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Monoclonal Antibodies against *Haemophilus* Lipopolysaccharides: Clone DP8 Specific for *Haemophilus ducreyi* and Clone DH24 Binding to Lacto-*N*-Neotetraose

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Mouse monoclonal antibodies (MAbs) DP8 [immunoglobulin G1(κ)] and DH24 [immunoglobulin M(κ)], which are specific for *Haemophilus ducreyi* lipopolysaccharide (LPS), were generated by fusing mouse myeloma NS0 cells with spleen cells of BALB/c mice immunized with a total membrane preparation of *H. ducreyi*. MAb DP8 reacted in whole-cell enzyme immunoassay (EIA) and colony dot immunoblotting with all 50 strains of *H. ducreyi* but not with any other bacteria tested, which suggests an exposed and species-specific epitope on the *H. ducreyi* cell surface. This conclusion was supported by the finding that DP8 bound to all six *H. ducreyi* LPSs tested but not to any of the *Haemophilus influenzae* or enterobacterial LPSs or synthetic glycoconjugates. The MAb DH24 bound to 43 of 50 strains of *H. ducreyi* and to few strains of *H. influenzae*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis*, as evaluated by whole-cell EIA and colony dot immunoblotting. The MAb DH24 reacted with five of the six *H. ducreyi* LPSs tested and with the lacto-*N*-neotetraose (Galβ1→4GlcNAcβ1→3Galβ1→4Glc) series of synthetic glycoconjugates, as determined by EIA. By using polysaccharides obtained after both mild acidic hydrolysis and strong alkali treatment and dephosphorylated samples as inhibitors of the MAbs binding to *H. ducreyi* LPS antigens, it could be shown that phosphate groups were essential for the binding of DP8 to LPS but that they did not affect antigenic recognition by DH24. None of the MAbs bound to isolated lipid A, but aggregation caused by the fatty acids of lipid A was essential for epitope recognition.

Haemophilus ducreyi is a fastidious microorganism that causes chancroid, a sexually transmitted disease characterized by genital ulcers and, in many cases, by abscessed inguinal lymph nodes. It is emerging as the major cause of genital ulcer disease in the developing world (1, 13, 23), and several reports suggest that chancroid is a risk factor for the sexual acquisition of the human immunodeficiency virus (27).

Recent studies suggest that *H. ducreyi* surface lipopolysaccharide (LPS) plays an important role in the infection process, but the molecular mechanism is not established (3). *H. ducreyi* LPS lacks the O-specific polysaccharide (PS) chains characteristic of enteric bacteria. In this aspect, *H. ducreyi* LPS is similar to those of other *Haemophilus*, *Neisseria*, and *Bordetella* species (2, 3, 17). Such LPSs consist of an oligosaccharide core ketosidically linked via a 3-deoxy-D-*manno*-octulosonic acid (Kdo) residue to the lipid A region. The *H. ducreyi* lipid A, like those from *Escherichia coli* or *Salmonella typhimurium*, is composed of two glucosamine (GlcN) residues, phosphate groups, and six long-chain fatty acids, often referred as to hexaacyl lipid A (10, 22). The GlcN residues are linked β1→6, and each carries one phosphate group (9, 10, 22). The oligosaccharide core characteristic of these bacteria has been divided into two regions: (i) an inner core, consisting of three L-*glycero*-D-*manno*-heptose (Hep) residues and one phosphorylated Kdo residue, and (ii) an outer core, with one or more heterogeneous oligosaccharide branches containing mainly galactose (Gal) and glucose (Glc) residues (9). The inner core has phosphate and phosphoethanolamine groups (PEt) substituted. The core oligosaccharide structures reported so far indicate that LPSs of different *Haemophilus* species have a common inner core (9, 22, 25, 26, 29, 30). This suggests that the frequent switching of LPS antigenic determinants (phase variation) observed for this genus may be a result of changes in the outer core, which in addition may be sialylated (9, 17, 21).

The structures of three *H. ducreyi* LPSs have been published (22, 29). Two of them have been reported to have a Galβ1→4GlcNAcβ1→3Galβ1→trisaccharide in the outer core. This trisaccharide is also found in *Haemophilus influenzae*, *Neisseria gonorrhoeae*, and *Neisseria meningitidis* LPSs and is expressed by a variety of human cells (17, 18, 33). This trisaccharide is recognized by a well-characterized murine monoclonal antibody (MAb), 3F11, raised against *N. gonorrhoeae* (18, 38, 39) and by murine MAb anti-My-28, obtained after immunization of mice with human granulocytes (33). The third LPS strain, ITM 4747 (29), has a saccharide structure similar to that of *H. influenzae* 2019 LPS (25).

The importance of broadly reactive MAbs for diagnostic and therapeutic purposes has been discussed previously for other bacteria (6).

This report describes the isolation and partial characterization of two *H. ducreyi* LPS-specific MAbs. The MAb DH24 is similar to the MAbs 3F11 and anti-My-28 (33, 38, 39) and recognizes lacto-*N*-neotetraose. The other MAb, DP8, recognizes a specific epitope present in all *H. ducreyi* strains but not in any other species, including the LPS from *H. influenzae* 2019.

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MATERIALS AND METHODS

Bacterial strains, LPS, and lipid A. All but one of the *H. ducreyi* strains were clinical isolates of the strain collection of the Institute of Tropical Medicine, Antwerp, Belgium. Strain ACY 1 is a clinical isolate provided by A. C. Yemane, Stockholm, Sweden. *H. influenzae*, *Haemophilus parainfluenzae*, *N. gonorrhoeae*, *N. meningitidis*, and *Branhamella* (*Moraxella*) *catarrhalis* clinical isolates were obtained from routine clinical specimens at Huddinge Hospital, Huddinge, Sweden. *H. influenzae* 2019 was obtained from M. A. Apicella, Iowa City, Iowa, *Aeromonas* sp. strain SJ-83 LPS was provided by D. H. Shaw, St. John's, Newfoundland, Canada. *Bordetella pertussis* 1414 LPS was obtained from M. Caroff, Orsay, France, and the remaining LPSs were obtained from the departmental collection of the Division of Clinical Bacteriology, Huddinge Hospital.

***H. ducreyi* culture conditions and LPS extraction.** *H. ducreyi* strains were cultured on plates as described previously (5). The plates were placed in jars containing CO_2 (anaerobic GasPak system; BBL Microbiology Systems) and incubated at 34°C for 48 h. Bacterial colonies were harvested, suspended in Luria-Bertani broth, and frozen for LPS extraction. LPS was extracted from washed lyophilized bacteria with phenol-water (36). Further purification of isolated LPS was done by treatment with RNAse (Sigma Chemical Co., St. Louis, Mo.), proteinase K (Sigma), and ultracentrifugation.

Synthetic oligosaccharides. Eleven synthetic glycoproteins with the oligosaccharides covalently linked to human serum albumin and containing 10 to 20 oligosaccharide eq per human serum albumin eq (see Table 4) were purchased from Accurate Chemical, Inc. (Westbury, N.Y.) or BioCarb Chemicals (Lund, Sweden).

Preparation of *H. ducreyi* total membranes used as immunogens. *H. ducreyi* bacteria were harvested from plates and suspended in 5 ml of phosphate-buffered saline (PBS [pH 7.4]). The suspension was incubated twice for 15 min at 30°C while shaking (200 rpm), and cells were pelleted by centrifugation at 30°C for 15 min at 900 × *g*. The final pellet was resuspended in 1 ml of PBS. Homogenization was done by ultrasonication (three times for 5 s each on ice) with a Branson Sonifier 250 (settings: duty cycle, 90; output, 1). Between sonications, the homogenate was allowed to cool down for 5 s. All subsequent steps were performed at 4°C. Unbroken cells were removed by centrifugation for 15 min at 900 × *g*. The pellet was washed with 3 ml of PBS and resedimented by centrifugation. The combined supernatants were called the homogenate. The total membrane fraction and the soluble fraction were separated by ultracentrifugation for 2 h at 200,000 × *g*.

Production of hybridomas. Ten BALB/c mice were each immunized with 20 μg of total membrane preparation in complete Freund's adjuvant by intrafootpad injection as described previously (12). After 10 days, the mice were sacrificed and the popliteal lymph nodes were collected. Hybridomas were produced by fusion of 5×10^7 lymph node cells with 10^7 nonsecreting NS0 myeloma cells provided by P. De Baetselier (V.U.B., Brussels, Belgium). Hybrids positive in an enzyme immunoassay (EIA) on 5 μg of total membrane preparation per well (28) were cloned by limiting dilution, expanded, and screened against a panel of LPS antigens by EIA. End point titers in EIA were determined in twofold dilutions of antibodies in serum-free culture fluid of 1/2 to 1/80,000. The values for the different dilutions were plotted, and the dilution giving an A_{405} of 0.3 after 60 min was extrapolated as the end point titer. MAb isotypes were determined with a Line immunoassay (Immunogenetics, Antwerp, Belgium).

