Structurally Defined Epitopes of *Haemophilus ducreyi* Lipooligosaccharides Recognized by Monoclonal Antibodies

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By use of enzyme-linked immunosorbent assay and immunoblotting techniques, the migration patterns and binding epitopes of lipooligosaccharides (LOS) from 10 *Haemophilus ducreyi* strains were investigated with two monoclonal antibodies (MAbs), MAHD6 and MAHD7, raised against LOS from *H. ducreyi* ITM 2665. Closely related LOS, with defined structures, from *Haemophilus influenzae*, *Bordetella pertussis*, *Aeromonas* spp., and synthetic glycoproteins were also included in the analyses. The MAbs bound to conserved epitopes of LOS exposed on the surface of *H. ducreyi*. The MAb MAHD6 reacted with 8 of the 10 LOS from *H. ducreyi* but with none of the other *Haemophilus* or *Bordetella* spp. with structurally defined LOS. It is suggested that MAb MAHD6 binds to a LOS epitope (-DD-Hepp-1→6-β-D-Glcp-). This LOS epitope is not present in the hexasaccharide structure of LOS from *H. ducreyi* ITM 4747 (E. K. H. Schweda, A. C. Sundström, L. M. Eriksson, J. A. Jonasson, and A. A. Lindberg, *J. Biol. Chem.* 269:12040–12048, 1994). Because MAb MAHD6 reacts with the epitope mentioned above, it also discriminates between the two LOS structures, the hexasaccharide group and the nonasaccharide group, of *H. ducreyi* strains. MAb MAHD7 recognizes the common conserved inner core region of the LOS because it reacts with all *H. ducreyi* strains and with LOS with minor components in the inner core epitope structure. Rabbit polyclonal sera raised against the LOS from strains CCUG 4438 and CCUG 7470 were tested with the 10 LOS from the *H. ducreyi* strains. The antiserum to CCUG 7470 reacted with all *H. ducreyi* strains as did MAb MAHD7, whereas the antiserum to CCUG 4438 reacted with only its homologous strain and strain ITM 4747. Also, the LOSs of our reference strains CCUG 4438 and CCUG 7470 were structurally analyzed by use of sugar analyses and electrospray ionization-mass spectrometry. The hexasaccharide and nonasaccharide structures obtained from LOS of strains CCUG 4438 and CCUG 7470 were identical to the described LOS structures from *H. ducreyi* ITM 4747 and ITM 2665, respectively. In conclusion, the MAb MAHD6 recognizes an epitope present in the nonasaccharide LOS group, whereas the MAb MAHD7 recognizes a conserved epitope on LOS of *H. ducreyi*, which is present in all strains of *H. ducreyi* tested. Two major groups of oligosaccharides were distinguished by their LOS structures and the reactivity of monoclonal as well as polyclonal antibodies. The majority of *H. ducreyi* strains possess a nonasaccharide structure of LOS.

Haemophilus ducreyi is the causative agent of chancroid, a sexually transmitted genital ulcer disease that is endemic in large parts of developing countries, and an increasing incidence in the United States and Canada has recently been reported (2, 11, 21, 24). Infection with *H. ducreyi* has been identified as a risk factor for the sexual transmission of the human immunodeficiency virus (13, 27).

Electrophoretic analyses of lipooligosaccharides (LOS) from *H. ducreyi* and other *Haemophilus* and *Neisseria* species show similar migration patterns, indicating that they lack the repeating polysaccharide O antigen that is characteristic of lipopolysaccharide from most gram-negative enteric bacteria (6, 8, 26). The LOS from *H. ducreyi* has been implicated as an important factor in the pathogenesis of chancroid, because intradermal injection of LOS from *H. ducreyi* into experimental animals resulted in skin lesions (7, 15, 16). It has been suggested that the LOS of some mucosal pathogens such as *Haemophilus* and

Neisseria species may play a role in the evasion of host immune responses by presenting a terminal trisaccharide that is antigenically similar to the terminal trisaccharide of paragloboside found in a variety of human cells (19, 36). In addition, sialic acid has been reported to be present in most of the LOS of these mucosal pathogens (19, 20).

The structures of six LOS from *H. ducreyi* strains have been reported (22, 31, 32). Five of these show a nonasaccharide in the outer core region. However, the reported structure of LOS from strain ITM 4747, a hexasaccharide, has a shorter oligosaccharide in the outer region than that of other LOS (32).

