Chemical structure and immunobiological activity of lipid A from *Serratia marcescens* LPS

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The chemical structure and immunobiological activities of *Serratia marcescens* lipid A, an active centre of LPS, were investigated. LPS preparations of *S. marcescens* were extracted using a hot phenol/water method, after which purified lipid A specimens were prepared by weak acid hydrolysis, followed by normal phase and gel filtration chromatographic separation. The lipid A structure was determined by MS to be a diglucosamine backbone with diphosphates and five C14 normal chain acyl groups, including two acyloxyacyl groups at the 2 and 3 positions of the non-reducing side. *S. marcescens* lipid A and *Escherichia coli*-type synthetic lipid A (compound 506) exhibited definite reactivity in *Limulus* amoebocyte lysate assays. The lethal toxicity of *S. marcescens* lipid A was nearly comparable to that of compound 506, and both induced nuclear factor-κB activation in murine cells via Toll-like receptor (TLR)4/MD-2 but not TLR2, as well as various inflammatory cytokines in peritoneal macrophages of C3H/HeN mice but not C3H/HeJ mice. Furthermore, *S. marcescens* lipid A induced nearly the same amounts of tumour necrosis factor alpha, interleukin-6, and nitric oxide production by the murine alveolar macrophage cell line MH-S as compared with compound 506. These results indicate that *S. marcescens* possesses a penta-acylated lipid A, which is nearly identical to *E. coli* lipid A in regard to biological activities, while it also may be a crucial virulence factor of the bacterium.

**INTRODUCTION**

*Serratia marcescens*, a Gram-negative bacillus of the *Enterobacteriaceae* family, is commonly found in soil, water and food (Hejazi & Falkiner, 1997), where it is considered to be a harmless indigenous bacterium, although it is also defined as a human opportunistic pathogen (Kwitko et al., 1977), as it has been recognized to be responsible for hospital-acquired infections over the past three decades (Hejazi & Falkiner, 1997). Furthermore, the bacterium has been demonstrated to possess several pathogenic factors, including adhesins (Reid & Sobel, 1987), cell surface hydrophobicity (Coria-Jimenez et al., 1998), extracellular enzymes (Fuchs et al., 1986) and LPS (Traub et al., 1987).

LPS is located in the outer membrane of Gram-negative bacteria and is a causative agent of lethal endotoxin shock (Raetz, 1990; Mayeux, 1997). It is known to mainly activate monocytes/macrophages to secrete various cytokines, including tumour necrosis factor alpha (TNF-α) and members of the interleukin (IL) family, leading to production of endogenous inflammatory mediators. It was recently demonstrated that lipid A, an active centre of LPS, derived from *Escherichia coli* and related species, induces mammalian cell activation through an innate immune receptor, Toll-like receptor (TLR)4, and its accessory protein, the MD-2 complex (Hoshino et al., 1999; Nagai et al., 2002). Thus, lipid A is a pivotal factor responsible for the pathogenicity of *E. coli*. We considered it important to clarify the chemical structure of *S. marcescens* lipid A for elucidation of the pathogenicity of this bacterium.

The chemical composition of *S. marcescens* lipid A was reported by Adams & Singh (1970); however, information regarding its chemical structure is lacking, and the immunobiological activities have not been demonstrated. In the present study, we elucidated the chemical structure of lipid A from *S. marcescens* and assessed its immunobiological activities.

**METHODS**

**Bacteria and reagents.** A non-pigmented strain of clinically isolated *S. marcescens* AU01 was grown aerobically in Luria–Bertani broth (BD Biosciences) at 37 °C for 48 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 hot phenol/water method (Westphal & Jann, 1965), and lipid A was prepared from the LPS preparations according to our previously described method (Ogawa et al., 2002). Briefly, hydrophobic products obtained by weak acid hydrolysis of the LPS preparation were...
subjected to silica gel and gel filtration column chromatography to yield the purified lipid A fraction. The lipid A specimens were dissolved at a concentration of 1 mg ml\(^{-1}\) in 0.1% (v/v) triethylamine aqueous solution. Compound 506 was chemically synthesized as described by Imoto et al. (1984) and Kotani et al. (1985). The bacterial synthetic lipopeptide Pam3CSK4 was obtained from EMC microcollections and dissolved at a concentration of 1 mg ml\(^{-1}\) in pyrogen-free distilled water. These stock solutions were kept at 4 °C and appropriately diluted with pyrogen-free PBS (Sigma) or cell culture medium before using in the assays.

**Analytical procedures.** Analytical TLC was performed on a TLC plate (no. 5715; Merck) using a solvent system consisting of chloroform/methanol/water (50:25:10, by vol.) and the spots were visualized with anisaldehyde/sulfuric acid reagent.