EIA. The EIA was done as described previously (14). The EIA inhibition test was performed by preincubation of twofold serial dilutions of inhibitors with antibodies in culture fluid for 2 h at 20°C. The antibody dilution used for the assay 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 LPS antigen. The preincubated mixture was then added to LPS-coated microtiter plates and incubated for 6 h at 20°C. The rest of the procedure was the same as that for the EIA described in reference 14. Inhibitory values (percent) were calculated in comparison with controls without inhibitor and plotted against the concentration of inhibitor. The concentration for 50% inhibition (IC_{50}) was determined for each LPS.

Whole-bacterium EIA. Bacterial suspensions in PBS were adjusted to an optical density of 0.2 at 595 nm. The bacteria were killed by the addition of 0.1% (wt/vol) sodium azide. The cell suspension was dispensed in 100-μl aliquots into 96-well microtiter plates (Costar, Cambridge, Mass.) and incubated overnight at 20°C. The rest of the EIA procedure was performed as described previously (14).

Colony dot immunoblotting. For colony dot immunoblotting, 22 colonies from each strain were individually suspended in 300 μl of PBS in a U-shape 96-well microtiter plate and dotted onto a nitrocellulose (0.45-μm pore size; BA-85; Schleicher & Schuell, Inc., Dassel, Germany) membrane filter placed in a Bio-Dot vacuum blotting apparatus (Bio-Rad Laboratories). After 60 min at 20°C, the wells were washed three times with 150 μl of PBS–0.5% (wt/vol) gelatine (Merck, Darmstadt, Germany)–0.05% (vol/vol) Tween 20 (PBS-G-T). The membrane was removed and left to air dry for 30 min. It was then incubated with 3% (wt/vol) gelatin in PBS for 2 h, followed by incubation for 5 h while rocking with the MAbs diluted 1/10 in PBS-G-T. After being washed three times with PBS-G-T, the membrane was incubated overnight with rocking in the presence of an alkaline phosphatase-rabbit anti-mouse immunoglobulin (Ig) conjugate (Dakopatts, AS, Glostrup, Denmark) appropriately diluted in PBS-G-T. The membrane was washed three more times as described above and developed for 3 to 4 min with Veronal acetate buffer (0.3 M [pH 9.6]) containing 0.01% (wt/vol) Nitro Blue Tetrazolium (Sigma), 0.005% (wt/vol) 5-bromo-4-chloro-3-indolyl phosphate (Sigma), and 0.004 M $MgCl_2$. All steps were performed at 20°C.

The results were scored as positive if more than 50% of the colonies were visually reactive, having phase variation if more than 10% but less than 50% of the colonies were visually reactive, and negative when at least 90% of the colonies were nonreactive.

SDS-PAGE. The LPS preparations from different *H. ducreyi* strains were analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 18% acrylamide resolving gel and with 0.45% bisacrylamide (11). The LPSs of *H. influenzae* and *S. typhimurium* SL1181 and SL1032 were used as controls. Samples (10 μg per well) were heated for 5 min at 100°C in sample buffer (11). Electrophoresis was run at a constant current of 40 mA per gel for about 9 h. Each gel was silver stained after SDS-PAGE (34).

Isolation of PS (PS-HOAc). *H. ducreyi* and *H. influenzae* LPSs (20 mg) in 1% acetic acid (HOAc) (6 ml) were sonicated in a bath for 10 min and hydrolyzed at 100°C for 90 min. After cooling, the mixture was centrifuged at 5,000 × *g* for 30 min at 4°C (Sorvall RC-5B rotor SS-34; Dupont, San Francisco, Calif.). The precipitated lipid A was washed twice with water, centrifuged and dried. The supernatant and washings were pooled and lyophilized. The PS-HOAc was dissolved in water and chromatographed on a Sephadex G-15 column (2.6 by 90 cm) (Pharmacia, Uppsala, Sweden) at a constant flow rate of 20 ml/h with water as the eluent. Fractions were analyzed by the phenol sulfuric acid assay to estimate neutral hexoses (7). Fractions containing saccharide were pooled and lyophilized.

Chemical modification of the LPS. (i) Dephosphorylation of LPS by hydrogen fluoride (LPS-HF). The LPS (10 mg) was treated with 48% (wt/vol) HF solution (1 ml [precooled at −20°C]) at 4°C for 48 h. HF was removed in vacuo with a polypropylene desiccator connected to a water aspirator with solid NaOH as a desiccant. After suspension of the residue in water, the mixture was dialyzed against distilled water with a Spectra/Por 2000 MW membrane (Spectrum, Houston, Tex.) and lyophilized. The phosphorus content before and after dephosphorylation was estimated (4).

(ii) De-O, N acylation of LPS by strong alkali treatment (PS-OH). Ester- and amide-bound fatty acids were cleaved from the LPS (10 mg) by hydrolysis in 4 M KOH (2 ml) at 100°C for 8 h (16, 40). The mixture was cooled to 20°C, neutralized with HOAc (50% [vol/vol]), and then desalted on a column of Sephadex G-15 with distilled water as the eluent. Fractions containing carbohydrate were collected, lyophilized, and immunochemically studied.

Treatment of LPS with neuraminidase. Neuraminidase treatment of antigens on EIA plates was done essentially as described previously (19–21). The EIA plates were coated with LPS (25 μg/ml of PBS) or with 100 μl of a bacterial suspension (unheated; $A_{595} = 0.2$) in PBS. After 2 h, the plates were washed with PBS (pH 6), and 25 μl of serially diluted (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*-acetylneuramic acid–aldolase, 0.024 U/mg of solid) was added to duplicate wells. Heat-inactivated neuraminidase was added at equal dilutions in parallel rows. PBS was added to duplicate wells as a negative control. After incubation for 2 h at 37°C, the enzyme solution was removed. The plates were blocked with PBS–0.5% (wt/vol) gelatin (PBS-G) and processed as described for the EIA. The antibody dilution used for the assay 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 LPS antigen (25 μg/ml in PBS). The conjugate did not bind to neuraminidase-treated antigens, as evaluated without addition of MAb to duplicate wells. The optimal concentration of enzyme was ≥50 mU/ml, as evaluated with a titration curve for the optimal binding of MAbs DP8 and DH24.

RESULTS

Characterization of MAbs by EIA and colony dot immunoblotting. A total of 24 MAbs were selected on the basis of their reactivity with *H. ducreyi* total membrane preparation as an antigen, but only DP8 [IgG1(κ)] and DH24 [IgM(κ)], bound to *H. ducreyi* LPSs in the EIA.

The MAbs were titrated against LPSs from *H. influenzae*, *H. ducreyi*, and rough mutants of *Salmonella* spp. by EIA (Table 1). DH24 reacted with five of the six *H. ducreyi* LPSs tested. The MAb DP8 reacted strongly with all six *H. ducreyi* LPSs (Table 1). Both of the MAbs failed to react with any of the LPSs or lipid A from 16 different *H. influenzae* strains or 8 different *Salmonella* strains. When tested against whole bacterial cells in EIA, DH24 was found to react with about 43 of 50 (86%) of the *H. ducreyi* clinical isolates (Table 2). It also bound to 2 of 4 *N. meningitidis* strains and 2 of 10 *B. catarrhalis* strains. DP8 bound to all of the *H. ducreyi* strains but did not react with any of the other 111 clinical isolates tested (Table 2). Similar

TABLE 1. EIA end point titers in supernatant fluid for the two *H. ducreyi* LPS-specific MAbs with *H. influenzae*, *H. ducreyi*, and *Salmonella* LPSs and isolated lipid A

LPS or lipid A[a]	End point titer of MAb supernatant[b]	
	DH24 (IgM)	DP8 (IgG1)
H. influenzae[c]	<2	<2
H. ducreyi		
ACY 1	1,800	15,000
ITM 5535	1,500	10,000
ITM 2665	2,200	10,000
ITM 4747	<2	2,200
ITM 3542	1,500	6,400
ITM 3147	640	6,400
Salmonella spp.[d]	<2	<2
Lipid A[e]	<2	<2

[a] Plates were coated with LPS or lipid A (10 μg/ml).
[b] Dilution of culture supernatant in coating buffer which gave an A_{405} of ≥0.3 after 60 min.
[c] LPSs from *H. influenzae* strains RM.7004, RM.7004 XP-1, RM.7004 AH1-2, RM.7004 XP-1 AH1-3, RM.7004 RVDEL8, RM.7004 *galE galK*, RM.118, Rd^-/b^+:01, Rd^-/b^+:02, RM.118-26, RM.7099, RM.7098, Morgan, Eagan, and Rd^-/b^+ I69.
[d] LPSs from *Salmonella* Ra to Re mutants derived from *S. typhimurium*: TV119 (Ra), SL733 (Rb1), TV161 (Rb2), TV148 (Rb3), SL805 (Rc), SL1032 (Rd1), SL1181 (Rd2), and SL1102 (Re).
[e] Lipid A isolated from *H. ducreyi*, *H. influenzae*, *S. typhimurium*, *S. minnesota*, and *E. coli.*

results were obtained by colony dot immunoblotting. DH24 showed positive reactions with 19 *H. ducreyi* isolates, and phase variation was observed in 25 of 50 *H. ducreyi*, 5 of 40 *H. influenzae*, 1 of 10 *N. gonorrhoeae*, and 2 of 4 *N. meningitidis* isolates tested (Table 3). DP8 only reacted with all 50 of the *H. ducreyi* strains, and phase variation was observed in 16 of them (Table 3 and Fig. 1).