Studies of the molecular structure and antigenic variation of *H. ducreyi* LOS may increase our knowledge about the role played by these components in the pathogenic and immunologic responses to *H. ducreyi* and may contribute to a better understanding of the epidemiology of chancroid.

Monoclonal antibodies (MAbs) that are capable of detecting different immunogenic components of LOS molecules may provide valuable information for understanding the kind of epitopes that may be responsible for specific detection of a pathogen or for protection. Recently, we reported the production of MAbs MAHD6 and MAHD7. These MAbs were found

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to be relatively, although not absolutely, specific and reacted with nearly all strains of *H. ducreyi* tested, i.e., 59 of 60 and 60 of 60 for MAHD6 and MAHD7, respectively (1). We have also demonstrated the diagnostic usefulness of MAb MAHD7 for direct antigen detection in clinical specimens (1).

The aim of this study was to determine the LOS epitopes recognized by the broadly reactive MABs MAHD6 and MAHD7, by using structurally defined LOS and synthetic glycoproteins, to classify different *H. ducreyi* strains on a structural basis, and to evaluate the antigenic reactivities of LOS.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and LOS preparation. The 10 *H. ducreyi* strains used in this study were isolates obtained from patients from different geographical locations. Six of the 10 *H. ducreyi* strains were kindly provided by P. Piot and E. Roggen, Institute of Tropical Medicine (ITM), Antwerp, Belgium, namely, strains ITM 2665, ITM 5535, ITM 3147, ITM 3542, ITM 4747, and ITM 3207. Strain ACY1 is a clinical isolate from Huddinge Hospital, Huddinge, Sweden. The remaining three strains were obtained from the culture collection at the University of Gothenburg, i.e., CCUG 7470 (=CIP 76118), CCUG 4438 (=CIP 542), and CCUG 10045. *H. influenzae* RM 7004 and Rb⁻/b⁺ 169 were kindly provided by E. R. Moxon (Oxford, United Kingdom). *H. influenzae* 2019 was provided by M. A. Apicella, Iowa City, Iowa. In addition, another *H. influenzae* noncapsulated variant of type b strain RAB (CCUG 17780) was included. *Bordetella pertussis* 1144 LOS was acquired from M. Caroff, Orsay, France, and the lipopolysaccharides from *Aeromonas* strains were obtained from D. H. Shaw, Northwest Atlantic Fisheries Centre, St. John's, Newfoundland, Canada. Table 1 shows all the chemically defined LOS included in this study.

H. ducreyi strains were cultured in large petri dishes on a medium containing 4.25% Columbia agar base (BBL Microbiology Systems), 5% fetal calf serum (Gibco BRL Life Technologies, Paisley, United Kingdom), 15% sheep blood, 3 mg of vancomycin (Sigma Chemical Co., St. Louis, Mo.) per liter, and 1.5% IsoVital X (BBL). To provide CO₂, the plates were incubated in jars containing the anaerobic GasPak system (BBL) for 48 h at 34°C as described previously (1). Bacterial colonies were harvested and suspended in Luria broth and frozen at -70°C for LOS extraction. The LOS was extracted from lyophilized bacteria by the phenol-water method (39).

MABs to LOS from *H. ducreyi*. The production, partial characterization, and specificity of the MABs to LOS from *H. ducreyi* have been reported earlier (1). MABs MAHD6 and MAHD7 were used for detection and characterization of LOS antigen from *H. ducreyi*.

