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Measurement of the Human Immune Response to Meningococcal Lipooligosaccharide Antigens by Using Serum To Inhibit Monoclonal Antibody Binding to Purified Lipooligosaccharide†

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We developed a human inhibition monoclonal enzyme-linked immunosorbent assay (HIMELISA) to investigate the human immune response to the lipooligosaccharides (LOS) of *Neisseria meningitidis*. Monoclonal antibodies (MAb) were used to define seven epitopes on four LOS molecules of a meningococcal strain (126E) previously shown to express immunogenic LOS epitopes. The assay could distinguish epitope-specific antibody within whole sera. Neither the specificity nor the amount of the antibody measured by HIMELISA in sera of vaccinates changed during the immune response to meningococcal capsular polysaccharides, a chemically unrelated antigen. By using the HIMELISA, it was determined that sera from adults convalescing from meningococcal disease strongly inhibited MAb binding to two of the seven defined epitopes. The 3.6-kilodalton LOS of strain 126E expressed both of these epitopes. In addition, one of the inhibited epitopes was also expressed on the 4.0-kilodalton LOS of strain 126E. The convalescent-phase sera inhibited MAb binding to these two epitopes when they were expressed on LOS of diverse meningococcal strains. An acute-phase serum blocked MAb to the two epitopes to a lesser degree than did a convalescent-phase serum from the same patient. Immunoblotting the sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated LOS with convalescent-phase sera confirmed the specificity of the human anti-LOS antibody identified by HIMELISA.

Considerable progress has been made in characterizing surface antigens of *Neisseria meningitidis* that are involved in various immunologic processes and in relating the manifestations of meningococcal disease to failures of immune effector mechanisms (9, 10, 13). We do not know, however, how to immunologically protect children younger than 2 years of age. Since capsular antibody is naturally acquired very slowly during infancy (29) and polysaccharide vaccines are either nonimmunogenic or poorly so in children younger than 2 years of age (8, 12, 22, 29, 30), alternative subcapsular immunogens that would induce broadly reactive bactericidal antibody in this age group have been sought. This study focuses on the immunogenicity of the lipooligosaccharides (LOS) present in the outer membrane complex of the meningococcus.

Young children can make antibody to meningococcal LOS. In a study of infants and children recovering from meningococcal disease, Griffiss et al. (12) determined that the purified LOS from an *N. meningitidis* group C strain (126E) was able to inhibit bactericidal activity in sera from children convalescing from group B disease, regardless of the outer membrane protein or LOS serotype of the infecting strain.

Neisseria spp. make from one to six different LOS molecules, with relative molecular weights (M_rs) of 3,200 to 7,100, that can be characterized by electrophoretic mobility demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (24, 26, 28, 35, 39). Differences in mobility appear to be due to heterogeneity of oligosaccharide chemical composition (16). These LOS are glycolipids that contain three domains: a basal oligosaccharide composed of 2-keto-3-deoxyoctulosonate (dOclA), heptose, and hexosamines; a segment of variable length that is composed of glucose or galactose; and a terminal polylactosamine structure that mimics those of human glycosphingolipids (17, 19–21). Serotypic diversity resides in differences in the composition of the basal oligosaccharide and the length of the intervening segment (17, 25). The LOS produced by a neisserial strain varies over growth time; stationary-phase organisms have LOS of higher molecular weight than do organisms in exponential phase (1, 31). This variation may be related to the capping or completion of the LOS molecule.

N. meningitidis 126E fails to cap its LOS with the polylactosamine terminus, thus exposing more basal structures and their epitopes (17, 23). Meningococcal strain 126E (C:L1,8) makes four LOS molecules of low M_r, ranging from 3,200 to 4,200 (23). We concluded from the study of pediatric immune response to meningococcal disease of Griffiss et al. (12) that one or more of the four LOS molecules of 126E may contain a common core epitope that induces broadly reactive bactericidal antibody in children. In addition, 126E makes a 3,600-M_r LOS molecule. A LOS molecule of this size, when present on the gonococcus, has been associated with serum resistance (33).

Several investigators have shown that neisserial LOS are also antigenically heterogenous. The techniques used by these investigators, including hemagglutination assays (44) and radioactive antigen binding assays (2), could not distinguish antigens on individual molecules. Neisserial LOS molecules separated by SDS-PAGE have been characterized by M_r as visualized by silver stain (24, 26, 28, 35, 39) and by

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binding of either monoclonal antibody (MAb) (17, 24, 26, 33, 37) or human sera (18, 36) on immunoblot. These techniques distinguished LOS molecules but not antigens on the individual molecules. In addition, separating the LOS by SDS-PAGE changed their native configuration, thus possibly altering their epitope expression.

MAb binding can also define an epitope which can be located on LOS molecules of different M_rs (17, 24, 26) either of the same or of a different meningococcal strain. We used MAb binding and the M_rs of the LOS molecules bound to define the epitopes on meningococcal LOS. We began with the LOS of strain 126E and then expanded to define epitopes on other meningococcal strains. We then adapted a solid-phase assay that keeps the LOS unseparated and in a more natural configuration in order to determine if these epitopes were bound by antibody in human convalescent-phase sera. Our purpose was to determine whether specific LOS molecules exist that would induce broadly reactive, bactericidal antibody against the meningococcus.

MATERIALS AND METHODS

***N. meningitidis* strains.** Strain 126E (C:L1,8) was isolated in 1964 in Germany from a patient with meningococcal disease (2, 23, 27, 44). Strain 8032 (group Y; undetermined serotype) was isolated from the blood of an asplenic patient with meningococcemia (15). Strain 7883 (A:L11) was isolated from an adult with meningococcal disease in the Pacific Northwest (4, 24). Strain 120M is serogroup A, serotype L9 (23, 24, 43). These strains were obtained from the Walter Reed Army Institute of Research, Washington, D.C. Two other strains, M978 (B:L3,8) (27) and M992 (B:L6) (23, 27), were kindly provided by Carl Frasch (Center for Biologics Evaluation and Research, Bethesda, Md.). Culture procedures have been previously described (35).

LOS. LOS were extracted from acetone-dried organisms by the hot phenol-water method (34, 40).

Sera. Sera previously collected from adults convalescing from disseminated meningococcal disease were studied. They had been stored frozen at −20°C. Serum 1 was collected on day 12 of illness from an individual infected with a group C, serotype II strain. Serum 2 was collected on day 8 from an adult with group B, serotype IV disease. Serum 3 was collected on day 10 from an individual with group C, serotype II disease. Sera 4a and 4c were drawn on days 1 and 7, respectively, of illness from an adult infected with a group C, serotype II-IV strain. The infecting strains have been described previously (14). Gold type II and II-IV strains accounted for epidemic group C meningococcal disease among military recruits in the late 1960s and early 1970s; type IV strains caused epidemic group B disease in the early 1980s (14).

