The Tetrasaccharide L- α -D-Heptose1 $\rightarrow$ 2-L- α -D-Heptose1 $\rightarrow$ 3-L- α -D-Heptose1 $\rightarrow$ (3-Deoxy-D-manno-Octulosonic Acid) and Phosphate in Lipid A Define the Conserved Epitope in *Haemophilus* Lipopolysaccharides Recognized by a Monoclonal Antibody

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Received 20 September 1994/Returned for modification 12 January 1995/Accepted 12 June 1995

A murine monoclonal antibody, MAHI 3 (immunoglobulin G2b), that is broadly reactive with *Haemophilus influenzae* lipopolysaccharides (LPSs) but nonreactive with all enterobacterial LPSs tested was generated by fusing mouse myeloma cells with spleen cells of BALB/c mice immunized with azide-killed *H. influenzae* RM.7004. MAHI 3 bound to all *H. influenzae*, all other human *Haemophilus* spp., all *Bordetella pertussis* and *Bordetella parapertussis*, and all *Aeromonas* spp. tested but not to any *Neisseria* or *Moraxella catarrhalis* strains, as determined by enzyme immunoassay, colony dot immunoblotting, and immunoblotting. In an inhibition enzyme immunoassay, MAHI 3 reacted with all 45 *H. influenzae* LPSs tested but not with the LPS from the rough mutant I69 Rd⁻/b⁺, which has only 3-deoxy-D-manno-octulosonic acid (P) [Kdo(P)] and lipid A. The antibody was not inhibited by *H. influenzae* lipid A or lipid-free polysaccharide isolated after mild acid hydrolysis. Only native LPSs show positive inhibitory activity, indicating that part of lipid A is involved in the binding of MAHI 3. From the results, it is indicated that the structural element recognized by MAHI 3 is Hep α 1 $\rightarrow$ 2Hep α 1 $\rightarrow$ 3Hep α 1 $\rightarrow$ Kdo together with part of lipid A, including the phosphate.

Haemophilus influenzae is common in the nasopharynx and often causes low-grade infections such as otitis media and exacerbations of chronic bronchitis (33). Some strains possess a polysaccharide (PS) capsule which confers increased virulence. In young children, capsulated *H. influenzae* type b (Hib) causes serious, sometimes life-threatening invasive diseases such as meningitis, epiglottitis, arthritis, cellulitis, pericarditis, and pneumonia. Diseases caused by noncapsulated, so-called nontypeable *H. influenzae* (NTHi) are seen at any age (33). The relative importance of NTHi has increased because of the general introduction of Hib PS-protein conjugate vaccines (54), which prevent invasive Hib diseases but have no effect on infections caused by NTHi or *H. influenzae* of other capsular types. For NTHi, much attention is being focused on surface antigens, such as the lipopolysaccharide (LPS) and the outer membrane proteins and fimbriae.

The LPS, which is a major component in the outer membrane of gram-negative bacteria, in general comprises three structural domains: lipid A, the core saccharide, and an O-antigenic saccharide chain. *H. influenzae* LPSs studied so far lack structures equivalent to the oligosaccharide repeating units which constitute the O antigen of members of the family *Enterobacteriaceae*. In this aspect, *H. influenzae* LPSs are sim-

ilar to those of *Neisseria*, *Moraxella*, and *Bordetella* spp. (18). They all consist of an oligosaccharide linked via a 3-deoxy-D-manno-octulosonic acid (Kdo) residue to a lipid A region (14, 15, 37). The *H. influenzae* lipid A, like those from *Escherichia coli* and *Salmonella typhimurium*, is composed of two glucosamine (GlcN) residues, phosphate, and six long-chain fatty acids (15, 39). The GlcN residues are β linked and 6 substituted, and each carries one phosphate group (one glycosidic phosphate and one at O-4 of the other GlcN residue) (15, 39). The core is often divided into two regions: an inner core consisting of a heptose (Hep) and a Kdo residue and an outer core containing galactose (Gal) and glucose (Glc). The inner core is often substituted with phosphate and phosphoethanolamine (PET) groups (15, 37). The analyses of the *Haemophilus* LPS core structures show that the inner core (14, 31, 35–37, 40, 41), as well as lipid A and Kdo (14, 15, 31, 45), is conserved. This suggests that the frequent switching of LPS antigenic structures in NTHi, referred to as phase variation (23), is a result of variations in the outer-core region.

The potential of broadly reactive and protective monoclonal antibodies (MAbs) as reagents for diagnostic and therapeutic purposes has been extensively discussed for members of the family *Enterobacteriaceae* (4, 9, 10, 19, 24, 34, 46). We here report on a MAb, MAHI 3, that recognizes a conserved epitope in *H. influenzae* LPSs and is therefore a possible candidate for such therapy. MAHI 3 also reacts with the six other human *Haemophilus* species and with the tested *Aeromonas* and *Bordetella* spp. To determine the epitope specificity, the inhibition experiments were performed with saccharide fractions isolated from LPSs. It is suggested that a Hep α 1 $\rightarrow$ 2Hep α 1 $\rightarrow$ 3Hep α 1 $\rightarrow$ Kdo tetrasaccharide element is the epi-

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tope recognized by MAHI 3. The lack of inhibitory activity observed with all of the dephosphorylated LPS samples and saccharide fractions tested furthermore implies that phosphate groups in the LPS are also important for efficient binding of MAHI 3.

(This work was presented at the 7th International Congress of Bacteriology and Applied Microbiology Division, Prague, Czech Republic, 3 to 8 July 1994.)

MATERIALS AND METHODS

Bacterial strains, LPS, and lipid A. Most of the *H. influenzae* and all of the *Haemophilus parainfluenzae*, *Haemophilus parahaemolyticus*, *Haemophilus haemolyticus*, *Haemophilus paraphrophilus*, *Haemophilus aphrophilus*, *Moraxella catarrhalis*, *Bordetella pertussis*, *Bordetella parapertussis*, *Neisseria gonorrhoeae*, *Neisseria lactamica*, and *Neisseria meningitidis* strains were clinical isolates obtained from the Microbiology Laboratory at Huddinge Hospital. *Haemophilus ducreyi* strains were clinical isolates provided by P. Piot, Antwerp, Belgium; NTHi biotype aegyptius and *H. influenzae* RM strains were provided by E. R. Moxon, Oxford, United Kingdom (27–29, 50, 51–53, 56). LPSs from *Aeromonas* SJ strains were from the collection of the Northwest Atlantic Fisheries Centre, St. John's, Newfoundland, Canada, and other *Aeromonas* isolates were provided by T. Wadström, Lund, Sweden. *H. influenzae* 2019 was obtained from M. A. Apicella, Iowa City, Iowa. All *S. typhimurium* rough mutant (Ra to Re), *E. coli* rough mutant (with the core types R1-R4 and K-12), and *H. ducreyi* LPSs and isolated lipid A were from the collection of the Division of Clinical Bacteriology, Huddinge Hospital. *B. pertussis* 1414 LPS was obtained from M. Caroff, Orsay, France.

