Studies on Lipopolysaccharides Isolated from Strains of *Neisseria gonorrhoeae*

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SUMMARY

Lipopolysaccharides, extracted by phenol-water from five strains of *Neisseria gonorrhoeae*, were purified by treatment with ribonuclease followed by multiple washes. These preparations were fatal to mice when administered in submicrogram amounts with actinomycin D, the LD₅₀ values varying from 4 to 16 µg/kg. Analyses showed that all preparations contained glucose, galactose, glucosamine, heptose, 2-keto-3-deoxyoctonic acid and phosphate. All the lipopolysaccharides contained the same fatty acids, namely β-OH-10:0, β-OH-12:0, β-OH-14:0, 12:0, 14:0, 16:0, 16:1, 18:0 and 18:1. We were unable to detect significant differences between the lipopolysaccharides of virulent and avirulent gonococci or between penicillin-sensitive and resistant strains. Gonococcal lipopolysaccharides appeared to lack O-antigen side chains.

INTRODUCTION

Despite extensive investigations on the endotoxic lipopolysaccharides (LPS) of the Enterobacteriaceae, there have been relatively few studies on the Neisseriaceae. *Neisseria* differ from other Gram-negative bacteria in their extreme sensitivity to penicillins. In *Pseudomonas aeruginosa* differing susceptibilities to penicillins have been correlated with LPS structure (Meadow, 1972). Perhaps the penicillin sensitivity of *Neisseria* is a reflection of a different type of LPS structure.

Lipopolysaccharide was the target antigen involved in the killing of *Neisseria gonorrhoeae* (gonococci) by antibody and complement (Glynn & Ward, 1970). These workers suggested that LPS might be used in the serotyping of gonococci. The O-antigen chains of LPS are responsible for serological specificity in *Salmonella* and are important in the serological diagnosis of enteric fever. There have been no reports of O-antigen chains on gonococcal LPS (Tauber & Garson, 1959; Maeland & Kristoffersen, 1971). Nevertheless, Watt, Ward & Glynn (1971) reported the use of latex particles coated with LPS as an antigen for the serological diagnosis of gonorrhoea. In order to pursue our investigations of the biological significance of gonococcal LPS, we have prepared, purified and analysed LPS extracted from both freshly isolated and old laboratory strains of gonococci.

METHODS

Bacterial cultures. We used *Neisseria gonorrhoeae* colony type 1 strains P9 and F62, colony type 4 strains G2, G4 and G50 (Kellogg *et al.* 1963), and strain A24 which was resistant to 0.5 µg penicillin/ml.

Cultivation and harvesting. Gonococci stored in liquid nitrogen (Ward & Watt, 1971) served as inocula for starter cultures on GC agar (Difco) grown overnight in a moist atmosphere containing 5% CO₂. Organisms from 50 plates were harvested aseptically by

suspension in 100 ml 1 % (w/v) proteose peptone No. 3 broth (Difco). Organisms were grown in 5 l volumes in a stirred jar fermenter (New Brunswick Scientific) in medium containing (per litre): proteose peptone, 15 g; NaCl, 5 g; K_2HPO_4 , 4 g; dextrose, 4 g; glutamine, 50 mg; Fe(NO), 10 mg; co-carboxylase, 0.2 mg. Polypropylene glycol 2000 (Shell Chemicals), 0.1 ml/l, was used as antifoam. Bacteria were grown at 37 °C and 15 % O_2 saturation, with stirring (300 rev./min), until the extinction of a 1:5 (v/v) dilution of the culture had reached 0.25, which took between 5 h for old laboratory strains to 8 h for freshly isolated ones. Then the culture was harvested at 22000 g using a continuous action rotor with a flow rate of 120 ml/min.