**MS and tandem MS (MS-MS).** Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS spectra were measured using a QSTAR XL (Applied Biosystems) instrument. Lipid A specimens were dissolved in chloroform/methanol (3:1, v/v) combined with 2,5-di hydroxybenzoic acid as a matrix, and then placed on a sample plate. Spectra were obtained in negative ion reflector modes. MS-MS spectra were obtained using the collision-induced dissociation mode with argon gas. The sample was irradiated randomly throughout the spot for 1.5 min. Raw data were analysed by using computer software provided by the manufacturer. Mass calibration was performed using the peptide [Glu]fibrinopeptide B (mass 1570.6) and computer software provided by the manufacturer.

**Animals.** C57BL/6, C3H/HeN and C3H/HeJ mice (8-week-old males) were obtained from Japan SLC. The mean body weights of C57BL/6, C3H/HeN and C3H/HeJ mice were 23.3 ± 0.9, 25.0 ± 1.1 and 25.1 ± 1.5 g, respectively. The animals received humane care in accordance with our institutional guidelines and the legal requirements of Japan.

**Limulus amoebocyte lysate (LAL) assay.** The indicated 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 quantitative chromogenic assays (Seikagaku Kogyo).

**Lethal toxicity in galactosamine-sensitized mice.** C57BL/6 mice were injected intraperitoneally with 0.5 ml test specimens at the indicated doses in 32 mg D-galactosamine hydrochloride (D-GalN) ml\(^{-1}\) (Wako Pure Chemicals) solution in PBS. Death of the mice was observed over a 1-week period. The 50% lethal dose (LD\(_{50}\)) for each group was calculated using the method of Kärber (1931).

**Luciferase assay.** IL-3-dependent murine Ba/F3 pro-B cells stably expressing p55LgLuc, as well as a nuclear factor (NF)-κB/DNA binding activity-dependent luciferase reporter construct (Ba/Nk), murine TLR2 and a p55LgLuc reporter construct (Ba/mTLR2), and murine TLR4/MD-2 and a p55LgLuc reporter construct (Ba/mTLR4/ mMD-2), were kindly provided by Dr K. Miyake (Institute of Medical Science, University of Tokyo, Japan). The cells were used to detect NF-κB-dependent luciferase activity, as described previously (Makimura et al., 2006).

**Murine cell culture and stimulation assay.** Peritoneal exudate macrophages were obtained from C3H/HeN and C3H/HeJ mice 72 h after intraperitoneal inoculation with 1.0 ml 3% (w/v) sterile Brewer modified thioglycollate medium (BD Biosciences). The cells were centrifuged and suspended in RPMI 1640 (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma), 50 U penicillin ml\(^{-1}\), and 50 μg streptomycin ml\(^{-1}\) (Gibco-BRL) at 1 × 10\(^6\) cells ml\(^{-1}\). These cells were distributed to each well of a 96-well flat-bottomed plate at 2 × 10\(^5\) cells per 200 μl, after which they were incubated at 37 °C for 2 h in a humidified 5% (v/v) CO\(_2\) incubator. Each well was then washed with PBS to remove non-adherent cells, and those attached to the culture plate served as peritoneal macrophages. A murine alveolar macrophage cell line, MH-S (European Collection of Cell Cultures, UK), was cultured in RPMI 1640 supplemented with 10% (v/v) FBS, 100 U penicillin ml\(^{-1}\) and 100 μg streptomycin ml\(^{-1}\). The cells were distributed to each well of a 96-well flat-bottomed plate at 2 × 10\(^5\) cells per 200 μl. After 24 h incubation at 37 °C in a humidified 5% (v/v) CO\(_2\) incubator, each well was washed with PBS. Peritoneal macrophages and MH-S cells were stimulated with various doses of the test specimens at 37 °C for 24 h, and the culture supernatants were collected and stored at −80 °C until the assays were performed. The production of TNF-α, IL-6 and KC was measured in the culture supernatants by means of a commercial ELISA kit system (from Endogen for TNF-α and IL-6 tests and from R&D Systems for KC tests). Each assay was performed according to the manufacturer’s instructions and the data were determined using a standard curve prepared for each one. Nitric oxide synthesis was determined as the stable production of nitrite (NO\(_2\)-) in the culture supernatant using Griess reagent (Weinberg et al., 1994).

**Statistical analysis.** Statistical significance between groups was evaluated by analysis of variance and Tukey multiple-comparison test using Excel 2004 (Microsoft) and STATCEL2 (OMS Publishing) software packages. Differences between groups were considered significant at the level of P<0.01. When an individual result is shown, it is representative of at least three independent experiments.

