN- $\alpha$ /IFN- $\beta$  receptor, which regulates IP-10/CXCL10 induction (39). The costimulatory molecules CD80 (B7-1) and CD86 (B7-2) are cell surface glycoproteins expressed on a variety of professional APCs, and those molecules play an important role in the stimulation of T cells. In addition, LPS signaling is well-known as an effective enhancer of CD80/CD86 expression in both MyD88- and TRIF/TICAM-1-dependent signaling pathways (40, 41). We examined the effects of FBS on lipid A-induced CD80/CD86 expression on the cell surface of mouse peritoneal macrophages (Fig. 6). In the absence of FBS, *F. nucleatum* lipid A as well as compound 506, induced weak and nearly the same expression of CD80/CD86, whereas *F. nucleatum* lipid A induced weaker CD80/CD86 expression as compared with compound 506 in the presence of 5% FBS. Induction of TRIF/TICAM-1-dependent cytokine, such as IFN- $\beta$  mRNA expression by both lipid A specimens in the presence and absence of 5% FBS also exhibited nearly the same patterns with lipid A-induced proinflammatory cytokines (Fig. 7A). *F. nucleatum* lipid A exhibited a comparable IP-10/CXCL10 production to compound 506 in the absence of FBS (Fig. 7B), whereas it showed weak IP-10/CXCL10 production in comparison with compound 506 in the presence of 5% FBS (Fig. 7C). A control TLR2 agonist Pam<sub>3</sub>CSK<sub>4</sub> induced no IP-10/

**FIGURE 8.** Contribution of sCD14 to lipid A-elicited NF- $\kappa$ B activation via human TLR4. Ba/hTLR4/hMD-2 cells were stimulated with the indicated doses of *F. nucleatum* lipid A (●) or compound 506 (○) for 4 h under the following culture conditions: medium alone (**A**), 1% FBS (**B**), 500 ng/ml CD14 (**C**), and 500 ng/ml CD14 together with 50 ng/ml LBP (**D**). NF- $\kappa$ B activation was measured using a luciferase assay. Results are shown as relative luciferase activity, which was determined as the ratio of stimulated to nonstimulated activity.







**FIGURE 9.** Binding of lipid A to sCD14. The indicated doses of lipid A specimens were incubated with 50 µg/ml sCD14 in the presence of 1.5 µg/ml LBP for 1 h at 37°C. Reactions were run on a 5–15% native PAGE gel. sCD14 was detected by Western blotting using a mouse anti-human CD14 Ab.

CXCL10 production (Fig. 7C). Thus, FBS appears to act effectively on compound 506, but not *F. nucleatum* lipid A in both cascades.

#### Effect of sCD14 on lipid A-induced cell activation

NF-κB activation in the human TLR4/MD-2-expressing cell line Ba/hTLR4/hMD-2 stimulated with *F. nucleatum* lipid A and compound 506 under various conditions was examined. Both lipid A specimens showed nearly the same NF-κB activation in serum-free culture conditions (Fig. 8A). However, compound 506 showed stronger NF-κB activation than *F. nucleatum* lipid A in FBS-supplemented conditions (Fig. 8B). We next assessed the effects of sCD14 on *F. nucleatum* lipid A- or compound 506-induced NF-κB activation of Ba/hTLR4/hMD-2 cells using recombinant human protein. Different levels of NF-κB activation were found between *F. nucleatum* lipid A and compound 506 in the presence of sCD14 or sCD14 together with LBP (Fig. 8, C and D). These results indicate that the difference in susceptibility to sCD14 between these lipid A specimens appears to cause the differences in cell activation.

#### Binding capacity of lipid A to sCD14

To address the binding of lipid A specimens to sCD14 directly, a native PAGE analysis was performed (Fig. 9). Incubation of sCD14 with the indicated doses of *F. nucleatum* lipid A and compound 506 in the presence of LBP caused a mobility shift, indicating that rigid complexes were formed. Compound 506 induced a more significant shift than *F. nucleatum* lipid A.