Extraction of LPS. Whole cells from strains P9, F62, G2, G4 and G50 were treated with 45 % (w/v) aqueous phenol (Westphal & Jann, 1965). The aqueous phases were centrifuged at 20000 g to remove contaminating peptidoglycan and dialysed for 3 days against tap water. Crude LPS was precipitated by 3 vol. of acetone (4 °C) in the presence of a trace of $MgCl_2$. After 2 h at 4 °C the precipitate was spun down at 2000 g, redissolved in water and dialysed for 24 h against distilled water. The non-diffusible material was treated with 1 mg pancreatic ribonuclease (Koch-Light)/ml at pH 7.2 for 16 h and centrifuged at 100000 g for 3 h to sediment the LPS. The pellet was washed 3 times with 0.1 % (w/v) NaCl and twice with distilled water. Loosely-bound lipids were removed by stirring the LPS for 2 h at room temperature (approx. 18 °C) with chloroform-methanol (2:1, v/v; 100 ml/20 mg LPS) as described by Wilkinson, Galbraith & Lightfoot (1973).

LPS was isolated from strains P9 and A24 by washing with chloroform-methanol (2:1, v/v; 100 ml/g bacteria) followed by the use of hydrolytic enzymes as described by Lehrer & Nowotny (1972). Lysozyme (from egg white), protease type VII (bacterial), alkaline phosphatase (EC. 3.1.3.1; from *Escherichia coli*) and phosphodiesterase type II (from *Crotalus adamanteus* venom) were all purchased from Sigma.

Removal of lipid A. To cleave LPS into its lipid A and polysaccharide moieties, 50 mg samples were hydrolysed with 1 % (w/v) acetic acid (5 ml) at 100 °C for 1.5 h under N_2 and chloroform-soluble and water-soluble products were separated as described by Wilkinson *et al.* (1973).

Qualitative analysis. The LPS preparations were hydrolysed in 2 M-HCl at 100 °C for 3 h under N_2 for neutral sugar analysis, and in 6.1 M-HCl at 105 °C for 4 h under N_2 for amino compounds. All hydrolysates were dried repeatedly *in vacuo* over P_2O_5 and KOH.

Neutral sugars were examined by descending paper chromatography on Whatman No. 1 paper using solvent systems butan-1-ol-pyridine-water (6:4:3, by vol.) and ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.) and detected by alkaline silver nitrate (Trevelyan, Proctor & Harrison, 1950). Hexosamines were detected using the Morgan-Elson reagent (Morgan & Elson, 1934) and ninhydrin (Consden & Gordon, 1948). Amino acids were similarly examined by paper chromatography in solvent system propan-1-ol-ammonia-water (6:3:1, by vol.) and were detected by ninhydrin reagent (Consden & Gordon, 1948).

Quantitative analysis. Phosphorus was determined by the method of Chen, Toribara & Warner (1956) and carbohydrate by the phenol-sulphuric acid reaction (Dubois *et al.* 1956). Heptose was measured as glucoheptose using the method of Osborn (1963), and 2-keto-3-deoxyoctonic acid (KDO) determined by the thiobarbituric acid reaction (Waravdekar & Saslaw, 1959) and the semicarbazide reaction (MacGee & Doudoroff, 1954). Protein was measured by the method of Lowry *et al.* (1951). Fatty acid esters were estimated by the method of Snyder & Stephens (1959), using tripalmitin for calibration. Glucosamine was measured as glucosamine-HCl (Kraan & Muir, 1967), and *N*-acetylglucosamine by the method of Reissig, Strominger & Leloir (1955).

Glucostat and Galactostat (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.) were used for quantitative analysis of glucose and galactose. Glucose and galactose ratios were also determined by preparation of alditol acetates (Shaw & Moss, 1969) analysed by g.l.c. on a Pye series 104 gas chromatograph using a flame ionization detector and a glass column (6 mm $\times$ 1.5 m) containing 3 % (w/w) ECNSS-M (Pye Unicam Ltd) on acid-washed diatomite 'c', operating at 190 °C. Nitrogen was used as carrier gas at a flow rate of 45 ml/min.

Identification of fatty acids. Methyl esters of fatty acids in loosely bound lipids were prepared by treatment with 0.2 M-NaOMe (Marinetti, 1962). Fatty acids were released from samples of lipid A by hydrolysis with 6.1 M-HCl at 105 °C for 4 h and were esterified using ethereal diazomethane. Fatty acids were examined by g.l.c. on a glass column (6 mm $\times$ 1.5 m) containing 15 % (w/w) polyethyleneglycol succinate on acid-washed diatomite 'c' at 175 °C, with nitrogen as carrier gas at a flow rate of 35 ml/min.