The hypogammaglobulinemic sera were drawn from two adults with acquired hypogammaglobulinemia. The sera contained no immunoglobulin G (IgG), IgA, or IgM antibody that bound to LOS from strains 126E, 120M, or M992 as assessed by enzyme-linked immunosorbent assay (ELISA).

For comparison, we also studied sera drawn from laboratory colleagues at the time of vaccination with tetravalent meningococcal capsular polysaccharide vaccine (Menomune-A/C/Y/W-135; Connaught Laboratories, Swiftwater, Pa.) and 11 days thereafter. None were aware of previous neisserial colonization or infection. The vaccine lot contained no proteins or LOS that could be detected by SDS-PAGE analysis. Each vaccinate responded appropriately to the group C polysaccharide (11).

Identification of antibody binding sites by SDS-PAGE and immunoblot analysis. Thirty-five murine MAbs previously determined to bind neisserial LOS were screened for binding to purified strain 126E LOS by solid-phase radioimmunoassay as described previously (41). Fifteen MAbs bound to strain 126E LOS and were characterized for this study.

Purified LOS from strains 126E, M978, 7883, and 8032 were separated by SDS-PAGE by using a modification of the method of Laemmli (33, 35). Briefly, LOS were diluted in a buffer consisting of 2% SDS in 60 mM Tris hydrochloride, 1.0 mM EDTA, 3.5% 2-mercaptoethanol, 18% glycerol, and 2% bromophenol blue and heated at 100°C for 5 min. The LOS samples (0.5 to 11.5 μg per well) were electrophoresed in duplicate discontinuous slab gels (3% acrylamide spacer gel; 12.9 to 13.1% resolving gel) at 10 mA per gel through the spacer gel and at 15 mA per gel through the resolving gel. The LOS bands were visualized by silver stain (38). The M_rs of the LOS bands of strains M978, 7883, and 126E were estimated by comparing their migration distances with those of the six LOS bands of strain 8032 whose M_rs have been determined (24, 33, 35). The LOS on the duplicate gel was electroblotted to nitrocellulose by applying 30 V overnight (3). The transblot was incubated in filler (1% casein dissolved in a solution of 0.01 M Tris hydrochloride, 0.15 M NaCl, 0.005 M $MgCl_2$, and 0.03 M NaN_3 [pH 7.4]) for 30 min, washed with phosphate-buffered saline (PBS), and incubated for 1 to 2 h in a dilution of 1 of the 15 MAb. After being washed, the transblot was incubated for 1 to 2 h in goat anti-mouse IgG or IgM alkaline phosphatase-conjugated antibody. After being washed, the transblot was developed with a solution of 50 mM Tris hydrochloride (pH 8.0), 0.1% naphthol AS-MX phosphate disodium salt (Sigma Chemical Co., St. Louis, Mo.), and 0.2% Fast Red TR Salt (Sigma). The procedure was repeated for each of the 15 MAb. Each blot contained a reference lane with LOS from strain 8032 that was reacted separately with MAb D6A. This MAb bound all six LOS molecules of strain 8032, providing an M_r standard for the blots as described above.

We used these blots to divide the 15 MAbs into seven binding patterns and then chose 1 representative MAb from each group to use as serologic probes in this study. MAb 6B7 was described previously (6). MAb 3E2, 3D4, 1B5, and 3C9 were kindly provided by M.A.J. Westerink (University of Buffalo, State University of New York, Buffalo, N.Y.). MAb D6A (24) and MAb MN13H21 (5) have been characterized, and J.T. Poolman, Rijksinstituut voor Volksgezondheid, Bilthoven, the Netherlands, kindly provided them.

HIMELISA. We adapted the protein-specific human inhibition monoclonal ELISA (HIMELISA) of Sarafian et al. (32) for use with LOS-specific MAb. Flat-bottom microdilution plates (Dynatech, Chantilly, Va.) were coated with 25 μg (75 μl) of 126E LOS per ml diluted in 50 mM $MgCl_2$ Dulbecco buffer. After 2 h, the plates were washed three times with PBS and the wells were filled with a buffer of 0.1% bovine serum albumin (Sigma) and 0.02% sodium azide in PBS to block nonspecific protein binding. After 2 h, the plates were washed with PBS. Serial doubling dilutions from 1:4 to 1:512 of a human convalescent-phase serum in the buffer used to block nonspecific protein binding were added to the wells and incubated overnight. (The serum was first heated at 56°C for 30 min.) After washing the plates, we then added 50 μl of each of the seven test MAbs diluted in the same bovine serum albumin buffer to duplicate rows and reacted them with LOS for 2 h. After washing away unbound MAb, we incubated all wells with 50 μl of a 1:200 dilution of biotin-conjugated goat antibody to mouse IgG (Caltag, San

Francisco, Calif.) for 2 h and then washed them. We then added to each well 50 μl of a preformed macromolecular complex of avidin and biotinylated alkaline phosphatase (Vectastain; Vector Laboratories, Burlingame, Calif.) diluted in 0.1% Tween 20-PBS (vol/vol), incubated the plates for 1 h, and washed the wells. The plates were then developed until positive control wells reached a predetermined absorbance with 75 μl of *p*-nitrophenyl phosphate per well (2 mg/ml in 100 mM bicarbonate buffer [pH 9.5] with 10 mM $MgCl_2$; Sigma). The absorbance was read at 405 nm with a model 2550 EIA Reader (Bio-Rad Laboratories, Richmond, Calif.). All incubations were at room temperature except the Vectastain step, which was at 37°C. We separately determined the binding capacity to strain 126E LOS of each MAb (uninhibited control). Determinations were done in quadruplicate. All steps were as described above except that hypogammaglobulinemic sera diluted 1:100 in 0.1% bovine serum albumin-0.02% sodium azide-PBS was substituted for the convalescent-phase serum. Wells that received all steps except MAb, all steps except serum and MAb, all steps except antigen, serum, and MAb, and all steps except antigen and serum (repeated for each of the seven MAb) were included as negative controls.

We used an ELISA to determine the optimal concentrations of antigen (LOS) and MAb to be used for the HIMELISA. A grid was constructed (26) by coating rows of wells with serial doubling dilutions of strain 126E LOS and testing each row with dilutions (50 μl) of MAb. The secondary antibody, Vectastain, and substrate steps were as described above for the HIMELISA. An antibody binding curve was generated by plotting absorbance against $\log_2$ dilution of the MAb for each LOS concentration. We chose from this curve an LOS concentration that resulted in absorbance that was 4 to 10 times background, but not in antigen excess, and a MAb concentration that was <60% of the saturation point for that concentration of LOS. The same LOS concentration was used for the HIMELISAs for all seven MAbs; the optimal concentration of each MAb was determined separately by grid titration.