Epitope analysis of LPS-reactive MAbs by using chemically defined LPSs and synthetic glycoconjugates. The titers of the DP8 and DH24 MAbs were determined by EIA against structurally defined *H. influenzae*, *H. ducreyi*, *Bordetella pertussis*, and *Aeromonas* LPSs and against synthetic glycoconjugates, which were selected on the basis of the saccharide being part of nonenteric gram-negative bacterial LPS (Fig. 2 and Table 4). The MAb DH24 bound to saccharides having in common a terminal or internal Galβ1→4GlcNAcβ1→3Galβ1→trisaccharide element (Table 4 and Fig. 2A). The MAb DP8 reacted only with *H. ducreyi* LPSs, suggesting that DP8 recognizes an epitope that is specific for *H. ducreyi* LPS.

EIA inhibition by purified LPS from *H. ducreyi* and *H. influenzae* LPS and by synthetic glycoconjugates. The epitope specificity of the MAbs was further investigated in EIA inhibition studies (Table 5 and Fig. 3). The five *H. ducreyi* LPSs that bound DH24 in a solid-phase EIA were also effective as inhibitors of the MAb binding to ITM 2665 LPS as an antigen, requiring an IC_{50} in the range of 0.15 to 1.0 μg/ml (Table 5 and Fig. 3A). None of the glycoconjugates tested showed any inhibition of binding of the MAbs to LPS at a concentration of 50 μg/ml.

The binding of DP8 was efficiently inhibited by all *H. ducreyi* LPSs, requiring an IC_{50} in the range of 0.7 to 2.0 μg/ml (Table 5 and Fig. 3B). The *H. influenzae* 2019 LPS, which has the same structure as *H. ducreyi* ITM 4747 LPS but a different LPS phosphorylation pattern, did not inhibit the binding of DP8 to ITM 4747 LPS (Table 5 and Fig. 3B).

Electrophoresis of LPS. The heterogeneity of the *H. ducreyi* and *H. influenzae* LPSs was revealed by silver staining after SDS-PAGE. Five of the *H. ducreyi* LPSs (those reactive with MAb DH24) yielded similar electrophoretic migration patterns, having higher heterogeneity and molecular weight (Fig. 4, lanes 2 to 7) than the rough ITM 4747 LPS (lanes 1 and 8). The band corresponding to *H. influenzae* 2019 LPS had an apparent molecular weight similar to that of ITM 4747 LPS but revealed a different staining pattern. The ITM 4747 LPS showed two distinct bands, while the 2019 LPS produced only one diffuse band, suggesting a higher degree of heterogeneity than that of ITM 4747 LPS (Fig. 4, lanes 8 and 9).

Analysis of *H. ducreyi* and *H. influenzae* PSs (PS-HOAc) and chemically modified LPSs as inhibitors of MAb binding. The

TABLE 2. Binding of MAbs DH24 and DP8 to whole gram-negative bacteria in EIA[a]

MAb	Organism	No. of samples[b]			
		Tested	Negative	Strongly positive	Weakly positive
DH24	*H. ducreyi*	50	7	22	21
	H. influenzae	40	40	0	0
	H. parainfluenzae	10	10	0	0
	N. gonorrhoeae	10	10	0	0
	N. meningitidis	4	2	1	1
	B. catarrhalis	10	8	0	2
	E. coli	4	4	0	0
DP8	*H. ducreyi*	50	0	49	1
	H. influenzae	40	40	0	0
	H. parainfluenzae	20	20	0	0
	N. gonorrhoeae	18	18	0	0
	N. meningitidis	4	4	0	0
	B. catarrhalis	25	25	0	0
	E. coli	4	4	0	0

[a] MAb culture supernatants diluted (1:20) were tested in duplicate EIAs. Data given are mean values of optical density (A_{405}) after 60 min.
[b] Scoring was done according to the reaction intensity compared with that of the substrate control as follows: $A_{405} < 0.2$, negative; $0.2 \leq A_{405} \leq 0.8$, weakly positive; $A_{405} \geq 0.8$, strongly positive.

TABLE 3. Binding of MAbs DH24 and DP8 to whole gram-negative bacteria in colony dot immunoblotting

MAb	Organism	No. of samples[a]			
		Tested	Negative	Positive	Showing phase variation
DH24	*H. ducreyi*	50	6	19	25
	H. influenzae	40	35	0	5
	H. parainfluenzae	10	10	0	0
	N. gonorrhoeae	10	9	0	1
	N. meningitidis	4	2	0	2
	B. catarrhalis	10	0	0	0
	E. coli	4	4	0	0
DP8	*H. ducreyi*	50	0	34	16
	H. influenzae	40	40	0	0
	H. parainfluenzae	20	20	0	0
	N. gonorrhoeae	18	18	0	0
	N. meningitidis	4	4	0	0
	B. catarrhalis	25	25	0	0
	E. coli	4	4	0	0

[a] Samples were scored as negative when at least 90% of the colonies were nonreactive, positive when there was phase variation for this epitope with more than 50% of variants reactive, and as showing phase variation for this epitope when less than 50% but more than 10% of the variants were reactive.

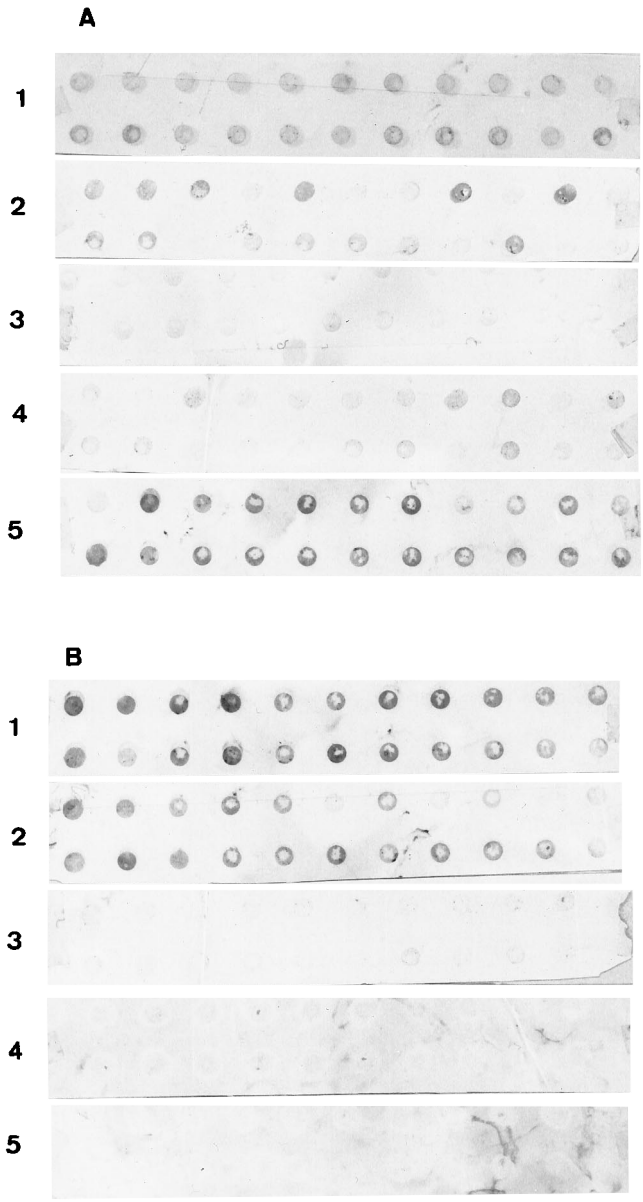

FIG. 1. Colony dot immunoblot showing reactivity of the MAbs against a representative panel of strains. (A) MAb DH24. Panels: 1, *H. ducreyi* positive; 2, *H. ducreyi* showing phase variation; 3, *H. ducreyi* negative; 4, *H. influenzae* showing phase variation; 5, *N. gonorrhoeae* showing phase variation. (B) MAb DP8. Panels: 1, *H. ducreyi* positive; 2, *H. ducreyi* showing phase variation; 3, *H. influenzae* negative; 4, *N. gonorrhoeae* negative; 5, *N. meningitidis*. Negative, positive, and phase variation are as defined in the text.