β -OH myristic acid was identified by comparison with that found in the LPS of *Escherichia coli* WF96. A plot of logarithm of retention time versus number of carbon atoms was used to identify β -OH-10:0 and β -OH-12:0 fatty acids.

Limulus lysate assay for endotoxin. Lipopolysaccharides were tested for gelling activity by the Limulus E-Toxate kit (Sigma). All tests were incubated at 37 °C for 30 min and a positive reaction was adjudged to be a firm gelation.

Mouse assay for endotoxin. Actinomycin D (Merck, Sharp & Dohme), 12.5 μ g in sodium phosphate-buffered 0.85 % NaCl, was injected intraperitoneally into 25 g TO white female mice using disposable syringes (Dowling & Feldman, 1970). After 3 h samples (0.1, 0.3 and 0.5 μ g) of LPS prepared by the phenol-water method from strains G2, G4, G50, F62 and P9, and LPS from strain A24 prepared by hydrolytic enzymes, were administered in phosphate-buffered saline to groups of six mice. Deaths were recorded and the LD₅₀ values calculated after 6 days by the method of Litchfield & Wilcoxon (1949).

RESULTS

Yields of N. gonorrhoeae and extracted LPS

Yields of *N. gonorrhoeae* grown in stirred culture were approximately 1 to 2 g wet wt/l for freshly isolated strains, and 3 to 4 g wet wt/l for old laboratory strains. Electron micrographs of the gonococci negatively stained with 1 % uranyl acetate show long projections, apparently derived from the outer envelope, extending from the bacterium (Fig. 1). Presumably these projections, which break off into vesicles, contain LPS. Yields of LPS from all strains were 0.1 % of the wet weight.

The purity of our preparations was illustrated when all LPS gave firm gelations with limulus lysate at 37 °C for 30 min at concentrations down to 30 ng/ml. All LPS preparations were fatal to mice when administered in submicrogram amounts together with the immunosuppressant actinomycin D; the LD₅₀ varied from 4 μ g/kg in the case of P9 to 16 μ g/kg for G50.

Spectrophotometric scans of LPS dissolved in 0.1 M-NaOH revealed negligible absorption at 260 and 280 nm. The Lowry *et al.* (1951) test indicated that the preparations contained 2.8 to 3.7 % (w/w) protein (Table 1), but acid hydrolysis revealed only traces of glutamic acid, alanine and serine. This protein could not be removed by repeated washing and may have been associated with the LPS, perhaps covalently linked to the hexosamine of lipid A (Wober & Alaupović, 1971). Our preparations were more pure than those reported by Maeland & Kristoffersen (1971), whose preparations contained 11 to 14 % (w/w) protein. No ethanolamine was detected; ethanolamine was also absent from other species of

Table 1. *Chemical composition (as % dry wt) of phenol-water-extracted lipopolysaccharides from five strains of N. gonorrhoeae*

	Strain				
	P9	G2	G4	G50	F62
Lipid A ₁ *	10.6	8.1	12.6	11.3	8.5
Lipid A ₂ †	33.1	33.4	26.2	26.2	29.7
KDO‡	5.8	2.1	2.2	3.6	3.6
KDO§	8.1	3.9	3.2	8.1	5.6
Heptose	3.8	3.7	2.5	3.0	3.1
Total carbohydrate	21.9	23.0	22.5	27.4	17.1
Phosphorus	3.0	2.4	1.5	2.5	2.9
Glucosamine	14.3	15.6	11.4	9.2	17.6
Protein	3.7	2.8	3.2	3.5	3.1

* Released by hydrolysis with 1.0 % (w/v) acetic acid at 100 °C for 1.5 h under N₂.

† Released by further hydrolysis with 2 M-HCl at 100 °C for 3 h under N₂.

‡ Measured by thiobarbituric acid procedure.

§ Measured by semicarbazide procedure.