Confirmation of inhibition by human sera of MAb-epitope binding. We used LOS made by strains 120M and M992 in HIMELISA to confirm the inhibition by human sera of MAb binding to its epitope. We based the choice of these two LOS on the MAbs they bound. As determined by ELISA, strain 120M LOS bound MAb D6A but not MAb 6B7 and strain M992 LOS bound MAb 6B7 but not MAb D6A. We used grid titration to determine optimal concentrations of LOS and MAb to be used in the HIMELISA.

Analysis of HIMELISA. For each serum-MAb HIMELISA with strain 126E LOS, we calculated the mean absorbance of the uninhibited control. We divided the replicate absorbances obtained at each serum dilution by this value. A simple linear regression with replication was performed for the linear portion of the curve for each MAb and serum by using $\log_2$ serum dilution versus absorbance divided by the mean of the control. $P < 0.05$ for a positive slope was considered significant inhibition. Curves with a mean percentage of control >98% at the highest serum concentration were considered negative and were not subjected to linear regression analysis.

The differences in absorbance among the duplicate wells at each serum dilution was ≤20% of the mean for nearly all HIMELISAs.

Immunoblots with convalescent-phase sera. Purified LOS from strains 126E, 7883, M978, and 8032 were separated by SDS-PAGE and transferred to nitrocellulose by using the same procedure as described above. The transblot was incubated in filler, washed with PBS, and incubated in a 1:20 dilution of the convalescent-phase serum for 4 h. The blot was reacted with a solution of alkaline phosphatase-conjugated goat antibody to human IgG, IgA, and IgM (Caltag) for 2 h. It was developed in the substrate previously described.

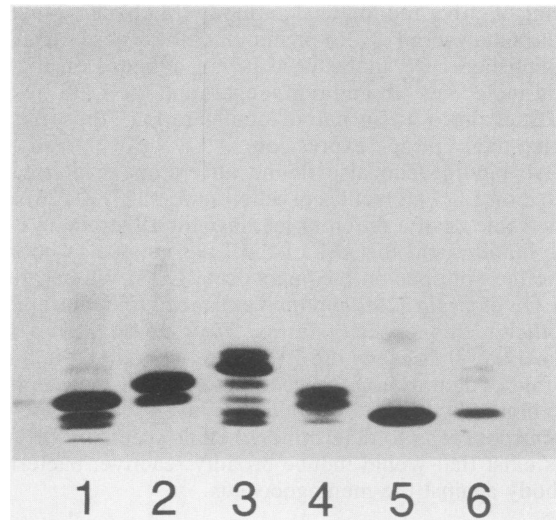

FIG. 1. Silver stain of SDS-PAGE-separated LOS of meningococcal strains M992 (lane 1), 120M (lane 2), 8032 (lane 3), 126E (lane 4), 7883 (lane 5), and M978 (lane 6).

RESULTS

Identification of LOS molecules. We separated and visualized by silver stain the LOS of meningococcal strains M992, 120M, 8032, 126E, 7883, and M978. The results of the silver stain are shown in Fig. 1, and a schematic of the individual LOS molecules of strains 8032, 126E, 7883, and M978 and the molecular weight standards are shown in Fig. 2.

Strains 8032, 126E, 7883, and M978 were used to characterize the MAbs used in the HIMELISA. Strain 126E is LOS type L1,8 and is the prototype L1 strain (23, 27). No other type L1 strains have been identified to our knowledge. Strain M978 is the L8 prototype strain (23, 27). Strain 7883 is an

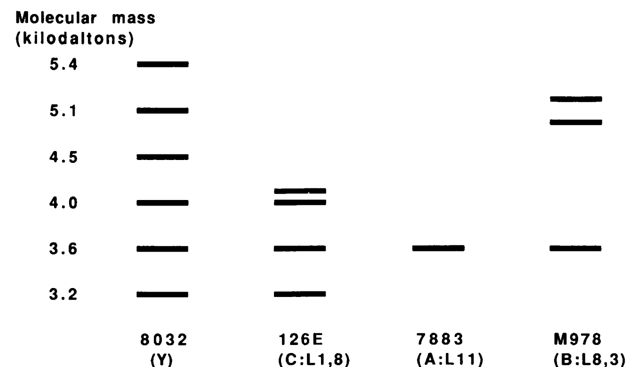

FIG. 2. Simplified schematic representation of positions of LOS molecules from strains 8032, 126E, 7883, and M978 as demonstrated by silver stain shown in Fig. 1, lanes 3 through 6, respectively. The six LOS of strain 8032 and their known molecular weights are shown as the standard.

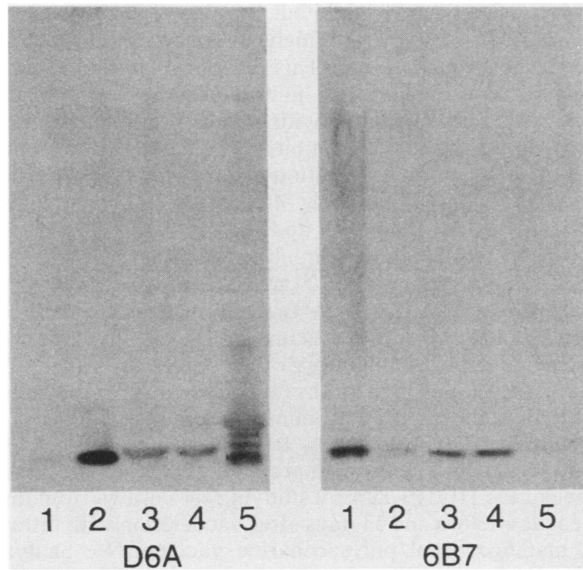

FIG. 3. Immunoblots of SDS-PAGE-separated LOS of meningococcal strains M978 (lane 1), 7883 (lane 2), 126E (lanes 3 and 4), and 8032 (lane 5) with MAb D6A and 6B7. MAb D6A bound an epitope expressed on the 3,600-M_r LOS of strain M978, the 3,600-M_r LOS of strain 7883, the 4,000- and 3,600-M_r LOS of strain 126E, and all of the LOS of strain 8032. MAb 6B7 bound an epitope expressed on the 3,600-M_r LOS of strains M978, 7883, 126E, and 8032.

L11 strain that was included for comparison with strain 126E because it was known to make a single 3,600-M_r LOS molecule (24). The six LOS molecules of strain 8032 served as an M_r standard. Each of the four test strains made a 3,600-M_r LOS molecule, and this was the only apparent molecule made by strain 7883. Strain M978 also made two molecules of greater molecular weight. In addition to the 3,600-M_r molecule, strain 126E also made molecules of M_rs of 3,200, 4,000, and 4,200.

Strains M992 and 120M were used to confirm the inhibition by human sera of MAb binding to defined epitopes (see below).