## Discussion

The structures of lipid As derived from several periodontopathic bacterial LPS have been reported. *Actinobacillus actinomycetem-comitans*, a bacterium associated with localized aggressive periodontitis, has a lipid A structure very similar to that of *E. coli* lipid A (Fig. 1C), except for replacement of dodecanoate (C<sub>12</sub>) with C<sub>14</sub> on the 2'-linked hydroxyl-tetradecanoic acid (42). The chemical structure of *Prevotella intermedia* has also been reported to have a characteristically diglucosamine backbone with a phosphate at the 4-position of the nonreducing side sugar and possesses five fatty acids containing branched long chains (43). Furthermore, *Porphyromonas gingivalis* LPS possesses tri-, tetra-, and penta-acylated heterogenous lipid As, which have a 1-phospho β-(1–6)-linked glucosamine disaccharide backbone (44–46). Thus, each periodontopathic bacterium has been demonstrated to have a characteristic structure and lipid A composition, leading to their distinct biological activities. In the present study, we found that an *F.*

*nucleatum* phenolic-phase LPS preparation possessed microheterogenous hexa-acylated lipid As with a chemical structure similar to that of *E. coli*-type lipid A. Both lipid As were shown to have two acyloxyacyl chains at the nonreducing terminal and two 3-hydroxyl acyl chains at the reducing terminal (Fig. 1, B and C). One of the differences between the *F. nucleatum* lipid A and compound 506 was acyl chain length, i.e., *F. nucleatum* lipid A is composed of C<sub>14</sub> and C<sub>16</sub> (Fig. 1B), as compared with compound 506, which is composed of C<sub>12</sub> and C<sub>14</sub> (Fig. 1C).

*E. coli* LPS elicited cytokine production by peritoneal macrophages derived from C3H/HeN but not C3H/HeJ mice, whereas the *F. nucleatum* phenolic-phase LPS preparation activated cells from both mouse strains (Fig. 3A). In addition, the *F. nucleatum* LPS preparation induced NF-κB activation in both Ba/mTLR2 and Ba/mTLR4/mMD-2 cells (Fig. 3B). It was previously demonstrated that *P. gingivalis* LPS-induced cell activation was not completely abrogated in TLR4-deficient mice (47), while another study indicated that TLR2 was the primary signal-transducing molecule for an LPS preparation from *P. gingivalis* (48). LPS preparations from some Gram-negative organisms, such as *Leptospira interrogans*, *Legionella pneumophila*, and *Helicobacter pylori*, have also been reported to activate cells through TLR2 (49–51). However, in a previous study we have demonstrated that highly purified natural lipid A derived from *P. gingivalis* as well as its synthetic counterpart elicited cell activation via only TLR4/MD-2, though a *P. gingivalis* LPS preparation activated the cells through both TLR2 and TLR4/MD-2 (25). Furthermore, we recently showed that a trace amount of contaminated lipoprotein in a *P. gingivalis* LPS preparation provoked strong cell activation through TLR2 (28). In the present study, a highly purified lipid A from an *F. nucleatum* LPS preparation elicited TLR4/MD-2- but not TLR2-dependent cell activation (Fig. 3A) and LPL treatment reduced *F. nucleatum* LPS preparation-elicited TLR2-dependent cell activation (Fig. 3C), suggesting that LPS preparations derived from *F. nucleatum* and *P. gingivalis* contain other molecules besides LPS, which activate host cells to use TLR2. Thus, to avoid TLR2 signaling, we examined innate immune response using a pure TLR4/MD-2 agonist, *F. nucleatum* lipid A, in the present experiments.

Our results demonstrated that *F. nucleatum* lipid A and compound 506 possess the same LAL activity (Fig. 4), though the concentration of *F. nucleatum* lipid A required for lethal toxicity against D-galactosamine-sensitized mice was ~8.4-fold greater than that of compound 506 (Table I). It was also indicated that *L. pneumophila* LPS-induced cytokine production was ~1000 times weaker than that of *Salmonella enterica* serovar Minnesota LPS, even though they showed the same responsiveness toward *Limulus* activity (33). Therefore, it is considered that *Limulus* reactivity is not necessarily associated with biological potency and other host molecules appear to play an important role in this biologic feature.

