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Characterization of an Antigenic Oligosaccharide from *Leptospira interrogans* Serovar pomona and Its Role in Immunity

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An antigenic oligosaccharide fraction derived from the lipopolysaccharide of *Leptospira interrogans* serovar pomona was isolated by endo-glycosidase H digestion and column chromatography. The oligosaccharide contained rhamnose, ribose, glucose, and glucosamine and inhibited the binding of opsonic, protective monoclonal antibodies directed against the lipopolysaccharide. When conjugated to diphtheria toxoid, the oligosaccharide elicited the production of agglutinating, opsonic antibodies.

Leptospirosis is a worldwide zoonosis, caused by infection with *Leptospira* serovars, which occurs as an acute, febrile illness in humans and animals. In temperate climates two of the serovars most commonly associated with disease are *L. interrogans* serovar pomona and *L. borgpetersenii* serovar hardjobovis, whose reservoirs are pigs and cattle, respectively (8, 14). Immunity to *Leptospira* infection depends on the production of circulating, agglutinating antibodies (4, 5). The most significant immunogenic antigen is leptospiral lipopolysaccharide (LPS), whose chemical and biological properties differ from those of the typical LPS of gram-negative organisms such as *Escherichia coli* (28, 29, 32) and which appears to be neither toxic nor pyrogenic. Monoclonal antibodies (MAbs) directed against a polysaccharide (PS) epitope of leptospiral LPS passively protected guinea pigs against leptospirosis (18), and MAbs directed against LPS determinants were opsonic (15). In addition, leptospiral LPS is a major antigen recognized by human antibodies produced in response to *Leptospira* infection (11). Immunization with leptospiral LPS or PS fraction of LPS from *L. interrogans* serovar copenhageni protected hamsters against homologous lethal challenge (17), suggesting that leptospiral LPS antigens could be used as immunizing agents against leptospirosis. However, the antigenic determinants of leptospiral LPS have not been defined despite extensive investigations using MAbs (1, 24) or antigenic fractions of the LPS (19, 25, 30). The aim of this study was to characterize a fraction of serovar pomona LPS containing a protective epitope(s) and to assay its ability to elicit the production of agglutinating, opsonic antibodies.

MATERIALS AND METHODS

Organisms, antigens, and serological methods. *L. interrogans* serovar pomona strain L10 was isolated from a human case of leptospirosis (12). Other leptospiral strains used were obtained from the *Leptospira* Reference Laboratory, Brisbane, Australia (*L. interrogans* serovars copenhageni L19 and australis L168 and *L. borgpetersenii* serovar tarassovi L169), the National Health Institute, Wellington, New Zealand (*L. borgpetersenii* serovar ballum L17), or Massey University, Palmerston North, New Zealand (*L. borgpetersenii* serovar hardjobovis L171). Cultivation of leptospires and the microscopic agglutination test were performed as described previously (3). Briefly, serial twofold dilutions of sera were incubated with viable leptospires at 28°C for 1 h and agglutination was observed by examining a loopful on a glass slide by dark-field microscopy. Enzyme immunoassays (EIA) were performed as described previously (2, 7), using LPS (10 μg) as the coating antigen. Inhibition EIA was performed by adding the inhibitor (leptospiral PS, oligosaccharide [OLS], or sugars) to each well immediately before the MAbs and incubating on a rotary shaker for 1 h at 37°C. The remainder of the test was performed as described previously (7). Chromatography-grade sugars (Mann Research Laboratories Inc. or Sigma) were used as inhibitors. The preparation and characteristics of the MAbs against LPS from serovars copenhageni (H1), pomona (SSF2-3), or hardjo (H4D4) were described previously (1, 6, 15, 18). A MAb against the LPS of *Pasteurella multocida* (T3F4), described previously (27), was used as a negative control.

