Ultrastructure and Chemical Composition of Lipopolysaccharide Extracted from *Leptospira interrogans* serovar *copenhageni*

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(Received 3 June 1985; revised 20 August 1985)

Lipopolysaccharide (LPS) from *Leptospira interrogans* serovar *copenhageni* was prepared from the aqueous phase of a phenol/water extract. Electron microscopic examination of negatively stained LPS showed a mixture of ribbon-like, round and ring structures. Carbohydrate analysis of the preparations revealed pentoses, hexoses, heptoses, hexosamines, and a 2-keto-3-deoxyonic acid which was chromatographically different from authentic 2-keto-3-deoxyoctonic acid (KDO). The major fatty acids of the LPS were hydroxylauric, palmitic and oleic acids. Although the leptospiral LPS preparations did not contain KDO or hydroxymyristic acid, they were otherwise morphologically and chemically similar to the LPS of other Gram-negative bacteria.

INTRODUCTION

The outer envelopes and cell walls of spirochaetes, including members of the genus *Leptospira*, are morphologically similar to those of other Gram-negative bacteria (Pillot & Ryter, 1965; Jackson & Zey, 1973; Azuma *et al.*, 1975). Jackson & Zey (1973) found that the ultrastructure of a lipopolysaccharide (LPS) extracted from a non-pathogenic treponeme described as a strain of *Treponema pallidum* resembled that of LPS from *Salmonella typhimurium* and *Escherichia coli*. However, endotoxic activity characteristic of Gram-negative bacterial endotoxin could not be demonstrated in studies of the biological properties of preparations of LPS extracted from *Leptospira interrogans* serovar *canicola* (Finco & Low, 1967; Johnson, 1976) or serovars *copenhageni* and *pomona* and from *Leptospira biflexa* serovar *patoc* (Faine *et al.*, 1974). However, no detailed studies of the morphology and chemical composition of leptospiral LPS have been reported.

In this paper, we describe the ultrastructure and the chemical composition of an LPS extracted from the aqueous phase of a phenol/water extract (Westphal & Jann, 1965) prepared from a virulent strain, *Leptospira interrogans* serovar *copenhageni*.

METHODS

Organisms. A strain of *Leptospira interrogans* serovar *copenhageni* (strain L45), virulent for guinea pigs (Faine & van der Hoeden, 1964), was used, grown and maintained in a virulent form as described by Vinh *et al.* (1984).

Preparation and chemical treatment of leptospiral LPS. LPS was extracted from lyophilized leptospires (serovar *copenhageni*) by the method of Westphal & Jann (1965). The lyophilized crude extract was dissolved in water and centrifuged at 105 000 *g* for 3 h to remove nucleic acids. The sediment was taken up in 1 ml of double glass-distilled water and lyophilized. Nucleic acids were not detected by UV absorption spectroscopy at 260 nm. The LPS obtained accounted for 3–5% of total dry weight. Alkali-treated LPS was prepared by treating LPS with 0.025 M-NaOH solution at a concentration of 5 mg ml⁻¹ at 37 °C for 60 min.

Detergent-treated LPS was prepared by dissolving LPS in a 0.1% (w/v) solution of SDS (BDH) at a concentration of 5 mg ml⁻¹.

Abbreviations: KDO, 2-keto-3-deoxyoctonic acid; LPS, lipopolysaccharide.

Electron microscopy. A drop of a solution of LPS which had been lyophilized or treated with alkali or SDS and dissolved in phosphate-buffered saline (pH 7.2) at a concentration of 5 mg ml⁻¹ was deposited on a carbon-coated 200-mesh copper grid. Excess fluid was removed with filter paper and the grid was air-dried, and negatively stained with 2% (w/v) sodium phosphotungstate for 30 s. Excess staining solution was removed as above and the grid again air-dried and examined using a Philips EM 300 electron microscope at 60 kV.

UV spectrum. The UV absorption spectrum of LPS solutions (100 µg ml⁻¹) was recorded using a Perkin-Elmer VIS/UV spectrophotometer model 552.

