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

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INTRODUCTION

Serratia marcescens, a Gram-negative bacterium 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 Pam\(3\)CSK\(4\) was obtained from EMC microcollisions 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-dihydroxybenzoic 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 laser replicate rate was 20 Hz. Ions were accelerated through 20 kV. 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 \(\alpha\)-galactosamine hydrochloride (\(\alpha\)-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 p55Ig\(k\) chain. Lipid A from closely related \(S.\) 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 1524.0 \([M1 + Na]\) and diphosphate 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 C\(_{14}\) acyl chains in the main peak structure was a substitute for the C\(_{13}\) acyl chain. Lipid A from closely related \(Enterobacteriaceae\) \(E.\) coli

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(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 and compound 506 (Imoto et al., 1984; Kotani et al., 1985) (b). M2=M1–(CH2)2.

**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–(CH2)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 LD$_{50}$ 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).

![Fig. 3. LAL activity of S. marcescens lipid A. The indicated doses of S. marcescens lipid A (●) or compound 506 (○) were mixed with LAL reagent, and LAL activity was determined using a quantitative chromogenic assay.](http://jmm.sgmjournals.org)

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

<table>
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<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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<td>5</td>
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<td>LD$_{50}$*</td>
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*LD$_{50}$ 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-, penta-, tetra-, and bi-acylated lipid As 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 diphosphates and five acyl groups, including two aclyoxyacetyl 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.

**ACKNOWLEDGEMENTS**

This work was supported by Grants-in-Aid for Scientific Research (B) (no. 17791317) from the Japan Society of the Promotion of Science and Miyata Research Foundation (A) of Asahi University (nos 05025 and 06036). We thank Ms Chieko Kanamori for her technical assistance, and Mr Mark Benton for his critical reading of the manuscript.

**REFERENCES**