The preparation of leptospiral LPS and the PS fraction of LPS was described previously (18), while leptospiral LPS prepared similarly from serovars other than pomona and hardjobovis was kindly provided by H. Jost. LPS from other gram-negative organisms was purchased from Sigma. For the production of OLS, PS was dissolved in 50 mM sodium acetate buffer, pH 7.2 (5 mg/ml), and digested with endo-glycosidase H (Boehringer-Mannheim, Mannheim, Germany) (2 mU/ml) overnight at 37°C. Further digestion was stopped by immersion in a boiling water bath for 4 min. The solution was applied to a Bio-Gel P-4 column and eluted with 0.01 M ammonium bicarbonate. Carbohydrate was detected in the column eluate by using a differential refractometer (Waters), and fractions containing carbohydrate were collected and dried. The antigenicity of the fractions containing OLS was assayed by EIA. The chemical composition of the OLS was determined by gas-liquid chromatography (GLC) as described previously (32), while the size of the OLS was determined by thin-layer chromatography (TLC). In both of these procedures chromatography-grade sugars (Mann Research Laboratories or Sigma) were used as standards. The OLS and standard sugars were spotted onto silica gel high-performance TLC aluminium-backed plates (Merck, Melbourne, Australia). The separation solvent consisted of equal volumes of 2-propanol, acetone, and 1 M lactic acid. Detection of saccharides was achieved by dipping the plate into detection reagent consisting of diphenylamine (0.4 g), aniline (0.4 ml), acetone (20 ml), and 85% (wt/vol) phosphoric acid (3 ml).

Preparation of OLS-DT. The procedure for preparation of OLS-diphtheria toxoid conjugates (OLS-DT) was based on the

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method of Peeters et al. (26). The OLS was dissolved in *N*-ethylmorpholine (Sigma)–water–12 N HCl (2.5:6.5:1 [vol/vol/vol], pH 8.5) in concentrations of approximately 24 μmol in 250 μl; 125 μmol of *N*-succinimidyl *S*-acetylmercaptoacetate (Sigma) in 250 μl of *N,N* dimethylacetamide (Sigma) was added to the OLS and mixed thoroughly. The reaction mixture was left at room temperature for 1 h with occasional shaking. Then, 125 μl of acetic acid and 5 ml of acetone were added sequentially and mixed. The precipitate was collected by centrifugation, washed with acetone, and lyophilized. To 10 mg of purified DT (Commonwealth Serum Laboratories, Melbourne, Australia) in 1.75 ml of 0.1 M sodium phosphate buffer (pH 7.8), 10 μmol of *N*-succinimidyl bromoacetate (Sigma) in 250 μl of *N,N* dimethylacetamide (Sigma) was added. After 1 h the reaction mixture was passed through a Sephadex P-10 column equilibrated in 0.1 M sodium phosphate buffer containing 5 mM EDTA. The solution of protein collected (3.5 ml) was added to the modified OLS and incubated at 22°C with 100 μl of 0.2 M hydroxylamine (Sigma) in 0.1 M sodium phosphate buffer containing 5 mM EDTA, pH 6.1. After 24 h the remaining bromoacetyl groups were blocked by the addition of 20 μmol of 2-aminoethanethiol (Sigma). After a further 24 h the conjugate was purified in two batches by using a Biogel P-4 column (buffer pH, 6.1) and then concentrated twofold in an Amicon P10 concentrator. The carbohydrate content was assayed by the phenol-sulphuric acid method of Dubois et al. (13), and the protein concentration was measured by the micromethod of Bradford (9).

Dot blotting. Solutions of LPS, PS, OLS-DT, DT, or pooled column eluates were dotted onto nitrocellulose (Schleicher and Schuell; 0.45 μm pore size) and immunostained with anti-LPS MAbs (1, 6, 15, 18, 27), mouse antisera against whole leptospires, or mouse antisera against PS-DT (22), as required. The secondary antibody consisted of peroxidase-conjugated rabbit anti-mouse immunoglobulins (Silenus Laboratories, Melbourne, Australia); development was done with 4-chloronaphthol (16). The procedure for Western blotting (immunoblotting) was described previously (23).

Immunization procedure. Groups of 6- to 8-week-old, inbred, female BALB/c mice were injected subcutaneously with OLS-DT conjugate containing 45 μg of protein and less than 1 μg carbohydrate in 0.05 ml of PBS with 0.05 ml of incomplete Freund's adjuvant on days 0 and 14 followed by a third injection of 0.1 ml of OLS-DT on day 35. The mice were bled at intervals from the orbital plexus.

