Relationship between lipopolysaccharide composition and virulence of *Haemophilus ducreyi*

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Summary. The relationship between lipopolysaccharide (LPS) composition and virulence of *Haemophilus ducreyi* strains was investigated. Glycoses identified in LPS by gas-liquid chromatography were glucose, galactose, and their amino derivatives glucosamine and galactosamine. Fucose was found in trace amounts but mannose and rhamnose, characteristic of the O-side chain of LPS in many species, were not detected. Qualitatively, the LPS composition of the eight strains examined was similar and differences were mainly quantitative. The total glycoside:KDO ratio of the LPS of virulent strains exceeded that of avirulent strains. All strains had similar fatty-acid composition but lacked lauric acid. SDS-polyacrylamide gel electrophoresis of the LPS of virulent and avirulent strains also revealed differences in their electrophoretic mobilities. The LPS profiles of avirulent strains were similar, but differed from those of virulent strains. These profiles lacked high mol. wt bands representing O-side chain repeating units. Thus, differences in the electrophoretic mobilities of the LPS of virulent and avirulent strains may reflect differences in the amount of carbohydrates associated with the core polysaccharide.

Introduction

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria. Structurally, it consists of three parts: lipid A, core polysaccharide and O-antigenic side chains. The O-antigenic side chains are found in smooth LPS, whereas rough LPS may contain lipid A and varying amounts of the core polysaccharide or lipid A and 2-keto-3-deoxy-octulosonic acid (KDO) only, as in the case of deep rough mutants (Luderitz *et al.*, 1971). The presence of O-antigenic side chains in the LPS of smooth strains of *Salmonella* spp. (Nelson and Roantree, 1967; Rowley, 1968; Olling, 1977), *Escherichia coli* (Mushel and Larsen, 1970; Taylor, 1975), and *Shigella* spp. (Reed and Albright, 1974) and the composition of the core polysaccharide of *E. coli* (Medearis *et al.*, 1968) and the LPS of *Neisseria gonorrhoeae* (Guymon *et al.*, 1982) have been associated with serum resistance and virulence.

In a previous study (Odumeru *et al.*, 1984), virulence of *Haemophilus ducreyi* was associated with resistance both to the bactericidal action of

human serum and to phagocytosis and killing by human polymorphonuclear leukocytes. The bactericidal effect of human serum was inhibited by the LPS of serum-sensitive strains but not by the LPS of serum-resistant strains (Odumeru *et al.*, 1985). The purpose of the present study was to determine whether there is a relationship between the chemical composition of the LPS of *H. ducreyi* strains and virulence and whether the gross chemical composition of *H. ducreyi* LPS resembled that of LPS from other gram-negative species.

Materials and methods

Bacterial strains and growth conditions

H. ducreyi strains used in this study were: reference strains A75, A77, and A76 (from the Pasteur Institute, Paris); strain 36-F-2 (from M. Kilian); strains 409, C148, 557, BG411 (from Kenya); strains 35000 and 78226 (from Winnipeg); and strain CH39 (from Thailand). Serum-resistant strain A77* was obtained by passage of strain A77 in the presence of increasing concentrations of pooled normal human serum until a 70% survival rate in 50% serum was reached.

The strains were kept as frozen-milk stock cultures at –70°C. Before use, they were cultured on haemoglobin agar composed of gonococcal medium base and 1% Isovitalex and incubated at 35°C with CO₂ 5% and high humidity for 24 h. All media were obtained from Gibco

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Laboratories, Madison, WI, USA. No visual evidence of capsulation was present.

Virulence tests

Virulence of *H. ducreyi* strains was tested by the intradermal injection (Hammond *et al.*, 1978) of 0.20 ml of Sheep Brain Heart Infusion Broth (Gibco) containing 10^9 cfu/ml of *H. ducreyi* into 2–3 kg, 1-year-old female rabbits. Induration and necrosis were determined daily from day 1 until day 11 after inoculation. The criteria for virulence were that induration exceeded 0.5 cm by day 4, and the lesion progressed to an eschar by day 11. *H. ducreyi* strains 409, 35000, A77*, C148, BG411, 557 and CH39 were virulent but strains A77, A75, A76, and 36-F-2 were avirulent by these criteria.

LPS isolation

The LPS of *H. ducreyi* strains was isolated by the rapid isolation micromethod described by Inzana (1983), and also from 18 g (wet weight) of stationary phase cells by the hot phenol-water procedure of Westphal and Jann (1965). All LPS preparations were lyophilised and stored at 4°C. The LPS of non-piliated *N. gonorrhoeae* P-6472 was supplied by Dr G. M. Wiseman. LPS of *E. coli* 0111B₄ was obtained from Difco Laboratories, Detroit, MI, USA.