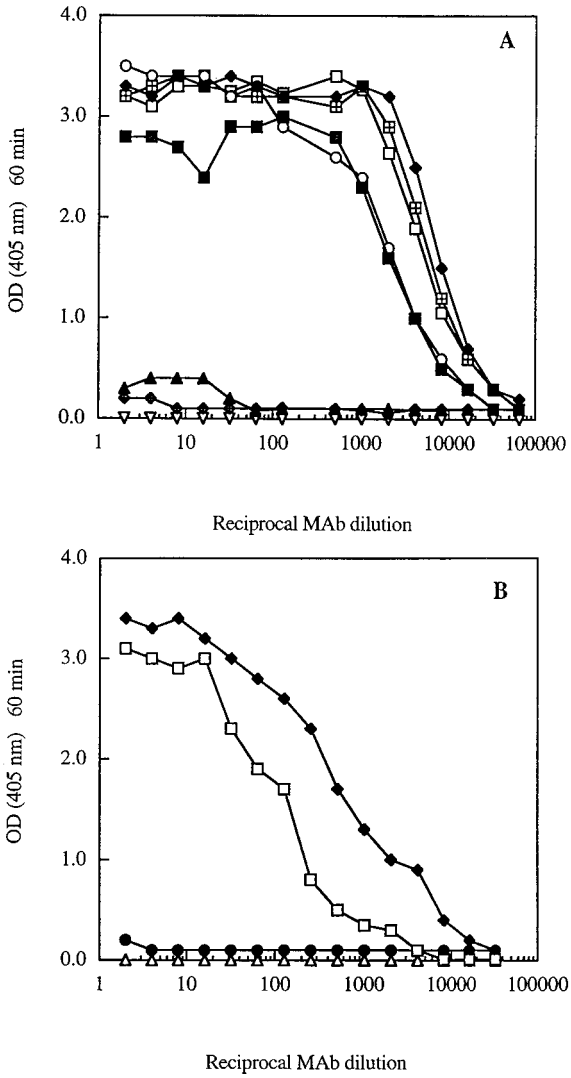

FIG. 2. Titration of the DH24 (A) and DP8 (B) MAbs against different antigens by EIA. The MAbs were serially diluted and then added to microtiter plates coated with LPS (10 μg/ml) or synthetic glycoconjugate (5 μg/ml). OD, optical density. (A) □, Lacto-*N*-neotetraose; ⊞, lacto-*N*-neohexaose; ○, lacto-*N*-hexaose; ◆, sialyllactose-*N*-neotetraose c; ■, *H. ducreyi* ITM 2665 LPS; ▲, *H. ducreyi* ITM 4747 LPS; ◆, lacto-*N*-tetraose; ▽, all other antigens. (B) ◆, *H. ducreyi* ITM 2665 LPS; □, *H. ducreyi* ITM 4747 LPS; ●, 2019 LPS; △, all other antigens.

LPSs from *H. ducreyi* ITM 2665, ITM 4747, and ITM 5535 and *H. influenzae* 2019, RM.7004 XP-1, and AH1-3 were dephosphorylated, and the product (LPS-HF) was found to be essentially phosphorus free. The ability of the LPS-HF samples to inhibit the MAbs was then studied (Fig. 5). Both ITM 2665 and ITM 5535 LPS-HF samples were effective as inhibitors of the binding of DH24 to ITM 2665 LPS as an antigen (Fig. 5A). None of the dephosphorylated samples tested caused good inhibition of the binding of DP8, including those from *H. influenzae* 2019 and *H. ducreyi* ITM 4747 (Fig. 5B). This suggests that the phosphate groups are not essential for the binding of DH24 but have an important role in the DP8 binding epitope.

PSs from both *H. ducreyi* and *H. influenzae* strains obtained after mild acid hydrolysis of LPSs (PS-HOAc) were next analyzed as inhibitors of the binding of MAbs DH24 and DP8. All of the PS-HOAc samples were poor inhibitors of the binding of both antibodies (Fig. 5). Some inhibition was observed for DP8 with the ITM 4747 PS-HOAc samples, which were about 50 times less effective as inhibitors than the native ITM 4747 LPS (IC_{50}, 50 μg/ml [Fig. 5B]). These data suggest that the micellar structure of the LPS molecule in the inhibition studies is important, since isolated lipid A did not inhibit the binding of either MAb to the LPS (data not shown).

The LPSs were de-O, N acylated in order to estimate the relative importance of the fatty acids in lipid A in recognition of the epitope by the MAbs. None of the de-O, N-acylated LPSs was effective as an inhibitor of any of the MAbs, even at the highest concentration tested (200 μg/ml [data not shown]).

TABLE 4. Reactivities of *H. ducreyi* LPS-specific MAbs against chemically defined *H. influenzae*, *B. pertussis*, *Aeromonas*, and *H. ducreyi* LPSs and glycoconjugates

Antigen (reference)[a]	Chemical structure[b]	End point titer of MAb supernatant[c]	
		DH24	DP8
LPSs			
H. ducreyi ITM 4747 (29)	Galβ1...4Glcβ1→4Hepα1–Kdo(P)–lipid A ↑ α1,3 Hepα1→2Hep	<2	2,048
H. ducreyi ITM 2665 (29)	Galβ1→4GlcNAcβ1→3Galβ1→4DDHepα1→6Glcβ1→4Hepα1–Kdo(P)–lipid A ↑ α1,3 Hepα1→2Hep	20,000	10,000
H. influenzae RM.7004 XP-1 AH1-3 (30)	Glcβ1...4Glcβ1→4Hep1–Kdo(P)–lipid A ↑ α1,3 Galβ1→2Hepα1→2Hep ↑ 6 PEt	<2	<2
H. influenzae 2019 (25)	Galβ1→4Glcβ1→4Hep1–Kdo(P)–lipid A ↑ α1,3 Hepα1→2Hep ↑ 6 ↑ 6 PEt PEt	<2	<2
B. pertussis 1414 (16)	Glcβ1→4Hep1→5Kdo(P)–lipid A ↑ α1,3 GlcAα1→2Hep ↑ α1,7 GlcN	<2	<2
H. influenzae Rd^-/b^+ I69 (10)	Kdo(P)–lipid A	<2	<2
A. salmonicida SJ-83 (31)	Hepα1→2Hep1α→3Hepα1-6Kdo*f*–lipid A	<2	<2
Glycoconjugates			
Lactose	Galβ1→4Glcβ1-*O*-PAP-HSA	<2	<2
T antigen	Galβ1→3GalNAcα1-*O*-APE-HSA	<2	<2
Globotriose	Galα1→4Galβ1→4(Glc)-APD-HSA	<2	<2
Lacto-*N*-tetroase	Galβ1→3Glc1NAcβ1→3Galβ1→4(Glc)-APD-HSA	<2	<2
Lacto-*N*-neotetraose[d]	Galβ1→4GlcNAcβ1→3Galβ1→4(Glc)-APD-HSA	30,000	<2
Globotetraose	GalNAcβ1→3Galα1→4Galβ1→4(Glc)-APD-HSA	<2	<2
Lacto-*N*-neohexaose	Galβ1→4GlcNAcβ1→[Galβ1→4GlcNAcβ1→6],3Galβ1→4(Glc)-APD-HSA	30,000	<2
Lacto-*N*-hexaose	Galβ1→4GlcNAcβ1→[Galβ1→3GlcNAcβ1→6],3Galβ1→4(Glc)-APD-HSA	20,000	<2
3′-Sialyllactose	Neu5Acα2→3Galβ1→4(Glc)-APD-HSA	<2	<2
6′-Sialyllactose	Neu5Acα2→6Galβ1→4(Glc)-APD-HSA	<2	<2
Sialyllacto-*N*-neotetraose c	Neu5Acα2→6Galβ1→4GlcNAcβ1→3Galβ1→4(Glc)-APD-HSA	30,000	<2

[a] EIA plates were coated with LPS (10 μg/ml) and glycoconjugates (5 μg/ml).