Rabbit immune sera. New Zealand White rabbits were immunized with sonic preparations from strains CCUG 4438 and CCUG 7470 as described earlier (15, 16). The rabbits were bled before and after immunization. One rabbit nonimmune serum, designated K 58, was used as a negative control. The rabbit immune sera were designated as follows: K 25 antiserum to strain CCUG 4438 and K 27 and K 55 antisera to strain CCUG 7470. Additionally, one rabbit was immunized with the oligosaccharide part of LOS conjugated to tetanus toxoid (TT) and prepared as follows. The LOS from *H. ducreyi* ITM 2665 was de-O-acetylated with anhydrous hydrazine as described previously, which resulted in the loss of lipid A activity as determined by the *Limulus* lysate assay (9). The de-O-acetylated LOS was conjugated to TT by use of a scheme closely similar to one used previously for conjugation of *Streptococcus* group B type III polysaccharide and TT (17). The oligosaccharide content in the conjugate was measured by the anthrone reaction (35), with de-O-acetylated LOS as a standard. The protein content was measured by the method of Bradford (5). The oligosaccharide/protein ratio in the conjugate was 1:4.5. One rabbit was injected at four injection sites subcutaneously with the conjugate mixed with Freund's complete adjuvant containing 50 µg of oligosaccharide. An additional 50 µg of oligosaccharide with Freund's incomplete adjuvant was given two times at 3-week intervals. A total of three doses of the conjugate were given to the rabbit. This antiligosaccharide serum was designated K 59.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed as described previously (12). The plates were coated with 100 µl of a 10-µg/ml concentration of LOS or 5 µg of the synthetic oligosaccharides mentioned below per ml, diluted in 0.05 M carbonate buffer (pH 9.6) (Merck, Darmstadt, Germany) and stored overnight at 4°C. Coated plates were blocked by incubation with 1% (wt/vol) bovine serum albumin (Sigma) in phosphate-buffered saline (PBS) for 2 h at room temperature. The plates were then washed three times with washing buffer (0.15 M NaCl and 0.05% [vol/vol] Tween 20; Merck). A 100-µl volume of serum-free culture supernatant of MABs MAHD6 and MAHD7, diluted 1:10 in PBS containing 0.05% Tween 20 or rabbit immune serum (started at a dilution of 1:200 and then diluted twofold in 0.1% BSA-PBS-Tween 20), was added per well, and the plates were incubated for 2 h at 37°C. As a negative control, a reference nonimmunized rabbit serum was used. After the plates were washed as before, 100 µl of alkaline phosphatase-labelled anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG (Jackson Immuno Research Lab, West Grove, Pa.) diluted 1:2,000 or 1:5,000, respectively, in BSA-PBS-Tween 20 was added per well, and the plates were incubated overnight at

room temperature. The plates were washed again and developed with 100 µl of substrate solution (1 mg of disodium *p*-nitrophenyl phosphate [Sigma] per ml in 1 M diethanolamine-0.5 mM MgCl₂ buffer [pH 9.8]) per well. The absorbance at 405 nm (*A*₄₀₅) was measured within 60 min with a Titertek Multiskan photometer (Flow Laboratories, Irvine, Scotland). The dilution giving an absorbance of approximately 0.2 greater than the background value at *A*₄₀₅ was determined as the endpoint titer.

Synthetic oligosaccharides. Ten synthetic glycoproteins (lactose, globotriose, lacto-*N*-tetraose, lacto-*N*-neotetraose, globotetraose, lacto-*N*-neohexaose, lacto-*N*-hexaose, 3'-sialyllactose, 6'-sialyllactose, and sialyllactose-*N*-neotetraose c) with the oligosaccharides covalently linked to human serum albumin and containing 10 to 20 equivalents per oligosaccharide per molecule of human serum albumin were purchased from Accurate Chemical, Inc. (Westbury, N.Y.), or Bio-Carb Chemicals (Lund, Sweden).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The LOS from *H. ducreyi* was solubilized and electrophoresed on a gel containing 14% acrylamide by the method of Laemmli (14). One gel was silver stained as described previously (37). Immunoblotting was performed as described previously (14). The MABs were diluted 1:5, and the rabbit serum was diluted 1:200. The antibodies bound were detected by horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Bio-Rad Laboratories, Richmond, Calif.) dilutions of 1:2,000 and 1:3,000, respectively, in BSA-PBS-Tween 20. For immunoblot development, the substrate color reagent 4-chloronaphthol (Bio-Rad) was used.

Antibody binding capacity after treatment of LOS with neuraminidase. The neuraminidase treatment of LOS from all 10 *H. ducreyi* strains was performed as described elsewhere (4). ELISA plates were coated with 25 µg of LOS per ml in PBS and incubated for 2 h. The plates were washed with PBS (pH 6) and incubated for 2 h with 25 µl of serially diluted (in PBS [pH 6], 0.05 to 100 mU/ml) *Clostridium perfringens* neuraminidase (Sigma type V neuraminidase, 1.0 U/mg of solid; proteinase, ≤0.001 U/mg of solid; *N*-acetylneuraminic acid aldolase, 0.024 U/mg of solid) per well. Heat-inactivated neuraminidase was added at equal dilutions in parallel rows; PBS was used as a negative control. After 2 h, the plates were blocked by incubation with 1% BSA-PBS and then processed as described for the ELISA. The antibody dilution used for this test was at the middle of the linear portion of the titration curve, which corresponds to an *A*₄₀₅ of about 1.0 when tested against the corresponding untreated LOS antigen (25 µg/ml in PBS).

