Studies on the Chemical Composition of Lipopolysaccharide from *Neisseria meningitidis* Group B

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A lipopolysaccharide was isolated from *Neisseria meningitidis* group B by phenol/water extraction and purified by differential ultracentrifugation. This preparation exhibited endotoxic properties as shown by the limulus-lysate assay. Mild acid hydrolysis of the lipopolysaccharide yielded a lipid A fraction and a polysaccharide fraction. The lipid A fraction contained fatty acids, phosphorus and glucosamine. Analysis of the polysaccharide fraction revealed the presence of glucose, galactose, glucosamine, 2-keto-3-deoxyoctonic acid and phosphorus. There was no heptose.

INTRODUCTION

The pathological and physiological effects of meningococcal infections have been associated with the endotoxic properties of the bacteria (Scherp, 1955; Thomas, 1954; Burrows, 1951). Although metabolic effects of meningococcal endotoxin have been reported (Kun, 1947; Kun & Abood, 1949), there are relatively few studies on the composition and structure of the lipopolysaccharide (LPS) of *Neisseriaceae*, whereas the LPS of the *Enterobacteriaceae* has been extensively studied. Boivin & Mesrobian (1933) first isolated a carbohydrate-lipid complex from meningococcus by trichloroacetic acid extraction. This substance was toxic for animals, and although protein-free it was antigenic and immunogenic. These investigators regarded the preparation as a somatic antigen and designated it antigen 'complet'. Boor & Miller (1944) isolated glycolipids which were both toxic and antigenic from various strains of *Neisseria*. They used three different extraction procedures: (i) trichloroacetic acid, (ii) 0.5 M-hydrochloric acid and (iii) diethylene glycol, all of which gave similar products. These were polysaccharides containing phosphorus and nitrogen bound to a phospholipid. Mergenhagen, Martin & Schiffmann (1963) isolated the LPS from *Neisseria meningitidis* group C, and found that it was 20% lipid and contained glucose, galactose, glucosamine and sialic acid. Yamakawa & Ueta (1964) studied the carbohydrate and fatty acid composition of whole organisms of *N. meningitidis* (groups A, B, C and D), *N. flavescens*, *N. perflava*, *N. haemolysans* and *N. gonorrhoeae*. *Neisseria haemolysans* differed from the other *Neisseria* species in the composition of both fatty acids and sugars. An extensive examination of the composition and structure of the LPS of *N. perflava*, *N. sicca* and *N. catarrhalis* (Adams, 1971; Adams *et al.*, 1968; Adams, Tornabene & Yaguchi, 1969) revealed marked differences among the LPS of the *Neisseriaceae*. Studies of LPS from different strains of *N. gonorrhoeae* (Stead *et al.*, 1975) showed that all had the same sugar and fatty acid composition. There was no significant difference between the LPS of virulent gonococci or between penicillin-sensitive and penicillin-resistant strains. Since very few studies (Frasch & Gotschlich, 1974) have been reported on *N. meningitidis* group B, the aim of this investigation was to determine some of the chemical properties of this LPS.

METHODS

Bacteria. The strain used was *N. meningitidis* group B, obtained from Dr Malcolm S. Artenstein, Walter Reed Army Institute of Research Collection, Washington, D.C., U.S.A.

Cultivation and harvesting. The culture was grown on five Mueller-Hinton agar plates (Mueller & Hinton, 1941) at 37 °C in a candle jar. After 16 h the bacteria were resuspended and used to inoculate 1 litre of medium (Berman *et al.*, 1970) in a 2 l Erlenmeyer flask which was incubated at 37 °C on a New Brunswick rotary shaker operating at 200 rev. min⁻¹. After 5 h the absorbance (A_{660}) reached 1.0 and the culture was inoculated into a 14 l fermenter (New Brunswick, model MA 114) containing 9 l of medium; the growth conditions were 37 °C, 300 rev. min⁻¹ and 2 l air flow min⁻¹. After the absorbance reached 1.0, the culture was transferred to a 250 l fermenter (New Brunswick, model FM 250) containing 190 l of medium; the growth conditions in this fermenter were 37 °C, 200 rev. min⁻¹ and 40 l air flow min⁻¹. The absorbance, read every 2 h, reached a maximum of 1.8 after 10 h incubation; the culture was then cooled to 20 °C and harvested into thimerosal (ethyl mercurithiosalicylic acid sodium salt, Fisher Scientific Co., Fairlawn, New Jersey, U.S.A.) at a concentration of 1 g per 5 l culture fluid. The bacteria were collected in a Sharples centrifuge operating at 28000 rev. min⁻¹.