Table 2. *Chemical composition (as % dry wt) of lipids A₁ and A₂ from five strains of N. gonorrhoeae*

	Strain									
	P9		G2		G4		G50		F62	
	A ₁	A ₂	A ₁	A ₂	A ₁	A ₂	A ₁	A ₂	A ₁	A ₂
Fatty acid ester	51.2	50.0	77.5	38.5	69.0	64.8	75.0	72.1	63.0	38.5
Phosphorus	0.9	1.5	0.3	1.2	0.2	1.9	0.6	1.5	0.5	1.4
Glucosamine	3.2	19.7	0.4	19.3	0.7	20.9	2.2	20.4	0.4	18.8

Neisseria (Adams, 1971). Strain G4 revealed traces of 2,6-diaminopimelic acid on paper chromatography of acid hydrolysates, indicating a slight degree of contamination with peptidoglycan.

Hydrolysis of polysaccharide from lipid A

To obtain information about the distribution of components in the lipopolysaccharides, mild hydrolysis with 1 % (w/v) acetic acid at 100 °C was performed. This treatment was expected to separate lipid A and polysaccharide moieties. During hydrolysis a white precipitate (lipid A) developed and hydrolysis was stopped after 1.5 h (Wilkinson *et al.* 1973). Only 8.1 to 12.6 % (w/w) of the LPS was released as lipid A (designated lipid A₁) (Table 1). The time taken fully to cleave lipid A from the polysaccharide has been shown to vary considerably in *Pseudomonas aeruginosa* (Chester, Meadow & Pitt, 1973). Stronger hydrolysis 2 M-HCl at 100 °C for 3 h, liberated a further 26.2 to 33.4 % (w/w) of the LPS as lipid A (designated lipid A₂) (Table 1).

Chemical analysis of lipid A

Lipid A contained all the fatty acid present in the LPS. Lipid A₁ contained 51.2 to 77.5 % (w/w) fatty acid, 0.2 to 0.9 % phosphorus and 0.4 to 3.2 % glucosamine. In contrast, lipid A₂ consisted of 38.5 to 72.1 % (w/w) fatty acid, 1.2 to 1.9 % phosphorus and 18.8 to 20.9 % glucosamine (Table 2). Thus mild hydrolysis partially cleaved LPS polysaccharide and lipid A moieties and liberated labile fatty acids, but failed to cleave the linkage of hydroxy fatty acids, which are therefore thought to be in the stable amide linkage. Combined results

Table 3. Fatty acid composition (as % dry wt) of lipids A_1 and A_2 from five strains of *N. gonorrhoeae*

Fatty acid*	Strain									
	P9		G2		G4		G50		F62	
	A_1	A_2	A_1	A_2	A_1	A_2	A_1	A_2	A_1	A_2
12:0	20.3	26.8	22.1	25.4	23.3	19.9	26.8	15.2	28.0	27.5
14:0	14.0	14.7	11.7	11.8	13.7	5.9	14.7	9.8	15.9	8.5
OH-10:0	—	20.2	—	10.1	—	13.2	—	14.5	—	8.1
UN	19.8	—	24.5	—	21.4	—	20.4	—	19.4	—
16:0	9.1	5.1	4.0	1.4	9.0	2.9	12.1	9.7	12.3	1.9
16:1	5.1	2.2	1.8	Tr	4.1	1.8	7.3	4.6	6.8	Tr
OH-12:0	6.0	3.0	2.8	4.0	5.1	6.0	3.8	4.7	3.5	4.5
18:0	9.2	5.4	13.3	18.7	10.4	20.1	8.1	12.1	5.6	20.7
18:1	11.6	2.0	17.1	Tr	12.0	Tr	1.2	2.7	6.5	Tr
OH-14:0	4.8	20.5	2.7	26.6	1.0	29.5	5.6	26.7	2.0	27.6

Tr, trace (< 1 %); —, not detected.

* The first number gives the chain length, the second the number of double bonds; UN, unidentified.