Chemical analyses. Protein was measured by the micromethod of Bradford (1976) using Bio-Rad protein assay reagents. Total carbohydrate content was measured by the phenol/sulphuric acid method (Dubois *et al.*, 1956) using glucose as standard. Hexoses, pentoses, heptoses and hexosamines were determined according to methods described by Nowotny (1979). To determine 2-keto-3-deoxyoctonic acid (KDO), 1 mg LPS was dissolved in 0.0125 M-H₂SO₄ and boiled for 20 min. The thiobarbituric acid reaction of Weissbach & Hurwitz described by Nowotny (1979) was used to measure the keto-deoxyoctonic acid in the hydrolysate using commercial KDO (Sigma) as standard. KDO was identified by ascending paper chromatography using Whatman no. 1 filter paper with ethyl acetate/pyridine/water (2:1:2, by vol.) as solvent, and the thiobarbiturate/periodate reaction described by Nowotny (1979) was used to detect spots.

Lipid, extracted by the chloroform/methanol method of Folch *et al.* (1957), was dried in a rotary evaporator under vacuum followed by a stream of dry nitrogen. Phosphorus was determined according to Chen *et al.* (1956).

GLC analysis of neutral sugars. Samples of LPS were dissolved in 0.5 M-H₂SO₄ at a concentration of 5 mg ml⁻¹ and heated at 100 °C for 4 h. The hydrolysate was neutralized by drying over sodium hydroxide pellets in a vacuum desiccator. Trimethylsilyl derivatives were prepared by adding to the dry sample 100 µl of a solution of TRISIL (Pierce Chemicals). The mixture was kept at room temperature for 30 min and then samples of 1 µl were injected into a Varian 3700 gas-liquid chromatograph equipped with a stainless steel 2.4 m × 0.6 cm column packed with 5% SE 30 on Chromosorb W-HP 80/100 mesh. Nitrogen carrier gas at a flow rate of 30 ml min⁻¹ and a flame ionization detector were used. The temperature programme started at 160 °C for 1 min, increasing to a maximum temperature of 230 °C at a rate of 2 °C min⁻¹. The injector and detector temperatures were 200 °C and 250 °C respectively. The sugars were identified by comparison with known standard sugars obtained from Mann Research Laboratory, New York, USA.

GLC analysis of fatty acids. Fatty acids were methylated as follows. The dried chloroform extract was treated with 5 ml of a solution of 5% (w/v) NaOH in 50% (w/v) methanol in water for 30 min at 100 °C, cooled to room temperature and 6 M-HCl was added to bring the mixture to pH 2.0, followed by 5 ml 10% (w/v) borotrichloride/methanol reagent (Applied Science Laboratories). The mixture was heated for 5 min at 80 °C to facilitate methylation. The methylated fatty acids were extracted with light petroleum (b.p. 40–60 °C, BDH) and concentrated to a volume of 50 µl by using a stream of dry nitrogen. A 3 µl sample was injected into a Packard 427 gas chromatograph containing a 1.8 m × 0.6 cm glass column packed with 3% Supelco GP SP2100 on 100/120 mesh Supelcoport. The temperature was programmed from 130 °C to 215 °C at a rate of 5 °C min⁻¹. The fatty acid profile was identified by comparison with known standard fatty acids.

RESULTS

Ultrastructure of leptospiral LPS

Negatively stained preparations of leptospiral LPS (extracted from *L. interrogans* serovar *copenhageni*) showed ribbon-like forms, some of trilaminar appearance, 10–12 nm wide, spread in sheets of closely-packed straight, looped, circular and round shapes (Figs 1 and 2).

After alkali treatment the LPS was transformed into a relatively homogeneous suspension of particles 2–5 nm in diameter (Fig. 3). Treatment of LPS with SDS induced the formation of short swollen strands and irregularly shaped strands, amongst which a few trilamellar straight fragments were seen (Fig. 4). The UV absorption maximum of leptospiral LPS was located sharply at approximately 192 nm (*A* 0.60) with a small shoulder at 235 nm (*A* 0.08). The absorbance above 250 nm was less than 0.04.

