

## Monoclonal Antibody Analysis of Lipopolysaccharide from *Neisseria gonorrhoeae* and *Neisseria meningitidis*

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A hybridoma produced by the polyethylene glycol fusion of the NS-1 variant of the P3x63Ag8 BALB/c plasmacytoma to splenocytes harvested from a BALB/c mouse immunized with whole gonococci was found to be producing antibody to a common region on gonococcal lipopolysaccharide (LPS). Enzyme-linked immunosorbent assay inhibition systems were established by utilizing this antibody, designated 3F11, and 100% inhibition occurred with both LPS and the LPS-derived polysaccharides from each of the six prototype strains. Meningococcal LPS and LPS-derived polysaccharides partially inhibited the enzyme-linked immunosorbent assay, whereas similar preparations isolated from *Escherichia coli* O:111, the J-5 mutant of this strain, and *Salmonella minnesota* Re595 failed to inhibit the assay. Studies utilizing whole gonococcal strains 4505 and the isogenic variant 4505r, which lacks both the LPS serotype and common determinants as inhibitors, demonstrated that the determinant recognized by the 3F11 antibody was present on the surface of 4505 and absent on 4505r. Inhibition studies were performed with  $\beta$ -glucose,  $\beta$ -galactose, D-glucosamine, D-galactosamine, heptose, 2-keto-3-deoxyoctanoate, N-acetylglucosamine, N-acetylgalactosamine,  $\alpha$ -lactose, and  $\beta$ -lactose. Complete inhibition of the enzyme-linked immunosorbent assay occurred with D-galactosamine, and partial inhibition was achieved with both  $\alpha$ -lactose and  $\beta$ -lactose. Based on these observations, the 3F11 antibody recognizes a site common to gonococcal LPS which is partially shared by meningococcal LPS. The chemical structure of the determinant appears to be a D-galactosamine-O-D-galactopyranosyl-(1-4)-D-glucopyranose. Additional specificity may be conferred by the steric relationship of the determinant on the intact LPS.

The antigenic structure of gonococcal lipopolysaccharide (LPS) is a complex of at least three major antigenic determinants (2, 4) which are contained on the carbohydrate portion of the macromolecule. These determinants consist of a specific serotype antigen and as many as two common antigens (4). Six antigenically distinct gonococcal serotype antigens have been described (4). In addition, phenotypic variation can occur within isogenic strains of gonococci, producing a distinct LPS with different antigenic characteristics (S. A. Morse and M. A. Apicella, submitted for publication).

To further elucidate the antigenic structure of gonococcal LPS and to correlate this structure with chemical composition, studies have been instituted which would identify hybridomas producing monoclonal antibody with specificity for LPS antigen sites. In this paper, the results of

the identification and specificity of such an LPS-specific antibody are described. This antibody identifies a common locus on gonococcal LPS. A portion of the site is present on meningococcal LPS. The antibody failed to recognize the site on LPS isolated from *Escherichia coli* O:111, the J-5 mutant of *E. coli* O:111, or *Salmonella minnesota* R595. Inhibition studies with a variety of sugars indicate that the antibody is inhibited by D-galactosamine and is partially inhibited by  $\alpha$ -lactose and  $\beta$ -lactose. Application of the monoclonal antibody technique has proven useful in defining an antigen locus common to all gonococcal LPSs.