Table 4. Fatty acid composition (% dry wt) from the chloroform-methanol washes of lipopolysaccharides from five strains of *N. gonorrhoeae*

Fatty acid	Strain				
	P9	G2	G4	G50	F62
12:0	1.0	2.0	2.0	2.9	Tr
13:0	5.8	6.8	7.8	15.3	3.6
?13:0 cyclopropane	48.9	Tr	Tr	Tr	Tr
14:0	3.9	11.7	9.8	11.0	6.8
14:1	Tr	Tr	Tr	Tr	Tr
16:0	9.8	7.0	13.0	19.7	12.3
16:1	3.5	6.9	4.9	6.5	Tr
18:0	2.6	4.1	17.2	11.6	36.0
18:1	9.4	11.4	17.3	12.8	27.8
20:1	14.1	49.0	28.0	20.0	12.3

Tr, trace (< 1 %).

showed total lipid A accounted for 37.5 to 43.7 % (w/w) of LPS (Table 1) and contained 43.9 to 73.0 % (w/w) fatty acid, 1.0 to 1.3 % phosphorus and 14.2 to 15.5 % glucosamine. Loosely bound lipids had been removed from LPS by prior washing with chloroform-methanol and it is therefore believed that all these fatty acids were constituents of the lipid A.

Fatty acids liberated from lipid A by acid hydrolysis were analysed as methyl esters. Lipid A_1 predominantly contained 12:0, 14:0, an unidentified acid, 16:0, 18:0 and 18:1, with hydroxy acids accounting for only 5.5 to 10.8 % (w/w) (Table 3). In contrast, fatty acids present in lipid A_2 were mainly hydroxy acids, comprising 40.2 to 48.7 % (w/w) of the total fatty acid (Table 3), together with lesser amounts of 12:0, 14:0 and 18:0, as has been found in other species of *Neisseria* (Adams *et al.* 1968; Adams, 1971). Associated lipids, removed from the LPS by prior washing with chloroform-methanol, represented 2 to 4 % of the dry weight of LPS. The fatty acid composition of the loosely bound lipids from strains G2, G4, G50 and F62 consisted predominantly of 16:0, 18:0, 18:1 and 20:1 (Table 4) which have been found in other strains of *N. gonorrhoeae* (Yamakawa & Ueta, 1964). In contrast, P9 consisted mainly of a fatty acid which has not been characterized completely but was almost certainly 13:0 cyclopropane, with lesser amounts of 16:0, 18:0, 18:1 and 20:1 (Table 4).

Chemical analysis of polysaccharide moiety

Paper chromatography revealed glucose, galactose, glucosamine, heptose and KDO, confirming earlier results with other strains of *N. gonorrhoeae* (Yamakawa & Ueta, 1964; Maeland & Kristoffersen, 1971). The heptose co-chromatographed with L-glycero-D-mannoheptose found in the LPS of *E. coli* WF96. Comparison of components present in the LPS preparations is shown in Table 1. Identification of galactose and glucose was performed by g.l.c. of their alditol acetates which gave a molar ratio of galactose to glucose of 2:1 in all strains. LPS from a duplicate culture of strain p9 analysed by Galactostat and Glucostat reagents contained 3.5 % (w/w) galactose to 1.8 % glucose, indicating consistent growth conditions. Measurement of the molar ratios of sugars in the LPS of p9 indicated glucose 1, galactose 2, glucosamine 4, heptose 2, KDO 3.

No evidence was obtained for the presence of any O-antigen chains. In *Citrobacter freundii*, LPS containing rhamnose and dideoxyglucose entered the phenol phase or was obtained from the interphase of phenol-water extraction (Raff & Wheat, 1968). By preparing enzyme-extracted LPS (Lehrer & Nowotny, 1972) from penicillin-sensitive and penicillin-resistant gonococci we have excluded the possibility of LPS being lost in this way. Protein and KDO determinations on the enzyme-isolated LPS from strains p9 and A24 revealed 3.6 and 4.4 % (w/w) KDO and 36 and 49.5 % (w/w) protein, respectively. Acid hydrolysis and paper chromatography revealed glucose, galactose, heptose, KDO and glucosamine, identical to those components present in phenol-water-extracted LPS. No deoxy-, dideoxy- or *N*-acetylated sugars, typical of O-antigen chains, could be detected.