***H. influenzae* culture conditions and LPS extraction.** Bacteria were cultivated to late logarithmic phase in brain heart infusion broth (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with yeast extract (0.3% [wt/vol]; Difco, Detroit, Mich.), hemin (10 µg/ml; Sigma Chemical Co., St. Louis, Mo.), and NAD (10 µg/ml; Sigma). Cultivation was done at a constant pH of 7.0 with aeration at 37°C in a 35-liter fermentor (Belach Bioteknik AB, Stockholm, Sweden). The bacteria were harvested by centrifugation with a Sorvall RC-5B (with rotor GS-3; Dupont, San Francisco, Calif.) at 4°C at 9,000 × g for 30 min, washed with deionized water, and lyophilized. LPS was extracted from lyophilized bacteria with phenol-chloroform-petroleum ether as described previously (13), but diethyl ether-acetone (1:5, vol/vol) was used instead of water (38). After 3 h at room temperature, the precipitated LPS was separated by centrifugation (Sorvall RC-5B, rotor SS-34) at 4°C at 6,000 × g for 30 min and was then washed twice with diethyl ether-acetone and once with acetone, centrifuged, and lyophilized. LPS dissolved in water was purified by ultracentrifugation (Beckman L8-55M; Beckman Instruments, Inc., Palo Alto, Calif.) at 150,000 × g at 4°C for at least 8 h. The supernatant was discarded and the pellet was lyophilized. Further purification of LPS was done by treatment with RNase (Sigma) and proteinase K (Sigma).

Isolation of PS. LPSs (20 mg) from *H. influenzae* or *Aeromonas* spp. in aqueous 1% acetic acid (HOAc; 6 ml) were kept in an ultrasonication bath for 10 min and then hydrolyzed at 100°C for 90 min. After it cooled, the mixture was centrifuged, and the precipitated lipid A was washed three times with water, centrifuged, and dried. The product, PS-HOAc (~10 mg), was purified by chromatography on a Sephadex G-15 column (2.6 by 90 cm; Pharmacia, Uppsala, Sweden) with water as the eluent. The eluate was monitored by a differential refractometer (Waters, Milford, Mass.), and fractions were analyzed by the phenol sulfuric acid assay to estimate neutral hexoses (11). Fractions containing PS-HOAc were pooled and lyophilized.

Chemical modification of the LPS. (i) **De-O-acylation of LPS by mild alkali treatment (LPS-OH).** *H. influenzae* LPS (20 mg) was treated with 0.25 M NaOH (2 ml) at 37°C for 16 h. After cooling to 20°C and neutralization with HOAc, the solution was dialyzed against distilled water with a Spectra/Por 2000 MW membrane (Spectrum, Houston, Tex.) and finally lyophilized.

(ii) **De-O,N-acylation of LPS-OH by strong alkali treatment (PS-OH).** Treatment of LPS-OH (10 mg) with 4 M KOH (2 ml) at 100°C for 4 h, cooling, neutralization with HOAc, and desalting on a Sephadex G-15 column gave PS-OH. Fractions were detected and isolated as described above.

(iii) **Dephosphorylation of LPS by hydrogen fluoride (LPS-HF).** LPS (10 mg) was treated with cooled 48% aqueous HF (1 ml) at 4°C for 48 h. The HF was removed in a polypropylene desiccator in vacuo, with solid NaOH as a desiccant. After dialysis, the phosphorus content before and after dephosphorylation was estimated (7).

Chemical analysis of LPS and PSs. LPS and derivatives (~1 mg) were hydrolyzed in 0.5 M trifluoroacetic acid (Merck, Darmstadt, Germany) at 100°C for 16 to 18 h to give a mixture of monosaccharides. Reduction with sodium borohydride and acetylation gave alditol acetates which were analyzed by gas-liquid chromatography. The alditol acetates were separated on a DB-5 fused silica capillary column with a flame ionization detector set for a temperature program of 180°C for 4 min and then rising to 240°C at 5°C/min. The different sugars were identified by their retention times according to standards.

Production and characterization of MAbs. MAb MAHI 3 was generated after

mouse myeloma cells (Sp2/0-Ag14) were fused with spleen cells of 8-week-old BALB/c mice immunized intraperitoneally with 10⁷ sodium azide-killed bacteria from Hib RM.7004 (27, 51). Cell culture media and fusion procedures were as described previously (8, 26). Screening was performed by enzyme immunoassay (EIA) at 15 to 20 days postfusion against the homologous LPSs as antigens. Cloning was done twice by limiting dilution, and clones were screened with a panel of LPS and lipid A from *H. influenzae*, *Salmonella* spp., *E. coli* strains, and *Aeromonas* spp. to determine cross-reactivity. The antibody class and subclass were determined by double radial immunodiffusion in which rabbit anti-mouse immunoglobulin 1, 2a, 2b, and 3 and µ heavy-chain antisera (Litton Bionetics, Kensington, Md.) were tested against the concentrated culture supernatant (26).