CL. Chemiluminescence (CL) assays to measure phagocytosis following opsonization of viable leptospires were performed as described previously (21) except that mouse peritoneal macrophages prepared as described previously (27) were used as the phagocytic cells.

RESULTS

Production of OLS. Endo-glycosidase H-digested PS could be separated into two fractions by Biogel P-4 gel filtration (Fig. 1). Fraction A was eluted at the void volume (>4,000 kDa), while fraction B eluted at a point similar to that determined in separate experiments for the trisaccharide raffinose. This fraction was designated OLS, and by TLC analysis it migrated to a distance equivalent to the tetrasaccharide stachyose (data not shown). The yield of OLS was approximately 5 to 10% of the weight of the original PS. Both the OLS and the PS recovered after digestion (peak A) reacted specifically in a dot blot with polyclonal pomona mouse antiserum and the serovar pomona LPS-specific MAb SSF2-3.

Chemical composition of OLS and PS. By GLC analysis

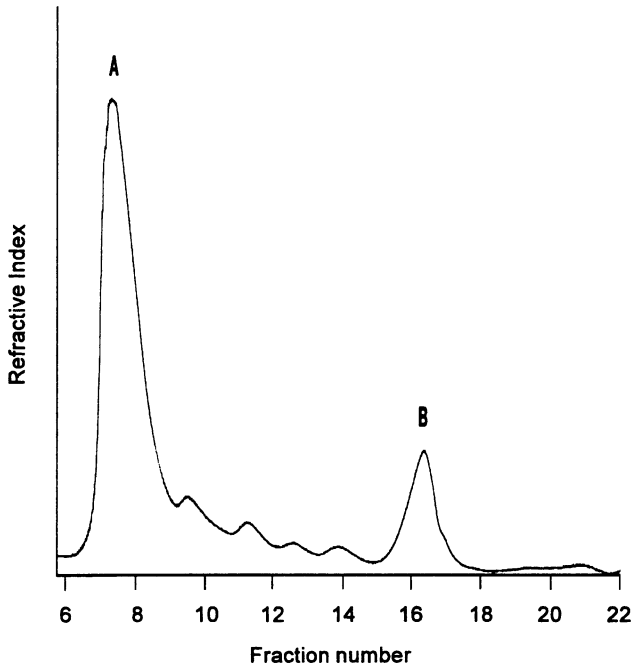

FIG. 1. Separation of fractions of endo-glycosidase H-treated PS from *L. interrogans* serovar pomona by Biogel P-4 chromatography. Peak A eluted at the void volume. Peak B contained the OLS fraction. The original was scanned using an Epson GT-8000 scanner and the Epson Scan! program. Labels and axes were added by using Corel Photopaint and Corel Draw.

pomona PS was found to contain rhamnose (24.3%), ribose (14.7%), mannose (10.0%), xylose (7.0%), glucose (5.6%), galactose (4.3%), and glucosamine (0.5%). Similar GLC analysis showed the OLS to contain rhamnose (33.5%), glucose (23%), ribose (15.5%), and glucosamine (14.6%). The OLS appeared to be moderately resistant to acid hydrolysis; 4 M HCl was required for hydrolysis of OLS as compared with 1 M HCl needed for hydrolysis of undigested PS.

Inhibition of EIA with PS, OLS, or sugars. In an inhibition EIA the OLS inhibited the reaction of the pomona PS-specific MAb SSF2-3 with serovar pomona LPS with activity similar to