### MATERIALS AND METHODS

**Strains.** Prototype strains representative of the six gonococcal LPS serotypes were obtained from our own collection. These were strain 1342 (serotype Gc<sub>1</sub>), strain 1291 (serotype Gc<sub>2</sub>), strain 4505 (serotype Gc<sub>3</sub>), strain 8551 (serotype Gc<sub>4</sub>), strain PID2 (serotype Gc<sub>5</sub>), and strain 3893 (serotype Gc<sub>6</sub>). Strain 4505r was also

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used in this study. This strain is an isogenic variant of Gc<sub>3</sub> strain 4505 and was selected by virtue of its resistance to pyocin 611 311. Studies of the antigenic and biological properties of the LPS derived from this variant have recently been described (10) and indicate that this LPS lacks the Gc<sub>3</sub> serotype antigen and a substantial portion of two common antigens found on the LPS of this gonococcal serotype. Meningococcal strains representative of serogroups A, B, C, and Y and *E. coli* O:111 were obtained from our own collection. *E. coli* J-5 and *S. minnesota* mutant 595 were obtained from James Nolan, Department of Medicine, State University of New York at Buffalo. All strains were preserved at -70°C in Mueller-Hinton broth containing 10% glycerol.

**Antisera.** Rabbit antisera to the six gonococcal LPS serotypes were prepared as previously described (2, 3). Rabbit anti-mouse immunoglobulin G1 (IgG1) for use in enzyme-linked immunosorbent assay (ELISA) studies was prepared by two subcutaneous immunizations with 0.25 mg of murine IgG1 (Bionetics, Kensington, Md.) in complete Freund adjuvant at 14-day intervals. Animals were bled for antibody 30 days after the last immunization. Sera were tested by immunodiffusion for the presence of precipitating antibody to murine IgG1. Affinity-purified murine IgG1 was accomplished as previously described (5), and the eluted antibody was conjugated to alkaline phosphatase for use in the ELISA studies (3, 4).

**ELISA and ELISA inhibition.** ELISAs for the detection of monoclonal antibody to gonococcal outer membranes and subsequently to LPS-derived polysaccharide were performed by a modification of the method of Voller et al. (13) in polyvinyl chloride microtiter V plates (Dynatech Laboratories, Inc., Alexandria, Va.). Rabbit antibody to murine IgG1 was conjugated to alkaline phosphatase for these experiments.

Each of the following reagents was added in 0.2-ml volumes to each well. Plates were coated with either 5 µg of 4505 Gc<sub>3</sub> polysaccharide per ml or 10 µg of 4505 outer membranes in 0.5 M carbonate-bicarbonate buffer (pH 9.6) at 37°C for 3 h and overnight at 4°C. The plates were washed three times with phosphate-buffered saline containing 0.05% Tween 20 at this time and before the addition of each of the subsequent reagents. After being washed, the wells were filled with serial twofold dilutions of hybridoma tissue culture or ascitic fluid and incubated for 24 h at 4°C. The initial dilutions of the tissue culture fluid and the ascitic fluid were 1:2 and 1:100, respectively. After washing, alkaline phosphatase-conjugated rabbit anti-murine IgG1 was added at a dilution of 1:400. The conjugated antibody was incubated in the microtiter plates overnight at 4°C. After the wells were washed, *p*-nitrophenylphosphate (Sigma Chemical Co., St. Louis, Mo.) in 1.0 M diethanolamine buffer (pH 9.8) containing 100 mg of MgCl<sub>2</sub> per liter was placed in each well. Color development was stopped at 30 min by the addition of 0.050 ml of 3 N NaOH to each well. The contents were read at 400 nm in a 2-mm cuvette with a 5-mm light path. Samples were read against a blank produced by following the same procedure as above, except that the wells were not coated with polysaccharide or membranes. Additional controls

consisted of incorporation of NS-1 tumor ascitic fluid in place of the hybridoma fluids. These controls gave readings of no greater than blank values (0.02 to 0.04 optical density unit at 400 nm).