EIA and inhibition studies. EIA was done as previously described (21). Briefly, 96-well microtiter plates (Costar, Cambridge, Mass.) were coated overnight at 20°C with LPS (10 µg/ml) in 0.05 M carbonate buffer (pH 9.6), blocked with 0.5% (wt/vol) gelatin (Merck) in phosphate-buffered saline (PBS) for 2 h at 20°C, and then washed three times with washing buffer (0.15 M NaCl and 0.05% [vol/vol] Tween 20). Hybridoma culture supernatant containing MAb was diluted with PBS containing 0.05% Tween 20, and a 100-µl portion was added to wells, incubated for 3 h at 20°C, and washed as before. This was followed by the addition of 100 µl of alkaline phosphatase-labelled rabbit antibody to mouse immunoglobulin (Dakopatts AS) per well appropriately diluted in PBS-Tween 20. The plate was incubated overnight at 20°C and washed again. Substrate solution (100 µl of *p*-nitrophenyl phosphate [Sigma] per well at 1 mg/ml in 1 M diethanolamine–0.5 mM MgCl₂ buffer [pH 9.8]) was added, the plate was incubated for 60 min at 20°C, and then the A₄₀₅ was read with a Titertek Multiskan photometer (Flow Laboratories, Irvine, Scotland). If the value was below 0.2, it was considered negative, 0.2 to 0.6 was considered weak, and above 0.6 was considered strong. EIA inhibition was performed by preincubating twofold serial dilutions of inhibitors with antibodies in serum-free culture fluid for 1 h at 20°C as described previously (26). The antibody dilution used for the assay was at the middle of the linear portion of the titration curve, which corresponded to an A₄₀₅ of about 1.0 when tested against the RM.7004 XP-1 AH1-3 LPS. The preincubated mixture was added to LPS-coated microtiter plates and incubated for 3 h at 20°C before the conjugate, appropriately diluted in PBS-Tween 20, was added. The rest of the procedure was as described above for the EIA assay. Percent inhibitory values were calculated, compared with those of the controls without inhibitor, and plotted against the concentration of inhibitor. The concentration for 50% inhibition (I₅₀) was determined for each LPS.

Whole-bacteria EIA. Concentrations of bacterial suspensions in PBS were adjusted to give an optical density of 0.2 at 595 nm. The bacteria were killed by addition of 0.1% sodium azide. The cell suspension was dispensed in 100-µl aliquots into 96-well microtiter plates (Costar) and incubated overnight at 20°C. PBS-gelatin was used as a blocking agent (at 100 µl/well for 2 h at 20°C). The rest of the EIA procedure was as described above.

Colony dot immunoblotting. For the colony dot immunoblotting assay, 22 single colonies from each strain were individually suspended in 300 µl of PBS in a U-shaped, 96-well microtiter plate and then were dotted onto a nitrocellulose membrane filter (pore size, 0.45 µm; BA-85; Schleicher & Schuell, Dassel, Germany) previously loaded in the blotting apparatus (Bio-Dot apparatus; Bio-Rad Laboratories, Richmond, Calif.). After 60 min at 20°C, the wells were washed three times with 150 µl of PBS–0.5% (wt/vol) gelatin–0.05% (vol/vol) Tween 20 (PBS-G-T) under vacuum. The membrane was removed and left to air dry for 30 min at 20°C. After that, the membrane was incubated with 3% (wt/vol) gelatin (Merck) in PBS at 20°C for 2 h, and this was followed by incubation with the culture fluid containing MAb MAHI 3 diluted (1/20) in PBS-G-T at 20°C for 3 h on a rocking device. After it was washed three times with PBS-G-T, the membrane was incubated overnight at 20°C with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin antibodies (Dakopatts) appropriately diluted with PBS-G-T. The membrane was washed three more times as described above and developed for 3 to 4 min at 20°C with 0.3 M veronal acetate buffer (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 MgCl₂ (1 M). The results were scored as positive if more than 50% of the colonies were visually reactive, for phase variation if more than 10 but less than 50% of the colonies were visually reactive, and negative if at least 90% of the colonies were constitutively nonreactive.

SDS-PAGE and immunoblotting. Whole bacterial lysate preparations from strains of *Haemophilus* spp., *Neisseria* spp., *M. catarrhalis*, *Bordetella* spp., *Aeromonas* spp., and *Enterobacteriaceae* spp. were analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 14% acrylamide resolving gel containing 0.45% bisacrylamide, prepared as previously described (17, 48). LPSs from *H. influenzae* RM.7004 and *S. typhimurium* SL 1032 and SL 1181 rough mutants were used as controls. Samples (10 µg of LPS per well or 30 µl of the whole bacterial lysate made with a bacterial suspension with an optical density adjusted to 0.8 at 595 nm per well) were heated for 5 min at 100°C in sample buffer as described previously (17). Electrophoresis was carried out at a constant current of 40 mA per gel for about 9 h. Electrophoretic transfer from SDS-PAGE gels was performed in a Trans Blot system (Bio-Rad Laboratories) immediately after the electrophoresis was finished. The transfer to a 0.2-µm-pore-size nitrocellulose membrane (Schleicher & Schuell) was done overnight at 110 mA. After the transfer, the membrane was soaked with 3% (wt/vol) gelatin (Merck) in PBS at 20°C for 2 h, and this was followed by

FIG. 1. Binding of MAHI 3 to isolated LPSs and free lipid A (10 μ g/ml) as determined by direct EIA. The numbered bars are as follows: 1, *H. influenzae* LPSs isolated from capsulated type a (Hia) strains (Morgan, RM.120, RM.7032, RM.7115, ATCC 9006, and RM.1185); 2, *H. influenzae* LPSs isolated from capsulated type b (Hib) strains (RM.7099, RM.7098, Eagan, *galE*, Eagan *galK*, Eagan *galE galK*, Rd⁻/b⁺:01, Rd⁻/b⁺:02, RM.1004, HS.9887, RM.7004-H5, RM.7004, RM.7004 AH1-2, RM.7004 XP-1, RM.7004 XP-1 AH1-3, RM.7004RVDEL8, RM.7004 *galE*, RM.7004 *galK*, and RM.7004 *galE galK*); 3, *H. influenzae* LPSs isolated from capsulated type c (Hic) strains (RM.8032 and RM.1166); 4, *H. influenzae* LPSs isolated from capsulated type d (Hid) strain ATCC 9008; 5, *H. influenzae* LPSs isolated from capsulated type e (Hie) strains (RM.1064, RM.1019, and HS.9583); 6, *H. influenzae* LPSs isolated from capsulated type f (Hif) strain RM.124; 7, *H. influenzae* LPSs isolated from uncapsulated (NTHi) strains (RM.118-26, RM.118-28, RM.118-1, RM.118-102, RM.118-201, RM.118, RM.7047, RM.118-26H4, HS.9764, HS.17013, HS.9495, 2019, and HS.10147); 8, *H. influenzae* pooled LPS batches isolated from capsulated type b strain I69 Rd⁻/b⁺; 9, *H. ducreyi* LPSs isolated from ITM 4747, ITM 2665, ITM 3542, ITM 3147, ITM 5535, and ACY1; 10, *S. typhimurium* LPSs isolated from rough mutant strains [TV119(Ra), SL733(Rb1), TV161(Rb2), TV148(Rb3), SL805(Rc), SL1032(Rd1), SL1181(Rd2), and SL1102(Re)]; and 11, *E. coli* LPSs isolated from rough strains [HF4707(R1), EH100(R2), F653(R3), F2513(R4), and W3110(K12)]. The numbers at the left are titers, and the error bars show standard deviations.