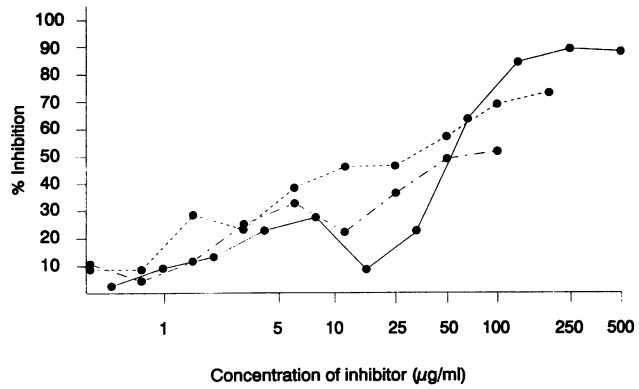

FIG. 2. Inhibition by OLS, HCl-digested OLS, or PS of the binding of serovar pomona LPS-specific MAb SSF2-3 to LPS from serovar pomona as measured by EIA. Inhibition by serovar pomona PS (——), serovar pomona OLS (– – – –), and serovar pomona OLS digested with 2 M HCl (— – — –).

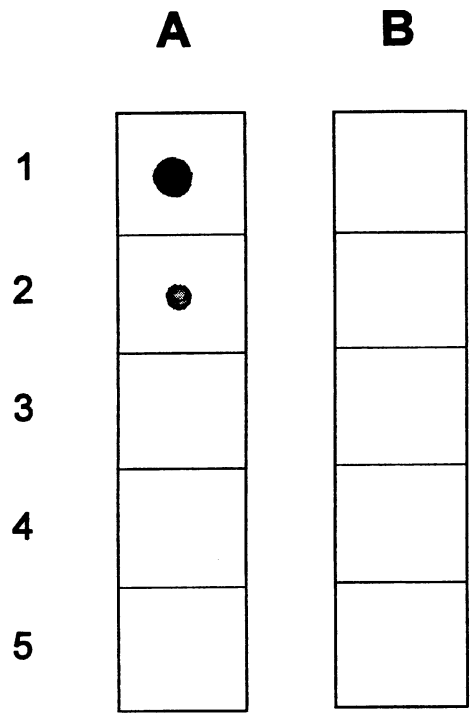

FIG. 3. Reactivity by dot blot of OLS-DT purification fractions with the serovar pomona LPS-specific MAb SSF2-3 and an unrelated MAb, T3F4. The original was scanned using an Epson GT-8000 scanner and the Epson Scan! program. Labels and axes were added by using Corel Photopaint and Corel Draw. (A) Serovar pomona LPS-specific MAb SSF2-3; (B) *P. multocida* LPS-specific MAb T3F4. Rows: 1, serovar pomona PS; 2, OLS-DT (peak A from OLS-DT purification chromatography); 3, peak B from OLS-DT purification chromatography; 4, peak C from OLS-DT purification chromatography; 5, DT.

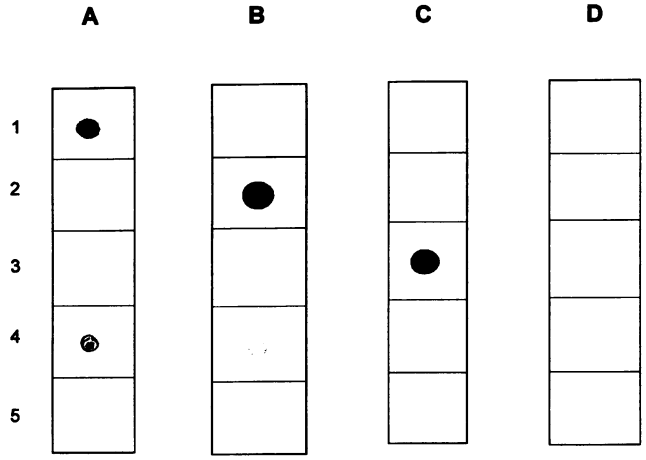

FIG. 4. Dot blot analysis of reactions between serovar pomona OLS-DT and MAbs specific for homologous (serovar pomona) or heterologous (serovars hardjo, copenhageni, or *P. multocida*) LPS. The original was scanned using an Epson GT-8000 scanner and the Epson Scan! program. Labels and axes were added by using Corel Photopaint and Corel Draw. (A) Serovar pomona LPS-specific MAb SSF2-3; (B) serovar hardjo LPS-specific MAb H4D4; (C) serovar copenhageni LPS-specific MAb H1; (D) *P. multocida* MAb T3F4. Rows: 1, serovar pomona LPS; 2, serovar hardjo LPS; 3, serovar copenhageni LPS; 4, serovar pomona OLS-DT; 5, DT.

that of undigested PS (Fig. 2). OLS hydrolyzed with 2 M HCl was also capable of inhibition but at lower levels than intact OLS. None of the monosaccharide components of PS or the short oligosaccharides (lactose, trehalose, raffinose, and stachyose) tested had any inhibitory activity.