**RESULTS AND DISCUSSION**

**Chemical structure of S. marcescens lipid A**

Environmental *S. marcescens* is often observed as a red-pigmented bacterium due to the production of prodigiosin (Thomson et al., 2000), whereas bacteria related to hospital infections are reported to be predominantly non-pigmented (Carbonell et al., 2000). Therefore, we extracted lipid A from a clinically isolated *S. marcescens* non-pigmented strain for this study. The molecular mass of lipid A was measured by MALDI-TOF MS in the negative ion mode. Ion peaks were observed as a sodium salt peak at m/z 1621.9 [M1 + Na], and diphosphatase and sodium salt peaks at m/z 1524.0 [M1-Pi + Na] and 1495.9 [M2-Pi + Na], at a relative intensity ratio of 0.4:1:0.1 (Fig. 1a). The results indicated that the lipid A structure corresponded to the GlcN2 backbone with diphosphates at the 1 position on the reducing side and at the 4 position on the non-reducing side, a hydroxyl fatty acid at the 2 position on the reducing side, and 2 acyloxyacyl groups at the 2 and 3 positions on the non-reducing side. Fatty-acid analysis also revealed that the component mainly contained 14:0 (3-OH) and 14:0 normal chains in a molar ratio of 3:2 at m/z 1621.9. The proposed chemical structure of lipid A is shown in Fig. 1(b), which was determined from the MS-MS spectra pattern (Fig. 2a). The chemical structure of the minor peak (M2: m/z 1495.9) indicated that one of the C14 acyl chains in the main peak structure was a substitute for the C12 acyl chain. Lipid A from closely related *Enterobacteriaceae E. coli*
(Takayama et al., 1983) and an opportunistic pathogen Pseudomonas aeruginosa (Ernst et al., 1999) have been reported to possess portions of consensus structures, that is, a GlcN2 backbone attached to diphosphate at the 1 position on the reducing side and at the 4 position on the non-reducing side, and normal chain fatty acids. S. marcescens lipid A is structurally different from compound 506, which is a classical lipid A of representative enterobacteria (Alexander & Zähringer, 2002) in regard to number of acyl chains (Fig. 1b). Several other peaks were indicated; however, the same major peaks were observed between S. marcescens lipid A and compound 506 at m/z 1621.9.

Fig. 1. MS spectrum of S. marcescens lipid A (a) and proposed chemical structures of S. marcescens lipid A at m/z 1621.9 and compound 506 (Imoto et al., 1984; Kotani et al., 1985) (b). M2=M1-(CH$_2$)$_2$.

Fig. 2. MS-MS spectra of S. marcescens lipid A at m/z 1524.0 (a) and compound 506 (b).
908.6 and 454.1, and similar peak patterns were also observed in S. marcescens lipid A (m/z 584.4, 1136.8) and compound 506 (m/z 556.4, 1108.8) (Fig. 2a, b). The differences were of molecular mass 28, which corresponded to 2 carbon atoms (-CH2-CH2-) in the fatty acid. These results suggest that the main formation of S. marcescens lipid A is similar to that of E. coli-type lipid A.

**Endotoxic activities**

To address the endotoxic activity of S. marcescens lipid A, we measured LAL activity and compared it with that of compound 506. The lipid A specimens showed LAL activity in a concentration-dependent manner (Fig. 3), and S. marcescens lipid A showed approximately the same LAL activity as compared to compound 506.

The lethal toxicity of S. marcescens lipid A and compound 506 in d-GalN-sensitized mice was also investigated (Table 1). Both showed severe lethality in d-GalN-sensitized C57BL/6 mice with an LD50 of 19.2 and 8.4 ng, respectively, per mouse. All the dead mice died within 48 h of peritoneal injection with a test specimen and no change in body weight of the dead mice was observed. These results suggest that the length of the acyl chain, as well as the presence of a branched acyl chain and phosphate group, in lipid A are important factors of endotoxic activity. It has been reported that an LPS preparation derived from clinically isolated S. marcescens showed nearly the same lethality in d-GalN-sensitized mice as E. coli O111:B4 LPS (Luchi & Morrison, 2000). Flavobacterium meningosepticum lipid A has a diglucosamine backbone with monophosphate and four branched chain C15 and C17 acyl groups, including an acyloxyacyl group at the 2 position on the non-reducing side and a 3-hydroxy normal chain C16 acyl group at the 3 position on the non-reducing side (Kato et al., 1998). This lipid A exhibited weaker LAL activity and lethal toxicity than Salmonella enterica subsp. enterica serovar Abortus equi LPS (Tanamoto et al., 2001).

**Table 1. Lethal toxicity of S. marcescens lipid A in d-GalN-sensitized C57BL/6 mice**

<table>
<thead>
<tr>
<th>Dose of lipid A (ng per mouse)</th>
<th>No. of dead mice/total no. of mice tested with:</th>
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<tr>
<td></td>
<td>S. marcescens lipid A</td>
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<tr>
<td>1</td>
<td>0/5</td>
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<tr>
<td>5</td>
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<td>5/5</td>
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<td>LD50*</td>
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*LD50 calculated by the method of Kärber (1931).