[b] Abbreviations: Glc, glucose; Gal, galactose; GlcN, glucosamine; GlcA, glucuronic acid; Hep, L-*glycero*-D-*manno*-heptose; DDHep, D-*glycero*-D-*manno*-heptose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Kdo, 3-deoxy-D-*manno*-octulosonic acid; Neu5Ac, *N*-acetylneuraminic acid (most common sialic acid); . . . , nonstoichiometrical substitution. The glycoconjugate structures, naming, and abbreviated forms are per the BioCarb catalog (1a): APD, acetylphenylenediamine; APE, aminophenylethyl; and PAP, *p*-aminophenyl (used as spacers to attach the carbohydrates to the human serum albumin [HSA]). Underlined portions correspond to common structure recognized by MAb DH24.

[c] Dilution of MAb culture supernatant which gave an A_{405} of ≥0.3 after 60 min.

[d] The prefix neo is used to denote differences in linkage (e.g. 1→4 versus 1→3) in otherwise identical structures.

This suggests that the degree of aggregation caused by the fatty acids in the lipid A moiety is important for MAb recognition.

Effect of neuraminidase treatment on the binding of MAbs DH24 and DP8 to whole bacteria and LPS. The binding of DH24 and DP8 to neuraminidase-treated, untreated, and inactivated neuraminidase-treated *H. ducreyi* bacteria was analyzed. It was found that for DH24, treatment with neuraminidase resulted in variable increases (from 0 to >100%). For binding of DH24, 6 of 21 strains showed an increment of <50%, 9 strains showed an increase of between 50 and 100%, and 5 strains showed an increase of >100%. For DP8, all strains but one showed an increase of <50%.

An analogous analysis with six *H. ducreyi* LPSs was also performed. LPSs from *H. influenzae* strains 2019 and Rd^-/b^+ I69 were used as negative controls. Sialyllactose-*N*-neotetraose c glycoconjugate was used as a positive control. For DH24, increases from 0 to 100% were observed. Four of the six LPSs (ITM 2665, ITM 5535, ITM 3542, and ITM 4747) showed an increase of ≥100% in binding of DH24; in two of the LPSs (ACY 1 and ITM 3147), the increase was between 78 and 86%, respectively, while with the Rd^-/b^+ I69 LPS, no increase was observed. For DP8, all of the LPSs but two showed an increase of between 80 and 100%: the exceptions were the LPSs from the *H. influenzae* 2019 and Rd^-/b^+ I69 strains. These data suggest that sialic acid is present, at least to some extent, in some of the *H. ducreyi* strains which express the DH24 epitope. For DP8, a small increase in binding after neuraminidase treatment of most of the strains was also observed.

DISCUSSION

The MAbs DP8 and DH24 recognize epitopes on the oligosaccharide moiety of the LPSs from *H. ducreyi* strains. However, the data indicate that the physical state of the LPS is

TABLE 5. EIA inhibition of anti-*H. ducreyi* LPS MAbs with *H. ducreyi* and *H. influenzae* LPSs and synthetic glycoconjugates

Inhibitor	MAb binding activity [IC_{50} (μg/ml)][a]	
	DH24 (antigen, ITM 2665 LPS [10 μg/ml])	DP8 (antigen, ITM 4747 LPS [10 μg/ml])
H. ducreyi LPSs		
ACY 1	0.6	0.8
ITM 5535	1.0	0.7
ITM 2665[b]	0.15	2.0
ITM 4747[b]	>50	1.0
ITM 3542	0.8	0.8
ITM 3147	0.3	2.0
H. influenzae LPSs		
RM. 7004 XP-1 AH1-3[b]	>50	>50
2019[b]	>50	>50
Glycoconjugates[b]		
Lacto-*N*-neotetraose	>50	>50
Lacto-*N*-hexaose	>50	>50
Lacto-*N*-hexaose	>50	>50
Sialyllactose-*N*- neotetraose c	>50	>50
Lacto-*N*-tetraose	>50	>50

[a] Mean values of at least two experiments are shown.
[b] Structure is shown in Table 4.

important for the inhibition of the binding of the MAbs, as indicated when the lipid A was removed and the polysaccharides were tested as inhibitors.

The MAb DP8 bound to an epitope expressed on the surface of *H. ducreyi* cells. It appears to be specific for *H. ducreyi*, since it did not bind to isolated LPSs or whole bacteria from 111 different strains of *H. influenzae*, *H. parainfluenzae*, *N. gonorrhoeae*, *N. meningitidis*, *B.* (*Moraxella*) *catarrhalis*, and members of the family *Enterobacteriaceae* (Tables 1 to 3 and Fig. 1). Moreover, DP8 did not bind to isolated lipid A from different sources: neither to the LPS from the deep rough mutant *H. influenzae* Rd^-/b^+ I69, which has only a phosphorylated Kdo and hexaacyl lipid A (10 [Table 4]), nor to the LPS from the core mutant *Aeromonas salmonicida* SJ-83, which has the structure Hepα1→2Hepα1→3Hepα1→Kdo–lipid A (31). This triheptosyl element and a hexaacyl lipid A have been indicated as being a conserved part of *Haemophilus* LPSs (references 9, 22, 25, 26, 29, and 30 and unpublished results). This suggests that the DP8 epitope is not defined by any of these conserved structures of the *H. ducreyi* LPS. In addition, DP8 did not bind to any of the 11 synthetic glycoconjugates tested (Tables 4 and 5), among them the glycoconjugate of lactose, a disaccharide that has been reported to be an element in the hexose part of one of the LPSs from *H. ducreyi* included in this study (strain ITM 4747 [reference 29 and Table 4]).

Interestingly, a lactosyl disaccharide element located on the conserved triheptosyl element of the *Haemophilus* LPS (unpublished data) defines a structure (i.e., native LPS from *H. ducreyi* 4747) which inhibits the binding of the DP8 to *H. ducreyi* LPS antigens.

The inhibition of the binding of DP8 to *H. ducreyi* LPS antigens by the six *H. ducreyi* LPSs tested demonstrated the presence of the MAb antigenic determinant in all of these LPSs. The two LPSs from *H. ducreyi* with known major core oligosaccharide structures included in this study were the ITM 4747 and ITM 2665 LPSs (structures shown in Table 4 and reference 29).

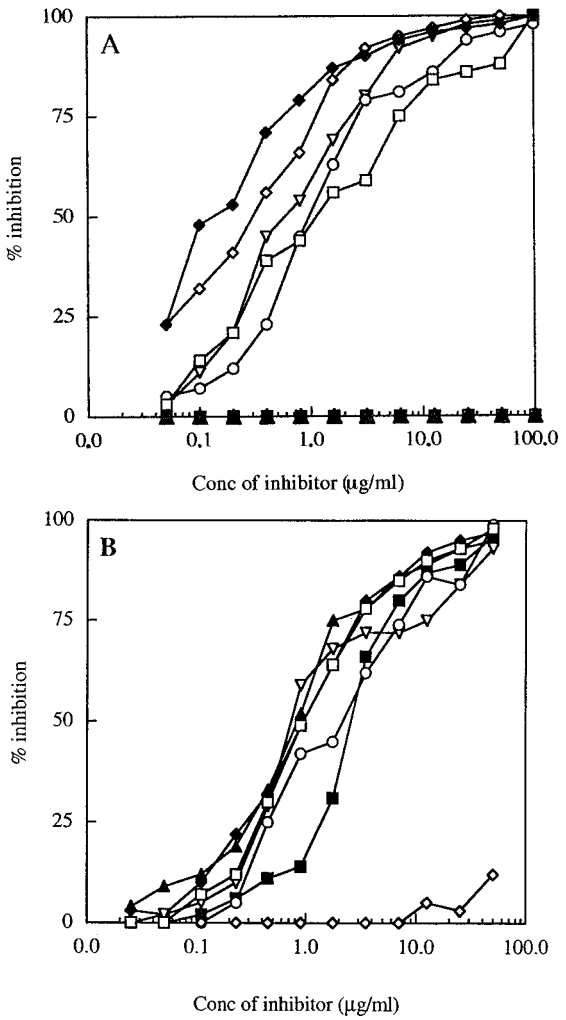

FIG. 3. Test of *H. ducreyi* and *H. influenzae* LPSs and synthetic glycoconjugates as inhibitors. The inhibitors were preincubated with DH24 (A) or DP8 (B) and then added to microtiter plates coated with *H. ducreyi* ITM 2665 LPS (10 μg/ml) (A) or ITM 4747 LPS (10 μg/ml) (B). (A) □, ITM 5535 LPS; ▽, ACY 1 LPS; ■, ITM 4747 LPS; ○, ITM 3542 LPS; ◆, ITM 2665 LPS; ◇, ITM 3147 LPS; ▲, 2019 LPS; ◩, all other inhibitors. (B) ▽, ITM 5535 LPS; ▲, ACY 1 LPS; □, ITM 4747 LPS; ◆, ITM 3542 LPS; ○, ITM 2665 LPS; ■, ITM 3147 LPS; ◇, 2019 LPS; —, all other inhibitors gave 0% inhibition and therefore align with the *x* axis. Conc, concentration.