Production of OLS-DT conjugate. The OLS-DT prepared as described in Materials and Methods eluted from a Biogel P-4 column in a broad peak in the void volume fraction (peak A). Two further peaks (peaks B and C) were also detected, probably containing residual chemicals from the conjugation. Because of the small size of the OLS no attempt was made to purify OLS-DT from unconjugated DT. The OLS-DT (peak A) reacted with a pomona PS-specific MAb, SSF2-3 (Fig. 3), by dot blot. A slight reaction was observed with peak B. It is likely that peak B, eluting at the same point as raffinose, contained residual unconjugated OLS. No reaction was seen with peak C. The pomona-specific MAb SSF2-3 did not react with DT. A MAb directed against the LPS of *P. multocida* (T3F4) did not react with OLS-DT or with OLS. The protein content of the OLS-DT was 895 μg/ml. The carbohydrate content of the conjugated OLS-DT was undetectable by the phenol-sulphuric acid method, indicating that the carbohydrate concentration of OLS-DT was less than 20 μg/ml. Thus, the proportion of carbohydrate in the OLS-DT was less than 0.25% of the protein content.

OLS-DT reacted with a serovar pomona specific MAb and also (to a lesser extent) a serovar hardjo LPS MAb when tested by dot blot. OLS-DT did not react with a serovar copenhageni-specific MAb (Fig. 4).

Immunization with OLS-DT conjugate. By week 8, four of the six mice that received three subcutaneous injections of 45 μg (based on its protein content) of OLS-DT had produced detectable amounts of agglutinating antibodies (Fig. 5). These antibodies were not pomona specific; they reacted with pomona LPS but also with LPS from some other leptospiral serovars (serovars hardjobovis, ballum, copenhageni, canicola, grippotyphosa, and batavia) and LPS from other bacteria

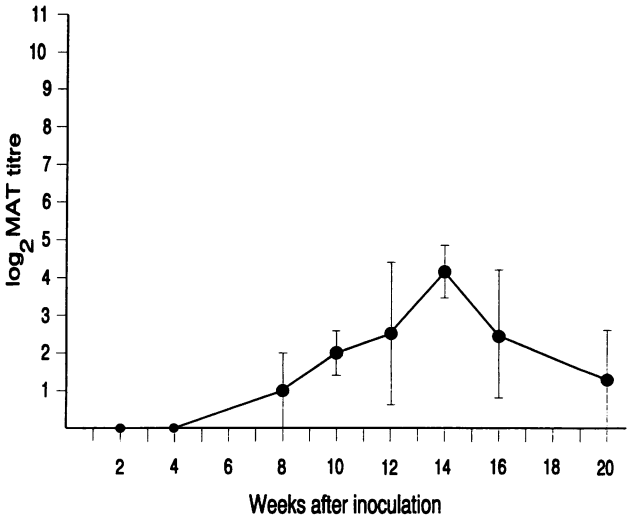

FIG. 5. Mean agglutinating antibody response in a group of six mice injected with serovar pomona OLS-DT at weeks 0, 2, and 4. The bars indicate one standard deviation. MAT, microscopic agglutination test.