Preparation of lipid A from LPS

Lipid A was prepared as described by Stead *et al.* (1975). About 30 mg of LPS was hydrolysed with 5 ml of 0.18 M acetic acid at 100°C for 2 h in a sealed ampoule. The precipitated lipid A was removed by centrifugation, washed with 0.18 M acetic acid, lyophilised and weighed.

Fatty acid analysis

Fatty acid methyl esters were prepared as described by Jennings *et al.* (1973). Lyophilised lipid A was dissolved in chloroform, reprecipitated in acetone (1 part lipid:3 parts acetone), and refluxed in methanolic HCl (5 parts methanol:1 part HCl) for 8 h. The methyl esters were extracted with ethyl ether-petroleum ether (1:1), concentrated, and analysed by gas-liquid chromatography (GLC) on a stainless-steel column (0.65 × 180 cm) prepacked with 10% diethylene glycol adipate (DEGA) and 2% phosphoric acid on 100–120-mesh Gas-Chrom Q. Oven temperature was programmed as follows: 120°C for 4 min, temperature rising at 6°C/min, and 250°C for 5 min. The detector was held at 250°C. Known standards were used for comparison in all GLC methods.

Analytical methods

Total carbohydrate was measured as described by Dubois *et al.* (1956), and total hexose was assayed by the

anthrone sulphuric acid method of Scott and Melvin (1953). Glucose was used as standard in both assays. The protein content was determined by the method of Bradford (1976) and the colorimetric method of Ceriotti (1952) was applied to the determination of deoxyribonucleic acid. Heptose was determined by the Dische method as modified by Wright and Rebers (1972) with glucoheptose as a standard. Aminoglycoses were quantitated by the method of Smith and Gilkerson (1979). N-acetylneuraminic acid (NANA) was determined by the periodate-resorcinol method of Jourdan *et al.* (1971) and phosphorus was determined as described by Fiske and Subbarow (1925). KDO was estimated by the thiobarbituric acid (TBA) method of Karkhanis *et al.* (1978), and also by the method of Brade *et al.* (1983).

Glycose analyses were performed as described by Wiseman and Caird (1977) with slight modifications. About 30 mg of LPS was hydrolysed with 1N H₂SO₄ for 4 h at 100°C in a sealed ampoule. The hydrolysate was neutralised with solid BaCO₃, centrifuged and the supernate was lyophilised. The lyophilised material was treated with 0.1 ml Sil-Prep reagent (Sigma Chemical Co., St Louis, MO, USA) (pyridine 0.5 ml; hexamethyldisilazane 0.15 ml; trimethylchlorosilane 0.1 ml) for each mg of material and shaken vigorously. The reaction was allowed to proceed for 60 min at room temperature and the trimethylsilylated (TMS) monosaccharides were analysed in a Pye 104 gas-liquid chromatograph equipped with dual hydrogen flame ionisation detectors. The separation of the various monosaccharides was achieved in a stainless steel column (0.65 × 180 cm) prepacked with 10% SE30 on 100–120-mesh Gas-Chrom Q in a temperature programme over the range 150–250°C and rising at 2°C/min. The detector temperature was 250°C.

Aminoglycoses were determined by GLC, as described by Laine *et al.* (1972).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The LPS preparations from *H. ducreyi* strains were analysed by SDS-PAGE with the discontinuous gel system described by Laemmli (1970). The gel was 15% polyacrylamide and contained 4 M urea. In the electrophoretic analysis of LPS preparations, 2 µg of purified LPS was loaded on to the gel and electrophoresed at 20 mA (constant current) per slab. After electrophoresis, the LPS bands were visualised by the silver staining method of Tsai and Frasch (1982). The electrophoretic mobility and profiles of LPS isolated by the methods of Inzana (1983) and Westphal and Jann (1965), were similar.

Infra-red spectrophotometry

Infra-red (IR) spectra of the LPS preparations were obtained with the assistance of the Chemistry Department, University of Manitoba. Lyophilised, dried LPS was ground with Nujol (mineral-oil), placed in cells and scanned.

Results

Chemical analysis of LPS

The chemical composition of the LPS preparations from virulent strains of *H. ducreyi* was compared with that of avirulent strains. The LPS of *E. coli* 0111B₄ and *N. gonorrhoeae* P-6274 were included for comparison. Qualitatively, the major components of all LPS preparations were similar (table I). These LPS preparations were characterised by a nucleic acid content of <1% and a protein content of ≤2%. The total carbohydrate, hexose, and NANA contents of virulent strains were relatively higher than those of the avirulent strains. In contrast, the lipid A content was higher in avirulent strains. Other LPS components varied between strains and there was no correlation between the amount and virulence.