In 1990, Wright et al. (35) identified the differentiation Ag CD14 on monocytes bound to LPS, which sensitizes host response to LPS by cooperating with LBP. Many other researchers have also indicated that CD14-deficient mice and their monocytes/macrophages express hyporesponsiveness to LPS challenge (52–54) and the degree of mCD14 expression was reported to correlate with LPS reactivity (55). Furthermore, mCD14 was demonstrated to play an important role in the LPS-induced TRIF/TICAM-1-dependent signaling pathway (56). To determine whether mCD14 can distinguish between *F. nucleatum* lipid A and compound 506, we assessed cytokine production by high mCD14-expressing human monocytes (Fig. 5A) and non-mCD14-expressing HGF (Fig. 5D). Both *F. nucleatum* lipid A and compound 506 showed TNF-α production by human monocytes in the absence of FBS (Fig. 5B), whereas no IL-8 production was observed in HGF (Fig. 5E). In

addition, the lipid A specimens elicited approximately the same TNF- $\alpha$  production (Fig. 5B), indicating that mCD14 alone could not differentiate those lipid A specimens. In contrast, *F. nucleatum* lipid A showed weak cytokine production as compared with that of compound 506 in the presence of 5% FBS regardless of the presence of mCD14 (Fig. 5, C and F). Similarly, the difference in CD80/CD86 expression, IFN- $\beta$  mRNA expression, and IP-10/CXCL10 production of peritoneal macrophages by these lipid A was observed under the serum-containing, but not serum-starved, condition (Figs. 6 and 7). Thus, serum factors, but not mCD14, may contribute to differentiation of the lipid A structure. It was previously reported that LPS preparations derived from various Gram-negative bacteria induced different levels of TNF- $\alpha$  production by mCD14-positive and -negative macrophages in the presence of 1% FBS (57). More recently, TNF- $\alpha$  production in response to wild-type smooth LPS, rough chemotypes Ra-LPS and Re-LPS were demonstrated to show different mCD14 dependency (58). These results suggested that each sCD14 and mCD14 recognized a different part of the LPS structure.

Various serum proteins that bind to LPS have been reported (10, 59–61). Among them, LBP and sCD14 in serum as well as mCD14 are well-known as LPS-sensitizer agents (10). Therefore, we examined the effects of sCD14 on lipid A-induced cell activation. *F. nucleatum* lipid A and compound 506 exhibited the same NF- $\kappa$ B activation in Ba/hTLR4/hMD-2 cells in a serum-depleted condition (Fig. 8A), which was the same as TNF- $\alpha$  production by human monocytes (Fig. 5B). In contrast, the addition of sCD14 together with or without LBP resulted in enhancement of the reactivity to lipid A specimens, though the *F. nucleatum* lipid A was ~100-fold less active than compound 506 (Fig. 8, C and D). LBP alone increased the sensitivity to lipid A, however, there were no differences between the lipid As (data not shown). These results suggest that recognition of the lipid A structure by a CD14 molecule has an effect on host cell response.

Low endotoxic LPS from *H. pylori* and *P. gingivalis* were demonstrated to possess scant transfer activity to sCD14 (62). Furthermore, it has been shown that lipid As derived from *H. pylori* and *P. gingivalis* possess longer chain fatty acids than compound 506, as well as weak cytokine-producing activities (63). In the present experiments, *F. nucleatum* lipid A exhibited a weaker binding activity to sCD14 than compound 506 (Fig. 9), which agrees with their cell-activating capacities, suggesting that the lipid A portion of LPS plays a critical role in binding to sCD14. These results also indicate that the chemical structure of compound 506 has good balance to bind to sCD14, while the binding capacity of the long-chain fatty acids of *F. nucleatum* lipid A may be inferior to those of compound 506.

We propose that sCD14, but not mCD14, is able to differentiate the slight structural differences between lipid As derived from *F. nucleatum* and *E. coli*, which cause distinct host cell activation. It was previously reported that the sCD14 concentration in serum was increased and mCD14 expression on monocytes was decreased in severe acute and systemic diseases, such as sepsis and trauma (64). Thus, it is possible that sCD14, which can distinguish a lipid A structure more sensitively than mCD14, plays an important role in host defense under these conditions. Furthermore, the present findings may provide a basis for understanding the mechanism involved in the interaction between LPS/lipid A and CD14.

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## Disclosures

The authors have no financial conflict of interest.

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