DISCUSSION

Of the strains of *N. gonorrhoeae* used in this study, strain F62 colony type 1 has been used to infect human volunteers (Kellogg *et al.* 1963) while strain p9 has caused an accidental eye infection in our laboratory. Under the conditions of growth described we were unable to detect significant differences in LPS composition between these strains and avirulent colony type 4 strains (G2, G4 and G50). The LPS of strain p9 consisted of lipid A covalently linked to 3 molecules of KDO, 2 of heptose, 1 of glucose, 2 of galactose and 4 of glucosamine. These ratios are compatible with a structure analogous to the basal region of the LPS of the Enterobacteriaceae (Osborn *et al.* 1964). Our analysis of LPS prepared by enzyme hydrolysis excluded the possibility that the failure to find evidence for an O-antigen side chain was due to loss during phenol-water extraction. Strain p9, like gonococci in the natural infection (Ward, Watt & Glynn, 1970) is relatively resistant to killing by antibody and complement. Gonococci therefore differ from *Salmonella*, where the O-antigen side chain of LPS is important both in virulence and resistance to killing by antibody and complement (Nelson & Roantree, 1967). Our analysis of LPS from strains G2, G4 and G50 showed no marked difference between the strains, yet antisera raised against G50 and G4 were type-specific in the bactericidal reaction whereas strain G2 was unusually sensitive to the natural antibodies of normal human serum (Glynn & Ward, 1970). Glynn & Ward (1970) suggested that the antigens involved were LPS. Structural differences in the simple LPS of gonococci could produce antigenic variations accounting for the four serological groups which they proposed. However, the absence of an O-antigen side chain would seem to preclude the possibility of devising a complex typing system comparable to the Kaufmann-White scheme (Kaufmann, 1966) successfully used in epidemiological studies of *Salmonella* infections.

Some 50 % of patients with gonorrhoea showed an increased titre of antibodies reactive

Fig. 1. Gonococcal strain F62 negatively stained with 1 % (w/v) uranyl acetate. Long projections derived from the outer envelope and presumably containing endotoxin extend from the bacterium. The projections have a tendency to break off into small vesicles.

with gonococcal LPS. However, 15 % of normal individuals had cross-reacting antibodies (Watt *et al.* 1971). The fact that the LPS of other *Neisseria* such as *N. perflava* (Adams *et al.* 1968) have essentially similar constituents to the LPS of *N. gonorrhoeae* suggests that production of the cross-reacting antibodies might have been stimulated by commensal *Neisseria* growing on mucosal surfaces. If further work confirms this, there can be little hope of developing a useful serological test for gonorrhoea based on LPS antigens.

The lipid A moieties in all five strains contained glucosamine to which was joined phosphate groups and fatty acids, principally hydroxy-acids which, in view of their resistance to acid hydrolysis, were presumed to be amide linked. The composition is comparable to that found in the commensal *Neisseria* (Adams *et al.* 1968; Adams, 1971) and is essentially similar to the lipid A of salmonellae (Rietschel *et al.* 1972). In *Salmonella* the determinant of endotoxicity of the LPS is lipid A, linked ketosidically to KDO, the remaining carbohydrate of the LPS being unimportant in toxicity (Rietschel *et al.* 1971). We have shown that the simple lipopolysaccharides of all the gonococcal strains tested were potent endotoxins. We do not know if this LPS plays a role in gonococcal virulence. However, growing gonococci bud off

large amounts of outer envelope which must contain the endotoxin (Fig. 1). This material could be involved in the interaction between host and pathogen.

Maness & Sparling (1973) have suggested that a single gene may control the response of *N. gonorrhoeae* to antibiotics, perhaps by altering the permeability of the cell envelope. In *P. aeruginosa*, resistance to penicillin has been correlated with the presence of a side chain containing rhamnose, glucosamine and mannose in the LPS (Meadow, 1972). This does not seem to be the case in *N. gonorrhoeae* A24, which had the same LPS carbohydrate content as the penicillin-sensitive strains.

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