incubation with MAb MAHI 3 diluted 1/20 in PBS-G-T at 20°C for 3 h on a rocking device. After it was washed three times with PBS-G-T, the membrane was incubated overnight at 20°C with alkaline phosphatase-rabbit anti-mouse immunoglobulin conjugate (Dakopatts) appropriately diluted in PBS-G-T. After the membrane was washed three more times as described above, the results of the reactions were visualized with the blue insoluble product of the phosphatase from the substrate Nitro Blue Tetrazolium in the same manner as for the colony dot immunoblotting described above.

RESULTS

Production and selection of MAb MAHI 3 specific for *H. influenzae* LPS. Immunization of mice with *H. influenzae* RM.7004, fusion, and initial screening by EIA against six different LPSs isolated from the RM.7004 series of *H. influenzae* strains (27–29, 50, 51–53) gave hybridoma MAHI 3. During subsequent recloning by limiting dilution, each culture supernatant was tested by EIA against LPSs from defined *H. influenzae*, *S. typhimurium*, and *E. coli* rough mutants and various lipid A preparations. MAHI 3 (immunoglobulin G2b) recognized all the *H. influenzae* LPSs, but no binding to the enterobacterial LPSs or any lipid A was observed (Fig. 1).

Binding of MAHI 3 to whole bacteria as determined by EIA and colony dot immunoblotting. MAHI 3 binding to any of a collection of azide-killed gram-negative bacteria, isolated from various sources from humans and fish, was tested by EIA. Binding, weakly and strongly positive, was obtained with *H. influenzae* and other *Haemophilus* species and *Aeromonas* iso-

FIG. 2. Binding of MAHI 3 to gram-negative bacteria as determined by EIA. MAb culture supernatants diluted 1:20 were tested by duplicate EIA. Data are mean values of optical densities (A_{405}) after 60 min for at least two experiments. Results were scored on the basis of A_{405} : negative, <0.2; weakly positive, 0.2 to <0.8; strongly positive, ≥ 0.8 . The numbers on the left are the number of samples.

lates (Fig. 2). For *Bordetella* strains, both negative binding and weakly and strongly positive binding were observed. The majority of the other bacteria were negative, with the exception of some *Neisseria* strains, which showed a weak positive reaction.

To further analyze MAHI 3 epitope expression within each strain of the same collection, a colony dot immunoblotting assay was performed. The observations were similar to those for binding by EIA, with positive reactions for *H. influenzae*, *H. ducreyi*, and *Aeromonas* spp. A mixed picture was observed for *Bordetella* and *Neisseria* isolates. None of the enterobacterial species was positive (Fig. 3). Phase variation was observed for *H. influenzae*, *H. ducreyi*, and *B. pertussis* isolates.

FIG. 3. Binding of MAHI 3 to whole gram-negative bacteria as determined by colony dot immunoblotting. Results were scored as follows: negative, at least 90% of the colonies were nonreactive; positive, more than 50% of the colonies were reactive; phase variation, more than 10 but less than 50% of the colonies were reactive. The numbers on the left are numbers of samples.

FIG. 4. Binding of MAHI 3 to lysates of whole gram-negative bacteria as determined by immunoblotting. The numbers on the left are the number of samples.

Immunoblotting of MAHI 3 against whole bacterial lysates. MAHI 3 was also tested against whole-cell lysates (Fig. 4) by immunoblotting to a nitrocellulose membrane after separation by SDS-PAGE (data not shown). The same strain collection as described above was used. MAHI 3 bound to all *Haemophilus*, *Aeromonas*, and *Bordetella* lysates. Differences in the characteristics of the bands were, however, observed with *Bordetella* strains, which had weaker reactions and different electrophoretic migration patterns for their lysates compared with those of the *Haemophilus* and *Aeromonas* lysates. None of the enterobacteria, *Neisseria*, or *M. catarrhalis* lysates reacted with MAHI 3. These data indicate that MAHI 3 binds to a major saccharide structure of the LPSs of the *Haemophilus*, *Aeromonas*, and *Bordetella* spp. tested.

Reactivity of MAHI 3 with LPS as determined by EIA. MAHI 3 reactivity with LPSs from three *H. influenzae*, two *H. ducreyi*, five *Aeromonas*, one *B. pertussis*, and eight *Salmonella* rough mutant strains, all with known saccharide structures, was tested (Table 1). In addition, MAHI 3 reactivity with LPSs isolated from 43 other *H. influenzae* strains was tested (Fig. 1). MAHI 3 strongly bound to 45 of the 46 LPSs from *H. influenzae* strains. It reacted weakly with the LPS from *H. influenzae* rough mutant I69 Rd⁻/b⁺, which has only a Kdo(P) residue and a lipid A. The binding of MAHI 3 to six *H. ducreyi* LPSs suggested the presence of a common epitope in these species (Table 1 and Fig. 1). MAHI 3 also reacted with all five *Aeromonas* LPSs and showed the highest titers against *Aeromonas* SJ-83 and SJ-25 LPSs (Table 1 and Fig. 1 and 5). MAHI 3 bound weakly to the *B. pertussis* 1414 LPS, but no binding to the *Salmonella* rough mutant LPSs or to different lipid As was observed (Fig. 1 and Table 1).