**Recognition receptor of S. marcescens lipid A**

TLR-mediated cell activation by S. marcescens lipid A was examined using stable transfected Ba/F3 cells expressing murine TLR2 or TLR4/MD-2. NF-κB activation by S. marcescens lipid A, as well as compound 506, was found in Ba/mTLR4/mMD-2 cells, but not in Ba/mTLR2 or Ba/κB cells (Fig. 4a, b), indicating that S. marcescens lipid A stimulates cells through TLR4 but not TLR2. Pam3CSK4, a positive control of the TLR2 agonist, induced NF-κB activation in Ba/mTLR2 cells (Fig. 4c).

Cytokine production by peritoneal exudate macrophages from LPS-responsive C3H/HeN and LPS-hyporesponsive C3H/HeJ mice was also investigated (Fig. 5). Both S. marcescens lipid A and compound 506 induced TNF-α, IL-6 and KC production by peritoneal macrophages from C3H/HeN mice in a dose-dependent manner, while the production of these cytokines by those cells from C3H/HeJ mice was completely abolished (Fig. 5a–f). However, Pam3CSK4 exhibited such cytokine production by both C3H/HeN and C3H/HeJ macrophages (Fig. 5g–i). Since C3H/HeJ mice have a dominant negative point mutation in the cytoplasmic domain of TLR4 (Poltorak et al., 1998), these results indicate that cell activation by S. marcescens lipid A is mediated through TLR4/MD-2, similar to compound 506.

**Activation of alveolar macrophages**

S. marcescens is well-known as an important opportunistic pathogen capable of causing pneumonia in humans (Sanders et al., 1970; Carlon et al., 1977). Alveolar macrophages, the resident mononuclear phagocytes in the lung, play a pivotal role in protecting lungs against invading microbial pathogens and particle dust (Maus et al., 1998). S. marcescens cells have been demonstrated to elicit acute lung inflammation due to their strong capability to induce proinflammatory cytokines, such as TNF-α and IL-6 (Nathan, 1987), and to release nitric oxide (Pendino et al., 1993). To clarify the contribution of S.
Fig. 4. TLR-dependent NF-κB activation of *S. marcescens* lipid A. Ba/κB (□), Ba/mTLR2 (○) and Ba/mTLR4/mMD-2 (●) cells were stimulated with the indicated doses of *S. marcescens* lipid A (a), compound 506 (b) or Pam3CSK4 (c) for 4 h. NF-κB activation was determined using a luciferase assay. Results are shown as relative luciferase activity, which was determined as the ratio of stimulated to non-stimulated activity.

Fig. 5. Cytokine production by peritoneal exudate macrophages from C3H/HeN (●) and C3H/HeJ (○) mice. Cells were cultured at 37 °C for 24 h in RPMI 1640 medium containing 10% (v/v) FBS, with or without the indicated doses of *S. marcescens* lipid A (a–c), compound 506 (d–f) or Pam3CSK4 (g–i). After incubation, the supernatants were collected and cytokine production was measured as described in Methods. Each assay was done in triplicate and the data expressed as the mean ± SD of those results. Differences between groups with and without the test specimens were considered significant (*) when *P* < 0.01.
Serratia marcescens lipid A to pulmonary inflammation, induction of inflammatory mediators by the murine alveolar macrophage cell line MH-S was examined. As shown in Fig. 6, S. marcescens lipid A specimens induced TNF-α, IL-6 and nitric oxide production by MH-S cells in a dose-dependent manner. Further, the lipid A caused nearly the same amount of induction of those mediators as compound 506, while both showed similar LAL activities (Fig. 3). These results suggest that S. marcescens lipid A is one of the essential factors of pneumonia caused by S. marcescens infection. It was previously demonstrated that maximal cytokine production from human peripheral blood mononuclear cells was related to bisphosphorylated and hexa-acylated lipid A, such as compound 506, while hepta-, octa-, penta-, tetra-, and bi-acyl lipid A are less active (Flad et al., 1993). The present findings indicate that the constitution of S. marcescens lipid A at the non-reducing side of GlcN is common to that of compound 506. Therefore, the fatty acid component at the non-reducing side of GlcN may be important for lipid A-induced cell-activating capacity.

Taken together, our results show that the chemical structure of S. marcescens lipid A is a diglucosamine backbone with phosphates and five acyl groups, including two acyloxyacyl groups at the 2 and 3 positions of the non-reducing side. The lipid A was also found to have a similar fatty acid length (C14 in the main) as compared to compound 506. Furthermore, S. marcescens lipid A was shown to be a TLR4/MD-2 agonist, and demonstrated highly endotoxic and immunobiological activities comparable to compound 506. These findings indicate that S. marcescens is likely a major virulence factor of opportunistic infectious diseases.

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REFERENCES