Chemical analysis of leptospiral LPS

Leptospiral LPS accounted for 3–5% of the total cellular dry weight. The general chemical composition of the leptospiral LPS was 5.4% protein, 33.3% lipid, 32% pentoses, 12% hexoses, 4.5% heptoses, 1% amino sugars, 0.45% 2-keto-3-deoxyonic acid and 0.4% phosphorus. When the 2-keto-3-deoxyonic acid detected by the thiobarbituric acid reaction was subjected to paper chromatography using authentic KDO as standard, its *R_F* value of 0.28 was different from the *R_F*

Fig. 1. Electron micrograph of leptospiral LPS negatively stained with 2% (w/v) phosphotungstic acid. Bar, 0.5 μ m.

Fig. 2. Electron micrograph of leptospiral LPS negatively stained with 2% (w/v) phosphotungstic acid showing trilaminar structure. Bar, 0.2 μ m.

value of 0.47 found for a sample of authentic KDO. The identity of this compound has not been established.

The results of GLC of trimethylsilyl derivatives of the sugars are shown in Table 1. The main group of sugars, the pentoses, comprised 83.9% of the total carbohydrates, while hexoses were 8.9% and amino hexoses were 7.5%.

Treatment of LPS with 0.2 M-H₂SO₄ resulted in the liberation of lipid A. The fatty acid composition of lipid A from leptospiral LPS is shown in Table 2. 3-Hydroxyauric acid (3-OH-C_{12:0}), palmitic acid (C_{16:0}) and oleic acid (C_{18:1}) were the main fatty acids of leptospiral LPS. Myristic (C_{14:0}) and 3-hydroxymyristic (3-OH-C_{14:0}) acids were not found.

Fig. 3. Electron micrograph of alkali-treated leptospiral LPS negatively stained with 2% (w/v) phosphotungstic acid. Bar, 0.1 μ m.

Fig. 4. Electron micrograph of SDS-treated leptospiral LPS negatively stained with 2% (w/v) phosphotungstic acid. Bar, 0.2 μ m

DISCUSSION

Electron microscopic examination of negatively stained LPS extracted from *L. interrogans* serovar *copenhageni* showed ribbon-like structures similar to that reported from LPS from *Escherichia coli* (Lopes & Inniss, 1970), *Salmonella typhimurium* (Shands *et al.*, 1967) and *Treponema pallidum* (Jackson & Zey, 1973). Nothing is known of 'smooth' and 'rough' equivalents in *Leptospira*, but it appears that the leptospiral LPS examined may be in a smooth form because its ultrastructure shows two dense lines, running parallel to the length of the ribbons, similar to the structure shown by Lopes & Inniss (1970) to represent the O-specific side chains which were not present in the rough form LPS of *E. coli*. The strain of leptospires used was maintained in a virulent state by frequent passage through guinea pigs.

Table 1. GLC analysis of trimethylsilyl derivatives of neutral sugars of LPS extracted from *L. interrogans* serovar *copenhageni* (strain L45)

Results are the means of two determinations $\pm$ the range and are expressed as a percentage of the total carbohydrate fraction.

Sugar	Percentage composition
Rhamnose	45.6 $\pm$ 1.0
Arabinose	13.5 $\pm$ 0.9
Fucose	9.1 $\pm$ 0.7
Xylose	24.9 $\pm$ 0.3
Mannose	3.4 $\pm$ 0.1
Galactose	2.3 $\pm$ 0.2
Glucose	5.0 $\pm$ 0.5
Galactosamine	2.3 $\pm$ 0.2
Glucosamine	7.5 $\pm$ 1.0

Table 2. GLC analysis of fatty acid composition of LPS extracted from *L. interrogans* serovar *copenhageni* (strain L45)

Results are the means of two determinations $\pm$ the range and are expressed as a percentage of the total lipid fraction.

Fatty acid	Percentage composition
Hydroxydecanoic (3-OH-C _{10:0})	7.1 $\pm$ 0.4
Lauric (C _{12:0})	2.5 $\pm$ 0.1
Hydroxylauric (3-OH-C _{12:0})	30.5 $\pm$ 0.9
Hydroxypentadecanoic (OH-C _{15:0})	4.6 $\pm$ 0.1
Palmitoleic (C _{16:1})	2.5 $\pm$ 0.2
Palmitic (C _{16:0})	17.0 $\pm$ 1.0
Hydroxypalmitic (2-OH-C _{16:0})	1.4 $\pm$ 0.1
Hydroxyheptadecanoic (OH-C _{17:0})	1.6 $\pm$ 0.2
Oleic (C _{18:1})	10.7 $\pm$ 0.1
Stearic (C _{18:0})	5.0 $\pm$ 0.2

Mild alkali treatment degraded leptospiral LPS into small particles, similar to those seen in alkali-treated Gram-negative LPS, which has a greater affinity than untreated LPS to bind to erythrocytic membranes (Ciznar & Shands, 1971). Alkali treatment was shown to enhance erythrocyte binding of a leptospiral LPS preparation (Faine *et al.*, 1974). Thus morphologically leptospiral LPS resembles typical LPS of Gram-negative enteric bacteria.