Structural analysis of LOS from CCUG 4438 and CCUG 7470. The *H. ducreyi* strains CCUG 4438 (=CIP 542) and CCUG 7470 (=CIP 76118) are reference strains in our laboratory representing nontoxigenic and toxigenic strains, respectively. The de-O-acetylation of LOS was performed as described elsewhere (9). Briefly, the LOS from *H. ducreyi* CCUG 4438 and CCUG 7470 was incubated with 2.5 ml of anhydrous hydrazine for 30 min at 37°C. The sample was cooled to -20°C, and acetone (2.5 ml, -20°C) was added to precipitate the de-O-acetylated LOS. After centrifugation, the supernatant was removed and the pellet was washed again with cold acetone and recentrifuged. The precipitate was then resuspended in water and lyophilized to give de-O-acetylated LOS, which was analyzed by electrospray ionization-mass spectrometry (ESI-MS) without further purification.

Analytical and spectroscopic methods. For gas-liquid chromatography, a Hewlett-Packard 5890 instrument fitted with a flame ionization detector was used. Separation of alditol acetates was performed on a DB-5 fused-silica capillary column with a temperature gradient of 160°C (2 min) to -220°C at 4°C/min. De-O-acetylated LOS from *H. ducreyi* CCUG 4438 and CCUG 7470 was analyzed by ESI-MS on a VG/Fisons Quattro II quadrupole mass spectrometer in the negative mode. The sample (1 mg) was first dissolved in 1 ml of a mixture of water and acetonitrile (1:1) with 0.1% ammonia solution added. Of this solution, 10 µl was injected via a syringe pump into a running solvent of H₂O-CH₃CN (1:1) at a flow rate of 5 µl/min.

Other analytical procedures. Sugars were identified as their alditol acetates by use of authentic standards (29). The relative proportions of various alditol acetates obtained in sugar analyses correspond to the detector response of the gas-liquid chromatography. For estimation of the sialic acid, the LOS was hydrolyzed in 0.05 M sulfuric acid for 60 min at 80°C. The liberated sialic acids were isolated by ion-exchange chromatography, and fractions were quantitated by the resorcinol assay (34).

RESULTS

Binding of MAB to LOS by ELISA. The binding of MABs MAHD6 and MAHD7 was tested against LOS prepared from 10 different *H. ducreyi* strains by ELISA. The chemical structures of 7 of the 10 LOS from *H. ducreyi* tested are shown in Table 1. MAHD6 reacted with eight LOS but not with the LOS from ITM 4747 and CCUG 4438. In contrast, MAHD7 reacted with all 10 LOS (Table 1). However, MAB MAHD7 reactivity to ITM 4747 and CCUG 4438 LOS was not as strong as the reactivity to other LOS. Further investigation of MAB binding

TABLE 1. ELISA reactivity of anti-LOS *H. ducreyi* MAbs MAHD6 and MAHD7 when tested against chemically defined structures of LOS from *H. ducreyi*, *H. influenzae*, *B. pertussis*, and *Aeromonas* strains