Definition of the binding specificity of MAHI 3. To further investigate the epitope specificity of MAHI 3, EIA inhibition studies were performed with LPS from *H. influenzae* RM.7004 XP-1 AH1-3 as the coating antigen. The inhibitory activities (given as I₅₀ values) of the LPSs and lipid As from 46 *H. influenzae*, 4 *H. ducreyi*, 5 *Aeromonas*, and about a dozen *Salmonella* and *E. coli* rough mutants were analyzed. Representative samples are presented in Fig. 6. The majority (98%) of the *H. influenzae* LPSs had values of <12 µg/ml, 84% of them had values of <1.0 µg/ml, and 22% of them had values of ≤0.1

µg/ml. No inhibitory activity was observed with the *H. influenzae* I69 Rd⁻/b⁺ LPS (Fig. 6B). Three of the *H. ducreyi* LPSs showed values of ≤3.0 µg/ml, while a threefold-higher concentration was required for *H. ducreyi* ITM 2665 LPS (Fig. 6C). The *Aeromonas* SJ-83 LPS was highly active, with an I₅₀ value of 0.1 µg/ml, while an 80-fold-higher concentration was needed for the *Aeromonas* SJ-25 LPS. The other *Aeromonas* LPSs were less active as inhibitors (Fig. 6D), and no inhibitory activity was observed with any of the LPSs from *Salmonella* and *E. coli* rough mutants and *B. pertussis* 1414 or with isolated lipid A, even at 100 µg/ml (data not shown).

Analysis of PSs and chemically modified LPSs as inhibitors of the binding of MAHI 3. In order to define the epitope specificity of MAHI 3 to RM.7004 XP-1 AH1-3 LPS, a series of LPS derivatives was made: (i) ester-linked fatty acids were removed (LPS-OH), (ii) ester- and amide-linked fatty acids were removed (PS-OH), (iii) the LPSs were dephosphorylated (PS-HF), and (iv) the LPSs were delipidated with acid (PS-HOAc). All values discussed are I₅₀ values.

Both LPS-OH and PS-OH strongly inhibited the binding of MAHI 3, with values of ≤2.0 µg/ml (Table 2 and Fig. 7A to D). The *H. influenzae* 2019 LPS-OH was the best inhibitor, with a value almost the same as that for the corresponding native LPS (0.9 and 0.6 µg/ml, respectively). Concentrations 2.5 to 3.3 times higher than those for the native LPSs were required for three other *H. influenzae* derivatives (Table 2 and Fig. 7A to D). The PS-HOAc obtained from both the *Aeromonas* and *H. influenzae* LPSs caused an inhibition of the binding of MAHI 3 below 50% at the highest concentration tested (50 µg/ml) (Table 2 and Fig. 7). None of the LPS-HF tested reached inhibition, even at a concentration of 200 µg/ml (data not shown). The results indicate that the phosphate group(s) of the LPS is important for the binding of MAHI 3.

DISCUSSION

The inhibition of the binding of MAHI 3 to *H. influenzae* LPS antigens by 45 *H. influenzae* LPSs demonstrated the presence of the MAHI 3 antigenic determinant in all these LPSs. The two LPSs from *H. influenzae* that had known major structures of the core saccharides (35, 40) and that were included in this study were as follows:

H. influenzae RM.7004 XP-1 AH1-3 (Hib)

H. influenzae 2019 (NTHi)

The common feature of these two LPSs is a pentasaccharide portion of the inner core, Hepα1→[PEt→6]2Hepα1→[Glcβ1→4]3Hepα1→Kdo(P), and a hexaacyl lipid A (14, 15, 37, 39). Furthermore, the inhibition with *H. ducreyi* LPSs suggests an epitope expressed also in this species. In line with this, other investigators have reported the presence of the same

TABLE 1. EIA reactivity of MAb MAHI 3 against *Aeromonas*, *B. pertussis*, *Haemophilus*, and *S. typhimurium* LPSs