The concentration of the components of the LPS of *E. coli* 0111B₄ was much higher than those of *H. ducreyi* strains and *N. gonorrhoeae* P-6472, except lipid A, NANA and phosphate concentrations which were lower than those of *H. ducreyi*.

The KDO content of the LPS of *H. ducreyi* strains and *N. gonorrhoeae* P-6472 was <1% when LPS was hydrolysed with 0.2 N H₂SO₄ (table II). Hydrolysis of LPS with 4 N HCl released KDO from the LPS in amounts similar to that released from *E.*

coli 0111B₄. The amount of KDO released from *N. gonorrhoeae* LPS by 4 N HCl was substantially higher than that from *E. coli* and *H. ducreyi*.

Identification of glycoses

Hexoses and hexosamines identified in the LPS of *H. ducreyi* strains by GLC were glucose, galactose, glucosamine and galactosamine (table III). Ribose in the concentration range 0.01–0.2 nmol/μg of LPS was present. Fucose was present in trace amounts but mannose and rhamnose were not detected. Rhamnose was also absent from LPS of *N. gonorrhoeae* P-6472 but these sugars were identified in *E. coli* 0111B₄. The total hexose:hexosamine concentration of the LPS of virulent strains 35000, 409, C148, and A77* exceeded that of avirulent strains A76, 36-F-2, A77, and A75.

Total glycoses:KDO ratios for virulent strains were also higher than those of avirulent strains (table II). This ratio for nonpilated *N. gonorrhoeae* P-6472 was in the same range as those of the avirulent *H. ducreyi* strains.

Identification of fatty acids

C14, C16, C18, and C20 fatty acids were identified by GLC in the lipid A moiety of *H. ducreyi*

Table I. Chemical composition of lipopolysaccharides isolated from *H. ducreyi*, *E. coli* and *N. gonorrhoeae*

Strain	Percentage (dry wt) of lipopolysaccharide*								
	Total carbohydrate	Total hexose	Hexosamine	Hep- tose	Pro- tein	Nucleic acid	Phos- phate	NANA	Lipid A
<i>H. ducreyi</i> (virulent)									
35000	25.6	20.3	7.3	7.1	2.0	0.3	4.5	3.7	41.3
409	26.8	20.0	5.4	6.5	1.8	0.4	4.8	3.7	32.6
C148	24.5	16.8	5.5	5.2	1.7	0.5	4.7	2.7	35.8
A77*	25.9	19.7	6.3	7.8	1.9	0.8	4.6	3.1	32.5
(avirulent)									
A77	20.7	11.8	5.8	8.0	1.8	0.3	4.8	2.8	47.5
A75	22.8	13.5	7.0	7.4	0.9	0.3	5.1	1.2	43.6
36-F-2	19.0	13.0	4.1	8.0	1.1	0.2	4.8	1.2	42.5
A76	20.3	12.6	5.6	7.8	1.3	0.3	5.3	2.2	48.5
<i>E. coli</i> 0111B ₄	44.5	34.7	23.3	12.5	0.8	0.9	3.5	0.6	25.1
<i>N. gonorrhoeae</i> P-6472	30.0	21.8	5.5	4.5	2.1	0.6	4.3	0.9	15.5

NANA = N-acetylneuraminic acid.

* Mean of three separate determinations.

Table II. KDO content of LPS preparations from *H. ducreyi*, *E. coli* and *N. gonorrhoeae*

Strain	Karkhanis Method*		Brade Method†	
	Per cent KDO	Ratio Glycose:KDO	Per cent KDO	Ratio Glycose:KDO
<i>H. ducreyi</i> (virulent)				
35000	0.7‡	2.8	4.0	0.49
409	0.6	3.7	4.0	0.57
C148	0.4	3.5	3.8	0.37
A77*	0.7	3.1	4.4	0.50
(avirulent)				
A77	0.7	1.3	4.4	0.20
A75	0.6	1.9	4.3	0.27
36-F-2	0.6	1.6	3.8	0.31
A76	0.6	1.9	4.2	0.23
<i>E. coli</i> 0111B ₄	3.4	0.9	4.3	0.71
<i>N. gonorrhoeae</i> P-6472	0.9	1.8	7.6	0.21

KDO = 2-Keto-3-deoxyoctulosonic acid.