The common parts of these two LPSs are the pentasaccharide portion of the inner core, Glcβ1→[Hepα1→2Hepα1→3]4Hepα1-Kdo(P), and a hexaacyl lipid A (9, 10, 29). We and other investigators have reported the presence of the same pentasaccharide element and lipid A as a part of the LPSs from *H. influenzae* strains A2, RM.7004 XP-1 AH1-3, and 2019 (9, 25, 26, 30 [Table 4]). However, DP8 did not bind to the LPSs from *H. influenzae* strains RM.7004 XP-1 AH1-3 and 2019. This suggests that the *H. ducreyi* LPS-specific MAb DP8 recognizes a unique epitope defined by the common part of the LPSs from the six *H. ducreyi* strains studied but not defined by similar elements present in the two LPSs from *H. influenzae* species. Interestingly, the only known difference between the major core oligosaccharide element of the LPS from *H. influenzae* 2019 and that from *H. ducreyi* ITM 4747 is the phosphorylation pattern of the inner core (25, 29). Thus, our first attempt was to evaluate dephosphorylated LPSs from both species as inhibitors. The lack of inhibitory activity of the

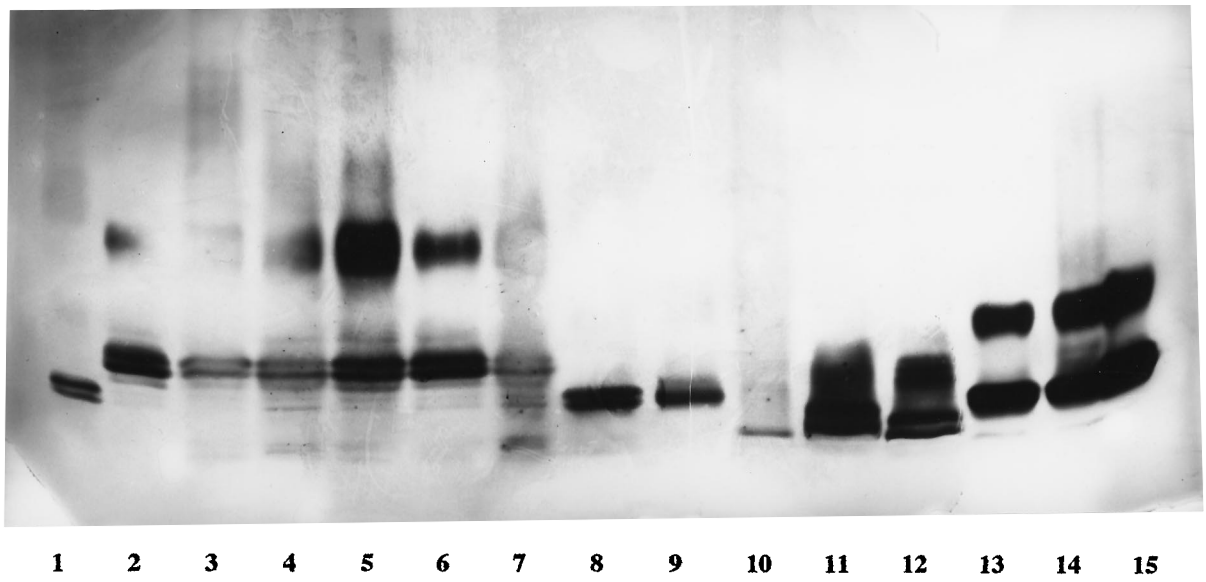

FIG. 4. SDS-PAGE and silver staining of isolated LPS samples. The samples (10 μg/ml unless otherwise noted) were prepared from the following: *H. ducreyi* ITM 4747 (5 μg/ml [lane 1]), *H. ducreyi* ITM 2665 (5 μg/ml [lane 2]), *H. ducreyi* ITM 5535 (lane 3), *H. ducreyi* ITM 3542 (lane 4), *H. ducreyi* ACY 1 (lane 5), *H. ducreyi* ITM 2665 (lane 6), *H. ducreyi* ITM 3147 (lane 7), *H. ducreyi* ITM 4747 (lane 8), *H. influenzae* 2019 (lane 9), Rd^{-}/b^{+} I69 (lane 10), *S. typhimurium* SL 1181 (lane 11), *S. typhimurium* SL 1032 (lane 12), *H. influenzae* AH1-3 (lane 13), *H. influenzae* XP-1 (lane 14), and *H. influenzae* AH1-3 (5 μg/ml [lane 15]).

dephosphorylated *H. influenzae* 2019 LPS and of the dephosphorylated ITM 4747 LPS, which should be chemically similar to the former, suggested the importance of the common phosphate groups (those from Kdo and the lipid A moiety) for MAb recognition. The absence of inhibitory activity was also observed with the two other dephosphorylated samples from *H. ducreyi* LPSs tested, which emphasizes the role of the phosphate groups for binding of DP8 (Fig. 5B).

The role of the lipid A or/and Kdo phosphate in the definition of the MAb specificity was further shown when *H. ducreyi* PSs obtained after mild acidic hydrolysis (PS-HOAc) were tested as inhibitors of the binding of DP8. None of the PS-HOAc samples were as effective (100-fold less active) as inhibitors as the native *H. ducreyi* LPS, which gave only about 40% inhibition at the highest concentration tested (100 μg/ml [Fig. 5B]). In line with this was the absence of inhibitory activity observed when de-O, N-acylated LPS samples (PS-OH [i.e., LPSs that have lost their amide- and ester-linked lipids]) were tested as inhibitors. This raises a question about the purity of the LPS preparations obtained by the phenol-water method, since ions such as Mg^{2+} or Ca^{2+} are always bound to LPS. Their presence strongly influences the physical properties of LPS, which may alter their biological activities (8, 32). One possible explanation may be that negatively and positively charged groups are located in or close to lipid A; intermolecular ionic interactions are present beside the hydrophobic interactions, causing aggregations of LPS to form micelles. The micellar structure is absent once the fatty acids are removed (8, 32).

The combined evidence leads us to suggest the following minimal structure as the DP8 saccharide epitope:

$$\begin{array}{l} \text{Glc}\beta1\rightarrow4\text{Hep}\alpha1\rightarrow\text{Kdo(P)} \\ \qquad\qquad\uparrow\alpha1,3 \\ \text{Hep}\alpha1\rightarrow2\text{Hep} \end{array}$$

The binding of DP8 to all of the LPSs from *H. ducreyi* tested, including ITM 2665, which has a larger core saccharide (see structure in Table 4), is easily explained, since the terminal glucose is 6-substituted (i.e., in the side chain), thereby masking the glucose residue only to a limited extent. Most of the residues should therefore be available for MAb recognition.

To determine whether the DP8 LPS epitope is accessible on the bacterial surface to MAb binding, *H. ducreyi* EIA plates were coated with whole cells. DP8 bound to all 50 of the strains tested, suggesting a surface-exposed LPS epitope. The colony dot immunoblotting results, in which all the strains were reactive, are in accord. However, phase variation was observed (Fig. 1); this may be explained as a consequence of the on-off expression of sugar residues in the outer core rather than a variation in the inner core moiety itself, since we and others have data which suggest that the *Haemophilus* inner core domain is conserved (references 9, 10, 22, 25, 26, 29, and 30 and unpublished results). When either the *H. ducreyi* LPSs or bacteria were treated with neuraminidase, the increase in MAb binding compared with that of untreated samples indicates that the sialic acid residues cause steric hindrance for the MAb to bind to the epitope, which was observed as phase variation in our colony dot blot assay.

The heterogeneity of the *H. ducreyi* LPSs was also shown in the silver-stained gel obtained after separation of LPSs by SDS-PAGE, with two sharp bands for the *H. ducreyi* ITM 4747 LPS and only a broad diffuse band for the *H. influenzae* 2019 LPS (Fig. 4).