Definition of strain 126E LOS epitopes. We found by solid-phase radioimmunoassay that 15 of 35 antineisserial LOS mouse MAbs bound to strain 126E LOS. We grouped these 15 MAbs by their patterns of binding to the LOS molecules of strains M978, 7883, 126E, and 8032 as seen on immunoblots (for examples, see Fig. 3). As some of these 15 MAbs bound the LOS molecules in the same pattern, a total of only seven binding patterns were seen, each of which defined an epitope on strain 126E LOS. One representative MAb was chosen for each of the seven patterns. These were 3D4, 1B5, 3E2, D6A, 6B7, 3C9, and MN13H21. Table 1 shows the presence of the seven MAb-defined epitopes on the LOS of the four meningococcal strains.

The relationship of an epitope to the LOS molecule(s) that bears it is illustrated (Table 1) by the epitopes found on the 3,600-M_r LOS of strain 126E. MAb 6B7, 3C9, and MN13H21 all bound the 3,600-M_r LOS of strain 126E and no other LOS molecules of this strain. These three MAbs could, therefore, not be differentiated by their binding to strain 126E alone. The epitopes defined by these MAbs could be distinguished by comparing their binding to the LOS molecules of strains M978, 7883, and 8032, as well as 126E. The epitope bound by MAb 6B7 was expressed on the 3,600-M_r LOS of all four strains; the epitope bound by MAb MN13H21 was expressed on the 3,600-M_r LOS of strains 126E and M978, but not on the other two; and the epitope bound by MAb 3C9 was expressed only on the 3,600-M_r LOS of strain 126E. Each of these three MAbs must define a different epitope on the 3,600-M_r LOS of strain 126E because their epitopes are not uniformly present on the 3,600-M_r LOS made by different strains.

Table 1 also shows that three of the seven MAb-defined epitopes were expressed on more than one LOS molecule of strain 126E (1B5, 3E2, D6A). The 4,200-M_r molecule of strain 126E expressed three epitopes, the 4,000-M_r molecule expressed three epitopes, and the 3,600-M_r molecule expressed four epitopes. No epitopes were identified on the 3,200-M_r molecule of strain 126E.

Determination of antibody in human convalescent-phase sera that bound defined 126E LOS epitopes. We performed a HIMELISA by using each of the seven test MAbs with each

TABLE 1. MAb and human antibody binding to SDS-PAGE-separated LOS of four meningococcal strains

Strain[a]	M_r of LOS molecule	Presence on LOS molecules of MAb-defined epitope[b]:							Presence of antibody to LOS molecules in convalescent-phase serum[c]:			
		3D4	1B5	3E2	D6A	6B7	3C9	MN13H21	1	2	3	4c
126E (C:L1,8)	4,200	++	+	++					++	++	++	
	4,000		++	++	++				++	++	++	
	3,600				+	++	++	++	++	++	++	++
	3,200								++	++	++	+
M978 (B:L8,3)	5,200								++	+		+
	4,800								++	++	+	++
	3,600				+	++		++	++	++	++	++
7883 (A:L11)	3,600				++	+			++	++	++	+
8032 (Y)	5,400				++				++	++	+	+/−
	5,100				++				++	++	+	+/−
	4,500				++				++	++	+	+/−
	4,000				++				++	++	+	+/−
	3,600				++	+/−			++	++	++	
	3,200				++				++	++	++	++

[a] LOS was extracted from the indicated strain. Serogroup and serotype are shown in parentheses.
[b] +/−, Weak binding; +, moderate binding; ++, strong binding.
[c] For characteristics of convalescent-phase sera, see Table 2; numerals are for identification only.

TABLE 2. Inhibition of MAb binding to strain 126E LOS by human serum

Serum	Day of illness	Sero-group[a]	Inhibition of binding of MAb:						
			D6A	6B7	MN13H21	3E2	3C9	1B5	3D4
1	12	C	+[b]	+	−	−	−	−	−
2	8	B	+	+	−	+	−	−	−
3	10	C	+	+	−	−	−	−	−
4a[c]	1	C	+	+	−	−	−	−	−
4c[c]	7	C	+	+	+	−	−	−	−

[a] Serogrup of infecting meningococcus.
[b] +, $P < 0.05$ for positive slope by simple linear regression with replication.
[c] Serum drawn from the same individual: a, acute phase; c, convalescent phase.

of the four convalescent-phase sera and strain 126E LOS. Three of the sera were drawn from individuals ill with group C disease, and one was drawn from an individual with group B disease. The sera were obtained 7 to 12 days after the onset of illness. Only two MAbs, D6A and 6B7, were significantly inhibited by all four of the convalescent-phase sera (sera 1, 2, 3, and 4c) (Table 2). MAb MN13H21 was inhibited by one of four sera (serum 4c), and MAb 3E2 was inhibited by one of four sera (serum 2).

Figure 4 shows the percentage of inhibition for the MAbs that were significantly inhibited. Binding of D6A was inhibited at least 70% by the highest concentration of each convalescent-phase serum. This level of inhibition was sustained for one $\log_2$ dilution in three of the sera and then decreased proportionately with serum dilution (Fig. 4A). MAb 6B7 was 49 to 66% inhibited at the highest concentrations of sera, and this inhibition also decreased proportionately with serum dilution (Fig. 4B). MAb 3E2 was inhibited to a lesser extent than D6A and 6B7 by serum 2 (Fig. 4C). MAb MN13H21 was also inhibited to a lesser extent by serum 4c (Fig. 4D). In Fig. 5 are shown, as examples, the results of the HIMELISA for three convalescent-phase sera (no. 1, 3, and 4c) and one acute serum (no. 4a) that were determined to be noninhibitory of the binding of MAb 3E2 to strain 126E LOS. Three MAbs (3C9, 1B5, and 3D4) were not inhibited by any of the four convalescent-phase sera.

