Electron Microscopic Studies of Endotoxins Treated with Alkaline and Acid Reagents

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Abstract  Endotoxins extracted from Salmonella minnesota wild strain and R mutants (Ra to Re) were treated with two different alkaline reagents. Treatment with diluted sodium hydroxide, which caused partial removal of O-ester linked fatty acids, changed the ultrastructures of endotoxins from an onion-like structure to monolayer particles (approximately 100 Å in diameter) except for endotoxic glycolipids from Rd2 and Re mutants which showed mixed ultrastructures of untreated and treated endotoxins. Treatment with alkaline hydroxylamine, which caused liberation of all O-ester linked fatty acids, changed the ultrastructures of all endotoxins to monolayer particles. The results suggested that the ultrastructures of alkaline-treated endotoxins were dependent on the degree of their hydrophobicity. On the other hand, the micrographs of acid treated endotoxins did not show a constant structure because of the high hydrophobicity.

Chemical modification of endotoxic lipopolysaccharide (LPS) has been used in several laboratories in attempts to dissociate its biological properties, especially the toxicity and other biological activities (20). One of the first modifications used was alkaline hydrolysis (20), which greatly reduced in vivo lethality (4, 5, 13, 14, 24), pyrogenicity (14, 23), and antitumor activity (4, 5, 23). Alkaline hydrolysis also cleaves the O-ester-linked fatty acids from the lipid A moiety (16), and reduces its particle size as determined by molecular weight and sedimentation velocity (12, 14, 24).

On the other hand, mild acid hydrolysis of LPS cleaves the linkage between 3-deoxy-D-manno-octulosonic acid (KDO) and the lipid A moiety. The lipid A moiety is generally accepted to be responsible for most of the biological properties of LPS. However, recent studies of Ribi and collaborators (4, 5, 15, 21) indicated that the toxicity of the endotoxin could be separated from the tumor regression activity using hydrochloric acid hydrolysis.

Electron microscopic studies of LPS treated with alkali and acid have been reported from several laboratories. Shands (19), and Goodman and Sultzer (9) showed the ultrastructures of alkali-treated LPS from Salmonella typhimurium and S. typhosa wild strain, respectively, which were found to split the bilayer structure of LPS into apparent monolayers. The electron micrographs of acid-treated LPS
(lipid A) from *S. typhimurium* G-30 and *S. minnesota* Re mutant reported by Rothfield and Horne (18) and Hamada (10), respectively, showed a cobweb appearance or a large monolayer disk.

In the present study, we examined the ultrastructures of endotoxins from the wild strain and R mutants of *S. minnesota* treated with different alkaline and acid reagents for the purpose of comparison of the aggregation forms before and after treatment and among different strains.

**MATERIALS AND METHODS**

*Microorganisms and extraction of endotoxins.* *S. minnesota* 1114 (wild strain), R60 (Ra mutant), R345 (Rb), R5 (Re), R7 (Rd1), R4 (Rd2), and R595 (Re) were kindly provided by O. Luderitz. The microorganisms were cultivated as described by Gmeiner et al (8). The endotoxins were extracted with phenol-water (PW) according to the method of Westphal et al (25), and phenol-chloroform-petroleum ether (PCP) according to the method of Galanos et al (7). The extracts (crude endotoxins) were purified by ultracentrifugation (100,000 ×g for 2 h at 4 C).

*Sodium hydroxide treatment of endotoxins.* The endotoxins were suspended for 30 min in 0.1 n NaOH in 99% ethanol at 37 C according to the method of Tanamoto et al (22). The mixtures were neutralized with 0.1 n HCl, dialyzed against distilled water and lyophilized. The residues were dissolved in distilled water, and free fatty acids were removed by extraction with chloroform (three times).

*Hydroxylaminolysis of endotoxins.* Almost all ester-linked fatty acids were released from the endotoxins by hydroxylaminolysis (2% NH₂OH in 4% NaOH in 99% ethanol at 65 C for 5 min) as described by Haskins (11). The free fatty acids were removed by three chloroform extractions after acidification and dialysis.

*Mild acid treatment of endotoxins.* The endotoxins were dissolved in 1% acetic acid and 0.1 n HCl and boiled for 2 h and 15 min, respectively. After cooling in an ice bath, the reaction mixtures were centrifuged at 2,000 ×g for 10 min. The precipitates were washed in distilled water three times and then lyophilized.

*Electron microscopy.* Uranyl formate (TAAB Laboratories, England) staining for electron microscopy was carried out as described by Fukushi et al (6). Uranyl acetate (TAAB Laboratories) staining was performed as described previously (2, 3). All specimens were examined with a Hitachi electron microscope type HU-12As operating with an accelerating potential of 100 kV.