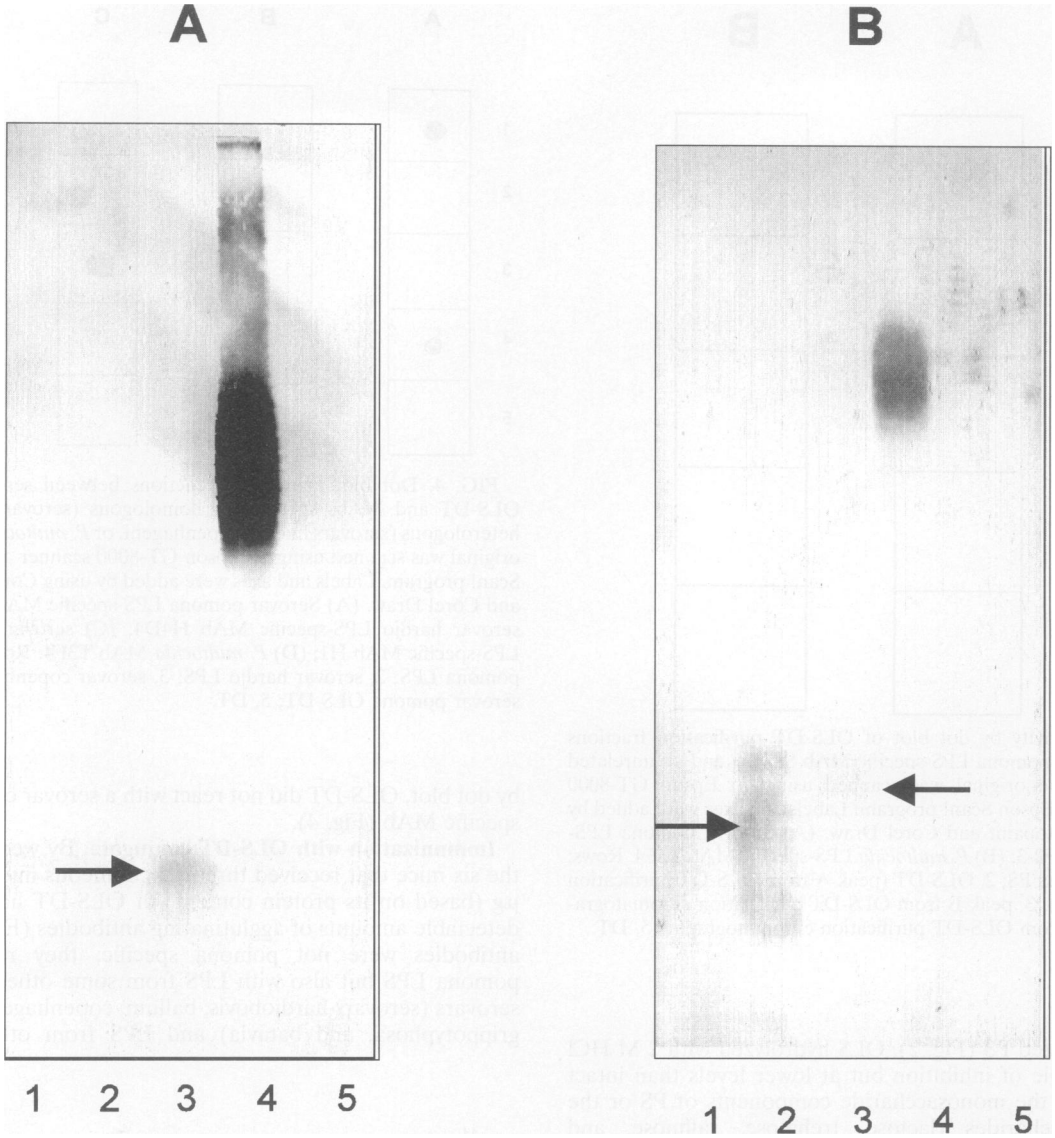

FIG. 6. Reactivity by immunoblot of mouse antiserum against serovar pomona OLS-DT followed by detection with immunoglobulin G-specific (A) or immunoglobulin M-specific (B) anti-immunoglobulins. The reactions with LPS are indicated by arrows. The original was scanned using an Epson GT-8000 scanner and the Epson Scan! program. Labels and axes were added by using Corel Photopaint and Corel Draw. Lane 1, serovar copenhageni LPS; lane 2, serovar hardjobovis LPS; lane 3, serovar pomona LPS; lane 4, purified DT; lane 5, serovar grippotyphosa LPS.

(*Salmonella typhimurium* and the Re mutant of *Shigella flexneri*) when tested by dot blot against purified LPS (Table 1).