Determination of antibody in normal human sera that bound strain 126E LOS epitopes. We evaluated the performance of the HIMELISA by studying sera drawn from three colleagues before and 11 days after vaccination with tetravalent meningococcal polysaccharide vaccine. We analyzed MAbs D6A and 6B7 only. The prevaccination sera of all three subjects showed measurable antibody that inhibited binding of MAb D6A (Fig. 6). The quantity of this antibody did not increase in any of the three subjects during the demonstrated response to a different glycose antigen of

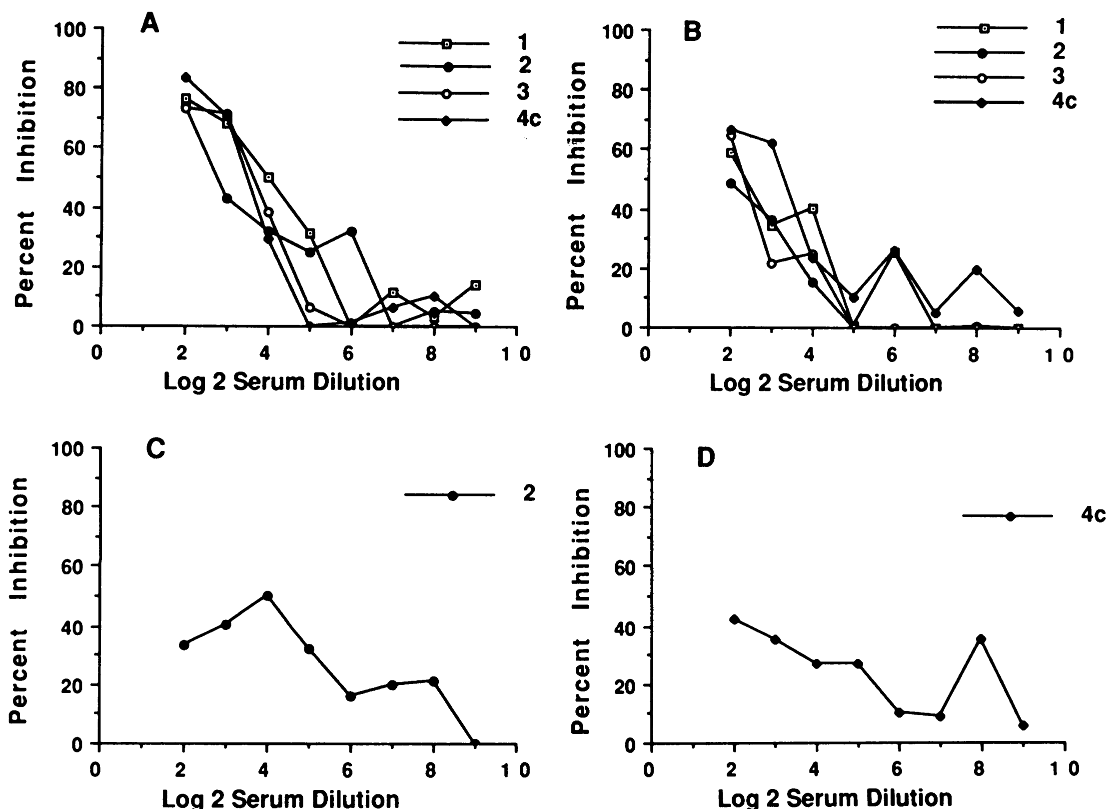

FIG. 4. Percent inhibition of MAb binding to strain 126E LOS by convalescent-phase sera as determined by HIMELISA. Graphs for all significant inhibitions are shown. Percent inhibition = [(absorbance in presence of control serum − absorbance in presence of convalescent-phase serum)/absorbance in presence of control serum] × 100. Curves show inhibition of the binding of MAb D6A to strain 126E LOS by the four convalescent-phase sera (no. 1, 2, 3, and 4c) (A), of MAb 6B7 to strain 126E LOS by the four convalescent-phase sera (no. 1, 2, 3, and 4c) (B), of MAb 3E2 to strain 126E LOS by serum 2 (C), and of MAb MN13H21 to strain 126E LOS by serum 4c (D). For characterization of sera, see Table 2. Negative percent inhibition values were graphed as zero. Only two MAb-defined epitopes (D6A and 6B7) were strongly inhibited by all four convalescent-phase sera.

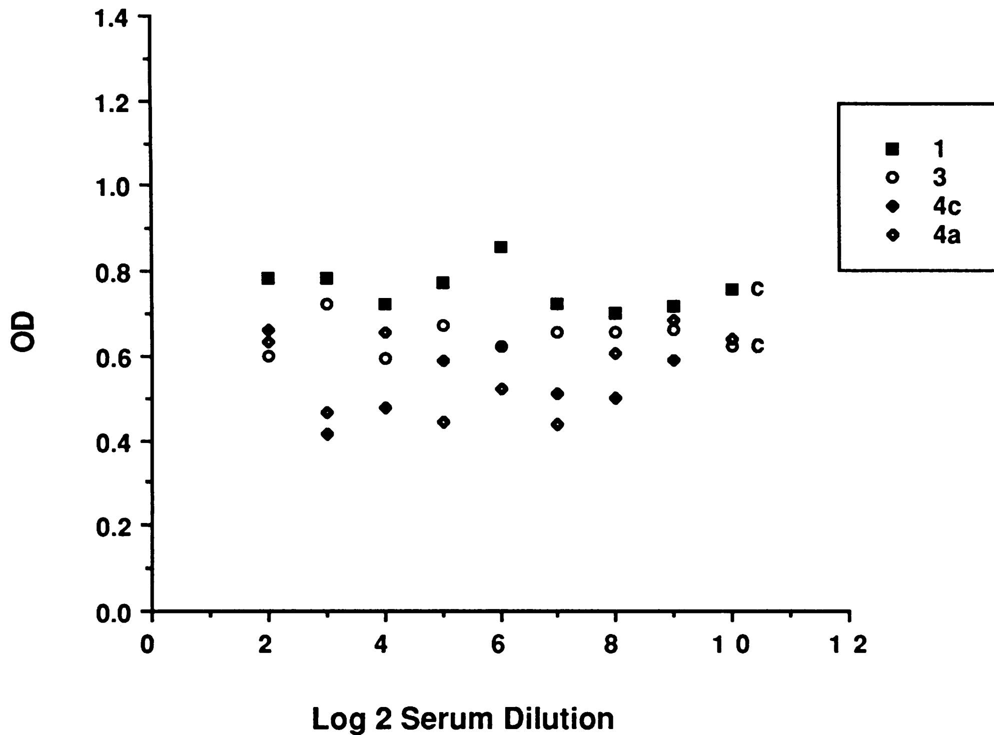

FIG. 5. Absence of inhibition by three convalescent-phase sera (no. 1, 3, and 4c) and one acute-phase serum (no. 4a) of the binding of MAb 3E2 to strain 126E LOS as measured by optical density in the HIMELISA. C indicates uninhibited control points; the lower two points are superimposed, and sera 4a and 4c have the same control point.

strain 126E, its capsular polysaccharide. The variation in inhibition at each dilution of the six serum samples (taken pre- and postvaccination from each subject) was similar to the variation in inhibition among replicate measurements of a single serum. All three prevaccination sera also inhibited the binding of MAb 6B7 to strain 126E LOS, and the degree