Source of LPS (reference)	Type	Structure of the major core oligosaccharide ^a	Mean A ₄₀₅ ^b
<i>H. influenzae</i> RM. 7004 XP-1 AH1-3 (40)	b	$\begin{array}{c} \text{Glc}\beta 1 \dots 4 \text{Glc}\beta 1 \rightarrow 4 \text{Hep}\alpha 1 \rightarrow \text{Kdo(P)} \\ \alpha \uparrow 1,3 \\ \text{Gal}\beta 1 \rightarrow 2 \text{Hep}\alpha 1 \rightarrow 2 \text{Hep} \\ \uparrow 6 \\ \text{PEt} \end{array}$	3.2
<i>H. influenzae</i> 2019 (35)	NT	$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{Glc}\beta 1 \rightarrow 4 \text{Hep}\alpha 1 \rightarrow \text{Kdo(P)} \\ \alpha \uparrow 1,3 \\ \text{Hep}\alpha 1 \rightarrow 2 \text{Hep} \\ \uparrow 6 \quad \uparrow 6 \\ \text{PEt} \quad \text{PEt} \end{array}$	3.1
<i>H. influenzae</i> I69 Rd ⁺ /b ⁺ (15)	b	Kdo(P)	0.2
<i>H. ducreyi</i> ITM 4747 (41)		$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{Glc}\beta 1 \rightarrow 4 \text{Hep}\alpha 1 \rightarrow \text{Kdo(P)} \\ \alpha \uparrow 1,3 \\ \text{Hep}\alpha 1 \rightarrow 2 \text{Hep} \end{array}$	3.4
<i>H. ducreyi</i> ITM 2665 (41)		$\begin{array}{c} \text{Gal}\beta 1 \rightarrow 4 \text{GlcNAc}\beta 1 \rightarrow 3 \text{Gal}\beta 1 \rightarrow 4 \text{DDHep}\beta 1 \rightarrow 6 \text{Glc}\beta 1 \rightarrow 4 \text{Hep}\alpha 1 \rightarrow \text{Kdo(P)} \\ \alpha \uparrow 1,3 \\ \text{Hep}\alpha 1 \rightarrow 2 \text{Hep} \end{array}$	1.7
<i>A. sobria</i> SJ-25 (3, 42)	III	$\begin{array}{c} \text{Glc} \\ \alpha \downarrow 1,6 \\ \text{Qui} p3 \text{NAc}\alpha 1 \rightarrow 3 \text{Gal}\alpha 1 \rightarrow 3 \text{Hep}\alpha 1 \rightarrow 2 \text{Hep}\alpha 1 \rightarrow 6 \text{Kdof} \\ \alpha \uparrow 1,4 \\ \text{GlcNAc}\alpha 1 \rightarrow 7 \text{Hep} \\ \alpha \uparrow 1,4 \\ \text{Glc} \end{array}$	2.9
<i>A. hydrophila</i> SJ-26 (2, 42)	II	$\begin{array}{c} \text{Hep} \\ \alpha \downarrow 1,6 \\ \text{Glc}\beta 1 \rightarrow 4 \text{Hep}\alpha 1 \rightarrow 2 \text{Hep}\alpha 1 \rightarrow 6 \text{Kdof} \\ \alpha \uparrow 1,3 \\ \text{GlcNAc}\alpha 1 \rightarrow 7 \text{Hep} \end{array}$	1.5
<i>A. hydrophila</i> SJ-55 (32, 42)	I	$\begin{array}{c} \text{Hep} \\ \alpha \downarrow 1,6 \\ \text{Gal}\beta 1 \rightarrow [\text{DDHep}\alpha 1 \rightarrow 6] 4 \text{DDHep}\alpha 1 \rightarrow 6 \text{Glc}\beta 1 \rightarrow 4 \text{Hep}\alpha 1 \rightarrow 2 \text{Hep}\alpha 1 \rightarrow 6 \text{Kdof} \\ \alpha \uparrow 1,3 \\ \text{GlcNAc}\alpha 1 \rightarrow 7 \text{Hep} \end{array}$	1.6
<i>A. salmonicida</i> SJ-83 (42, 44)		Hep $\alpha 1 \rightarrow 2$ Hep $\alpha 1 \rightarrow 3$ Hep $\alpha 1 \rightarrow 6$ Kdof	3.0
<i>A. salmonicida</i> SJ-15 (42, 43)		$\begin{array}{c} \text{Hep} \\ \alpha \downarrow 1,6 \\ \text{Gal}\alpha 1 \rightarrow 4 \text{GalNAc}\alpha 1 \rightarrow 6 \text{Glc}\alpha 1 \rightarrow 4 \text{Hep}\alpha 1 \rightarrow 2 \text{Hep}\alpha 1 \rightarrow 3 \text{Hep}\alpha 1 \rightarrow 6 \text{Kdof} \\ \beta \uparrow 1,4 \\ \text{GlcNAc}\alpha 1 \rightarrow 7 \text{Hep} \end{array}$	0.7
<i>B. pertussis</i> 1414 (25)		$\begin{array}{c} \text{Glc}\beta 1 \rightarrow 4 \text{Hep}\alpha 1 \rightarrow 5 \text{Kdo(P)} \\ \uparrow \alpha 1,3 \\ \text{GlcA}\alpha 1 \rightarrow 2 \text{Hep} \\ \uparrow \alpha 1,7 \\ \text{GlcN} \end{array}$	0.6
<i>S. typhimurium</i> ^c TV119 (16)	Core Ra	$\begin{array}{c} \text{GlcNAc} \\ \downarrow \alpha 1,2 \\ \text{Glc}\alpha 1 \rightarrow 2 \text{Gal}\alpha 1 \rightarrow 3 \text{Gal}\alpha 1 \rightarrow 3 \text{Gal}\alpha 1 \rightarrow 3 \text{Hep}\alpha 1 \rightarrow 3 \text{Hep}\alpha 1 \rightarrow (\text{Kdo})_2 \rightarrow \\ \text{Rb}_1 \quad \text{Rb}_2 \quad \text{Rc} \quad \text{Rd}_1 \quad \text{Rd}_2 \quad \text{Re} \\ \text{Rb}_3 \end{array}$	<0.15

^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; Qui, quinosamine; ..., nonstoichiometrical substitution.

^b Each reading represents the average of triplicate determinations taken after 60 min.

^c *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).

FIG. 5. Titration EIA of MAHI 3 against different *Aeromonas* spp. LPS antigens (10 μ g/ml each) are as follows: ■, SJ-25 LPS; ▲, SJ-26 LPS; ◆, SJ-83 LPS; □, SJ-55 LPS; and ●, SJ-15 LPS.

pentasaccharide (31, 41) and lipid A (31) as a part of the LPS from *H. ducreyi* 35000, ITM 2665, and ITM 4747. Moreover, the binding of MAHI 3 to the five *Aeromonas* LPSs with known saccharide structures suggests that elements of these LPSs are involved in the MAHI 3 epitope. Common features among the *Aeromonas* LPSs are the disaccharide element Hep α 1 $\rightarrow$ 2Hep found either terminally or internally, a single nonphosphorylated Kdo residue, and a triphosphorylated hexaacyl lipid A (2, 3, 32, 42–44). However, neither the LPS from the *H. influenzae*

rough mutant I69 Rd $^-$ /b $^+$, which has only Kdo(P) and hexaacyl lipid A (15), nor purified hexaacyl lipid A from *S. typhimurium*, *E. coli*, *H. influenzae*, or *Aeromonas* spp. was able to cause any inhibition of the binding of MAHI 3 to *H. influenzae* LPS antigens. This suggests that the immunodominant groups which are defining the specificity of the MAb are mainly in the saccharide moiety of the LPS from both *Haemophilus* and *Aeromonas* species. The binding of MAHI 3 to *Aeromonas* SJ-83 LPS, which has the saccharide part Hep α 1 $\rightarrow$ 3Hep α 1 $\rightarrow$ 6Kdo, and to the LPSs from *H. influenzae* RM.7004 XP-1 AH1-3 and 2019 (Tables 1 and 2) suggested that the tetrasaccharide Hep α 1 $\rightarrow$ 2Hep α 1 $\rightarrow$ 3Hep α 1 $\rightarrow$ Kdo is the essential part of the MAb epitope. It is essential to point out here the importance of the inhibition experiments for the definition of epitope specificity. Binding data are indicative, but inhibitory data are much more conclusive. However, the strong binding of MAHI 3 to *Aeromonas sobria* SJ-25 LPS, in which the Hep α 1 $\rightarrow$ 3Hep linkage is absent and the Hep α 1 $\rightarrow$ 2Hep disaccharide element is highly substituted (Table 1), was surprising. The results of the inhibition studies using *Aeromonas* LPSs as inhibitors (Table 2 and Fig. 6D) suggested a partial recognition of the epitope in the SJ-25 LPS (which is 80 times less efficient as an inhibitor than the SJ-83 LPS). The combined evidence leads us to conclude that the MAb epitope recognizes the tetrasaccharide Hep α 1 $\rightarrow$ 2Hep α 1 $\rightarrow$ 3Hep α 1 $\rightarrow$ Kdo.