Source of LOS (references)	Chemical structures of major core oligosaccharides ^a	Mean A_{405} ^b	
		MAHD6	MAHD7
<i>H. ducreyi</i> ITM 2665, ITM 3147, ITM 5535, ACY1, CCUG 7470, and 35000 ^c (22, 31, 32)	Gal β →4GlcNAc1→3Gal β 1→4DDHep α 1→6Glc β 1→4Hep α 1-Kdo(P)-lipid A α ↑ 1,3 Hep α →2Hep	>3.0	>3.0
<i>H. ducreyi</i> ITM 3542, CCUG 10045, and ITM 3207	ND ^d	ND >1.0–3.0	ND >1.0–3.0
<i>H. ducreyi</i> ITM 4747 and CCUG 4438 (32)	Gal β 1→4Glc β 1→4Hep α 1→Kdo(P)-lipid A α ↑ 1,3 Hep α 1→2Hep	<0.2	>1.0
<i>H. influenzae</i> 2019 nontypeable (25)	Gal β 1→4Glc β 1→4Hep α 1→Kdo(P)-lipid A α ↑ 1,3 Hep α 1→2Hep ↑ 6 ↑ 6 PEt PEt	<0.2	>1.0
<i>H. influenzae</i> RM 7004, XP-1, and AHI-3 (30)	Gal β 1→4Glc β 1→4Hep α 1→Kdo(P)-lipid A α ↑ 1,3 Gal β 1→2Hep α 1→2Hep ↑ 6 PEt	0.1	0.1
<i>H. influenzae</i> Rb ⁻ /b ⁺ 169 (10)	Kdo(P)-lipid A	<0.1	<0.1
<i>H. influenzae</i> CCUG 17780	ND	<0.2	<0.2
<i>B. pertussis</i> 1414 (18)	Glc β 1→4Hep α 1→5Kdo(P)-lipid A α ↑ 1,3 GlcA α 1→2Hep α ↑ 1,7 GlcN	<0.1	<0.1
<i>A. hydrophila</i> SJ-26 (23)	Hep α ↓ 1,6 Glc β 1→4Hep α 1→2Hep α 1→6Kdof-lipid A α ↑ 1,3 GlcNAc1-7Hep	>3.0	>3.0
<i>A. hydrophila</i> SJ-55 (3)	Hep α ↓ 1,6 Gal β 1→[DDHep α 1→6]4DDHep α 1→6Glc β 1→4Hep α 1→2Hep α 1→6Kdof-lipid A α ↑ 1,3 GlcNAc1-7Hep	>3.0	>3.0
<i>A. salmonicida</i> SJ-83 (33)	Hep α 1-2Hep α 1-3Hep α 1-6Kdof-lipid A	<0.2	<0.2

^a Abbreviations: Gal, galactose; Glc, glucose; Hep, L-glycero-D-manno-heptose; DDHep, D-glycero-D-manno-heptose; GlcN, glucosamine; GlcNAc, N-acetylglucosamine; GlcA, glucuronic acid.

^b Culture supernatants of MAbs MAHD6 and MAHD7 were diluted 1:10. An A_{405} of ≤ 0.2 after 60 min was considered negative.

^c *H. ducreyi* 35000 is not included in this study; however, we used the published chemical structure in the table to indicate that the majority of chemically defined LOS from *H. ducreyi* strains bear nonsaccharide structures in their LOS.

^d ND, not done.

to LOS epitopes was carried out with other chemically defined LOS (Table 1). Four *H. influenzae* strains, including strain 17780, which is not structurally characterized, one *B. pertussis* strain, three *Aeromonas* strains, and 10 synthetic glycoproteins with oligosaccharide covalently linked to human serum albumin were tested by ELISA. All of the LOS from *H. influenzae* and *B. pertussis* failed to react with MAb MAHD6, and only

the LOS of *H. influenzae* 2019 reacted with MAb MAHD7 (Table 1). The chemical structures of LOS from *H. influenzae* 2019 are similar to that of LOS from ITM 4747. Reactions were also observed with both MAbs with two of the three *Aeromonas* LOS (Table 1). None of the MAbs bound to any of the 10 oligosaccharides conjugated to human serum albumin (data not shown).

TABLE 2. ELISA reactivity of K 25 antiserum to strain CCUG 4438, K 27 and K 55 antisera to strain CCUG 7470, K 59 antioligosaccharide to LOS 2665, and nonimmune serum K 58 tested against 10 LOS from *H. ducreyi* strains

LOS from <i>H. ducreyi</i> strain	Mean A_{405} ^a				Nonimmune serum K 58
	Rabbit hyperimmune sera				
	K 25	K 27	K 55	K 59	
ITM 2665	1.5	2.6	2.3	2.0	0.2
ITM 3147	1.0	2.5	2.1	2.0	0.2
ITM 3207	1.4	2.4	2.2	1.4	0.1
ITM 3542	1.6	2.6	2.3	1.8	0.2
ITM 4747	2.6	1.6	1.4	0.4	0.1
ITM 5535	1.0	2.6	2.5	1.4	0.1
ACY1	1.5	2.6	2.5	2.1	0.2
CCUG 4438	2.8	1.2	0.6	0.3	0.1
CCUG 7470	0.7	2.7	2.5	1.5	0.2
CCUG 10045	0.8	1.7	0.8	0.7	0.1

^a Rabbit hyperimmune and nonimmune sera were diluted 1:200. An A_{405} of ≤ 0.2 after 60 min was considered negative.