Inhibition of ELISA was done in triplicate at inhibitor concentrations of 1,000, 500, 250, 100, 10, 2.5, and 0.15 µg/ml in a manner similar to that described by Zollinger and Mandrell for the solid-phase system (15). Dilutions of inhibiting antigens in phosphate-buffered saline-Tween 20 were added to a fixed dilution of hybridoma ascitic fluid (1:640). This mixture was then placed in the outer membrane-coated microtiter wells and incubated at 4°C overnight, after which the plate was washed and the standard ELISA procedures again were followed. Ten duplicate, uninhibited, diluted-hybridoma ascitic-fluid controls were also performed to determine the range of values for the uninhibited ELISA. Antigen inhibition curves were calculated as a percentage of the control by using the preprogrammed linear-regression function of a TI-59 programmable calculator (Texas Instruments, Lubbock, Tex.). Confidence limits around the calculated lines were determined as described by Steel and Torrie (12).

**Development of hybridomas producing antibodies to gonococcal LPSs. (i) Immunization of spleen cell donor mice.** BALB/c mice were immunized intraperitoneally with 0.5 ml of 10<sup>9</sup> heat-killed gonococcal strain 4505 cells (LPS serotype Gc<sub>3</sub>) on days 0, 13, and 21. Mice were bled by the orbital route on day 28, and sera were tested for the presence of anti-LPS antibody by immunodiffusion and ELISA studies. Animals with high-titer ELISA antibody (optical density of greater than 0.5 at 1:20,000) and demonstrable precipitating antibody were selected as donors. On day 32 after the initial immunization, selected animals were sacrificed with chloroform, their spleens were removed, and splenic lymphocytes were harvested by perfusion of splenic pulp with minimum essential medium (MEM).

**(ii) Fusion of donor spleen cells to the NS-1 nonsecreting clone of the P3x63Ag8 BALB/c plasmacytoma.** To achieve fusion of the donor spleen cells to the NS-1 (nonsecreting variant of the IgG1 BA/c plasmacytoma P3xAg8) plasmacytoma cells (obtained from the Salk Institute for Biology under National Cancer Institute contract NO1-CB-23886), 30% polyethylene glycol was used in a modification of the procedure of Kennett (9). Briefly, 10<sup>8</sup> spleen cells were combined with 10<sup>7</sup> NS-1 cells in MEM with serum. The cells were centrifuged at 500 × *g* for 15 min at 100°C. All of the supernatant was removed, and the pellet was tapped to loosen. One milliliter of 30% polyethylene glycol 4000 (Sigma Chemical Co.) in MEM without serum was added, and the mixture was stirred lightly and left at 37°C. After 1 min, 10 ml of MEM with 20% serum was layered over the polyethylene glycol. The cells were pelleted at 500 × *g* for 2 min. The supernatant was discarded, and the medium addition and centrifugation were repeated. Five milliliters of growth medium (MEM with high glucose and 20% bovine fetal serum) was added without disturbing the cell pellet. The cells were incubated at 37°C for 45 min. At this time, the cell pellet was resuspended and transferred to a 50-ml Erlenmeyer flask containing 25 ml of growth medium. The cells were stirred lightly

and distributed in 0.05-ml samples into microtiter wells. At 24 h after the polyethylene glycol fusion, 0.05 ml of hypoxanthine-aminopterin-thymidine (HAT) medium was added to each well. The microtiter plates were placed in a tissue culture incubator at 85% humidity under 5% carbon dioxide. Fresh HAT medium was added at day 7, and plates were checked for macroscopic plaques after day 10. The supernatant from all wells was tested for the presence of antibody by using an ELISA system in which 10  $\mu$ g of strain 4505 outer membranes per ml was coated to the microtiter plates. The alkaline phosphatase-conjugated rabbit anti-murine IgG1 was used to detect the presence of antibody binding to the outer membranes. Wells which contained hybridomas producing antibody to outer membranes were retested against microtiter plates coated with strain 4505 LPS-derived polysaccharide to identify those clones producing antibody to LPS. These clones were propagated by subsequent transfer to larger tissue culture wells. Large quantities of antibody were produced by the intraperitoneal injection of  $10^5$  cells from the LPS antibody-producing clone into pristane (2,6,10,14-tetramethylpentadecane)-primed BALB/c mice. The resulting ascitic fluid was harvested in 3 to 4 weeks, and the ascitic fluid was tested for specificity of the respective antibody. Similar peritoneal tumors were established by using NS-1 cells. The resultant fluid was used as a control.