Finally, we should emphasize that MAb DP8 did not react with whole bacteria or LPSs from different strains of *H. influenzae*, *H. parainfluenzae*, *N. gonorrhoeae*, *N. meningitidis*, *B. catarrhalis*, and members of the family *Enterobacteriaceae*, which suggests a broadly expressed epitope within *H. ducreyi.*

The MAb DH24 bound to the LPS from *H. ducreyi* ITM 2665 but not to the LPS from *H. ducreyi* ITM 4747, both with the reported major core oligosaccharide structures (29) shown in Table 4. This indicates reactivity involving the outer core region. The ITM 2665 LPS has the terminal tetrasaccharide Galβ1→4GlcNAcβ1→3Galβ1→4DDHepα1→6Glc, while ITM

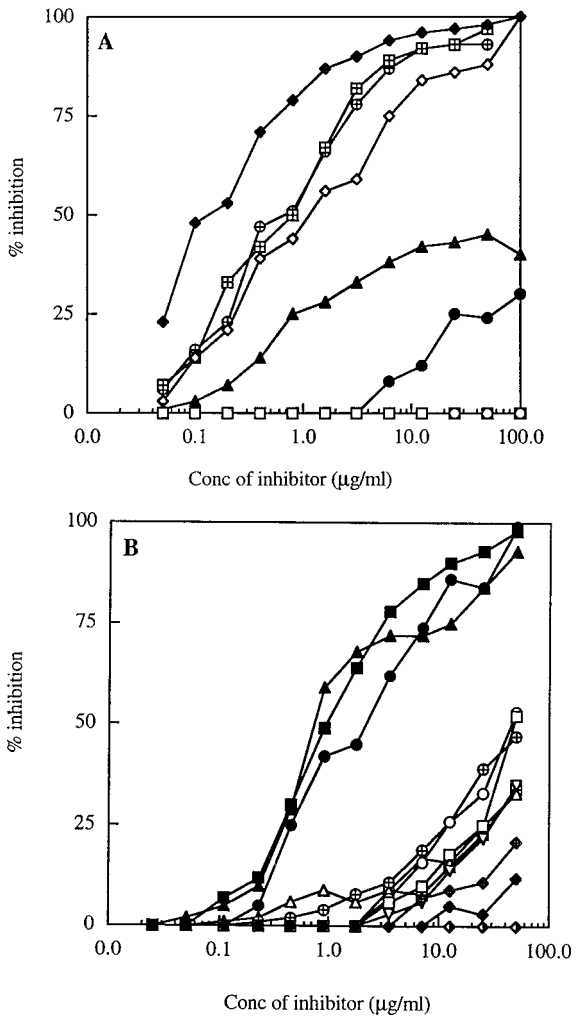

FIG. 5. Test of *H. ducreyi* and *H. influenzae* LPS, chemically modified LPS, and PS as inhibitors. The inhibitors were preincubated with DH24 (A) or DP8 (B) and then added to microtiter plates coated with *H. ducreyi* ITM 2665 LPS (10 μg/ml) (A) or ITM 4747 LPS (10 μg/ml) (B). (A) ◆, ITM 2665 LPS; ◇, ITM 5535 LPS; ⊕, ITM 5535 LPS-HF; ⊞, ITM 2665 LPS-HF; ▲, ITM 2665 PS-HOAc; ●, ITM 5535 PS-HOAc; □, all other inhibitors. (B) ●, ITM 2665 LPS; ▲, ITM 5535 LPS; ■, ITM 4747 LPS; ⊕, ITM 2665 LPS-HF; ○, ITM 2665 PS-HOAc; △, ITM 5535 PS-HOAc; ▽, ITM 5535 LPS-HF; □, ITM 4747 PS-HOAc; ◆, 2019 LPS-HF; ◆, 2019 LPS; ◀, 2019 (LPS-HF) PS-HOAc; ⊞, ITM 4747 LPS-HF; —, all other inhibitors gave 0% inhibition and therefore align with the *x* axis. Conc, concentration.

4747 LPS has a terminal Galβ1→4Glc disaccharide element. The binding of DH24 to the lacto-*N*-neotetraose series of synthetic glycoconjugates, all having as a common denominator the trisaccharide sequence Galβ1→4GlcNAcβ1→3Galβ1, which is also present in ITM 2665 LPS, suggests that DH24 recognizes this trisaccharide element. Interestingly, MAb DH24 also bound to the sialyllacto-*N*-neotetraose c synthetic glycoconjugate Neu5Acα2→6Galβ1→4GlcNAcβ1→3Galβ1, where the trisaccharide in common is internally placed. Since the terminal galactose is 6-substituted (i.e., in the side chain), a large part of the residues are still available.

The role of the sialic acid (Neu5Ac) in the epitope was analyzed by treatment of LPSs and bacteria with neuraminidase. An increase (between 50 and 100%) in DH24 binding with neuraminidase-treated LPSs, compared with untreated LPSs, was observed. This suggests that the sialic acid is not involved in the epitope and does not entirely cap the MAb binding site. The picture is, however, complicated in the LPSs by the fact that data from ESI-MS from the authors' laboratory indicate that substitution is partial.

Binding of DH24 was efficiently inhibited by dephosphorylated LPSs (LPS-HF [Fig. 5A]), in accord with the expectation of an outer core epitope. Neither the delipidated LPS obtained after mild acidic hydrolysis (PS-HOAc) nor the de-O, N-acylated LPS (PS-OH) caused any remarkable inhibition (Fig. 5A). This significantly less efficient inhibition of binding or absence of inhibition by the PS-HOAc or PS-OH samples stresses the importance of (i) the degree of aggregation of LPS (explained above) relevant for an IgM antibody and/or (ii) the fatty acid residues in the lipid A, which could influence the mode of presentation of the LPS molecules, as suggested previously (15, 38, 39). This was also evident when the synthetic lacto-*N*-neotetraose series of glycoconjugates were tested as inhibitors, since none of them inhibited the binding of DH24.

To determine whether the DH24 epitope is presented on the cell surface, *H. ducreyi* bacteria were assayed by EIA and colony dot immunoblotting. The results showed that 43 of 50 *H. ducreyi* strains tested expressed the DH24 epitope and that it was subject to phase variation. DH24 also bound to strains of *H. influenzae*, *N. gonorrhoeae*, and *N. meningitidis* in the colony dot immunoblotting assay and showed phase variation. The latter may partially be explained by sialylation, which could mask the MAb binding sites.

The present results and those of others (17, 21, 22, 29, 30, 37) indicate that the majority of *H. ducreyi* strains, like those of the *Haemophilus* and *Neisseria* genera, contain saccharide structures identical to those of lactoneo series gangliosides present on human cells and in secretions. Through this molecular mimicry, the bacteria can avoid the host defense mechanisms as has previously been reported (17, 21, 35).

The specificity of these MAbs for *H. ducreyi* LPS, especially that of DP8, should allow the development of sensitive immunodiagnostics for chancroid. The possibility of developing a synthetic DP8 epitope or alternatively, production of an anti-idiotype MAb having the internal image of the DP8 epitope, and thus the antigenic determinant, opens new strategies for the study of the still largely unknown immunological response to chancroid that predisposes the patient to infection by human immunodeficiency virus, and thus to AIDS (24).

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REFERENCES

1. **Albritton, W. L.** 1989. Biology of *Haemophilus ducreyi*. Microbiol. Rev. **53:**377–389.
1a. **BioCarb Chemicals.** 1988. BioCarb Chemicals catalog. BioCarb Chemicals, Lund, Sweden.
2. **Campagnari, A. A., S. M. Spinola, A. J. Lesse, Y. A. Kwaik, R. E. Mandrell, and M. A. Apicella.** 1990. Lipopolysaccharide epitopes shared among gram-negative non-enteric mucosal pathogens. Microb. Pathog. **8:**353–362.
3. **Campagnari, A. A., L. M. Wild, G. E. Griffiths, R. J. Karalus, M. A. Wirth, and S. M. Spinola.** 1991. Role of lipooligosaccharides in experimental dermal lesions caused by *Haemophilus ducreyi*. Infect. Immun. **59:**2601–2608.
4. **Chen, P. S., T. Y. Toribara, and H. Warner.** 1956. Estimation of organic phosphorus. Anal. Chem. **28:**1756–1761.
5. **Clarridge, J. E., R. Shawar, and B. Simon.** 1990. *Haemophilus ducreyi* and chancroid: practical aspects for the clinical microbiology laboratory. Clin. Microbiol. Lett. **18:**137–139.
6. **Di Padova, F. E., H. Brade, G. R. Barclay, I. R. Poxton, E. Liehl, E. Schuetze, H. P. Kocher, G. Ramsay, M. H. Schreier, D. B. L. McClelland, and E. T. Rietschel.** 1993. A broadly cross-reactive monoclonal antibody binding to *E. coli* and *Salmonella* lipopolysaccharides. Infect. Immun. **61:**3863–3872.
7. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. A colorimetric method for the determination of sugars and related sub-