Extraction and purification of LPS. The procedure for the extraction of LPS was that of Frasch & Gotschlich (1974). Organisms (about 60 g wet wt) were suspended in a mixture of 600 ml distilled water and 600 ml 88% liquid phenol, and extracted at 70 °C for 20 min using a Sorvall Omnimixer. The homogenate was cooled in ice, centrifuged at 2000 g for 20 min and the aqueous phase was removed taking care not to include the interphase material. The crude LPS was precipitated from the aqueous phase with acetone at -20 °C (2 vol.) in the presence of sodium acetate (20 mg per 600 ml). The precipitate was collected by centrifugation (2000 g for 20 min), washed several times with cold acetone, redissolved in distilled water, dialysed for 24 h at 4 °C with two to three changes of distilled water and lyophilized. The lyophilized LPS was suspended in distilled water (80 ml) and centrifuged in the Beckman L2-75B ultracentrifuge at 80000 g for 1 h; the absorbance of the supernatant was read at 260 nm and 280 nm to determine contamination with nucleic acid and protein. This procedure was repeated several times until the supernatant gave a negligible absorbance reading at 260 nm and 280 nm. The final glassy pellet was resuspended in distilled water and lyophilized.

Hydrolysis and fractionation of LPS. To cleave LPS into lipid A and polysaccharide, samples (5 mg ml⁻¹) were hydrolysed with 1% (w/v) acetic acid at 100 °C for 1 to 1.5 h until the precipitation of lipid A was complete (Wilkinson, Galbraith & Lightfoot, 1973). The water-insoluble lipid A was removed from the hydrolysate by centrifugation at 2000 g. The pellet was washed several times with water to remove contaminating polysaccharide and finally resuspended in water and lyophilized. The washings were pooled and combined with the water-soluble material (containing degraded or cleaved polysaccharide) and the solution was lyophilized.

High-voltage paper electrophoresis of LPS. LPS was hydrolysed with 0.25 M-H₂SO₄ for 24 h at 100 °C under N₂. After hydrolysis, sulphate ions were precipitated by adding saturated Ba(OH)₂ until the pH of the solution reached 5 to 5.5. The insoluble barium sulphate was removed by filtration and the filtrate was evaporated to dryness. Samples of this hydrolysate, together with appropriate standards, were subjected to electrophoresis for 3 h at 3000 V (120 mA) on Whatman no. 1 paper (18 × 42 in) using pyridine/acetic acid/water (1:10:189, by vol.) pH 3.7 as the solvent. Neutral sugars were detected by alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950), hexosamines by ninhydrin (Consden & Gordon, 1948), and phosphates by the method of Hanes & Isherwood (1949).

Thin-layer chromatography. Acid-hydrolysed samples of the cleaved polysaccharide (1 M-HCl at 100 °C for 30 min) were chromatographed on plates of silica gel GF (Analtech) in methanol/pyridine/10 M-HCl/water (32:4:1:7, by vol.) (Wilkinson & Galbraith, 1975). Standards of 2,6-diaminopimelic acid and ethanolamine were run with the samples. Acid ninhydrin (Consden & Gordon, 1948) was used to detect 2,6-diaminopimelic acid and ethanolamine. In addition, ethanolamine was detected by spraying the plates with a 20% solution of fluorescamine in acetone and visualizing with ultraviolet light (Udenfriend *et al.*, 1972).