**Preparation of LPS, LPS-derived polysaccharides, and gonococcal outer membranes.** Gonococcal LPSs were produced by a modification of the phenol-water method described by Perry et al. (11). LPS-derived polysaccharides were produced by the alkaline digestion of phenol-water LPS, followed by diethylaminoethyl chromatography as previously described (2, 3). Outer membranes were produced by the method of Johnston (8).

**Other immunological studies.** Immunodiffusion and immunoelectrophoresis studies were performed as previously described (2-4) to determine the immunoglobulin class of the monoclonal antibody. For these studies, antisera specific for murine IgG1, IgG2a, IgG2b, and IgG3 were obtained from Bionetics, Kensington, Md. Complement-mediated hemagglutination lysis studies were performed by methods described by Maeland et al. with LPS-derived polysaccharide-coated sheep erythrocytes (10).

## RESULTS

The fusion of spleen cells from a BALB/c mouse hyperimmunized with Gc<sub>3</sub> gonococcal strain 4505 to the NS-1 variant of the P3x63Ag8 BALB/c plasmacytoma resulted in the production of 192 hybridomas in 576 wells. Initial screening of these hybrids indicated that 21 were producing antibody to the outer membrane complex of strain 4505 by direct ELISA. Four hybridomas were producing antibody to LPS in an ELISA system. One of these hybridomas, designated 3F11, produces a monoclonal antibody which recognized LPS from all six gonococcal serotypes. This antibody was evaluated and is the subject of this report.

Immunodiffusion and immunoelectrophoresis studies indicated that the 3F11 antibody was a kappa chain IgG1 murine immunoglobulin. In addition, by using a complement lysis assay with sheep erythrocytes coated with LPS-derived polysaccharide from strain 4505, the antibody induced specific hemolysis at high antibody-antigen concentrations. Whether this was due to poor complement binding or the steric relationship of the LPS antigen on the erythrocyte surface is not known.

Peritoneal tumors of hybridoma 3F11 were induced in BALB/c mice primed with pristane. Ascitic fluid was harvested at 3 to 4 weeks, and this material was the source of antibody in this study. Control ascitic fluid was obtained from peritoneal exudates produced in BALB/c mice with NS-1 plasmacytomas. Figure 1 demonstrates a direct ELISA study showing reactivity of the 3F11 ascitic fluid with Gc<sub>3</sub> strain 4505 outer membranes and an LPS-derived polysaccharide. Studies which used NS-1 ascitic fluid against the same microtiter well coats are also shown. The 3F11 ascitic fluid recognized both 4505 LPS-derived antigen and membranes with titers three standard deviations above the background (absorbance of 0.03 at 400 nm) at 1:2,560 and 1:10,240, respectively, whereas the NS-1 ascitic fluid against 4505 membranes reached this level at 1:80 and against LPS-derived polysaccharides showed no reactivity at any dilution.

Inhibition studies were established by using the direct ELISA as a basis. After initial experiments demonstrated that 4505 LPS-derived pol-