On the basis of the majority of these data, the LOS epitopes recognized by MAb MAHD6 and MAHD7 are suggested to be, respectively, as follows:

and

where Kdo is 3-deoxy-D-manno-octulosonic acid.

Binding of rabbit polyclonal sera to *H. ducreyi* LOS tested by ELISA. To investigate the reactivity of polyclonal sera, rabbit sera raised against different *H. ducreyi* strains, as well as a nonimmune serum (K 58) as a negative control, were tested with 10 LOS from the *H. ducreyi* strains by the ELISA. No reactivity of the nonimmune serum was noted in the ELISA (after the dilution of 1:100), whereas the hyperimmune sera reacted in dilutions of up to 1:8,000. The hyperimmune sera raised against CCUG 7470 (K 27 and K 55) detected all 10 LOS, although the absorbance values to LOS from CCUG 4438 and ITM 4747 were considerably lower. Table 2 shows the ELISA reactivity of the rabbit polyclonal sera. Antiserum to CCUG 4438 LOS (K 25) emitted a stronger reaction to the LOS of its homologous strain CCUG 4438 and to the LOS of ITM 4747 than antisera to the other eight LOS. Antiserum to the oligosaccharide part of LOS from *H. ducreyi* ITM 2665 (K 59) bound strongly to eight LOS but not to LOS from CCUG 4438 and ITM 4747 (Table 2). When this serum, K 59, was tested against de-O-acylated LOS from strain 2665 in the ELISA, it gave an absorbance value of 0.21, indicating that the inner core structure may have been influenced by the de-O-acylation procedure.

SDS-PAGE silver staining and immunoblot analysis. Figure 1 depicts the silver staining of nine LOS from *H. ducreyi*. Strains CCUG 4438 and ITM 4747 showed some heterogeneity with bands of apparently lower molecular weight representing LOS having approximately three fewer sugar residues than the remaining seven LOS. The results of SDS-PAGE and immunoblotting of LOS with MAHD6 and MAHD7 are shown in

FIG. 1. SDS-PAGE and silver staining of LOS from *H. ducreyi* strains. LOS preparations (10 $\mu\text{g/ml}$) from *H. ducreyi* CCUG 10045, ACY1, CCUG 7470, ITM 5535, CCUG 4438, ITM 4747, ITM 2665, ITM 3147, and ITM 3542 (lanes 1 to 9, respectively) are shown.

Fig. 2. MAb MAHD6 bound to eight of the LOS but not to the LOS from CCUG 4438 and ITM 4747, as shown in Fig. 2A. This suggests that the LOS epitope recognized by this MAb is not present in these LOS. MAb MAHD7 bound to all LOS from *H. ducreyi*. However, the bands detected for LOS from CCUG 4438 and ITM 4747 appear to have a lower molecular weight than that of the reacted bands of the other LOSs (Fig. 2B).

The rabbit immune serum K 25 bound only to the homologous strain CCUG 4438 and to strain ITM 4747 (Fig. 3A), which is in contrast to rabbit polyclonal sera K 27 and K 55, which reacted with all of the *H. ducreyi* strains (Fig. 3B). The rabbit polyclonal serum to the oligosaccharide part of LOS from ITM 2665 (K 59) bound to eight LOS but not to LOS from CCUG 4438 and ITM 4747. This also indicates that the de-O-acylation might have changed the inner core structure of

A

B

FIG. 2. Immunoblot analysis of LOS from *H. ducreyi* strains tested with MAb MAHD6 (A) and MAHD7 (B) raised against LOS ITM 2665. LOS preparations (10 $\mu\text{g/ml}$) from *H. ducreyi* ITM 3207, CCUG 10045, ACY1, CCUG 7470, ITM 5535, CCUG 4438, ITM 4747, ITM 2665, ITM 3147, and ITM 3542 (lanes 1 to 10, respectively) are shown.