Quantitative analysis. For amino acid analysis, the LPS preparation was hydrolysed in constant-boiling 6 M-HCl at 110 °C for 24 h under N₂. After removal of HCl *in vacuo*, the amino acids were analysed on a Perkin Elmer KLA-5 amino acid analyser using ligand exchange chromatography. Protein was determined by the method of Lowry *et al.* (1951). For neutral sugar analysis, LPS was hydrolysed with 0.25 M-H₂SO₄ for 24 h at 100 °C under N₂, and then sulphate ions were removed as described for the acid hydrolysate used for high-voltage paper electrophoresis. Neutral sugars were identified and quantified by gas-liquid chromatography (g.l.c.) of their alditol acetate derivatives (Kim *et al.*, 1967) on a Perkin-Elmer 900 gas chromatograph. Total carbohydrates were determined by the phenol-sulphuric acid method (Dubois *et al.*, 1956). Hexosamines were determined by the method of Gatt & Bermann (1966) and identified by g.l.c. of their alditol acetate derivatives using polyamide as the stationary phase (Neidermeier & Tomana, 1974). Heptose and 2-keto-3-deoxyoctonic acid (KDO) were determined by the method of Osborn (1963). In addition, heptose

Table 1. *Chemical composition (as % dry wt) of phenol/water-extracted lipopolysaccharide of N. meningitidis group B*

	Total LPS	Polysaccharide	Lipid A
Lipid A	35.0	—	—
Fatty acid	26.6	—	64.7
Phosphorus (PO ₄ ³⁻)	3.7 (11.1)‡	4.0 (12.1)‡	3.9 (9.3)‡
Protein	1.0	—	—
Carbohydrate*	15.4	21.4	—
Carbohydrate†	20.8	27.0	—
Galactose	10.8	14.0	—
Glucose	10.0	13.0	—
Glucosamine	16.5	9.7	14.6
KDO	3.0	6.1	—

—, Not detected.

* Measured by the phenol-sulphuric acid procedure.

† Measured by g.l.c. of alditol acetate derivatives.

‡ Number in parentheses represents the percentage of phosphates.

Table 2. *Fatty acid composition of lipid A and LPS and their corresponding molar ratios*

Fatty acid*	Percentage of total lipid		Molar ratio		Percentage of dry weight	
	Lipid A	LPS	Lipid A	LPS	Lipid A	LPS
12:0	22.6	23.2	1.9	2.3	14.6	6.1
14:0	13.7	11.6	1.0	1.0	8.8	3.1
OH-14:0	21.0	21.0	1.6	1.9	13.6	5.5
15:0	1.5	tr	—	—	tr	tr
16:0	9.7	7.3	0.6	0.6	6.3	1.7
16:1	8.9	10.6	0.6	0.8	5.7	2.8
18:0	tr	tr	—	—	tr	tr
18:1	21.8	23.2	1.3	1.6	14.1	6.1

tr, Trace (= < 1 %); —, not detected.

* The first number indicates the carbon chain length; the second number is the number of double bonds.

was determined by the g.l.c. of its alditol acetate derivative (Holme *et al.*, 1968), phosphorus by the method of Chen, Toribara & Warner (1956) and sialic acid by the method of Warren (1959). The total fatty acid content of LPS was determined using the titrimetric procedure of Lauwerys (1969). Individual fatty acids were identified by g.l.c. of their methyl ester derivatives using a 183 × 0.2 cm glass column containing 20% DEGS, at column, manifold, and injector temperatures of 170, 230 and 200 °C, respectively; helium was the carrier gas (Schlenk & Gellerman, 1960).

Assay for endotoxin activity. LPS was tested for endotoxin activity by the limulus-lysate assay (Yin *et al.*, 1972). Tubes were incubated at 37 °C for 30 min.