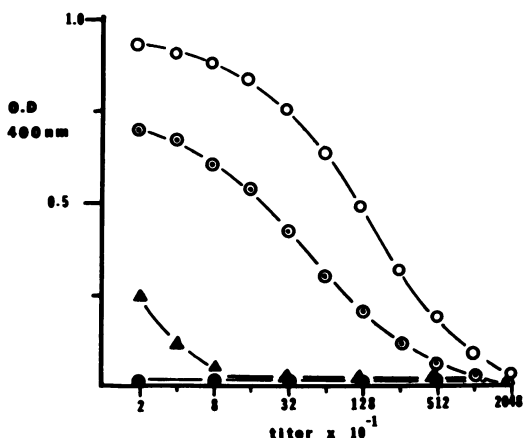


FIG. 1. Microtiter ELISA, using the 3F11 monoclonal antibody with Gc<sub>3</sub> 4505 outer membrane-coated wells (10  $\mu$ g/ml, ○) or 4504 LPS-derived polysaccharide-coated wells (5  $\mu$ g/ml, ○). Similar studies were performed with ascitic fluid formed from the NS-1 Gc<sub>3</sub> parent plasmacytoma. Symbols: ▲, 4505 outer membrane-coated wells; ●, 4505 LPS-derived polysaccharide-coated wells. O.D., Optical density.

ysaccharide inhibited the 3F11 ELISA directed against 4505 outer membranes (Fig. 2), this cell component was used as the well coat in subsequent experiments. LPS-derived polysaccharide was a significantly more effective inhibitor of the 3F11 ELISA than was native phenol water LPS from strain 4505 (Fig. 2). LPS and LPS-derived polysaccharide from *E. coli* O:111 and the J-5 mutant of this strain, as well as the LPS from the Re chemotype mutant of *S. minnesota* R595, failed to inhibit the antibody. Studies with LPS and LPS-derived polysaccharides from the five other gonococcal LPS serotypes, including Gc<sub>1</sub> strain 1342, Gc<sub>2</sub> strain 1291, Gc<sub>4</sub> strain 8551, Gc<sub>5</sub> strain PID2, and Gc<sub>6</sub> strain 3893, showed that the 3F11 antibody was inhibited in the same fashion (Fig. 2). The slopes of the inhibition curves of these materials fell within the 95% confidence limits of the LPS-derived and LPS preparations. Meningococcal LPS-derived polysaccharides partially inhibited the 3F11 ELISA (Fig. 3). Studies with 12 LPS-derived preparations isolated from group A (1), group B (7), group C (2), group X (2), and group Y (2) all showed partial inhibition of the system within the 95% confidence limits defined by the inhibition slope of LPS-derived polysaccharide from meningococcal strain S4812Y (Fig. 3).

The antigen site which is recognized by the 3F11 antibody is present on the surface of the gonococcus. The absorption of the 3F11 antibody with slightly over  $10^7$  organisms per ml resulted in almost complete inhibition (92%) of the ELISA (Fig. 4), whereas the LPS mutant 4505R, which lacks LPS serotype specificity and common LPS antigens (10), but retains outer

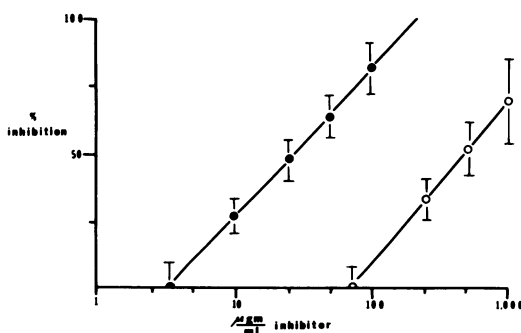


FIG. 2. Inhibition of the 3F11 monoclonal antibody ELISA by 4505 LPS-derived polysaccharide (●) and 4505 LPS (○). Microtiter wells were coated with  $10 \mu\text{g}$  of 4505 outer membrane per ml, and the 3F11 antibody final dilution was 1:1,280. Brackets define the 95% confidence limits around each datum point. Plots were constructed by using linear-regression analysis of six inhibitor concentrations performed in triplicate. The *r*-value for both plots was greater than 0.934.