The UV absorption spectrum indicated a single peak (absorption maximum 0.60 at 192 nm) and a small shoulder at 235 nm (absorption maximum 0.08) which appears to correspond to an absorption maximum similar to the TM antigen described by Shinagawa & Yanagawa (1972).

Leptospiral LPS has a chemical composition similar to most Gram-negative bacterial LPS, except for the absence of hydroxymyristic acid (3-OH-C₁₄), a common marker from enteric group LPS, and KDO. The 2-keto-deoxy sugar acid found in leptospiral LPS was not KDO because of its different migration on paper chromatography. Since all 2-keto-3-deoxy sugar acids react with periodic and thiobarbituric acids giving a pink colour absorbed at 549 nm (Ghalambor *et al.*, 1966; Luderitz *et al.*, 1971), the corresponding deoxy sugar has to be determined in order to identify a reactive 2-keto-3-deoxy sugar acid. These observations confirm those of Shimizu *et al.* (1984) who also found an unidentified KDO-like component in LPS isolated from *L. interrogans* serovar *canicola*. Kasai & Nowotny (1967) also found 3 thiobarbituric acid-positive spots on high voltage paper electrophoresis while studying the sugar composition of a *Salmonella minnesota* glycolipid. 2-Keto-deoxy acids or their phosphorylated forms have been shown to be intermediates in the metabolism of carbohydrates by bacteria.

Examples are 2-keto-3-deoxygluconic acid 6-phosphate, an intermediate in the metabolism of glucose (Entner & Doudoroff, 1952; Wood & Schwerdt, 1954); and 2-keto-3-deoxyheptonic acid 7-phosphate, an intermediate in aromatic amino acid biosynthesis (Weissbach & Hurwitz, 1959).

No attempt has been made to identify all the components of LPS. Ono *et al.* (1984) described components of an antigenic fraction (TM) of *L. interrogans* serovar *canicola*, related to LPS (Adler *et al.*, 1980), emphasizing the difficulties arising from the small amounts of material available, even though they extracted 1000 l of culture.

The absence of endotoxic activity such as pyrogenicity, mouse lethality and cytotoxicity for cultured L-cell fibroblasts (Vinh *et al.*, 1986) confirms the observations of Finco & Low (1967) and Johnson (1976) for leptospiral LPS. Hardy & Levin (1983) were unable to demonstrate endotoxic activities in *Borrelia* and *Treponema pallidum* infections.

KDO is a linkage between lipid A and polysaccharide chains in LPS from other Gram-negative bacteria, but plays no role in the endotoxic activity which is entirely dependent on lipid A (Luderitz *et al.*, 1971; Amano *et al.*, 1983). Furthermore the removal from LPS of lauryl, myristyl and 3-hydroxymyristyl groups by alkaline hydroxylaminolysis was associated with the reduction of pyrogenicity and low toxicity for mice and dogs (McIntire *et al.*, 1967).

It is not known whether the absence of myristic acid (C_{14:0}) and 3-hydroxymyristic (3-OH-C_{14:0}) acids, which are the main fatty acids of the other Gram-negative bacterial LPS (Galanos *et al.*, 1977), is the reason why leptospiral LPS lacks endotoxic activity characteristic of LPS from enteric bacteria. Toxic activity similar to some characteristics of typical Gram-negative bacterial LPS was found in another type of preparation from leptospires (Vinh *et al.*, 1986).

This work was supported by a grant from the National Health and Medical Research Council, Canberra, Australia. We thank Dr Peter Coloe, Veterinary Research Institute, for the fatty acid analysis and Mr Wayne Christopher for excellent technical assistance.

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