* KDO released by 0.2 N H₂SO₄ (Karkhanis *et al.*, 1978).† KDO released by 4 N HCl (Brade *et al.*, 1983).

‡ Each figure represents the mean of three separate determinations.

Table III. Monosaccharide content of the LPS from *H. ducreyi*, *N. gonorrhoeae* and *E. coli*

Strain	Glycose concentration* (nmol/μg of LPS)							
	Glu-cose	Galac-tose	Man-nose	Rham-nose	Fucose	Gluco-samine	Galacto-samine	Total
<i>H. ducreyi</i> (virulent)								
C148	0.41	0.30	0	0	0.04	0.42	0.25	1.42
409	0.81	0.53	0	0	0.04	0.51	0.38	2.27
35000	0.71	0.48	0	0	0.01	0.47	0.30	1.97
A77*	0.79	0.41	0	0	0.06	0.52	0.41	2.19
(avirulent)								
A77	0.27	0.19	0	0	0.01	0.32	0.12	0.91
A75	0.48	0.33	0	0	0.01	0.20	0.16	1.18
A76	0.34	0.21	0	0	0.005	0.24	0.16	0.955
36-F-2	0.40	0.26	0	0	0.01	0.31	0.21	1.19
<i>N. gonorrhoeae</i> P-6472	0.64	0.44	0.02	0	0.15	0.31	0.17	1.73
<i>E. coli</i> 0111B ₄	1.10	0.69	0.36	0.03	0.04	0.56	0.31	3.09

* Determined by GLC as TMS derivatives.

Table IV. Lipid A fatty acid content of *H. ducreyi* and *E. coli* O111B₄

Strain	Concentrations (nmol/ μ g of lipid A) of fatty acids of chain length				
	C12	C14	C16	C18	C20
<i>H. ducreyi</i>					
409	0	0.61†	0.06†	0.10	0.05
35000	0	0.65	0.04	0.12	0.05
A77*	0	0.72	0.03	0.14	0.03
A77	0	0.68	0.04	0.10	0.07
A75	0	0.70	0.04	0.14	0.07
36-F-2	0	0.75	0.06	0.16	0.07
A76	0	0.61	0.04	0.16	0.08
<i>E. coli</i>					
111B ₄	0.17	0.58	0.22	0.09	0.05

† Values represent the fatty acid concentrations (nmol/ μ g lipid A), as determined by GLC.

strains (table IV). C₁₂ (lauric) acid, was identified in *E. coli*, but was absent in *H. ducreyi* strains. OH-substituted C₁₄ (β -hydroxymyristic) acid was identified tentatively in *E. coli* and *H. ducreyi*, based on the relative retention time of this acid. However, no

standard was available at the time of analysis to confirm the identification.

Quantitatively, there was little difference in fatty acid content between virulent and avirulent *H. ducreyi*. It has been suggested (Rietschel *et al.*, 1984) that C18 and C20 acids in *E. coli* O111B₄ commercial LPS preparations are phospholipid contaminants, but this cannot be ascertained with certainty for the *H. ducreyi* strains.

SDS-Page of LPS

The electrophoretic profiles of the LPS of virulent and avirulent *H. ducreyi* are shown in fig. 1. Patterns of the avirulent strains (lanes A–D) appeared similar to each other but differed from the patterns of the virulent strains (lanes E–K). The LPS profile of serum-resistant isogenic strain A77* in lane E differed from that of serum-sensitive parent strain A77 in lane D. The isogenic strain was virulent in the rabbit model, whereas the parent strain was avirulent.

The LPS profiles of all *H. ducreyi* strains examined lacked the high mol. wt subunits present in the LPS of *E. coli* (lane I) (fig. 2). Such bands apparently represent the O-side chains of smooth LPS (Jann *et al.*, 1975).

Fig. 1. Electrophoretic profiles of the LPS of *H. ducreyi*. Avirulent strains: lanes A, 36-F-2; B, A76; C, A75; D, A77. Virulent strains: lanes E, A77*; F, 35000; G, 557; H, CH39; I, BG411; J, 409; K, 78226.

Fig. 2. Electrophoretic profiles of the LPS of *H. ducreyi* strains and *E. coli* O111B₄. *H. ducreyi* strains: lanes A, C148; B, 35000; C, A77*; D, 409; E, A77; F, A75; G, A76; H, 36-F-2; lane I, *E. coli* O111B₄. Each well contained 2 µg of LPS.