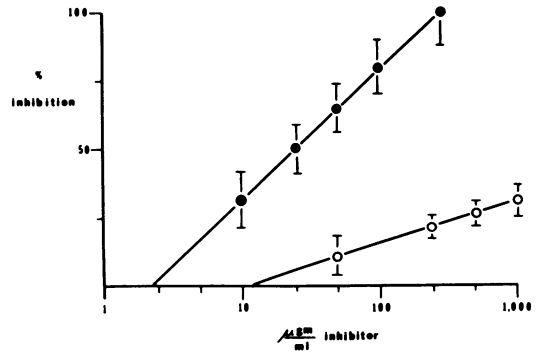


FIG. 3. Inhibition of the 3F11 monoclonal antibody ELISA by 4505 LPS-derived polysaccharide (●) and meningococcal LPS-derived polysaccharide S-4812Y (○). Microtiter wells were coated with  $10 \mu\text{g}$  of 4505 outer membranes per ml, and the 3F11 antibody final dilution was 1:1,280. Brackets define the 95% confidence limits around each datum point. Plots were constructed by using linear-regression analysis of six inhibitor concentrations performed in triplicate. The *r*-value for both plots was greater than 0.961.

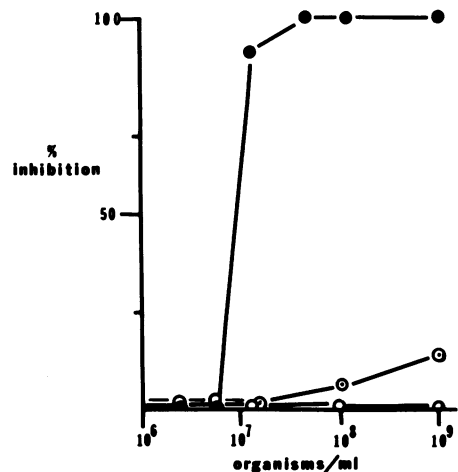


FIG. 4. Inhibition of the 3F11 monoclonal antibody ELISA with whole-organism 4505 (●), 4505r (○), and J-5 (○). Microtiter wells were coated with  $10 \mu\text{g}$  of 4505 outer membranes per ml, and the 3F11 antibody dilution was 1:1,280.

membrane proteins of 4505, inhibited to the 14% level at  $10^9$  organisms per ml. *E. coli* O:111 and the J-5 mutant failed to inhibit this ELISA in concentrations as high as  $10^9$  organisms per ml.

Inhibition of the ELISA with a series of sugars was performed. Figure 5 shows the results of inhibition with D-galactosamine, which resulted in complete inhibition at concentrations of 14 mg/ml, and  $\beta$ -lactose, which gave partial inhibition (83%) to a concentration of 100 mg/ml.

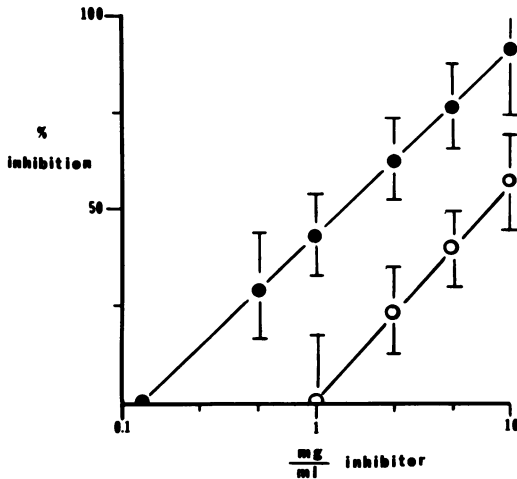


FIG. 5. Inhibition of the 3F11 monoclonal antibody ELISA with D-galactosamine (●) and  $\alpha$ -lactose (○). Brackets define the 95% confidence limits around each datum point. Plots were constructed by using linear-regression analysis of six inhibitor concentrations performed in triplicate. The  $r$ -value for both plots was greater than 0.942.

No inhibition occurred with D-glucosamine, N-acetylglucosamine, N-acetylgalactosamine, glucosamine-6- $\text{PO}_4$ , 2-keto-3-deoxyoctanoate, D-glucoheptose,  $\alpha$ -glucose,  $\beta$ -glucose,  $\beta$ -galactose, or  $\beta$ -hydroxymyristic acid. Partial inhibition identical to that seen with  $\beta$ -lactose occurred with  $\alpha$ -lactose. All sugars cited above were studied in concentrations as high as 100 mg/ml.