RESULTS

The yield of LPS was 0.22 % (w/w) of the wet organisms or cell paste. The LPS preparation gave firm gelation in the limulus-lysate assay similar in degree to that given by the standard *E. coli* LPS at 5 ng ml⁻¹ (Mallinckrodt, St Louis, U.S.A.) which was used as a positive control.

Analysis of lipopolysaccharide

The LPS preparation contained negligible amounts of nucleic acid as shown by the amount of ribose present (0.05 %, w/w) and the very low absorbance at 260 nm. The protein content of the LPS was 1.0 % (w/w) and on acid hydrolysis revealed a trace amount of alanine. Electrophoresis of acid-hydrolysed LPS revealed the presence of neutral sugars, glucosamine and a phosphate-containing compound. The neutral sugars were subsequently identified by g.l.c. as glucose and galactose. Thin-layer chromatography of the polysaccharide fraction

revealed trace amounts of 2,6-diaminopimelic acid indicating a slight degree of contamination with peptidoglycan. However, ethanolamine was not detected. The chemical analysis of the LPS is given in Table 1. Heptose was not detected either by g.l.c. or by the cysteine-H₂SO₄ reagent. G.l.c. revealed a trace amount of methyl pentose. Neutral sugars, identified as their alditol acetate derivatives, were present in a molar ratio of galactose to glucose of 1:1. Sialic acid was not detected.

Lipid A and partly degraded polysaccharide

Mild acid hydrolysis of LPS with 1% acetic acid at 100 °C cleaved lipid A from the polysaccharide moiety. The lipid A released was approximately 35% of the total LPS based on the weight of the insoluble white precipitate obtained. The fatty acid compositions of lipid A and whole LPS were identical (Table 2) whereas the soluble cleaved polysaccharide was lipid-free. Polysaccharide recovered from the acid hydrolysate comprised 62.5% of the LPS (based on the weight of freeze-dried material) and had glucosamine and phosphorus contents of 9.7% and 4.0% respectively (Table 1). Fatty acids liberated from lipid A by alkaline hydrolysis (4 M-NaOH for 4 h at 100 °C) were analysed and identified as their methyl ester derivatives by g.l.c. The major fatty acids present were β -hydroxymyristic acid, lauric acid, oleic acid and myristic acid. Palmitic acid and palmitoleic acid were present in smaller amounts (Table 2).

DISCUSSION

The LPS isolated from *N. meningitidis* group B contained glucose, galactose, glucosamine, KDO, lipid A and phosphorus. The fatty acid composition showed a high proportion of fatty acids with even numbers of carbon atoms, as is in other Gram-negative bacteria. One of the major fatty acids present, second only in amount to β -hydroxymyristic and lauric acids, was oleic acid, C_{18:1}. This, and β -hydroxymyristic and lauric acids, seem to be fatty acid components common to all Neisseriaceae (Yamakawa & Ueta, 1964).

The presence of galactose in the isolated LPS distinguishes it from the LPS of both *N. perflava* (Adams *et al.*, 1968) and *N. sicca* (Adams, 1971) which lack this sugar. Galactose is, however, a component of LPS of other *Neisseria* species such as *N. catarrhalis* (Adams *et al.*, 1969), *N. meningitidis* group C (Mergenhagen *et al.*, 1963) and *N. gonorrhoeae* (Stead *et al.*, 1975). Sialic acid was not present in the isolated LPS, in contrast to the LPS of *N. meningitidis* group C, although both group B and C have sialic acid as the major constituent of their capsular polysaccharide. Sialic acid was present in the crude LPS but was removed by ultracentrifugation. Another feature of this LPS was the absence of heptose and in this respect it resembles the LPS of *N. sicca* (Adams, 1971) and *N. catarrhalis* (Adams *et al.*, 1969).

Examination of the LPS of *N. meningitidis* group B has shown that its composition is markedly different from that of *N. meningitidis* group C, and is also different from the LPS of *N. perflava*, *N. sicca*, *N. gonorrhoeae* and *N. catarrhalis*. Thus no common pattern exists among the LPS of Neisseriaceae.

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