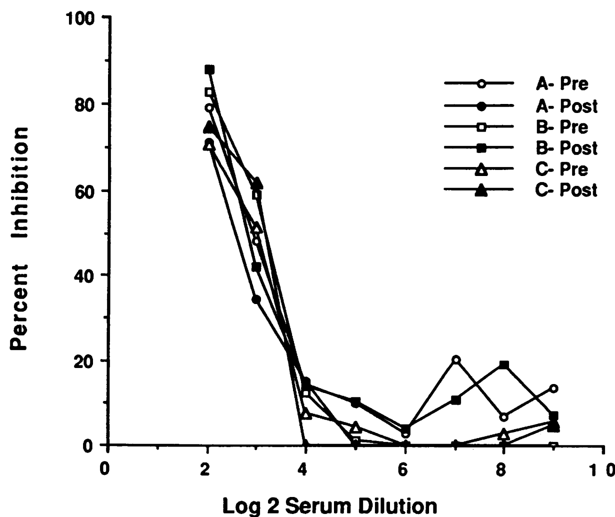

FIG. 6. Percent inhibition of MAb D6A binding to strain 126E LOS by prevaccination and 11-day postvaccination sera of three adults. The adults (designated A, B, and C) were vaccinated with a tetravalent meningococcal capsular polysaccharide vaccine.

of inhibition did not change in response to the vaccine. The degree of inhibition of MAb 6B7 was less (31 to 40% at the lowest dilution) than that of MAb D6A (73 to 86%) for all three pairs of sera.

Comparison of antibody in acute- and convalescent-phase sera that bound defined 126E LOS epitopes. We performed a HIMELISA with paired acute- and convalescent-phase sera (sera 4a and 4c, respectively) (Table 2). The acute-phase serum was drawn on day 1 of illness, and the convalescent-phase serum was drawn on day 7 of illness. The acute-phase serum was able to significantly inhibit the binding of two MAbs, D6A and 6B7, to strain 126E LOS. The inhibition of the binding of these two MAbs increased from the acute-phase serum to the convalescent-phase serum (Fig. 7). The values for duplicate wells are shown, and the change in inhibition was not due to variability between them. The antibodies that were able to bind to the 6B7- and D6A-defined epitopes expressed on strain 126E LOS were present in the acute-phase serum of the individual and increased either in quantity or affinity during the convalescent phase.

Ability of human convalescent-phase sera to inhibit MAb binding to epitopes on LOS from other meningococcal strains. To evaluate the reproducibility of the HIMELISA, we used LOS from two other meningococcal strains. Purified LOS from strain 120M expressed the D6A epitope but not the 6B7 epitope in ELISA. LOS from strain M992 expressed the 6B7 but not the D6A epitope. The silver stain of the SDS-PAGE-separated LOS of these two strains is shown in Fig. 1. When an immunoblot (not shown) of these SDS-PAGE-separated LOS was performed, MAb D6A bound weakly to a 3,600- and a 3,200-M_r molecule made by strain 120M and did not bind to LOS made by strain M992. Conversely, MAb

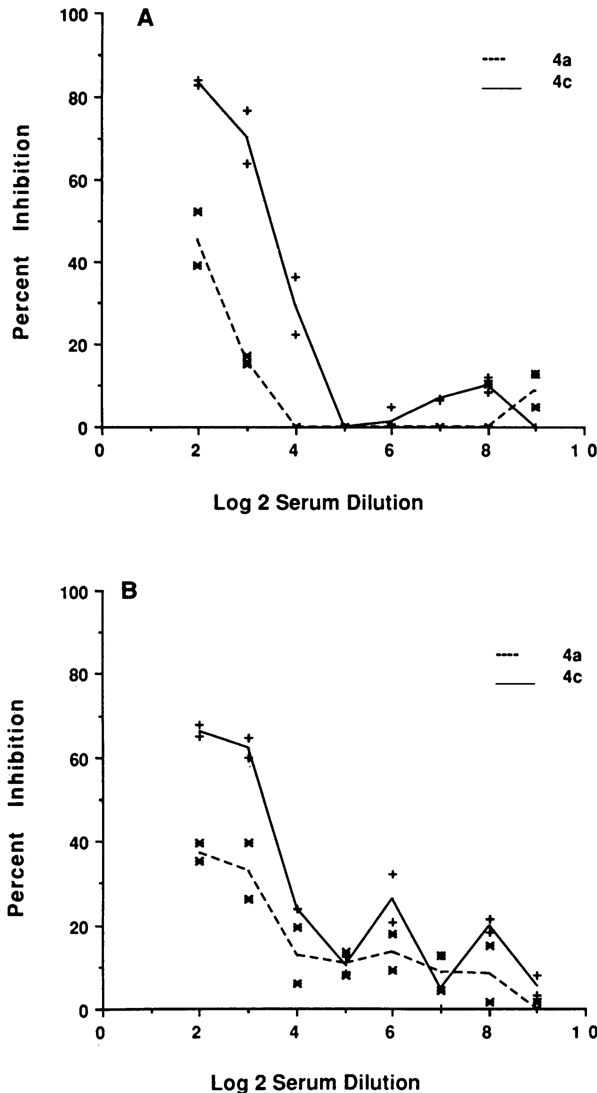

FIG. 7. Percent inhibition of MAb binding to strain 126E LOS by acute-phase (day 1) versus convalescent-phase (day 7) sera drawn from an individual infected with a group C meningococcus (Table 2, sera 4a and 4c). Curves show inhibition of binding of MAb D6A (A) and MAb 6B7 (B). 4a, Acute-phase serum; 4c, convalescent-phase serum. Values for the duplicate wells are shown for acute- (*) and convalescent- (+) phase sera; mean values are also shown (----, acute phase; ———, convalescent phase).

6B7 bound weakly to a 3,600-M_r molecule made by both strains.

We performed a HIMELISA of MAb D6A binding to strain 120M LOS and of MAb 6B7 binding to strain M992 LOS with the four convalescent-phase sera. All four convalescent-phase sera inhibited the binding of each MAb to its respective LOS (Fig. 8).

The four sera were very similar in their maximum inhibition of binding of MAb 6B7 to M992 LOS (83 to 91%) (Fig. 8B). For each convalescent-phase serum, the inhibition was greater for the MAb 6B7 epitope when expressed on strain M992 LOS than when expressed on 126E LOS, probably reflecting the lower affinity with which 6B7 bound to M992 LOS, as judged by immunoblot (Fig. 9A).