FIG. 3. Immunoblot analysis of LOS from *H. ducreyi* strains tested with polyclonal sera K 25 (A) and K 27 (B) raised against *H. ducreyi* CCUG 4438 and CCUG 7470, respectively. LOS preparations (10 µg/ml) from *H. ducreyi* ITM 3207, CCUG 10045, ACY1, CCUG 7470, ITM 5535, CCUG 4438, ITM 4747, ITM 2665, ITM 3147, and ITM 3542 (lanes 1 to 10, respectively) are shown.

the LOS, and the immunoblotting results in all analyses are consistent with those obtained by the ELISA.

Sialic acid concentration in LOS and effect of neuraminidase treatment of LOS for antibody binding capacity. Six of 10 *H. ducreyi* LOS and one *H. influenzae* LOS were analyzed for sialic acid content by the resorcinol method. All LOS preparations from *H. ducreyi* were shown to contain sialic acid. The LOS from ITM 4747 had the highest sialic acid content, and the LOS from CCUG 10045 had the lowest (47 and 5 µmol/g of LOS extract, respectively). No sialic acid could be detected in LOS of noncapsulated *H. influenzae* (Table 3). After LOS treatment with neuraminidase, no significant change in the

TABLE 3. Concentration of sialic acid in LOS from six *H. ducreyi* strains and an *H. influenzae* strain

Organism	Sialic acid ^a (µmol/g of LOS)
<i>H. ducreyi</i>	
ITM 4747	47
CCUG 4438	35
ITM 2665	21
CCUG 7470	13
ITM 3542	12
CCUG 10045	5
<i>H. influenzae</i> noncapsulated CCUG 17780	Not detectable

^a The sialic acid from LOS was liberated by hydrolysis in 0.05 M sulfuric acid, isolated by ion-exchange chromatography, and quantitated with the resorcinol assay (34).

TABLE 4. Sugar analyses of LOS from *H. ducreyi* CCUG 4438 and CCUG 7470^a

Monosaccharide	Molar ratio ^b	
	CCUG 4438	CCUG 7470
Glc	1	1
Gal	2.6	7
GNAc	0.4	0.9
LD-Hep	1.6	1.3
DD-Hep		0.7

^a Determined by Glc analysis of derived alditol acetates.

^b Molar ratio relative to Glc (taken as 1).

binding capacity of the MAbs was found as described above. These results suggest that the epitopes recognized by these MAbs are not affected by the presence or absence of sialic acid in LOS from *H. ducreyi*. However, in rabbit hyperimmune sera K 27 and K 55, about a 30% lower absorbance (0.5) was observed for the neuraminidase-treated LOS than for the non-treated LOS (1.5).

Structural analysis of LOS from *H. ducreyi* CCUG 4438 and CCUG 7470. The *H. ducreyi* strains CCUG 4438 and ITM 4747 differ in growth in solid media from the other *H. ducreyi* strains. The growth is slower and the colonies are somewhat smoother and less dry for these strains than for the majority of the *H. ducreyi* strains observed in our laboratory. Also, the colonies have a more polymorphic appearance. With respect to these differences, LOS structural analyses were undertaken. Sugar analyses of LOS preparation from *H. ducreyi* CCUG 4438 and CCUG 7470 were performed (Table 4). A hydrolysate of *H. ducreyi* CCUG 4438 contained glucose, galactose, 2-amino-2-deoxyglucose (GNAc), and L-glycero-D-manno-heptose (LD-Hep) in the relative proportions 1:2.6:0.4:1.6 and analyzed as alditol acetates. A hydrolysate of CCUG 7470 contained glucose, galactose, GNAc, LD-Hep, and D-glycero-D-manno-heptose (DD-Hep) in the relative proportions 1:6.6:0.9:1.3:0.7 and analyzed as alditol acetates.