The role of the Hep α 1 $\rightarrow$ 3Hep disaccharide element in the MAHI 3 epitope was analyzed by the binding of MAHI 3 to LPSs, PSs, and synthetic saccharides (34) from *Salmonella* core mutants with known saccharide structures (16) as inhibitors.

FIG. 6. *H. influenzae*, *H. ducreyi*, and *Aeromonas* LPSs as inhibitors. The inhibitors were preincubated with MAHI 3 and then were added to microtiter plates coated with 10 μ g of *H. influenzae* RM.7004 XP-1 AH1-3 LPS per ml. (A) LPSs isolated from *H. influenzae* strains: ◆, Morgan (Hia); □, RM.118-26 (NTHi); ■, RM.124 (Hif); ▲, ATCC 9008 (Hid); ●, RM.1064 (Hie); and ○, RM.1166 (Hic). See the legend to Fig. 1 for an explanation of the abbreviations. (B) LPSs isolated from capsulated type b strains: ■, RM.7004; ◆, RM.7004 XP-1; ●, RM.7004 AH1-2; ▲, RM.7004 XP-1 AH1-3; □, RM.7004 RVDEL8; ◆, RM.7004 galE galK; ▽, Rd $^-$ /b $^+$:01; ▽, Rd $^-$ /b $^+$:02; and ○, I69 Rd $^-$ /b $^+$. (C) LPSs isolated from *H. ducreyi* strains: ■, ITM4747; ◆, ITM2665; ●, ITM3147; and ▲, ITM3542. (D) LPSs isolated from *Aeromonas* spp.: ■, SJ-15; ◆, SJ-25; ▲, SJ-26; ●, SJ-55; and ○, SJ-83.

TABLE 2. Inhibition of MAHI 3 by *H. influenzae* and *Aeromonas* PSs and LPSs^a

Strain and inhibitor	Sugar composition ^b			Phosphorus content (%) ^c	I ₅₀ of inhibitor for MAHI 3 ^d
	Glc	Gal	Hep		
<i>H. influenzae</i>					
RM.7004 XP-1 AH1-3					
LPS	2.0	1.0	2.0	3.8	0.1
LPS-OH ^e	1.8	1.0	2.0	1.2	0.25
PS-OH ^f	1.7	1.0	1.6	0.9	0.9
PS-HOAc ^g	2.1	1.0	1.7	1.0	>50
LPS-HF ^h	2.0	1.0	2.0	<0.1	>50
RM.7004 XP-1					
LPS	2.0	1.0	2.0	3.9	0.1
LPS-OH	1.6	1.0	1.2	1.0	0.3
PS-OH	2.1	1.0	1.6	1.1	0.5
PS-HOAc	2.2	1.0	2.0	1.1	>50
LPS-HF	1.5	1.0	1.6	<0.1	>50
RM.7004 AH1-2					
LPS	1.3	1.0	0.5	4.5	0.3
LPS-OH	1.4	1.0	0.5	1.6	1.0
PS-OH	1.4	1.0	0.8	1.5	2.0
PS-HOAc	1.4	1.0	0.8	1.6	>50
LPS-HF	1.3	1.0	0.6	<0.1	>50
2019					
LPS	1.0	1.0	0.8	4.4	0.6
LPS-OH	1.0	0.9	0.8	nd ⁱ	0.9
PS-OH	nd	nd	nd	nd	0.8
PS-HOAc	1.0	1.0	0.8	1.8	>50
LPS-HF	nd	nd	nd	<0.1	>50
<i>Aeromonas</i> strains					
SJ-25					
LPS	2.0	1.0	2.0	2.7	8.0
PS-HOAc	2.0	1.0	2.0	<0.1	>50
LPS-HF	nd	nd	nd	<0.1	>50
SJ-26					
LPS	1.0		4.5	2.8	6.0
PS-HOAc	1.1		5.0	<0.1	>50
LPS-HF	nd	nd	nd	<0.1	>50
SJ-55					
LPS	1.1	1.0	5.2	2.6	8.0
PS-HOAc	1.0	1.6	6.2	<0.1	>50
LPS-HF	nd	nd	nd	<0.1	>50
SJ-83					
LPS			1.0	2.6	0.1
PS-HOAc			1.0	<0.1	20.0
LPS-HF	nd	nd	nd	<0.1	>50
SJ-15					
LPS	1.0	1.2	3.6	2.7	>50
PS-HOAc	1.0	1.0	4.0	<0.1	>50

^a As determined by EIA. Coating antigen, RM.7004 XP-1 AH1-3 LPS (10 µg/ml).^b The sugar content of each LPS and PS was analyzed by gas-liquid chromatography on the basis of their alditol acetates. Results are given relative to those for galactose. Only the compositions of Glc, Gal, and Hep were taken into account in the case of the *Aeromonas* strains.^c The phosphorus content was determined according to the method of Chen et al. (7).^d Mean values from at least two experiments.^e De-O-acylated LPS.^f De-O,N-acylated LPS.^g Delipidated LPS.^h Dephosphorylated LPS.ⁱ nd, not determined.

We were not able to find any inhibition at concentrations of up to 100 µg/ml of each inhibitor (data not shown).

The binding of MAHI 3 was efficiently inhibited by de-O-acylated (LPS-OH) and de-O,N-acylated (PS-OH) LPSs, i.e., LPSs that have lost their ester-linked lipids and ester- and amide-linked fatty acids, respectively. The higher amount required by the PS-OH for I₅₀ indicated that micelle formation of the LPS was essential for effective inhibition (Table 2 and Fig. 7A to D). As was expected, the native LPSs were potent

inhibitors, since LPS in aqueous solution exists as aggregates with a multivalent presentation of the epitope. Neither the *H. influenzae* nor the *Aeromonas* PSs obtained by mild acid hydrolysis (PS-HOAc) caused remarkable inhibition; however, a low inhibitory value (about 40%) was observed at the highest concentration (50 µg/ml). This significantly less efficient inhibition of binding or absence of inhibition by the *Haemophilus* PSs (PS-HOAc) may indicate the importance of an intact Kdo residue (1) and/or of the fatty acid residues in lipid A which