OLS-DT antiserum agglutinated serovar pomona with an average maximum titer of 24 at week 14. The antiserum could also agglutinate other leptospiral serovars (hardjobovis and copenhageni) at lower titers (microscopic agglutination test titers of 8 and 2, respectively) than pomona. It did not agglutinate serovars australis, grippotyphosa, ballum, or tarassovi. Serovars proechimys, kennewicki, and tropica (members of the Pomona serogroup) were agglutinated by OLS-DT antiserum at titers of between 2 and 8, while monjakov, kunming, and tsaratsovo (also members of the Pomona serogroup) were not agglutinated. Antiserum raised against DT alone did not agglutinate leptospires, nor did it react with any LPS preparations in dot blot assays.

By Western blot analysis (Fig. 6) both the IgG and IgM antibody fractions of OLS-DT antiserum reacted with the upper band of serovar pomona LPS (arrow) while the IgM fraction also reacted with serovar hardjobovis LPS (arrow). Neither the IgG nor IgM fraction reacted with LPS from serovars copenhageni or grippotyphosa. Both isotypes reacted with DT. OLS-DT antiserum reacted by EIA with the LPS of serovars pomona (absorbance of 0.9 at a serum dilution of 1/100), hardjobovis (absorbance of 0.5 at a serum dilution of 1/100), and grippotyphosa (absorbance of 0.65 at a serum dilution of 1/100). There was no reaction with the LPS of serovars ballum, illini, bim, canicola, or batavia (data not shown).

The incubation of serovars pomona and hardjobovis bacterial cells with OLS-DT antiserum induced a similarly high CL response in mouse macrophage monolayers (Fig. 7), while serovar copenhageni cells and OLS-DT antisera induced no

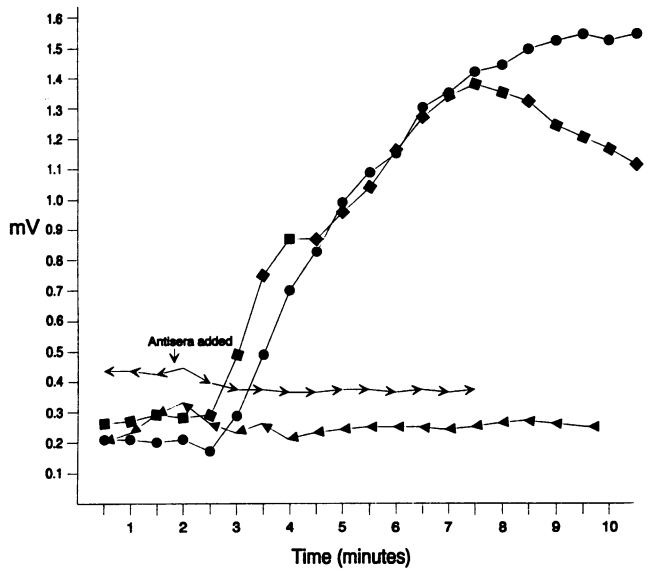

FIG. 7. CL responses of mouse macrophages after interaction with leptospiral cells and antisera against either *L. interrogans* serovar pomona OLS-DT or DT. ●, OLS-DT antiserum and serovar pomona cells; ■, OLS-DT antiserum and serovar hardjobovis cells; >, OLS-DT antiserum and serovar copenhageni cells ▶, DT antiserum and serovar pomona or hardjobovis cells.

CL response. Anti-DT antiserum was similarly negative. Similar results were obtained when phagocytosis was assessed by immunofluorescence tests in which both pomona and hardjobovis bacterial cells were opsonized by PS-DT antiserum while serovars copenhageni and ballum were not (data not shown).

DISCUSSION

Studies by other workers (19, 24, 30) have reported the isolation of antigenic fragments from leptospiral LPS by acid or alkaline hydrolysis. These fragments ranged in size from 4 to less than 1 kDa. The size of the OLS isolated in this study was comparable to the approximately tetrasaccharide-size fragment produced from serovar canicola (24). Inhibition of activity was seen with a MAb (SSF2-3) specific for serovar pomona (1, 6). As no sugar or combination of single sugars could inhibit the binding of the pomona MAb SSF2-3 to LPS, it is reasonable to assume that linkages of monosaccharides are important in determining the reactive epitopes.