stances. Anal. Chem. **28:**350–356.
8. **Galanos, C., and O. Lüderitz.** 1984. Lipopolysaccharide: properties of an amphipathic molecule, p. 46–58. *In* E. T. Rietschel (ed.), Handbook of endotoxin: chemistry of endotoxin, vol. 1. Elsevier Science Publishing, Inc., New York.
9. **Gibson, B. W., W. Melaugh, N. J. Phillips, M. A. Apicella, A. A. Campagnari, and J. M. Griffiss.** 1993. Investigation of the structural heterogeneity of lipopolysaccharides from pathogenic *Haemophilus* and *Neisseria* species and of R-type lipopolysaccharides from *Salmonella typhimurium* by electrospray mass spectrometry. J. Bacteriol. **175:**2702–2712.
10. **Helander, I. M., B. Lindner, H. Brade, K. Altmann, A. A. Lindberg, E. T. Rietschel, and U. Zähringer.** 1988. Chemical structure of the lipopolysaccharide of *Haemophilus influenzae* strain I69 Rd−/b+: description of a novel deep-rough chemotype. Eur. J. Biochem. **177:**483–492.
11. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
12. **Holmdahl, R., T. Moran, and M. Anderssen.** 1985. A rapid and efficient immunization protocol of monoclonal antibodies reactive with autoantigens. J. Immunol. Methods **85:**379–384.
13. **Jonasson, J. A.** 1993. *Haemophilus ducreyi*. Int. J. Sex. Transm. Dis. AIDS **4:**317–321.
14. **Karlsson, K., M. Granström, and A. A. Lindberg.** 1986. *Salmonella* sp. antibodies, p. 85–98. *In* V. Verlagsgesellschaft (ed.), Antigens and antibodies, vol. XI. Bergmeyer, Weinheim, Germany.
15. **Kim, J. J., N. J. Phillips, B. W. Gibson, J. M. Griffiss, and R. Yamasaki.** 1994. Meningococcal group A lipooligosaccharides (LOS): preliminary structural studies and characterization of serotype-associated and conserved LOS epitopes. Infect. Immun. **62:**1566–1575.
16. **Lind, S. M., L. Kenne, and A. A. Lindberg.** 1991. Mapping of the binding specificity for five monoclonal antibodies recognizing 3-deoxy-D-*manno*-octulosonic acid in bacterial lipopolysaccharides. J. Immunol. **146:**3864–3870.
17. **Mandrell, R. E., and M. A. Apicella.** 1993. Lipooligosaccharides (LOS) of mucosal pathogens: molecular mimicry and host-modification of LOS. Immunobiology **187:**382–402.
18. **Mandrell, R. E., J. M. Griffiss, and B. A. Macher.** 1988. Lipooligosaccharides (LOS) and *Neisseria meningitidis* have components that are immunochemically similar to precursors of human blood group antigens. J. Exp. Med. **168:**107–126.
19. **Mandrell, R. E., J. J. Kim, C. M. John, B. W. Gibson, J. V. Sugai, M. A. Apicella, J. M. Griffiss, and R. Yamasaki.** 1991. Endogenous sialylation of the lipooligosaccharides of *Neisseria meningitidis*. J. Bacteriol. **173:**2823–2832.
20. **Mandrell, R. E., A. J. Lesse, J. V. Sugai, M. Shero, J. M. Griffiss, J. A. Cole, N. J. Parsons, H. Smith, S. A. Morse, and M. A. Apicella.** 1990. In vivo and in vitro modification of *Neisseria gonorrhoeae* lipopolysaccharide epitope structure by sialylation. J. Exp. Med. **171:**1649–1664.
21. **Mandrell, R. E., R. McLaughlin, Y. A. Kwaik, A. Lesse, R. Yamasaki, B. Gibson, S. M. Spinola, and M. A. Apicella.** 1992. Lipooligosaccharides (LOS) of some *Haemophilus* species mimic human glycosphingolipids, and some LOS are sialylated. Infect. Immun. **60:**1322–1328.
22. **Melaugh, W., N. J. Phillips, A. A. Campagnari, R. Karalus, and B. W. Gibson.** 1992. Partial characterization of major lipooligosaccharide from a strain of *Haemophilus ducreyi*, the causative agent of chancroid, a genital ulcer disease. J. Biol. Chem. **267:**13434–13439.
23. **Morse, S. A.** 1989. Chancroid and *Haemophilus ducreyi*. Clin. Microbiol. Rev. **2:**137–157.
24. **Pepin, J., F. A. Plummer, R. C. Brunham, P. Piot, D. W. Cameron, and A. R. Ronald.** 1989. The interaction of HIV infection and other sexually transmitted diseases: an opportunity for intervention. AIDS **3:**3–9.
25. **Phillips, N. J., M. A. Apicella, J. M. Griffiss, and B. W. Gibson.** 1992. Structural characterization of the cell surface lipooligosaccharides from a nontypeable strain of *Haemophilus influenzae*. Biochemistry **31:**4515–4526.
26. **Phillips, N. J., M. A. Apicella, J. M. Griffiss, and B. W. Gibson.** 1993. Structural studies of the lipooligosaccharides from *Haemophilus influenzae* type b strain A2. Biochemistry **32:**2003–2012.
27. **Piot, P., and M. Laga.** 1989. Genital ulcers, other sexually transmitted diseases and the sexual transmission of HIV. J. Br. Med. **298:**623–624.
28. **Roggen, E. L., G. Hoofol, E. Van Dyck, and P. Piot.** 1994. Enzyme immunoassays (EIAs) for the detection of anti-*Haemophilus ducreyi* serum IgA, IgG and IgM antibodies. Sex. Transm. Dis. **21:**36–42.
29. **Schweda, E. K., A. C. Sundström, L. M. Eriksson, J. A. Jonasson, and A. A. Lindberg.** 1994. Structural studies of the cell envelope lipopolysaccharides from *Haemophilus ducreyi* strains ITM 2665 and ITM 4747. J. Biol. Chem. **269:**12040–12048.
30. **Schweda, E. K. H., O. Hegedus, S. Borrelli, A. A. Lindberg, J. N. Weiser, D. J. Maskell, and E. R. Moxon.** 1993. Structural studies of the saccharide part of the cell envelope lipopolysaccharide from *Haemophilus influenzae* strain AH1-3 (*lic*3+). Carbohydr. Res. **246:**319–330.
31. **Shaw, D. H., M. J. Squires, E. E. Ishiguro, and T. J. Trust.** 1986. The structure of the heptose-3-deoxy-D-mannooctulosonic-acid region in a mutant form of *Aeromonas salmonicida* lipopolysaccharide. Eur. J. Biochem. **161:**309–313.
32. **Shnyra, A., K. Hultenby, and A. A. Lindberg.** 1993. Role of the physical state of *Salmonella* lipopolysaccharide in expression of biological and endotoxic properties. Infect. Immun. **61:**5351–5360.
33. **Tsai, C.-M., and C. I. Civin.** 1991. Eight lipooligosaccharides of *Neisseria meningitidis* react with a monoclonal antibody which binds lacto-*n*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc). Infect. Immun. **59:**3604–3609.
34. **Tsai, C. M., and C. E. Frash.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Chem. **119:**115–119.
35. **Virji, M., J. N. Weiser, A. A. Lindberg, and E. R. Moxon.** 1990. Antigenic similarities in lipopolysaccharides of *Haemophilus* and *Neisseria* and expression of a digalactoside structure also present on human cells. Microb. Pathog. **9:**441–450.
36. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharide: extraction with phenol-water and further applications of the procedure, p. 83–91. *In* R. L. Whistler (ed.), Methods in carbohydrate chemistry, vol. 5. Academic Press, New York.
37. **Yamasaki, R., J. M. Griffiss, K. P. Quinn, and R. E. Mandrell.** 1993. Neuraminic acid is ∝2→3 linked in the lipooligosaccharide of *Neisseria meningitidis* serogroup B strain 6275. J. Bacteriol. **175:**4565–4568.
38. **Yamasaki, R., W. Nasholds, H. Schneider, and M. A. Apicella.** 1991. Epitope expression and partial structural characterization of F62 lipooligosaccharide (LOS) of *Neisseria gonorrhoeae*: IgM monoclonal antibodies (3F11 and 1-1-M) recognize nonreducing termini of the LOS components. Mol. Immunol. **28:**1233–1242.
39. **Yamasaki, R., H. Schneider, J. M. Griffiss, and R. Mandrell.** 1988. Epitope expression of gonococcal lipooligosaccharide (LOS). Importance of the lipoidal moiety for expression of an epitope that exists in the oligosaccharide moiety of LOS. Mol. Immunol. **25:**799–809.
40. **Zähringer, U., V. Sinnwell, J. Peter-Katalinic, E. T. Rietschel, and C. Galanos.** 1993. Isolation and characterisation of the tetrasaccharide (bis) phosphate from the glycosyl backbone of *Salmonella minnesota* and *Escherichia coli* Re-mutant lipopolysaccharides. Tetrahedron **49:**4193–4200.