**RESULTS**

**Ultrastructure of Endotoxins Treated with Mild Alkali**

Figure 1 shows the micrographs of endotoxins treated with 0.1 n NaOH at 37 C for 30 min. The ultrastructures of alkali-treated endotoxic glycolipid from Rb mutant (RbGl-NaOH) stained with uranyl formate showed small circular forms, with a diameter between 85–105 Å, which seems to be a monolayer (Fig. 1a). When stained with uranyl acetate, RbGl-NaOH showed spherical structures approximately
Fig. 1. Electron micrographs of NaOH-treated endotoxins from *Salmonella minnesota* R mutants. a and b, RbGl-NaOH; c, RcGl-NaOH; d, Rd1Gl-NaOH; e, Rd2Gl-NaOH. a was stained with uranyl formate and b to e were stained with uranyl acetate.
100 Å in diameter (Fig. 1b). Both findings were obtained from NaOH-treated endotoxins (LPS from the wild strain and RGL from R mutants (Ra, Rc, and Rdl)) (Fig. 1, c and d). They could be interpreted as aggregates of monolayers similar to micelles. However, the micrographs of Rd2Gl-NaOH and ReGl-NaOH showed different structures from those of other NaOH-treated endotoxins. Both products showed mixtures of spherical, stick, and amorphous structures, in addition to onion-
like structures of untreated glycolipids from R mutants extracted with PW (Fig. 1e, 2a, and 2b) (1–3, 6). The micrographs of ReGl-\textit{NaOH} especially showed a bilayer structure (Fig. 2, a and b) as described previously (1–3, 6). These data indicated that the endotoxic glycolipids from deep rough mutants (Rd2 and Re) are still hydrophobic after release of about one-third of the fatty acids from the glycolipids with mild alkali-treatment used above (4, 5). On the other hand, the endotoxins from the wild strain and the other rough mutants (Ra to Rd1) became more hydrophilic with mild alkali-treatment. These ultrastructures of NaOH-treated endotoxins were independent of the method used for extraction (PW and PCP methods).

**Ultrastructure of Endotoxins Treated with Hydroxylamine**

The endotoxins were hydrolyzed with alkaline NH$_2$OH to remove almost all O-ester linked fatty acids from the endotoxins (4, 5), and examined with an electron microscope (Fig. 3). The micrograph of RbGl treated with NH$_2$OH (RbGl-NH$_2$OH) stained with uranyl acetate showed small spherical forms which were similar to those of RbGl-NaOH, but the structures were indistinct (Fig. 3a). Similar findings were obtained from other endotoxins (wild strain and Ra to Rd1 mutants). On the other hand, the micrograph of ReGl-NH$_2$OH indicated small spheres which seemed to adhere one another (Fig. 3b).

**Ultrastructure of Endotoxins Treated with Mild Acid**

We treated ReGl with mild HCl or acetic acid to isolate lipid A. Because such lipid A preparations were insoluble in water, these preparations were dissolved in 1% triethylamine solution for examination by electron microscopy. The micrographs of lipid A (ReGl-HCl) stained with uranyl acetate showed partial multilayers among the amorphous structure (data not shown). After dialysis, lipid A had various forms (\textit{i.e.}, small particles, disks, doughnuts, and sticks) (Fig. 4). Similar findings were obtained from the other endotoxins treated with mild HCl or acetic acid (data not shown).
DISCUSSION

By alkali-treatment, many biological activities of the endotoxin from gram-negative bacteria were changed because of the removal of O-ester linked fatty acids and less hydrophobicity other than the original material. Moreover, Niwa et al (14) and Tripodi et al (24) reported that the molecular weight of endotoxin was reduced when treated with alkali. These results suggest changes in aggregating form of the endotoxin. Shands (19) and Goodman et al (9) reported that the ultrastructure of endotoxic lipopolysaccharide from *Salmonella* wild strain was changed to small monolayers by alkali-treatment.

In this study, we compared the ultrastructures of endotoxins from *S. minnesota* wild type and R mutants (Ra to Re) treated with different alkaline reagents. The endotoxins treated with mild alkali (0.1 M NaOH) showed particles of monolayer approximately 100 Å in diameter except for Rd2Gl and ReGl. Tanamoto et al (22) and Amano et al (4, 5) revealed that this alkali-treatment led to removal of about one-third of the fatty acids from the endotoxins. We assume that the monolayer particle of alkali-treated endotoxins consists of a hydrophobic portion (lipid A moiety) which faced the center of the particle and a hydrophilic portion (polysaccharide moiety) which faced the outside of the particle. This particle seems to be a 'spherical micelle' with a monolayer, but not a 'liposome' with a bilayer. On the other hand, Rd2Gl and ReGl formed stick and onion-like structures, besides monolayer particles, when treated with the mild alkali. This result indicated that both treated glycolipids still had some hydrophobicity. However, when treated with 2% NH₂OH, ReGl formed small particles (approximately 100 Å in diameter) which appeared to be similar to the monolayer particles of the other NaOH-treated endotoxins. It is well known that the hydroxylaminolysis of endotoxin led to complete de-O-acylation (4, 5, 11, 16, 22).

Although acid treated endotoxin is usually called lipid A, Rosner et al (17) revealed that the lipid A obtained from ReGl by sodium acetate (pH 4.5) lost only the KDO moiety and lipid A obtained from ReGl by 0.1 M HCl treatment lost the KDO moiety and acid-labile glycosidic phosphate. Amano et al (4, 5, 15) noted that ReGl treated with sodium acetate retained toxicity and antitumor activity, and ReGl treated with mild HCl was rendered non-toxic but retained antitumor activity. In this study, we observed with an electron microscope lipid A (0.1 M HCl) which was insoluble in water and was dissolved in 1% triethylamine. The micrographs of lipid A showed several forms.

These results suggested that the ultrastructures of chemically treated endotoxins were dependent on solubility in water. Thus, we assume that the monolayer particle formation in endotoxin treated with alkali results from reduction of the steric hindrance by fatty acids for aggregation, in addition to the reduction of the hydrophobicity.

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