TABLE 1. Reactivity by dot blot of antiserum against serovar pomona OLS-DT with the LPS of other serovars and other bacterial species.

Bacterial LPS	Dot blot result
L. interrogans serovar pomona	+
L. borgpetersenii serovar hardjobovis	+
L. borgpetersenii serovar ballum	+
L. interrogans serovar copenhageni	+
L. interrogans serovar canicola	+
L. kirschneri serovar grippotyphosa	+
L. interrogans serovar batavia	−
L. illini	−
S. minnesota	−
S. minnesota Rc mutant	−
S. minnesota Re mutant	−
S. minnesota Rd mutant	−
S. flexneri	−
S. flexneri Re mutant	+
S. typhimurium	+

As the size and carbohydrate nature of the OLS suggested that it was unlikely to be immunogenic, it was conjugated to DT to produce an immunoconjugate. This immunoconjugate stimulated the production of antibodies which agglutinated homologous and heterologous *Leptospira* serovars. This is the first report of an immunogenic OLS from leptospiral LPS, but fractions of the *Haemophilus influenzae* capsule (26) and OLS derived from *Neisseria meningitidis* LPS (31) induced the production of specific antibodies when conjugated to proteins.

The OLS appeared homogeneous in both column chromatography and TLC assays. Despite the small size of the OLS, the antiserum raised against it reacted with a range of leptospiral serovars when tested by dot blot or EIA, suggesting that the OLS contained either multiple epitopes or a single epitope common to several serovars. The dot blot and EIA appeared to be more sensitive than the Western blot for the detection of such heterologous reactions. Alternatively the cross-reactive epitope(s) may have been affected by the denaturing conditions used in polyacrylamide gel electrophoresis for Western blotting.

The reaction of OLS-DT with both pomona MAb SSF2-3 and the hardjo MAb H4D4 (neither of which cross-reacted by agglutination or EIA with the heterologous LPS [1, 15]) and the ability of anti-OLS-DT serum to agglutinate and opsonize both pomona and hardjobovis suggest that at least one of the OLS epitopes is common to the two serovars. There is a high degree of cross-reactivity between leptospiral serovars (including pomona, copenhageni, and hardjobovis) (10) attributed at least in part to carbohydrate antigens. Those cross-reactions were detected by Western blotting but not by agglutination, which is usually more specific (10). When antibodies produced against the PS fraction of LPS from serovars pomona and hardjobovis were tested by the microscopic agglutination test, the agglutinating antibodies reacted only with homologous organisms (22). We therefore suggest that in the whole cell or PS (when conjugated to DT), the cross-reacting epitope(s) induces antibodies that do not agglutinate the heterologous serovars, but in OLS conjugated to DT, the epitope or epitopes are exposed, arranged, or altered in such a way as to induce the production of cross-reacting agglutinating antibodies. The reactivity of antiserum against OLS-DT with the Re mutant of *S. flexneri* (which contains only 2-keto-3-deoxyoctulosonic acid [KDO] linked to lipid A) suggested that the altered or exposed epitopes in the conjugated OLS resembled components of the lipid A or KDO. Possibly the reaction was with the disaccharide of glucosamine contained in the lipid A and exposed only in the Re mutants. Although the core region of *S. flexneri* is reported to be similar if not identical to that of salmonellae (20) and OLS-DT antiserum did not react with the Re mutant of *Salmonella minnesota*, substituents of phosphate groups and phosphorylethanolamine may be found in the core region (20) and these additions may be important in epitope recognition.

The stimulation of cross-reactive agglutinins is noteworthy because agglutinating antibodies play a key role in protection from infection (4, 5). This correlated with the results of the opsonization assays in which leptospires of both serovars pomona and hardjobovis were opsonized, suggesting that cross-protection between the two serovars may be possible with a vaccine containing appropriate cross-reactive LPS epitopes such as the OLS antigen identified in this study.

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