The de-O-acetylated LOS of *H. ducreyi* CCUG 4438 and CCUG 7470 were analyzed by negative-ion ESI-MS. The data are summarized in Table 5. The ESI spectrum of *H. ducreyi* CCUG 4438 showed inter alia ions at *m/z* 716.7, 757.8, 813.8, and 855.6 and their sodium adducts at *m/z* 724.2, 765.2, 821.3, and 862.3. It was evident from the spectrum that the ions were triply charged and that the ion *m/z* 716.7 corresponded to a molecular mass of 2153.3 Da and was thus consistent with the structure Hex₂Hep₃Kdo(P)-de-O-acetylated-lipid A. *H. ducreyi* de-O-acetylated-lipid A is known to be composed of a bis-phosphorylated β-1,6-glucosamine disaccharide substituted by 3-hydroxytetradecanoamide groups at C-2 and C-2' (32). The ion at *m/z* 813.8 corresponded to a molecular mass of 2,444.5 Da, which indicated the presence of a sialylated compound with the structure Neu5AcHex₂Hep₃Kdo(P)-de-O-acetylated-lipid A. The ions at *m/z* 757.8 and 855.6 corresponded to molecular ions of 2,276.4 and 2,569.8 Da, respectively, and might be rationalized with Hex₂Hep₃KdoPPEA-de-O-acetylated-lipid A and Neu5AcHex₂Hep₃KdoPPEA-de-O-acetylated-lipid A, respectively. The ESI spectrum of de-O-acetylated *H. ducreyi* CCUG 7470 differed significantly from that of *H. ducreyi* CCUG 4438 and was virtually identical to the corresponding ESI-MS spectrum of *H. ducreyi* ITM 5535 (31). The spectrum showed triply charged major ions at *m/z* 904.0 and 1000.2 and their sodium adducts (*m/z* 909.7 and 1006.9), which corresponded to molecular masses of 2,715.1 and 3,003.0 Da, respectively, and could be rationalized with Hex₃HexNacHep₄Kdo(P)-

play a role in the evasion of host immune and defense responses. Studies of pathogenic *Neisseria* spp. have indicated that sialylation of LOS provides serum resistance to strains that were previously sensitive to serum (28).

Sugar analyses and ESI-MS of LOS preparations from *H. ducreyi* CCUG 4438 and CCUG 7470 gave similar results as those obtained from *H. ducreyi* ITM 4747 and ITM 2665, respectively (32), indicating the presence of identical major structures between strains CCUG 4438 and ITM 4747 and between strains CCUG 7470 and ITM 2665. Six of the eight LOS from *H. ducreyi*, all of which are chemically defined (22, 31, 32), have identical nonasaccharide structures in their LOS, while the remaining two have identical hexasaccharide structures.

Based on the present results and those of others (22, 31, 32), there are two major groups of LOS from *H. ducreyi*. One group, a nonasaccharide LOS group, possesses a major oligosaccharide in the outer core region like those presented by strains 35000 (18), ITM 2665, ITM 3147, ITM 5535, ACY1 (16, 17), and CCUG 7470. The second group, a hexasaccharide LOS group, exhibits a shorter oligosaccharide in the outer core region, as seen in LOS CCUG 4438 and ITM 4747 (32). In this study, 7 of the 10 strains investigated, as well as strain 35000 (22), have long oligosaccharides, and all of them but strain ITM 3542 are toxin producers. On the other hand, the two strains (CCUG 4438 and ITM 4747) with the short oligosaccharides are nontoxicogenic. Further studies are needed to investigate the possible relation between toxin production, growth appearance, and LOS structural composition.

Judging from the number of structurally defined LOS and the reactivity of MAb MAHD6 in the whole-cell ELISA with the 64 *H. ducreyi* strains tested (1), it seems that the majority (62 of 64) of *H. ducreyi* strains bear nonasaccharide structures in their LOS. Thus, the LOS variation of *H. ducreyi* is suggested to be limited relative to the high rate of phase variation observed among other mucosal pathogens such as *Neisseria* spp. and other *Haemophilus* spp. (38). The antigenic determinants residing on LOS are often exposed on the surface of the bacteria and are accessible to the host for immune recognition. MABs, which are capable of detecting different immunogenic components of LOS molecules, may provide valuable information for a better understanding of the kind of epitopes that may be accountable for diagnostic specificity or therapeutic activities. The usefulness of MAB MAHD7 for diagnostic purposes in detecting *H. ducreyi* antigen, in an immunofluorescence assay, has been demonstrated earlier (1). Further studies to evaluate the assay mentioned above and to develop less-complex diagnostic tests are planned. In this study, the ability of the MABs MAHD6 and MAHD7 to aid in the classification of *H. ducreyi* for epidemiological purposes has been demonstrated.

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