## DISCUSSION

The ability of monoclonal antibodies to be utilized in the analysis of antigenic structure is just now beginning to be appreciated in microbiology. In this study, we selected a hybridoma produced by the fusion of spleen cells from a BALB/c mouse hyperimmunized with a Gc<sub>3</sub> LPS gonococcal serotype strain to a BALB/c mouse plasmacytoma line. This fusion resulted in the selection of a hybridoma clone which is producing a monoclonal antibody to a common site on the carbohydrate portion of gonococcal LPS. We tested this monoclonal antibody against prototype strains from six gonococcal LPS serotypes. These strains represent serotypes into which approximately 90% of gonococci can be typed (2, 4). The monoclonal antibody recognizes a determinant on each of these LPS moieties. Inhibition studies utilizing meningococcal LPS and LPS-derived polysaccharide indicated that only partial inhibition occurs utilizing this antisera, whereas studied with *E. coli* O:

111 and the J-5 mutant of this *E. coli* serotype failed to inhibit this antiserum. Similarly, LPS isolated from *S. minnesota* mutant R595 failed to inhibit the 3F11 monoclonal antibody ELISA system. Studies with a wide variety of carbohydrates, including  $\beta$ -galactose,  $\alpha$ -glucose,  $\beta$ -glucose, 2-keto-3-deoxyoctanoate, heptose, D-glucosamine, N-acetylglucosamine, glucosamine-6- $\text{PO}_4$ , and N-acetylgalactosamine, failed to inhibit this ELISA. Studies with D-galactosamine indicated that complete inhibition was achievable at relatively high carbohydrate concentrations.  $\alpha$ -Lactose and  $\beta$ -lactose partially inhibited the antibody. These studies suggest that this antibody is recognizing a common moiety present on gonococcal LPS which is composed at least partially of D-galactosamine-galactose-glucose, with the galactose and glucose in a (1-4) pyranosyl linkage. This is suggested by the complete inhibition of the 3F11 antibody by D-galactosamine and the partial inhibition of the 3F11 antibody by lactose [4-O-D-galactopyranosyl-(1-4)-D-glucopyranose]. The importance of the (1-4) linkage is evident from the failure of glucose and galactose alone or in combination to inhibit the antiserum. The high concentrations of carbohydrates necessary to inhibit this system lead one to the conclusion that an additional steric confirmation may also be necessary and that this is lacking when the component monosaccharides and disaccharides are employed.

Based on these studies, a schema for the common region of gonococcal and meningococcal lipopolysaccharides can be proposed. Previous studies have indicated that the J-5 mutant of *E. coli* O:111 contains a glucosamine-glucose-galactose-heptose-2-keto-3-deoxyoctanoate moiety as a common portion of its core region (7). It is also suggested that this section may be common to all *Enterobacteriaceae* and gram-negative bacteria, including *Neisseria* spp. (6, 15). From the studies with antibody 3F11, it appears that the gonococcal common region contains a D-galactosamine-O-D-galactopyranosyl-(1-4)-D-glucopyranose moiety, whereas meningococcal LPS contains only a portion of this component. Based on the small amount of D-galactosamine found in meningococcal LPS (3) when compared with gonococcal LPS (1), it is attractive to suggest that D-galactosamine is the missing portion of the moiety. These studies also suggest that the common portion of the LPS shared by enterobacterial and neisserial LPS is different than the core carbohydrate structure found in J-5 (7) and may reside in the 2-keto-3-deoxyoctanoate-lipid A portion of the molecule.

These studies have demonstrated the application of monoclonal antibodies for the definition of the macromolecular structure of gono-

coccal LPS. Future studies are planned with this antibody in an attempt to define the biological function of LPS.

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