The four convalescent-phase sera varied in their maximum inhibition of binding of MAb D6A to 120M LOS (42 to 87%)

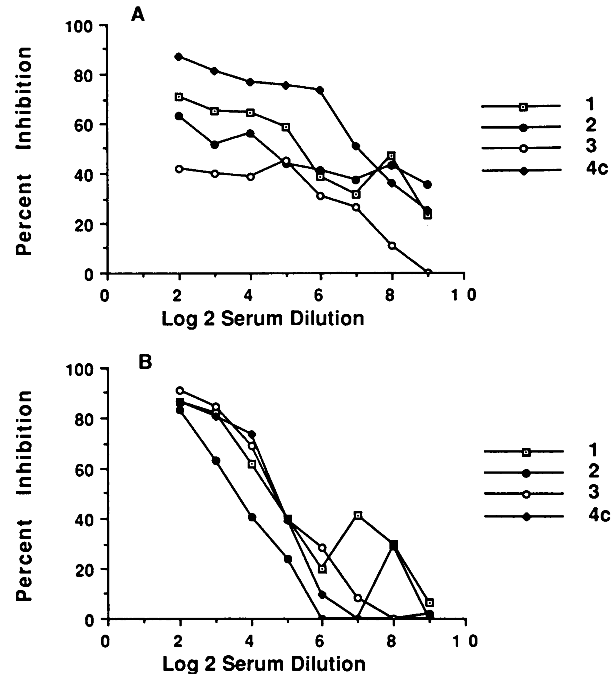

FIG. 8. Percent inhibition of MAb D6A binding to strain 120M LOS and of MAb 6B7 binding to strain M992 LOS by the four convalescent-phase sera (no. 1, 2, 3, and 4c). Curves show the inhibition of MAb D6A binding to 120M LOS (A) and of MAb 6B7 binding to M992 LOS (B). The four convalescent-phase sera inhibited the D6A- and 6B7-defined epitopes when they were expressed on LOS produced by diverse meningococcal strains.

(Fig. 8A). Three of the sera inhibited the binding of D6A to 120M LOS to approximately the same degree as they inhibited its binding to 126E LOS, but the titer of inhibition was greater for 120M LOS (Fig. 9B). In contrast, one serum (no. 3) failed to completely inhibit the binding of D6A to 120M LOS (Fig. 9C). The relatively high titer of the partial inhibition suggested that human antibody bound to only one of the epitopes recognized by D6A on this LOS (17).

Immunoblot analysis of antibody in human convalescent-phase sera capable of binding LOS molecules. We performed an immunoblot with each convalescent-phase serum and SDS-PAGE-separated LOS from strains 126E, M978, 7883, and 8032 (Fig. 10). Table 1 summarizes the results of these immunoblots with human antibody and shows the MAb binding patterns.

By immunoblot, sera 1 and 2 contained antibody to all LOS molecules of the four test strains. Serum 3 lacked antibody only to the 5,200-M_r LOS of strain M978. Serum 4c contained antibody to the 3,600- and the 3,200-M_r LOS of 126E, all LOS of M978 and 7883, and all LOS of 8032 except the 3,600-M_r LOS. All four convalescent-phase sera contained antibody to the 3,600-M_r LOS of strain 126E. This LOS molecule expressed the MAb D6A-and 6B7-defined epitopes which were inhibited by four of four convalescent-phase sera by HIMELISA. Three of four convalescent-phase sera contained antibody to the 4,000-M_r LOS of strain 126E, which also expressed the D6A-defined epitope.

All four sera contained antibodies to LOS molecules that were not identified by MAb (Table 1; 126E [M_r, 3,200] and M978 [M_r, 4,800]). The sera also contained antibodies that did not inhibit MAb binding to the same LOS molecules. For example, Table 1 shows serum 1 containing antibodies to the

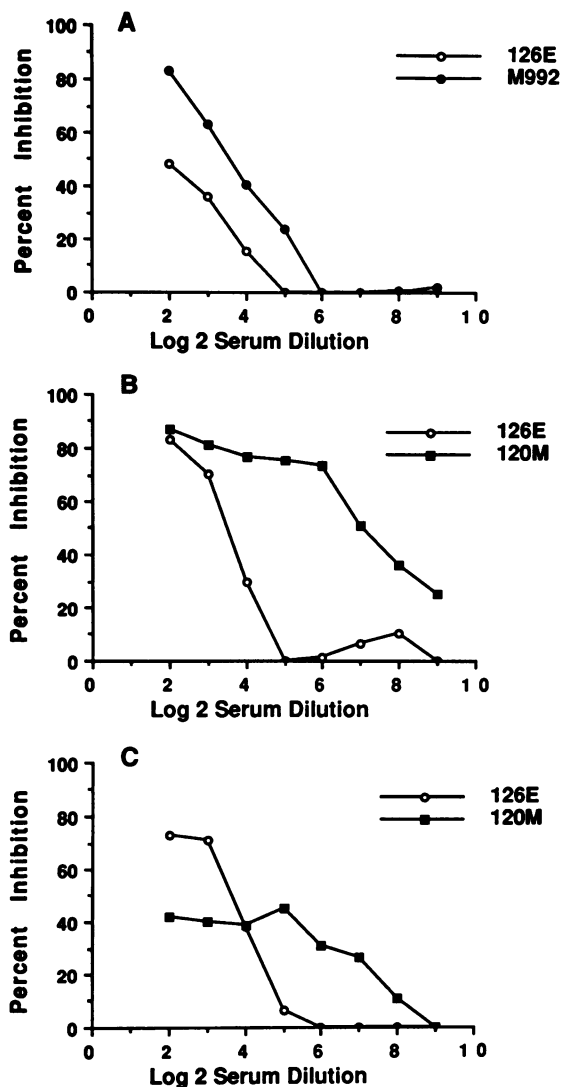

FIG. 9. Comparison of the abilities of convalescent-phase sera to inhibit MAb binding to its epitope when that epitope was expressed on LOS made by different meningococcal strains. Curves show the inhibition of MAb 6B7 binding to strain 126E LOS versus strain M992 LOS by serum 2 (A), of MAb D6A binding to strain 126E LOS versus strain 120M LOS by serum 4c (B), and of MAb D6A binding to strain 126E LOS versus strain 120M LOS by serum 3 (C). The degree of inhibition of MAb binding to its LOS epitope varied depending on the strain of origin of the LOS.

4,200-M_r LOS of strain 126E. MAb 3D4, 1B5, and 3E2 all bound this LOS molecule. Table 2 shows that serum 1 did not inhibit these three MAbs as determined by HIMELISA.

DISCUSSION

In this study, we identified seven MAb-defined epitopes on meningococcal LOS, confirming the antigenic heterogeneity of these molecules that was previously shown. The HIMELISA permitted epitope-specific discrimination among LOS antibodies in whole sera and demonstration of their relationship to individual LOS molecules. Although adult vaccinates had measurable antibody specific for two of the epitopes, the levels of the antibody were higher than those in acute-phase serum and lower than those in convalescent-phase sera. Thus, the HIMELISA can provide useful information about the LOS epitope-specific composition of antibody in a serum, estimate changes in the levels of antibody, and detect the induction of antibody of new specificities, as shown for the MAb MN13H21-defined epitope in the convalescent-phase serum 4c.