Infra-red spectra

The IR spectra of the LPS of *H. ducreyi* A76, 35000, 409, and A77* were identical and were similar to those of *E. coli* O111B₄ and *N. gonorrhoeae* P⁻ 6274 (fig. 3). The spectra contained broad absorption peaks at 3350/cm attributed to hydroxyl

radicals characteristic of carbohydrates. Absorption bands at 1650 and 1510 to 1550 were due to monosubstituted amide groups ($\text{—NH}\cdot\text{CO}\cdot\text{CH}_3$). The presence of an ester carbonyl band at 1720/cm and the broad absorption in the range from 1000/cm to 12000/cm characterised the LPS preparation as containing fatty acid esters. IR scans of *H. ducreyi* LPS lacked the sharp absorption bands at 1275/cm and the fatty acid ester band at 1300/cm⁻¹, which were present in the *E. coli* LPS scan.

Discussion

The gross chemical composition of the LPS of *H. ducreyi* strains was similar to that of other gram-negative bacteria, as indicated by IR spectra and chemical analysis. Although there were no qualitative differences in the LPS of virulent and avirulent *H. ducreyi* strains, the total glucose:KDO ratio of the LPS of virulent strains exceeded that of avirulent strains. Glycoses such as rhamnose and mannose which are characteristic of the O-specific chains of the LPS of many gram-negative bacteria were not identified in *H. ducreyi*. Thus, differences appear to reside in the core polysaccharide. Virulent strains, in contrast to avirulent strains, have been shown previously (Odumeru *et al.*, 1984) to be resistant to complement-mediated bactericidal action of human and rabbit serum. Strain C148 which has a lower glucose:KDO ratio (table II) than virulent strains 35000, 409, and A77 was less resistant to the bactericidal action of serum than these strains (Odumeru *et al.*, 1984). Strains A77, A74, and A76 which had the lowest glucose:KDO ratios, were susceptible to the bactericidal action of serum (Odumeru *et al.*, 1984). Thus, differences in the serum-sensitivity of strains may be associated with the amount of carbohydrate present in the core region of the LPS.

The KDO content of *H. ducreyi* and *N. gonorrhoeae* P⁻ 6472 was low (< 1%), compared with that of *E. coli* O111B₄ (3.5%), when 0.2N H₂SO₄ was used in hydrolysis. Low KDO content has been reported for *H. influenzae* types a–f (Zoon and Scocca, 1975; Fleisher and Insel, 1978). However, Parr and Bryan (1984) detected KDO in three strains of *H. influenzae* at levels similar to that in a strain of *S. typhimurium* (c. 3%), by hydrolysing LPS with 4 N HCl, as described previously by Brade *et al.* (1983). Hydrolysis of *H. ducreyi* LPS with 4 N HCl yielded more TBA-reactive material producing KDO levels similar to that of *E. coli* O111B₄. This procedure frees C-4 and C-5 substituted KDO from LPS (Brade *et al.*, 1983), indicating that KDO in *H. ducreyi* is highly substituted. The same is true of *N.*

Fig. 3. Infra-red spectra of the lipopolysaccharides of *H. ducreyi* strains: A77* (A), A76 (B), 35000 (C), 409 (D); *N. gonorrhoeae* P⁻6472 (E); and *E. coli* O111B₄ (F).

gonorrhoeae P⁻6472 LPS, in which KDO at levels higher than *E. coli* and *H. ducreyi* was detected after strong acid hydrolysis.

SDS-PAGE analysis of LPS revealed differences in the electrophoretic profiles of virulent and avirulent strains. The profiles of avirulent strains were similar, but differed from those of virulent strains as exemplified by serum-resistant isogenic strain A77* which is virulent, and serum-sensitive parent strain A77 which is avirulent in the rabbit model. *H. ducreyi* strains contained LPS of low mol. wt, suggesting that the O-side chains typical of smooth LPS of *E. coli*, *Salmonella* spp., and *Shigella* spp. (Luderitz *et al.*, 1971; Jann *et al.*, 1975) are not present in these strains. These observations support the chemical data that glycoses characteristic of O-side chain LPS such as mannose and rhamnose were not detected. However, the non-pilated *N. gonorr-*

hoeae 6472 contained a trace amount of mannose. Thus, differences in mobility of the LPS of virulent and avirulent strains may reflect differences in the amount of carbohydrates associated with the core polysaccharide. Other gram-negative bacteria such as *N. meningitidis*, *N. gonorrhoeae*, *Bordetella pertussis*, *H. influenzae*, and also *Chlamydia* spp. have a "rough type" LPS (Tsai and Frasch, 1982; Inzana, 1983; Caldwell and Hitchcock, 1984; Logan and Trust, 1984; Peppler, 1984), indicating that "smooth" type LPS is not a prerequisite for virulence of these organisms.

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