FIG. 7. Test of chemically modified *H. influenzae* and *Aeromonas* LPSs as inhibitors. The inhibitors were preincubated with MAHI 3 and then were added to microtiter plates coated with 10 μ g of *H. influenzae* RM.7004 XP-1 AH1-3 LPS per ml. (A) RM.7004 XP-1 AH1-3 LPS. (B) RM.7004 XP-1 LPS. (C) RM.7004 AH1-2 LPS. (D) NTHi 2019 LPS. The symbols for panels A to D are as follows: ■, LPS; ◆, de-O-acylated LPS; ●, de-O,N-acylated LPS; ▲, delipidated LPS; and □, dephosphorylated LPS. (E) *Aeromonas* LPSs and PS-HOAc. ■, SJ-25 LPS; ◆, SJ-25 delipidated LPS; ◇, SJ-25 dephosphorylated LPS; □, SJ-26 LPS; ▽, SJ-26 delipidated LPS; *, SJ-26 dephosphorylated LPS; ♦, SJ-83 LPS; ⊕, SJ-83 delipidated LPS; ▣, SJ-83 dephosphorylated LPS; ●, SJ-55 LPS; ▲, SJ-55 delipidated LPS; ▤, SJ-55 dephosphorylated LPS; —, SJ-15 LPS.

could influence the mode of presentation of the LPS molecules, as was suggested previously (55).

The importance of the phosphate groups in MAb binding was evident, since none of the dephosphorylated LPSs (LPS-HF), *Aeromonas* SJ-83 PS-HOAc, or SJ-83 LPS-HF, all devoid of phosphate, was an inhibitor (2, 3, 31, 42–44) (Table 2 and Fig. 7D). The phosphate group in lipid A is presumably that at O-4 of the GlcN residue linked to Kdo. The weak binding of the LPS from *H. influenzae* I69 Rd[−]/b⁺ that was observed ($A_{405} = 0.2$, but no inhibition) also supports the role played by the phosphate in the lipid A in the MAb epitope. That the Kdo residue in *Aeromonas* spp. is furanosidic and 6-substituted obviously has no bearing on the binding capacity.

The MAHI 3 epitope remained accessible in the majority of *Haemophilus* strains tested, as evaluated with whole bacteria (Fig. 2 and 3). The binding to whole-cell lysates (Fig. 4) con-

firmed that the MAb epitope is a major conserved structure of the *Haemophilus* LPS, which seems to be an invariant feature of this bacterial genus, although some human and animal species listed in Bergey's Manual (22) have not been tested yet. These results are in line with the limited phase variation observed by colony dot immunoblotting (Fig. 3). Thus, phase variation seems to be a consequence of the on-off expression of other sugars in the outer core rather than a variation of the inner-core moiety itself.

The high percentage of nonreactive *H. ducreyi* as determined by the whole-cell EIA may be explained as a consequence of a steric hindrance, i.e., glycosyl capping of the inner core that sterically affects the accessibility of the MAb to its epitope. This correlates well with the reactivity found by immunoblot analysis, in which the steric hindrance does not appear to play any role. Assay-dependent differences were evident (Fig. 2 to

4), supporting the broader reactivity of MAHI 3 as determined by colony dot immunoblotting. The nonreactivity of four *H. influenzae* strains as determined by EIA may be explained on the same basis.

MAHI 3 did not bind to *Neisseria* or *M. catarrhalis* whole-cell lysates. The Hep region in known neisserial LPSs (14, 20, 37, 49) lacks the Hep α 1 $\rightarrow$ 2Hep disaccharide element, which may explain the absence of reactivity. For *M. catarrhalis* LPS, which is devoid of Hep residues (12, 30), the nonreactivity is in accord with expectations.

Interestingly, MAHI 3 bound to *B. pertussis* 1414 LPS, although no inhibition was observed. It also reacted with whole cells as determined by EIA and colony dot immunoblotting and with all 53 *B. pertussis* lysates tested by an immunoblotting assay (Fig. 4). Differences in the intensities and characteristics of the bands, compared with those of the *Haemophilus* and *Aeromonas* lysates, were observed (data not shown). This may indicate a partial recognition of the epitope in the *B. pertussis* lysates by MAHI 3. Szabó and Szabó (47) and recently Lebbar et al. (25) reported on two LPSs of *B. pertussis* (LPS A and B) that both have two Hep residues, one phosphorylated Kdo residue, and a pentaacyl lipid A (1, 5, 6). The saccharide part has GlcA α 1 $\rightarrow$ 2Hep and GlcNAc1 $\rightarrow$ 7Hep elements instead of Hep α 1 $\rightarrow$ 2Hep and PET $\rightarrow$ 6Hep. It is worth noting that α -GlcA and α -D-Hep have the same ring conformation and anomeric configuration and therefore exhibit fair similarity with each other. The reported structure (20, 25) is as follows:

This shows that the Hep α 1 $\rightarrow$ 3Hep1 $\rightarrow$ 5Kdo(P) trisaccharide element and lipid A are a common denominator for *Haemophilus* and *Bordetella* LPSs. However, the lack of inhibition indicates that this trisaccharide element, as a part of *B. pertussis* LPSs, was not enough to inhibit the binding of MAHI 3 to *H. influenzae* LPS antigens.

These results lead us to conclude that (i) the Hep α 1 $\rightarrow$ 2Hep disaccharide element plays an essential role in MAHI 3 binding, (ii) the Hep α 1 $\rightarrow$ 2Hep α 1 $\rightarrow$ 3Hep α 1 $\rightarrow$ Kdo tetrasaccharide element is recognized by the MAb epitope, and (iii) the phosphates of the lipid A moiety are important for the definition of MAHI 3 specificity, although the MAb does not bind to isolated lipid A.

MAHI 3 demonstrates that a conserved epitope is not only exposed and accessible in LPSs from different strains of *Haemophilus* spp. but is also immunogenic. This is, to the best of our knowledge, the first broadly reactive, *Haemophilus* LPS-specific MAb that recognizes a chemically defined epitope.

There is a potential for the use of MAbs in the therapy of diseases caused by gram-negative bacteria, as has been discussed for, e.g., *E. coli* (10). LPSs and cytokines are responsible for the intermediate stages of the pathogenic process and ought to be suitable therapeutic targets. It would be most valuable if a broadly cross-reactive, anti-LPS MAb like MAHI 3 could be produced. The common epitope, if recognized, is also a suitable epitope to be included in a future vaccine against NTHi.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Agency for Research Co-operation with Developing Countries (SAREC) and the Swedish Medical Research Council (grant 16x-09496).

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