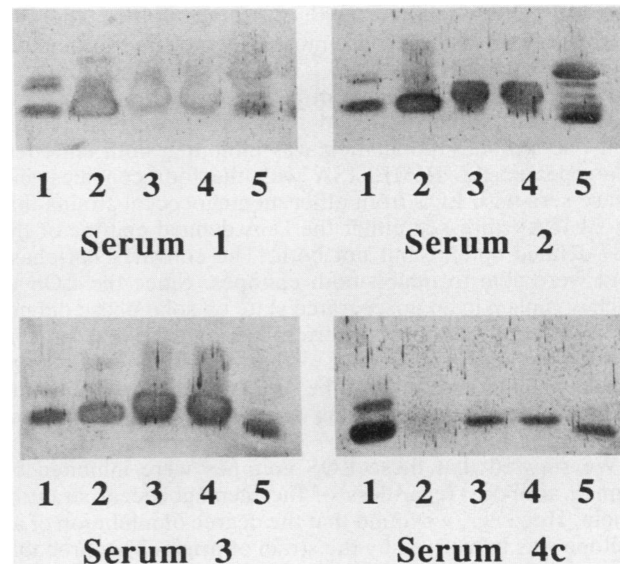

FIG. 10. Immunoblots with the four convalescent-phase sera (no. 1, 2, 3, and 4c) of SDS-PAGE-separated LOS of meningococcal strains M978 (lane 1), 7883 (lane 2), 126E (lanes 3 and 4), and 8032 (lane 5). For characterization of convalescent-phase sera, see Table 2.

Only two of the seven epitopes were strongly inhibited by human convalescent-phase sera. One of these (D6A) was expressed on both the 3,600- and 4,000-M_r LOS molecules of meningococcal strain 126E. The other epitope (6B7) was expressed on the 3,600-M_r LOS of strain 126E. Since the D6A-defined epitope was expressed on two LOS molecules of 126E of different M_rs, determining the relationship of this immunogenic epitope to the chemical structure of the LOS molecules on which it is expressed will be important in understanding the human immune response to meningococcal LOS.

When we investigated the expression of the D6A epitope on other meningococcal strains, we found that it was expressed on LOS of different M_rs depending on the strain of origin (M_r range, 3,200 to 5,400), but all strains that bound it made a 3,600-M_r LOS that expressed the epitope. Since this epitope was expressed on a molecule with as small an M_r as 3,200 (Table 1, strain 8032), we conclude that it must be in a core region of the LOS molecule. Kim et al. (24) found that the epitope defined by D6A was highly conserved on *N. meningitidis* group A strains and was expressed on multiple LOS molecules of variable M_r. It was also expressed on some group B, C, and Y strains (24).

The 6B7-defined epitope was restricted to the 3,600-M_r molecule of all meningococcal strains that we investigated. Dudas and Apicella (6) found that the 6B7-defined epitope was not expressed on the higher-molecular-weight (M_r, 4,700) parent LOS molecule of a gonococcal strain but was expressed on lower-molecular-weight LOS of pyocin-resistant strains (M_rs, 4,000 and 3,600, respectively). They concluded that it was a cryptic epitope that was exposed when sugar residues were removed from the parent LOS molecule.

Our data are consistent with the concept that the two epitopes, D6A and 6B7, are most likely located in the core region of the LOS molecule.

Because each convalescent-phase serum inhibited both the D6A- and the 6B7-defined epitopes, we were concerned that one antibody population was inhibiting both epitopes. We repeated the HIMELISA with the four convalescent-phase sera with LOS from other meningococcal strains that by ELISA expressed either the D6A-defined epitope or the 6B7-defined epitope but not both. The convalescent-phase sera were able to inhibit both epitopes. Since the LOS of each organism in an unseparated state on solid phase did not express both epitopes, interference or blocking of one epitope by antibody binding to the other seemed unlikely. These results indicated that the sera contained two antibody populations, one recognizing the D6A epitope and one recognizing the 6B7 epitope.

We showed that these LOS epitopes were inhibited by human antibody regardless of the meningococcal strain of origin. However, we found that the degree of inhibition of an epitope was influenced by the strain of origin. This probably was a result of differences in affinity of the MAb and/or human antibody for the epitope expressed on different LOS molecules. Griffiss et al. also found that the affinity of a MAb binding to its epitope was dependent on the LOS repertoire of the strain of origin (17). Interestingly, SDS-PAGE appeared to expose the 6B7 epitope on the LOS of strain 120M that was not apparent by ELISA. This demonstrated the influence of the physical state of LOS on epitope expression.

If the LOS repertoire of a strain and the physical state of LOS strongly influence epitope expression, it becomes important to know the M_r of the molecule expressing that epitope. We were able to define antibody binding sites on meningococcal LOS by their MAb binding and the M_r of the molecule that expressed them. The HIMELISA was capable of identifying a potentially immunogenic epitope, but it could not differentiate LOS molecules when that epitope was expressed on molecules of different M_rs of a strain. To investigate whether the binding of human antibody to an epitope varied by the M_r of the LOS molecule on which it appeared, we tested the convalescent-phase sera for binding to the SDS-PAGE-separated LOS molecules of our test strains. These immunoblots identified human antibody to LOS molecules only, not to epitopes, so we compared the immunoblots with the binding patterns of the MAbs to the same LOS. The immunoblots were consistent with the results of the HIMELISA but were not helpful in identifying individual, immunogenic epitopes.

We conclude that the immunogenicity of an epitope may vary when expressed on LOS molecules of different M_rs, that the physical state of LOS influences epitope expression, and that the affinity of an antibody to its epitope is dependent on the M_r of the molecule on which the epitope is located, the strain of origin, and method of LOS presentation (SDS-PAGE versus solid-phase ELISA).

An LOS serotyping system for *N. meningitidis* strains that used hemagglutination and solid-phase radioimmunoassay inhibition was described previously (7, 27, 42, 43). Strain 126E is type L1,8; it is the prototype L1 strain. No other L1 strains have been identified, so we do not know if any of our MAb-defined epitopes reflect the L1 antigen. Strain 126E is also type L8, as is strain M978, which is the L8 prototype strain. Kim et al. (24) found that MAb 2-1-L8 was serotype specific for L8 and bound to a 3,600-M_r LOS. In our study, MAb MN13H21 bound the same pattern of LOS molecules as did MAb 2-1-L8 and may reflect the L8 antigen. Kim et al. also found that MAb 4C4 was L11 serotype specific. Strain 7883 is type L11 and makes a single LOS molecule of 3,600 M_r. We found that, although MAb 4C4 bound to strain 7883, it did not bind to strain 126E and so was not used in our inhibition studies.

Poolman et al. (31) found that MAb D6A was bactericidal for a group B meningococcal strain (2996). LOS molecules bearing epitopes that induce broadly reactive, bactericidal antibody in children could be incorporated into a meningococcal vaccine. We have identified two potential molecules, the 3,600- and 4,000-M_r LOS of *N. meningitidis* strain 126E, for this use. The techniques described in this study will be used to investigate the immune response to neisserial LOS in a pediatric population.

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